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Contribution of vascular resident mesenchymal stromal cells to abdominal aortic aneurysm pathogenesis: increased MMP-9 expression and ineffective immunomodulation

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LIST OF ABBREVIATIONS

AAA: Abdominal Aortic Aneurysm

AAA-MSCS: Abdominal Aortic Aneurysm-Derived Mesenchymal Stromal Cells

AAAP-40: Aortic Aneurysm Antigenic protein-40

ADAMT: A Disintegrine and Metalloproteinase with Thrombospondin domain

Ag: Antigen

ASCs: Adult Stem Cells

BP: Base Pair

BrdU: 5-Bromo-2'-Deoxyuridine

CAD: Coronary Artery Disease

CD: Cluster of Differentiation

cDNA: Copy of DNA

CFU-f: Colony Forming Unit -Fibroblast

cMSCs: Control healthy Mesenchymal Stromal Cells

CNS-SC: Neural Crest Derived-Stem Cells

COPD: Chronic Obstructive Pulmonary Disease

CRF: Chronic Renal Failure

CSPG: Chondroitin Sulfate Proteoglycan

CT: Computational Tomography

CVD: Cerebro-Vascular Chronic Disease

DAAA: Abdominal Aortic Aneurysm Diameter

DCs: Dendritic Cells

DMEM: Dulbecco's Modified Eagle Medium

DSPG dermatan sulfate proteoglycan

ECM: Extracellular Matrix

ECs: Endothelial Cells

EGCs: Embryonic Germ Stem Cells

EGF: Epidermal- derived Growth Factor

EMMPRIN: Extracellular Matrix Metalloproteinases Inducer

EPCs: Endothelial Progenitor Cells

ESCs: Embryonic Stem Cells

ESR: Erythrocyte Sedimentation Rate

FBS: Fetal Bovin Serum

FCS: Fetal Calf Serum

FITC: Fluorescein Isothiocyanate

GAG: Glycosaminoglycan

GAPDH: Glyceraldehyde 3-Phosphate Dehydrogenase

GAS: IFN-γ Activated Site

GUS: β-Glucoronidase

GVHD: Graft Versus Host Disease

H/E: Hematoxylin/Eosin

H_2O_2: Hydrogen Peroxide

HDL: High Density Lipoprotein

HGF: Heaptocyte Growth Factor

HLA: Histocompatibility Complex

HSCs: Heamtopoietic Stem Cells

HSP heparan sulfate proteogly

IAAA: Inflammatory Abdominal Aortic Aneurysm

ICAM-1: Intracellular Adhesion Molecule-1

ICM: Inner Cell Mass

IDO: Indoleamine 2,3-Dioxygenase

IFN-γ: Interferon-γ

IHC: Immunohistochemistry

IL-1, -8, -10, -β : Inteurlekin-1, -8, -10, -β

ILT: Intraluminal Thrombus

ISCT: International Society for Cell Therapy

ISREs :IFN-stimulated Response Elements

KSP keratan sulfate proteoglycan

LDL: Low Density Lipoprotein

LPS: Lypopolysaccharide

MAGP-1: Microfibril-Associated Glycorprotein

MAPCs : Multipotent Adult Progenitor Cells

MMPs: Matrix Metalloproteinases

MRI: Magnetic Resonance Imaging

MSCs: Mesenchymal Stromal (Stem) Cells

NKs: Natural Killer Cells

NO: Nitrix oxide

O_2^-: Anion Superoxide

Oct-4: Octamer binding Transcription Factor-4

PAGE: Polyacrylamide Gel Electrophoresis

PAMPs: Pathogen Associated Mmolecular Patterns

PAOD: Peripheral Arterial Obstructive Disease;

PBMCs: Peripheral Blood Mononuclear Cells

PBS: Phosphate Buffer Saline

PCR: Polymerase Chain Reaction

PDGF-B: Platelet-Derived Growth Factor-B

PE: Phycoerythrin

PGE2: Prostaglandin E2

PHA: Phytohaemagglutinin

qPCR: quantitative PCR

RNS: Reactive Nitrogen Species

ROS: Reactive Oxygen Species

RT-PCR: Reverse-Transcription-PCR

SDS: Sodium-Dodecyl-Sulfate

SMCs: Smooth Muscle Cells

Sox-2: SRY: Sex determining Region Y- Box 2

SSEA-3, -4: Stage Specific Embryonic Antigen

SSRE: Shear Stress Rresponsive Elements

STAT-5: Signal Transducer and Activator of Transcription 5

T regs: T regulatory cells

TGF-β: Transforming Growth Factor-β

TIMPs: Tissue inhibitor of Matrix Metalloproteinase**s**

TLR: Toll-Like Receptors

TNF-α: Tumour Necrosis Factor-α

t-PA: Tissue-Plasminogen Activator

TRA1-60, -1-8: Keratane Sulphate

u-PA: Urokinase- Plasminogen Activator

UT: Ultrasonography

VCAM-1: Vascular Cell Adhesion Molecule-1

VEGF: Vascular Endothelial Growth Factor

ABSTRACT

Background. Ageing and inflammation are critical for the occurrence of aortic diseases. Extensive inflammatory infiltrate and excessive ECM proteloysis, mediated by MMPs, are typical features of abdominal aortic aneurysm (AAA). Mesenchymal Stromal Cells (MSCs) have been detected within the vascular wall and represent attractive candidates for regenerative medicine, in virtue of mesodermal lineage differentiation and immunomodulatory activity. Meanwhile, many works have underlined an impaired MSC behaviour under pathological conditions. This study was aimed to define a potential role of vascular MSCs to AAA development.

Methods. Aortic tissues were collected from AAA patients and healthy donors. Our analysis was organized on three levels: 1) histology of AAA wall; 2) detection of MSCs and evaluation of MMP-9 expression on AAA tissue; 3) MSC isolation from AAA wall and characterization for mesenchymal/stemness markers, MMP-2, MMP-9, TIMP-1, TIMP-2 and EMMPRIN. AAA-MSCs were tested for immunomodulation, when cultured together with activated peripheral blood mononuclear cells (PBMCs). In addition, a co-colture of both healthy and AAA MSCs was assessed and afterwards MMP-2/9 mRNA levels were analyzed.

Results. AAA-MSCs showed basic mesenchymal properties: fibroblastic shape, MSC antigens, stemness genes. MMP-9 mRNA, protein and enzymatic activity were significantly increased in AAA-MSCs. Moreover, AAA-MSCs displayed a weak immunosuppressive activity, as shown by PBMC ongoing along cell cycle. MMP-9 was shown to be modulated at the transcriptional level through the direct contact as well as the paracrine action of healthy MSCs.

Discussion. Vascular injury did not affect the MSC basic phenotype, but altered their function, a increased MMP-9 expression and ineffective immunmodulation. These data suggest that vascular MSCs can contribute to aortic disease. In this view, the study of key processes to restore MSC immunomodulation could be relevant to find a pharmacological approach for monitoring the aneurysm progression.

Keywords: AAA; MSCs; inflammation; MMP-9; immunomodulation

LIST OF ORIGINAL PUBLICATIONS

Some of the data presented in this thesis have been object of two original articles:

Valente S., Alviano F., Ciavarella C., Buzzi M., Ricci F., Tazzari P. L et al. (2014). Human cadaver multipotent stromal/stem cells isolated from arteries stored in liquid nitrogen for 5 years. Stem Cell Res Ther 5(1), 8.

Ciavarella C., Alviano F., Gallitto E., Ricci F., Buzzi M., Velati C., Stella A., Freyrie A., Pasquinelli G. Human Vascular Wall Mesenchymal Stromal Cells contribute to Abdominal Aortic Aneurysm pathogenesis through an impaired immunomodulatory activity and increased levels of MMP-9. Circulation Journal, in press.

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INTRODUCTION

HUMAN AORTA: STRUCTURAL AND BIOMECHANICAL PROPERTIES IN HEALTH AND DISEASE

The aorta is the largest artery of the human vasculature system and it is designated to deliver blood from heart to the systemic circulation. The human aorta arises from the left ventricle and goes down to the abdomen; it can be distinguished into distinct segments, each of one has a different susceptibility to pathological changes:

- Ascending aorta, between the left ventricle and the aortic arch;
- Aortic arch;
- Descending thoracic aorta, above the diaphragm;
- Descending abdominal aorta, below the diaphragm.

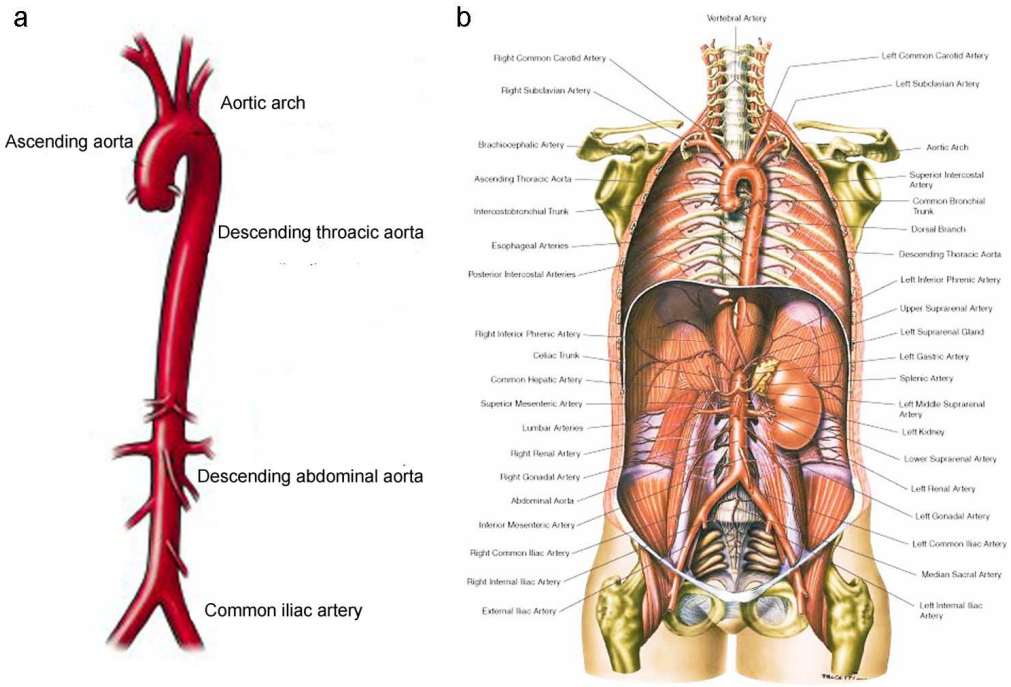


Figure 1. (a) Illustration of the human aorta regions and (b) scheme of abdominal aorta branches.

The aorta belongs to the elastic arteries, large-diameter vessel (larger than 2.5 cm) with close localization to the heart. Healthy aorta of an adult has a 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.

Pulmonary trunk, carotid and iliac arteries are also included in the elastic arteries category.

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 deliver blood to the skeletal muscle and inner organs. Muscular arteries are medium-size vessels (~ 0.4 cm) and are mainly composed of smooth muscle. This structure is functional to the muscular artery ability to change its size, depending on the blood pressure, and regulate blood flow to each organ (Abramson and Dobrin, 1984; Martini, 2007).

Structural features of human arterial wall

The idealized healthy arterial wall is composed of three layers, called tunics: the intima, the media and the adventitia (Gasser et al, 2006).

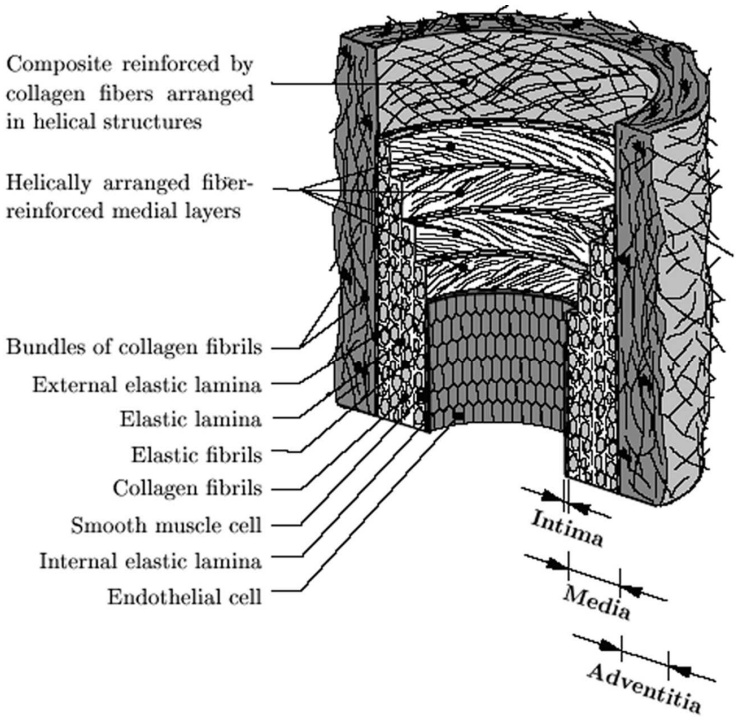


Figure 2. Histological composition of idealized healthy elastic arterial wall (Holzapfel and Gasser, 2000)**.**

Tunica intima

The 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 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 and are designated to control the endothelium permeability to circulating cells; *iii)* gap junctions, communication structures designated to the ions and metabolite exchange between cells (Bazzoni and Dejana, 2004). ECs posses several structural and regulatory roles, participating to the vascular homeostasis and vessel wall integrity. ECs are responsible for the maintenance of a selectively permeable and non-thrombogenic barrier, regulate the smooth muscle cell tone through the secretion of vasocostrictors and vasodilatators, modulate the immune response through the expression of chemotactic and adhesion molecules. ECs are placed on the basal lamina, composed of collagen type IV, proteoglycans, laminin and fibronectin. As regards more specifically the abdominal aorta, the intima layer is mainly composed of type I collagen (16%), while type III and type IV collagen are present in a less 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 trough 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 (Ross et al, histology; Abramson, blood vessels and lymphatics in organ system).

Tunica media

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 of smooth muscle cells (SMCs), organized in a complex network of elastin and collagen,

mainly of type I, III, IV and V (Zarins and Glagov, 1987). An extracellular matrix (ECM) containing proteoglycans surrounds the cellular and elastic components. SMCs are spindle shaped, with an elongated nucleus and assume a specific orientation in elastic and muscular arteries. In elastic arteries, SMCs are arranged into 5-15 µm thick concentric structures, separated by elastin. At physiological distending pressures, the aortic smooth muscle, elastin and collagen are well 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, 1967). 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 human aortic media; the number as well as the orientation and the composition of these units are functional to the uniform distribution and magnitude of the tensile stress along the vessel wall (Clarke and Glagov, 1985).

Medial SMCs are essential to the correct functionality of the vasculature; indeed, their contraction and relaxation modulate the arterial structural changes and diameter, to adapt to the blood flow dynamics and keep blood pressure. In addition, SMCs are responsible of the synthesis of ECM components. SMCs are susceptible to phenotype changes, shifting from the contractile to the synthetic one, characterized on the basis of different cell shape and structure, marker expression, proliferative and migration rates (Rensen et al, 2007). Contractile SMCs display an elongated, spindle-shape morphology, substituted by a less elongated, epitheloid-like shape in synthetic SMCs. The synthetic phenotype is characteristic for the presence of an high number of organelles appointed to protein synthesis, as well as for an high proliferative rate. In contractile SMCs, biosynthetic organelles are replaced for contractile filaments; in addition, the contractile SM phenotype presents increased contractile markers, such as alpha-smooth muscle actin, smoothelin, smooth muscle heavy chain, that are not represented in synthetic SMCs (Rensen et al, 2007). SMC heterogeneity exists not only between different tissues and vascular districts, but also in the same vessel, suggesting a genetic basis. Moreover, SMC phenotype changes in dearly development: during blood vessel formation, SMCs assume a contractile phenotype, corresponding to a reduced ECM production and an increased myofilament

formation, to allow the vessel contraction and dilation under the effects of blood flow and pressure.
A reversal of SMC phenotype, from contractile into synthetic, induces cell proliferation and invasion of the intima tunica, characteristic of vascular disorders.

Some differences regarding 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 the 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, here 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 and Glagov, 1969; Wolinsky and Glagov, 1970; Halloran et al, 1995; Ruddy et al, 2008).

The media of muscular arteries has a less-defined structure, predominantly made of a 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, contributes to the artery strength and force.

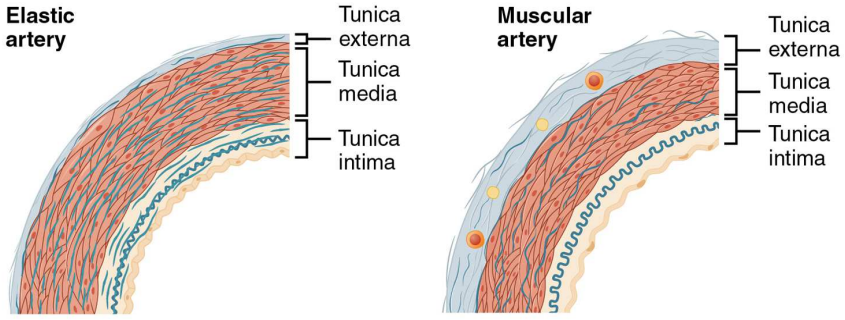


Figure 3. Elastic artery wall versus muscular artery wall.

Tunica adventitia

The adventia 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: fibroblasts, macrophages, collagen, groundmatrix. The adventitia displays 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 adventitia providing support and strengthen to the arterial wall, preventing the excessive distension of the vessels. Nerves and vasa vasorum are also characteristic of adventitial structure. Nerves contribute to the regulation of medial smooth muscle activity, trough 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 (Humphrey, 2002).

The structural changes that affect the arterial wall go under the term of *vascular remodelling*, which interests larger elastic arteries more than smaller and muscular ones. Ageing represents the main decisive factor for arterial remodelling, 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 both on the structural and functional hand by ageing process. Firstly, they 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 SMC infiltration. These alterations characterize the atherosclerotic process.

Extracellular Matrix (ECM): composition and turnover

Extracellular matrix (ECM) is synthesized by medial SMCs and adventitial fibroblasts, and constitutes the skeleton 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, 2007). 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 prevent vessel wall excessive distension; about 26 members have been identified and their expression and distribution depend on the cell type. As regard vascular cells, type I and type III collagen are the most common types, representing the 30% and 60% of the vascular wall; the remaining 10% is composed of type V, XII and XIV collagen types (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-transcriptional modifications are required before collagen maturation into a functional protein. Crosslinking mediated by the enzyme lysil-oxidase increases collagen insolubility and tensile strength

Elastin is the most prominent component of the large arteries wall and, together with the *microfibrils*, forms the structure of the elastic fiber. Microfibrils are 10-15 nm filaments that represent the 10% of the elastic fiber and contribute to elastin assembly (Ross, 1973; Xu J. and Shi G-P,2014). The elastin gene is located on chromosome 7 and its expression is high during pre- and neo-natal development (Arribas et al, 2006). Its activity is restricted to the early life of humans, after that the mature form of elastin has a half-life of 40 years and its degradations starts with aging and diseases. Elastin gene encodes for a precursor form of the functional protein, the *tropoelastin*. This is a monomeric protein (64-72 kDa), whose structure is characterized by the alternance of hydrophobic domains and lysine residues with cross-linking motifs. When tropoelastin is released in the extracellular space, its lysine residues undergo modifications to form covalent links with elastin molecules. As

for collagen, crosslinking is mediated by lysil-oxidase and takes to a sequence reactions terminating with the functional and insoluble form of elastin. Differentially from elastin that has 15-20 cross-links per unit, collagen only contains 1-4 cross-links. This high number is critical for elastin recoil property, as well as for insolubility and high half-life (Eyre, 1984). Vascular elastin is produced by SMCs in the media and fibroblasts in the adventitia. Elastin confers elasticity to the vessel wall, the most essential property in large-diameter arteries which are subjected to an high pressure generated by blood flow, defining their ability to contract and distend according to the cardiac cycle. In addition, elastin has been shown to regulate SMC proliferation and phenotype.

Glycoprotein family include *fibrillins* and *microfibril-associated glycorproteins* (MAGP-1, MAGP-2). These are large glycoproteins that mainly compose microfibrils, largely contributing to their structural integrity. Particularly, fibrillin-2 and MAGP-1 are believed to be crucial for aorta formation during embryonic and fetal stage. Other glycoproteins mainly represented in the vascular wall include: fibronectin, laminin, tenascin, thrombospondin, osteopontin. These glycoproteins can associate each other and contain multiple domains to interact with multiple ECM members, contributing to ECM structure and function. For example, they contribute to the mechanotransduction mediated through ECM: glycoproteins can interact with integring, triggering the signalling cascade in vascular cells to respond to biomechanical forces that act on vessel wall.

Proteoglycans are complex macromolecules, whose structure is composed of a protein core, associated to glycosaminoglycan (GAG) chains, through covalent links between the O-glycosidic domain and the serine residues of the protein core. Glycosaminoglycans consist of disaccharide units, containing an amino-sugar (i.e. N-acetylglucosamine, N-acetylgalactosamine) and an uronic acid. According to the glycosaminoglycan type, proteoglycans are divided into four classes: chondroitin sulfate proteoglycan (CSPG), dermatan sulfate proteoglycan (DSPG), heparan sulfate proteoglycan (HSPG), and keratan sulfate proteoglycan (KSPG) (Wight, 1989). Proteoglycans participate to many biological functions in the vascular wall: ECM assembly and cell proliferation, differentiation, adhesion and migration. In addition, proteoglycans regulate vascular permeability, haemostasis and lipid metabolism, due to the interaction with lipoprotein. Since they participate to key processes for atherosclerosis development, proteoglycans can be crucial

regulator of both physiological and pathological vascular conditions (Wight, 1989). Proteoglycans present in the vascular wall are: aggregan and versican (large aggregating proteoglycans); decorin and fibromodulin (small aggregating proteoglycans); syndecan, fibroglycan, glypican (cell-associated proteoglycans) (Jacob et al, 2001). The presence of proteoglycans is not uniform within the vascular wall: for example, versican and glypican are abundant in the intima and media, while decorin is mainly concentrated in the advential collagen.

ECM is a dynamic structure, constantly subjected to remodelling through the synthesis and degradation of the main components, or changes in the organization level of ECM architecture (Daley, 2007). These processes are functional to ECM regulation of many biological processes, under physiological conditions, and they are mediated through ECM interactions with resident cells or signalling molecules. On the other hand, keeping the ECM integrity is necessary to prevent definitive perturbations, as occurs in many cardiovascular disease (i.e., aneurysm, hypertension). Many human diseases are associated with the loss of function or expression as well as excessive degradation of ECM proteins.
ECM component degradation is mediated by many types of proteolytic enzymes, with a certain degree of specificity. The most known enzymes are *matrix-metalloproteinases* (MMPs), a disintegrine and metalloproteinase with thrombospondin domain (ADAMTS). Other proteinases families include serine, aspartic and cysteine proteinases.
Plasmin belongs to the serine proteinases and degrade fibrin, fibronectin and laminin.
MMPs degrade a large range of ECM members, including porteoglycans and glycoprotein; in addition, specific enzymes are targeted against the GAG polysaccharide chains in a specific manner. For example, sulfatases SULF-1 and SULF-2 degrade the 6-O-sulfates from the heparin sulphate proteoglycans (Proillet, 1998).

Matrix Metalloproteinases (MMPs) and their inhibitors (TIMPs)
MMPs are a family of Zn^{2+} and Ca^{2+} endopeptidases and, as proteolytic enzymes of the ECM components, regulate and participate to many biological processes, such as vascular remodelling, cell proliferation, migration and adhesion (Raffetto and Khalil, 2008).
The family of MMPs presents a conserved structure, which is generally composed of:

1. a prodomain at the N-terminal extremity, containing a cysteine switch motif that chelates the zinc active site and keeps MMPs in their precursor form (zymogens);
2. a catalytic domain, containing a zinc-binding motif;

3. a hemopexin domain, at the C-terminal extremity (Visse and Nagase, 2003; Raffetto and Khalil, 2008).

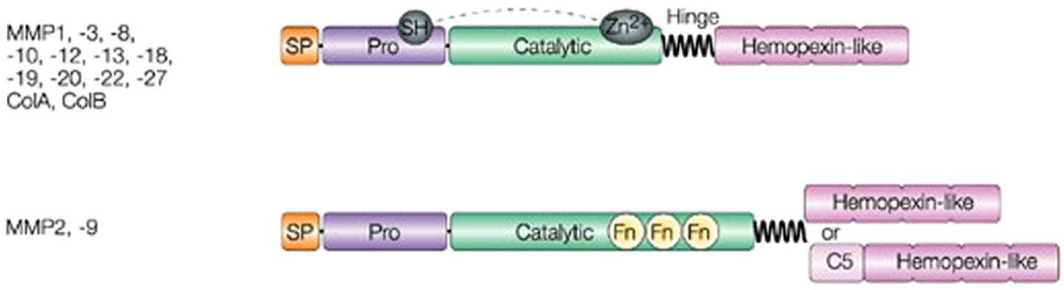


Figure 4. Structural domain of MMPs (Parks et al. 2004)**.**

MMP family comprises 26 members, that can be distinguished into 6 groups according to the substrate specificity:

1. Collagenases (MMP-1, -8, -13) cleave type I, II and III collagens.
2. Gelatinases (MMP-2, -9) cleave heat denatured collagen (gelatine); they share the MMP structure, with some variations: the catalytic domain contains three repeats of type III fibronectin that bind to collagen and laminin.
3. Stromelysins (MMP-3, -10).
4. Matrilysins (MMP-7, -26), lacking of the hemopexin domain.
5. Membrane-Type MMPs (MT-MMPs, that include: MMP-14, -15, -16, -17, -24, -25), that digest type I, II, III collagen and activate MMPs from precursor proMMPs.
6. Other MMPs, that are not classified in the described classes, including metalloealastase (MMP-12).

MMPs are involved in many physiological processes, such as angiogenesis, placental remodelling during pregnancy (Raffetto and Khalil, 2008); in these conditions, MMPs are tightly regulated through different mechanisms, including transcriptional and post-transcriptional modifications, activation of the precursor form of zymogens, interaction with ECM components and inhibition by specific mediators, known as *tissue inhibitors of MMPs* (TIMPs) (Visse and Nagase, 2003).

Most MMPs are transcriptionally modulated, except MMP-2 which is often consitutively expressed (Sternlicht e Werb, 2009). In addition, MMP genic expression is regulated by many cytokines and growth factors, such as VEGF, TNF-α, TGF-β, IL-1β and the

extracellular matrix metalloporteinases inducer (EMMPRIN, or CD147), which is a glycoprotein involved in MMP induction in tumoral and physiological/pathological conditions (Fini et, 1998; Gabison, et al, 2005).

The vertebrate family of TIMPs represents a class of endogenous inhibitor of MMPs and is composed of four members (TIMP-1, -2, -3, -4) (Gomez et al, 1997) of 21-29 kDa, structurally divided in two domains:

1. the N-terminal domain, consisting of 125 amino acids and responsible of MMP inhibition;
2. the C-terminal domain, consisting of 65 amino acids, that drives the link between MMPs and TIMPs (Lu et al, 2011).

TIMPs act in a specific manner and use the C-terminus to bind MMPs at a stechiometric ratio 1:1 (Visse and Nagase, 2003; Lu et al, 2011).

A lack of the above described regulatory dynamics, is responsible of MMP alterations, hyperexpression and hyperactivity, leading to pathological conditions that occur in many human diseases; among these, inflammation, malignant disorders and vascular diseases have been associated with an increased MMP activity.

Biomechanical properties of human arterial wall

The arterial tree is continuously exposed to a wide range of hemodynamic forces of various magnitude, frequency and direction. The fluid mechanic laws cannot be simply applied to explain these mechanics because of the pulsatile nature of blood and the complexity of the arterial system, being composed of various size vessels, branches and bifurcations (Resnick et al, 2003). The biomechanical forces that act on arterial wall are essentially two: the stretch and the shear stress. The stretch is a tensile stress, determined by the blood pressure and consists of a mechanical strain that creates radial and tangential forces, aimed to balance the intraluminal pressure.

The shear stress is determined by the friction of the blood on the vessel surface and has a parallel direction to the vessel wall. Although both the two forces induce biomechanical changes that involve the whole arterial wall, shear stress mainly affects the ECs. Conversely, the mechanical stretch interests both SMCs and ECs. Vascular cells respond to biomechanical alterations through different mechanism, involving morphogical as well as biomolecular aspects, mediated by cytoskeleton rearrangement. 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).

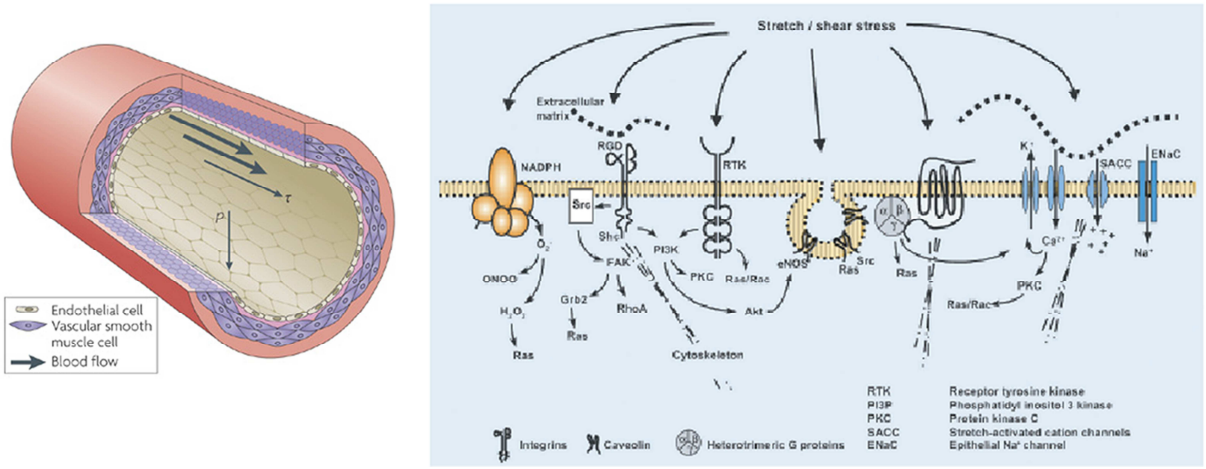


Figure 5. Biomechanical forces acting on vascular wall (Hahn and Schwartz, 2009) **and molecular signal transduction** (Lehoux et al, 2006).

As described above, the endothelium owns several biological functions, first of all it works as a selectively permeable barrier that regulate the macromolecules entrance from the blood into the vessel wall, such as low-density lipoprotein (LDL). In this view, endothelium has a protective function, together with an anti-thrombogenic property; moreover, it orchestrates the inflammatory process and drives the SMC contraction through the release of vasocostrictors and vasodilatators. Any impairment in these activities is crucial to the development of pathological processes, especially related to pro-atherogenic and pro-thrombotic events.

Each shear stress variation is sensed by ECs, that undergo several mechanism 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. ECs with an elongated shape and aligned with blood flow reflect a laminar and unidirectional flow; closely to side branches, bifurcations and curvatures, such as the aortic arch, the flow becomes turbulent and disrupted, 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. Moreover, several investigations pointed the genic expression following blood flow alterations and revealed the presence of *cis*-elements in the promoter of

different genes in ECs able to respond to shear stress (Li et al, 2005); these sequences were defined as shear stress responsive elements (SSRE) and they were firstly observed in the promoter of the gene PDGF-B (sequence: GAGACC, or GGTCTC) (Resnick et al, 1993). The introduction of the 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, pro-atherogenic and pro-inflammatory genes were stimulated in response to a disturbed 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, 1991), 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. Moreover, these steady conditions stimulate SMC apoptosis. All these data are concurrent with the belief that SMC under physiological stretch are differentiated and contractile. 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), undergo hypertrophy, increased proliferation and enhanced synthesis of collagen and elastin (Chapman et al, 2000; Lehoux et al, 2006).

ABDOMINAL AORTIC ANEURYSM (AAA)

Definition, epidemiology and management of AAAs

Definition and epidemiology

The term "aneurysm" is used to define a permanent focal dilatation of the vessel wall. Mc Gregor et al defined the abdominal aortic aneurysm (AAA) as an aortic segment with a diameter larger than 3 cm (McGregor et al, 1975); under this term, aneurysms of the infrarenal, intrarenal and suprarenal aorta are included (Sakalihasan and Limet, 2005). True 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. Dissecting aneurysms (or aortic dissections) are distinct from true aneurysms and are characterized by a longitudinal splitting of the aorta that separates the intima tunica from the adventitia.

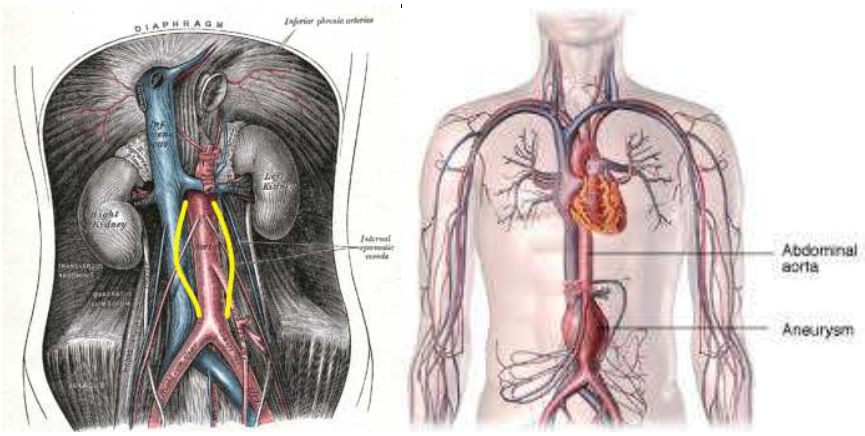


Figure 6. Localization of abdominal aortic aneurysm (AAA). Yellow lines indicate the infrarenal location of AAA.

AAA incidence is recently increased, as a consequence of the ageing of the population, the increasing number of smokers and also the improved screening and diagnostic tools (Aggarwal et al, 2011). Major risk factors for the occurrence of AAA are male gender, age, smoking, pre-existent cardiovascular affections, such as hypertension and atherosclerosis. Most aneurysms are small in size and they do not need to be repaired by surgery, but the

increase in the aortic diameter can lead to rupture, which can be fatal. The mortality rate associated with AAA rupture is about 65-85%.

Atherosclerotic abdominal aortic aneurysm

Except in specific cases of aneurysms associated with inflammatory diseases, connective tissue affections or traumatic event, the most common form of this dilatative affection rises consequently to atherosclerotic plaque disease, although many researches suggest that aneurysm formation is due to a combination of genetic, environmental and immune factors (Sakalihasan and Limet, 2005).

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 validated theory, the "atherosclerotic aneurysm" develops as a complication to the pathological processes deriving from the atherosclerotic plaque development: matrix remodelling, 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 (Tonar et al, 2010). In any case, according to the 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.

Inflammatory abdominal aortic aneurysm (IAAA): definition, incidence and pathogenesis

The concept of inflammatory aortic aneurysm (IAAA) was firstly defined by Walker et al in 1972, observing unusual clinical features on a group of 19 patients. The presence of very thick aortic wall, surrounded by a dense extended fibrosis of white aspect, involving the adjacent organs were recognized as common features of a distinct form of AAA (Walker et al, 1972).

At present, based on the original description by Walker, IAAAs are characterized by three constant traits:

- Thickened aortic wall;
- Extended peri-aneurysmal and retroperineal fibrosis;
- Dense adhesion of adjacent abdominal organs to the aortic wall (Pennel et al, 1985; Crawford et al, 1985).

IAAAs represent the 3-10% of all aneurysm cases, with an higher prevalence rate among males between 62 and 68 years; smoke and family history are additional risk factors (Tang et al, 2005). A case control study by Nitecki et demonstrated a more elevated genetic predisposition in IAAAs (17%) versus non non-inflammatory aneurysms (1.7%) (Nitecki et al, 1996). The same work described the three main symptoms affecting IAAA patients: back pain, weight loss and high erythrocyte sedimentation rate (ESR) (Nitecki et al, 1996).
Many histological evidences report the presence of a chronic inflammatory infiltrate, composed of macrophages, T-lymphocytes and B-lymphocytes, in all AAA, mainly interesting the adventitia layer (Pennel et al, 1985; Pasquinelli et al, 1993). A marked and more extended infiltrate distinguishes IAAA from non-inflammatory aneurysms and some clinical studies concord to consider IAAA as a progressive development from atherosclerotic AAA (Pennel et al, 1985; Sterpetti et al, 1989; Latifi et al, 1992). Several mechanism are responsible for the occurrence of inflammation, among which the immune response to an unknown antigen is the most validated theory. Infection by a viral antigen can also be involved; indeed, a more prominent presence of herpes simplex virus and cytomegalovirus was observed in inflammatory aneurysm wall, compared to ordinary non-inflammatory aneurysms.

Symptoms, diagnosis and management

Differently from IAAAs, non-inflammatory aneurysms are generally asymptomatic; only in 8-18% of AAA cases, patients have reported symptoms. For this reason, unruptured aneurysms are often recognized following clinical investigation in presence of other cardiovascular diseases, or during screening test. Conversely, rupture of aneurysm is accompanied by abdominal pain, shock and a pulsatile abdominal mass. Aneurysm evolution and possible complications are generally evaluated by diameter measure, which represent the major index of aortic rupture. 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 secure to decide drug therapy or surgical interventation. Matrix metalloproteinase 9 (MMP-9) may be represent one of the promising

candidates, since it results higher in patients with AAA and its serum levels correlate with aneurysm size and expansion (Lindholt et al, 2000).

About 30% of asymptomatic aneurysms are recognized by physical palpation as a pulsatile abdominal mass, but this type of inspection conveys of the variability inter-operator.

Preferential diagnostic tool to identify aneurysm is represented by ultrasonography (UT), also applied to follow-up, surveillance of asymptomatic aneurysms and screening. UT has an high sensitivity and sensibility, without high costs. When the management of an aneurysm is such to require surgical procedure, computational tomography (CT) is performed and it can be helpful to define what type of surgical treatment is the best. CT gives more detailed information about the aortic diameter, the shape of the aneurysm, the thickness of intraluminal thrombus, together with the presence of blood within it. In presence of an IAAA, CT also indicates the extent of inflammation. Magnetic resonance Imaging (MRI) is an alternative and more accurate diagnostic procedure, but it is expensive.

The treatment selection depends on the aortic size and diameter (Sakalihasan and Limet, 2005):

- aortic diameter less than 5 cm: follow-up through UT;
- aortic diameter between 5 and 5 cm: follow-up or surgery (the latter in case of female patient, familial history, high serum markers)
- aortic diameter larger than 5.5 cm: surgery.

Surgical treatment of AAA is performed through open or endovascular repair. Open repair has been performed since 1950s and consists of a large abdominal incision, followed by the removal of the affected aortic segment and the insertion of a fabric graft (DuBost, 1952).

A less invasive option is given by the endovascular stenting, first described by Parodi in 1991 (Parodi et al, 1991) and consisting 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, that excludes the damaged aortic segment from the normal blood flow, and prevents aneurysm progression and rupture. This reparative alternative does not require the surgical incision of the abdomen, thus avoiding complications consequent to open surgery; in addition, it allows a shorter recovery time, reduced hospital stay and implies a lesser blood loss (Paravastu et al, 2014). In some cases, the blood can flow into the aneurysm sac, external to the graft, and this condition can lead

to aneurysm expansion and rupture. This condition is defined by the term “endoleak” and it is the major endovascular repair failure. This condition can be detected by angiography, CT scan or duplex ultrasound imaging (White G. H. et al, 1997). Endoleaks can be classified according to the timing of development: primary endoleak (early endoleak) include all cases that occur during the 30 day perioperative period; secondary endoleak (late endoleak) represent a late complication of the correct seal (White G. H. et al, 1997). Endoleaks are further distinguished into: *type I endoleak*, when the seal between the ends is not complete or ineffective, interesting the distal and proximal attachment sites; *type II endoleak*, in presence of blood flow into the aneurysm sac from collateral arterial branches, such as patent lumbar or inferior mesenteric artery, not depending on the graft seal; *type III endoleak*, derived from a mechanical failure or a structural defect of the graft; *type IV endoleak*, consequent to a graft wall porosity (White G. H. et al, 1998; Baum R.A. et al, 2003).

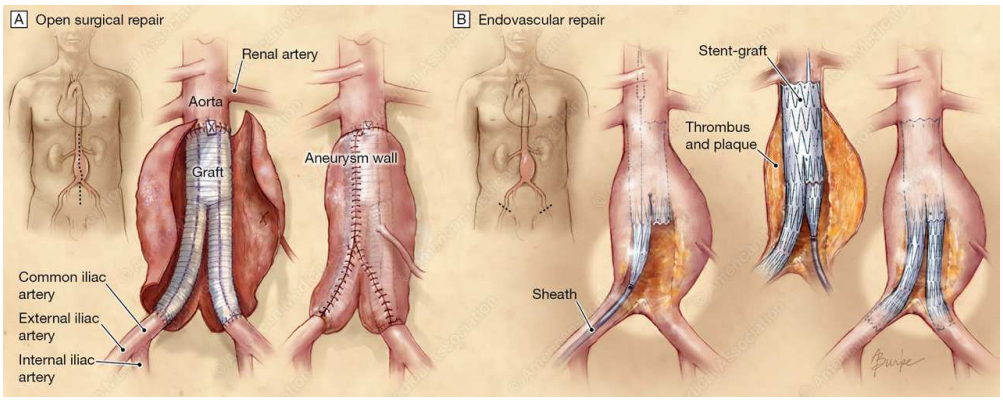


Figure 7. Open surgical repair versus endovascular repair (American Medical Association, 2009).

Histological and molecular features of AAAs

The human abdominal aorta is well organized into three concentric layers, or tunica, displaced in the following order, starting from the lumen: intima, media and adventitia. The histological composition, the cell types and the surrounding extracellular matrix have been described in the previous chapter. The main fibrous component of the aortic wall are represented by elastin and collagen, contributing to the aorta viscoelasticity and tensile strength respectively, and assuring an equal distribution of the tensile stress along the vessel. In this way, the healthy aorta is able to counteract the pulsatile pressure of the blood flow. The typical laminar architecture of the aortic wall is lost in the AAA wall: analyzing the AAA structure from the inner side, its characteristic hallmarks are the presence of the intraluminal thrombus (ILT), tunica media degradation, marked by a decrease elastin content and SMCs apoptosis, and adventitial inflammatory infiltrate, together with a thickening of the intima. The presence of intimal lesions have supported the hypothesis of aneurysm development from the atherosclerotic disease, as suggested by many laboratory and clinical data. Currently, the link between aneurysm formation and atherosclerosis is not completely defined.

Medial degradation: EMC remodelling and SMC apoptosis

The destruction of the lamellar architecture of the aortic media is the predominant histopathological feature of aneurysm disease. As described in the previous chapter, ECM represents the dynamic structure that supports the aortic wall, driving its main functions. ECM remodelling is strictly regulated under physiological conditions and abnormal expression and activity of the main ECM remodelling mediators are characteristic of many vascular disorders. The elastin fragmentation represents an early event in AAA formation, being cause of wall weakening; the degradation of the fibrillar collagen type I and III, occurs in a later phase of the AAA expansion, leading to the loss of aortic tensile strength and being a decisive step in the aortic rupture.

Many experimental data, obtained from tissue studies of human aneurysm specimens, as well as from *in vitro* and *in vivo* animal models, have highlighted the MMP involvement in ECM degradation, evidenced by the increased expression of these proteolytic enzymes during aneurysm initiation and/or progression, together with a correlation with aneurysm size. MMPs are secreted by medial SMCs, adventitial fibroblast and inflammatory cells;

MMP hyper-expression, in terms of mRNA, protein as well as enzymatic activity, is not only associated with an increased production and secretion, but is also the result of the unbalanced MMPs/TIMPs ratio. MMP-2 and MMP-9 are the MMP members most commonly associated with aneurysm disease and degrade both 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 to 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 aneurysm. In addition to MMPs, other proteases localize at aneurysm wall: u-PA (urokinase-Plasminogen 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 remodelling mediators, like MMPs and their tissue inhibitors TIMPs. The importance of SMC behaviour in the aneurysm development was explored by Lopez-Candavalez 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).

The elastin degradation is tightly related to the abnormal hemodynamic shear stress that influences aneurysm growth. The SMCs respond to the 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.

Aortic inflammation and neo-angiogenesis

In addition to the loss of structural integrity, the aortic aneurysm is characterized by an intensive transmural inflammation. The presence of the inflammatory infiltrate may be due to the elastin fragmentation: this process releases soluble peptides exerting a chemotactic effect that recruits inflammatory cells, which further secrete proteolytic enzymes, thus amplifying the ECM degradation (Satta et al, 1998). 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 formation of neovessels; in addition, CD3+ stimulate the IFN-γ release, which in turn induces MMP expression.

The presence of cells presenting antigen in aneurysm wall takes to suggest 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 the 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 remodelling and inflammation: immunohistochemical and *in-situ* hybridization data revealed an intensive MMP-9 expression by aneurysm-infiltrating macrophages (Thompson 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 production and neoangiogenesis.

Neoangiogenesis 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 the AAA wall has been demonstrated by Thompson 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. Not only, increased neovascularisation and expression of angiogenic cytokines were detected at the site of rupture, indicating an involvement of the angiogenic response into aneurysm rupture (Choke et al, 2006).

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 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 (Pfeffer, 2003). As a pro-inflammatory cytokine, TNF-α is involved in the pathogenesis of several inflammatory affections, such as Chron's disease (Danese et al, 2006) and rheumatoid arthritis (Matsuki et al, 2005). 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 observation suggested a role for TNF-α to early aneurysm pathogenesis; this association was further confirmed by studies on animal models, showing that the inhibition of TNF-α protein was able to prevent aortic dilatation (Hingorani et al, 1998).

Macrophages an 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 (Saren et al, 1996 Cohen et al, 2006).

Biomechanical wall stress

Distinctive structural and hemodynamic features make the infrarenal aortic segment more prone to the aneurysm development. As described in the previous chapter, the elastic media of infrarenal aorta is not reached by adventitial vasa vasorum and, compared to thoracic aorta, it receives a lesser nourishment. In addition, abdominal aortic media has a decreased elastin content, corresponding to a shorter number of lamellar units. These

anatomical parameters imply a major thickness and an increased wall tension per lamellar unit. Many studies of the blood flow dynamics, revealed the presence of an oscillatory and disturbed flow, together with an increased wall tension in the infrarenal aorta. These factors contributes to aneurysm formation, as well as to accelerate its expansion and rupture.

Intraluminal thrombus (ILT)

The ILT is considered as a neo-tissue, of various thickness, exerting a decisive role on aneurysm fate and closely related to its rupture. The ILT structure is generally organized into three layers (Michel et al, 2011):

- the luminal layer is in contact with the blood flow and has a red aspect, due to the high content of erythrocytes; moreover, it is the site of platelet aggregation, leucocytes and fibrin network;
- the middle layer has a white-yellow aspect and it is generally devoid of intact erythrocytes, rarely it contains leukocyte infiltration;
- the abluminal layer is brown, lacking of alive cells and is adjacent to the aneurysm wall; it is characterized by degraded fibrin and a weak gelatinous substance (Wilson et al, 2013).

The origin of the ILT is mainly ascribed to the abnormal hemodynamic characteristic of aneurysm disease; compared to the normal aorta, the blood flow along aneurysm lesion is disturbed and turbulent, creating vortices and recirculation zones with high shear stress that promotes platelet activation. Along the most dilated areas of the aneurysm, the shear stress is lower than normal aorta, facilitating the platelet adhesion to the endothelium (Biasetti et al, 2010; Folkesson et al, 2011).

Many studies addressing the composition and molecular behaviour of aneurysms containing or not ILT, highlighted different aspects between them, suggesting the ILT participation to the aneurysm development and rupture. At first, the presence of ILT resulted associated with a thinner aortic wall, a decreased elastin content as well as an enhanced fragmentation, accompanied by a prominent inflammation and increased depletion of SMCs, showing a synthetic phenotype (Kazi et al, 2003). In addition, the presence of ILT reduces the oxygen delivery to the aneurysm wall and generates an hypoxic environment, which in turn exacerbates inflammation and neo-vascularisation, increasing the risk of wall rupture (Vorp et al, 2001; Kazi et al, 2003). The mural thrombus

constitutes a source of circulating inflammatory cells that invade the aneurysm wall and enhance the ECM remodelling through the production and release of proteases, like MMP-9 and urokinase-plasminogen activator (u-PA) (Fontaine et al, 2002).

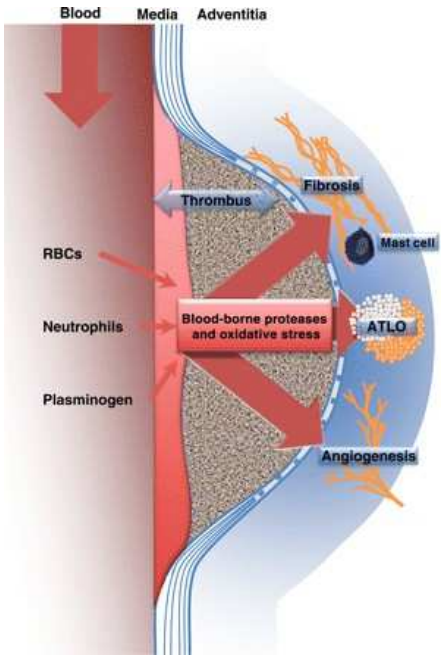


Figure 8. Schematic representation of ILT contribution to inflammation and proteolytic process in aneurysm wall (Michel et al, 2010).

Oxidative stress

The existence of oxygen and nitrogen reactive species (ROS, RNS) in the aneurysm wall has been widely demonstrated. The oxidative stress include a series of reactions 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 evidence of ROS and RNS implication in AAA pathogenesis derive from works of Dubick et al, that demonstrated a reduced activity of superoxide dismutase in aneurysm tissues, versus normal aorta. 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 segment; moreover, the O_2^-

increase was associated with inflammation, SMC depletion and elastin degradation (Miller et al, 2002).

The cells of the inflammatory infiltrate constitute the main source of oxidative stress, especially macrophages generating large amounts of superoxide (O_2^-) and hydrogen peroxide (H_2O_2), thus further contributing to the inflammation and the progression of the disease. Another source of reactive oxygen species is represented by the vascular cells, including ECs, SMCs and adventitial fibroblasts. In particular, the NADPH oxidase seems to play a key role in generating ROS and its activity may be influenced by several and different patwhays, including cytokines released by inflammatory cells, lipid mediators, such as leucotryenes, oxidized LDL and growth factors. In addition, the mechanical stretch also exert a decisive role, since it can stimulate the 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 remodelling and dilatation, including osteopontin upregulation by ECs; MMP activation; SMC apoptosis.

The molecular mechanisms participating to AAA pathogenesis are summarized in Figure 9.

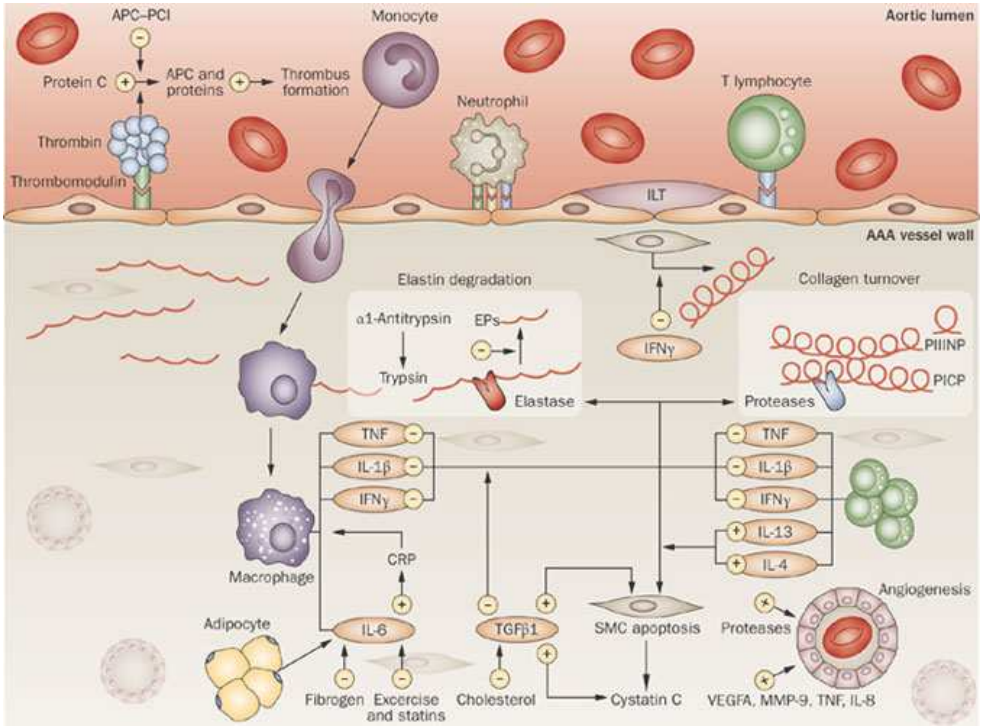


Figure 8. Molecular pathways leading to aneurysm formation and progression. (Kotze and Ahmed 2011).

STEM CELLS

Stem cells: definition, properties and classification

Stem cells are defined as undifferentiated cells capable to indefinitely *self-replicate*, giving origin to an identical daughter cell at the same undifferentiated status. Under the appropriate condition and stimulation, stem cells can also give rise to differentiated mature cells, of all the organism types (*multilineage differentiation*). The property of a stem cell to give origin to two daughter cells with different fates, goes under the termn of *asymmetric division*.

Stem cells can be distinguished according to their differentiation potential, which is functional to the development stage of individual life, and classified as totipotent, pluripotent, multipotent and unipotent (Wagers and Weissman, 2004).

Totipotent stem cells have the ability to form cells of the whole organism and represent the top of the stem cell hierarchy. The fertilized egg is a totipotent stem cell, leading to the formation of germ layers (endoderm, mesoderm and ectoderm) and throphoblast, necessary to support and protect the embryo. *Pluripotent* stem cells reside in the inner mass of blastocyst and retain the capacity to differentiate into the cells of the three germ layers (endoderm, ectoderm, mesoderm), but not the extra-embryonic components. Embryonic stem cells (ESCs) and embryonic germ cells (EGCs) belong to this category, whereas stem cells residing in adult tissues and able to differentiate into a more restricted subset of cell lineages are defined as *multipotent.*

Stem cells belonging to adult tissues and able to differentiate only into a specific mature cell type are called *unipotent*.

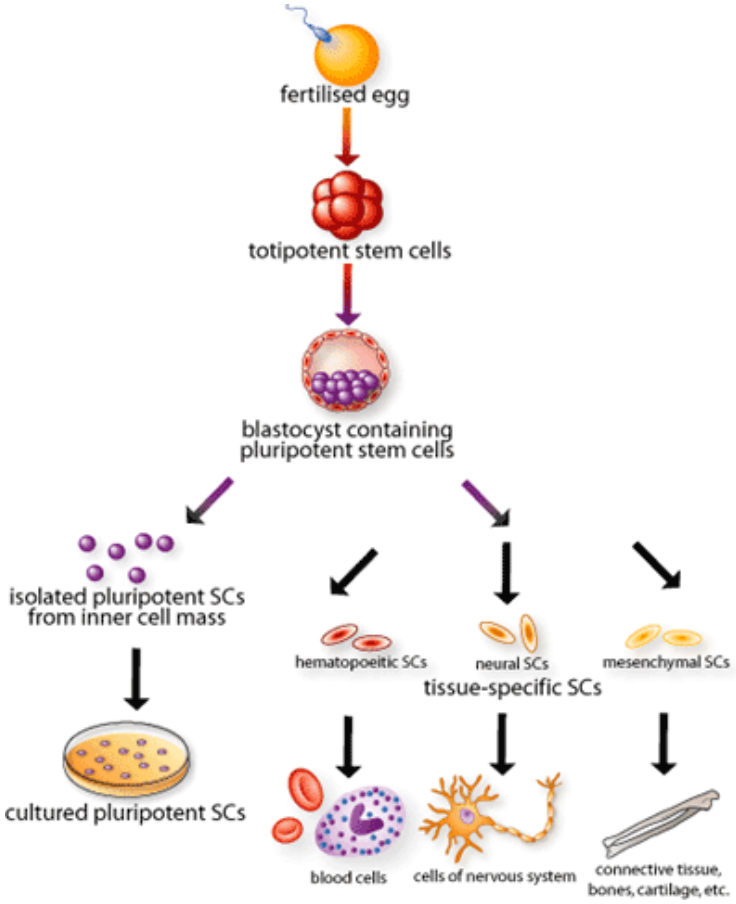


Figure 10. The stem cell hierarchy.

A major distinction may be done between embryonic and adult stem cells, according to the tissue source and the developmental stage: embryonic stem cells are pluripotent and derive from embryonic tissues, whereas the adult stem cells have a restricted differentiation potential and are present in many adult tissues including skin, bone, fat, cartilage, intestine, liver.

Figure 11 illustrates the main differences between embryonic and somatic stem cells.

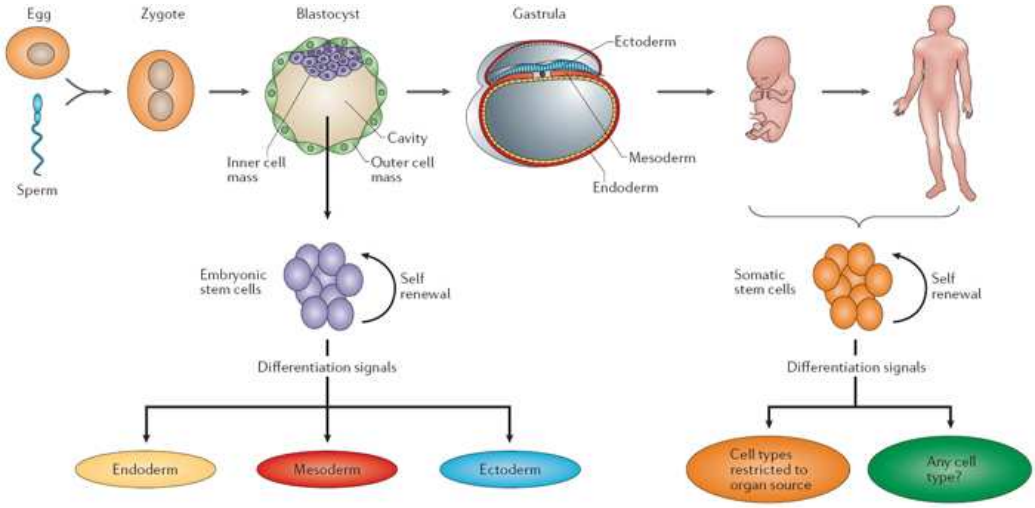


Figure 11. Major differences between Embryonic Stem Cells (ESCs) and Adult Stem Cells (ASCs) (O'Connor, 2008)**.**

Embryonic stem cells (ESCs)

Blastocyst is an early stage of embryo life: it consists of 150-200 cells and is composed of the Inner Cell Mass (ICM), source of Embryonic Stem Cells (ESCs), and the external trophoblast, that forms the placenta. Differently from totipotent fertilized egg, ESCs differentiate into the three embryo lines, but do not form extra-embryonal tissues. ESCs have been characterized as immortal stem cells, capable to indefinitely replicate themselves. Mouse ESCs were detected for the first time in 1981 (Evans and Kaufman, 1981; Martin et al, 1981) and, many years later, Thomson and his team of investigators were able to isolate ESCs from non-human primate and human blastocyst (Thomson et al, 1995; Thomson et al, 1998).

As described by Thomson et al, human embryos, donated after *in-vitro* fertilization, were cultured to the blastocyst stage and 14 inner mass cells were isolated. Five embryos were obtained and ES cell lines were isolated from each of them. Morphological characteristics were comparable to rhesus monkey ESCs previously isolated (Thomson et al, 1995): human ESCs displayed high nucleus to cytoplasm ratio, prominent nucleoli and kept a normal karyotype. ESCs also expressed high levels of telomerase activity, index of cell line immortality. Indeed, telmorase is a ribonucleoprotein which protect chromosome ending with repeated telomeric sequence, thus preventing DNA rupture for each replicative cycle and cell senescence. ESCs were established in *in vitro* cultures, showing prolonged growing rates. ESCs showed the ability to self-renew and, under the appropriate culture

condition, to aggregate into typical embryoid bodies and differentiate into many cell types, like nerve cells, muscle cells and pancreatic isle cells.

Surface markers identificative of ESCs include: Stage Specific Associated Antigen (SSEA-4 and SSEA-3); keratane sulphate antigens TRA-1-60 and TRA-1-8; alkaline phosphatises (Thomson et al, 1998; Chambers et al, 2003).

ESC molecular profile was also investigated and revealed the presence of a transcriptional core, composed of Nanog, Oct-4 and Sox-2, regulating the expression of downstream genes. Genome scale location studies demonstrated that these three transcription factors are physically associated and occupy the promoter region of many genes regulating the developmental pathway (Boyer et al, 2005). This regulatory core may activate genes involved in pluripotency and self-renewal, and repress genes promoting differentiation and development of extra-embryonic, endoderm, mesoderm and ectoderm lineages.

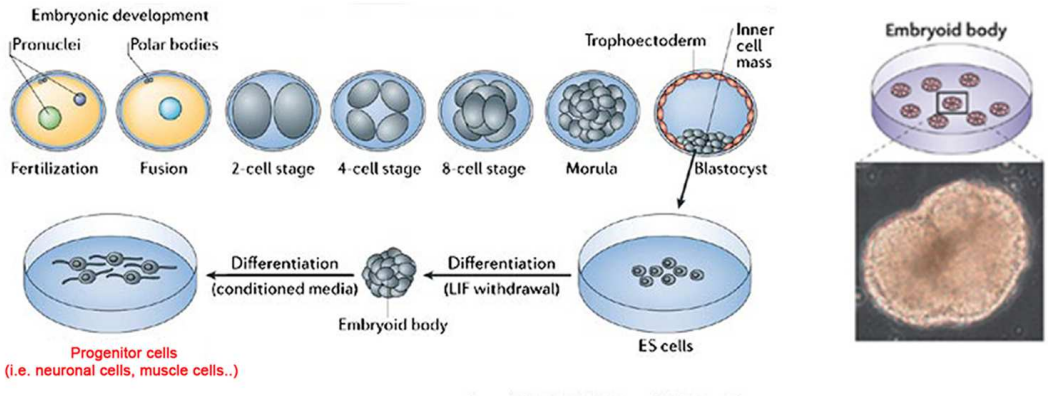


Figure 12. ESC development and differentiation; embryoid bodies; transcriptional profile of ESCs (adapted by Meshorer and Misteli, 2006; Nikishawa et al, 2007)**.**

The high proliferative potential and differentiation ability make ESCs an important resource for many research aims, from the transplantation medicine to toxicological studies. However, major factors, including ethical questions, limit the use of ESCs for clinical applications. Indeed, ESCs have been shown to induce, after transplatantion, the formation of teratoma, which is a tumor originating from all the germ line cells. In addition ESCs have displayed high immunogenicity, which could activate immune rejection in heterologous transplant (Reubinoff et al, 2000).

Adult stem cells (or Somatic stem cells)

The ethical debate and the disease risk associated with the clinical use of ESCs, have addressed the regenerative medicine toward the study and the application of Adult Stem Cells (ASCs).

ASCs, or Somatic Stem Cells, are undifferentiated cells, owning the self-renewal and differentiation properties, like other stem cells. Unlike ESCs, ASCs are tissue-specific and reside in post-natal differentiated tissues, but their origin is not completely identified and this constitutes research field under current investigation. ASCs have been detected in many post-natal organs and tissues, including bone-marrow, brain, peripheral blood, vascular wall, muscle, skin and liver. ASCs reside inside a stem cell *niche*, which provides support and drives stem cell fate and behaviour through many pathways and signalling molecules. ASCs can differentiate into the specific mature cell types of the organ or tissue they belong to; this property ensures the normal tissue homeostasis, as well as the tissue repair when injured. Research on ASCs started in 1960 and, currently, the best characterized ASC populations are represented by Hemopoietic Stem Cells (HSCs) (Spangrude et al, 1988) and the Mesenchymal Stem Cells (MSCs) (Friedenstein, 1970), both isolated from bone marrow.

Recently, many investigative studies have challenged the adult stem cell biology dogma, introducing the concept of ASC *plasticity*. Indeed, different experimental findings suggest that multipotent ASCs can overtake the lineage specific barrier and give origin to cell types distinct from the origin tissue (Goodell, 2005). In this context, HSCs were shown to differentiate into nonhematopoietic cells, such as liver cells; moreover, cultured neurosphere cells (CNS-SC) were shown to contribute to hematopoiesis, when injected into lethally irradiated mice. Also muscle mononuclear cells demonstrated hematopoietic activity (Lakshmipathy and Verfaillie, 2005).

The possible explanations of ASC plasticity that have been proposed can be summarized in the following mechanisms (Kapp and Mertelsmann, 2001):

- Direct or indirect transdifferentiation: a committed cell can differentiate into a cell type specific of a distinct tissue through the direct activation of an alternative differentiation lineage, or through a *dedifferentiation* program, which consists in

the specialized stem cell return to a more primitive phenotype and consequent change in lineage commitment.

- Coexistance of multiple stem cells within an adult organ or tissue, each of one owns a different lineage conversion.
- Presence of a pluripotent stem cell in bone marrow and other tissues, able to differentiate into all organism cell types; this theory has been supported by the isolation of Multipotent Adult Progenitor Cells (MAPCs) from mouse and human bone marrow, able to generate mesoderm, neuroectoderm and endoderm cells in vitro; these cells, which have also been proved to give rise to most of somatic tissues after implanation into early blasocyst, were also discovered in brain and muscle tissues (Jang et al, 2002a, 2002b).
- Cell-cell fusion, leading to an heterokaryon formation, whose differentiation pattern is the result of the two fused cells. This process has been observed at a very low frequency and it has been mainly associated with a reparative function on tissue injury.

Data supporting the ASC plasticity are still controversial, since they lack of standardized detection method and are of difficult reproducibility between different experimental models as well as different laboratories. The limits in using ASCs as substitutes of ESCs for transplantation medicine are due to the restricted multilineage poltential and the lesser content of ASCs in adult tissues; in addition, ESCs are more easily to be expanded in vitro, with an higher proliferative potential, which is essential to guarantee a cell number sufficient for transplantation uses. Thus, optimizing and standardizing the detection methods and the experimental models, together with the understanding of cellular and molecular pathways driving the ASC plasticity, are necessary to extend the knowledge on ASCs and improve their clinical applications.

Stem cell niche

The hypothesis on the existence of the stem cell niche was introduced by Schofield in 1978 (Schofield, 1971) who proposed that stem cells reside in a defined structure designated to control the stem cell behaviour. The niche is an anatomical structure which regulates many stem cell functions, including proliferation and differentiation, and prevent cell death. These activities are elaborated trhough surface molecules and signalling transduction pathways. Distinct and specialized stem cell niches have been detected in many tissues

and an unbalance in the niche regulation or structure may be responsible of tissue damage with the occurrence of different diseases (Jones and Wagers, 2008).

Beside the variability existing between niche belonging to different tissues, the niche generally comprises the following components:

- Stromal support cells, appointed to communicate with stem cells trough the expression of specific surface molecules, gap junctions and the release of soluble factors.
- ECM, which exerts a structural support and a regulatory role of the signalling mechanisms inside the niche.
- Vascular network and neural inputs.

The hypothetical scheme of how is organized the stem cell niche is illustrated in Figure 13.

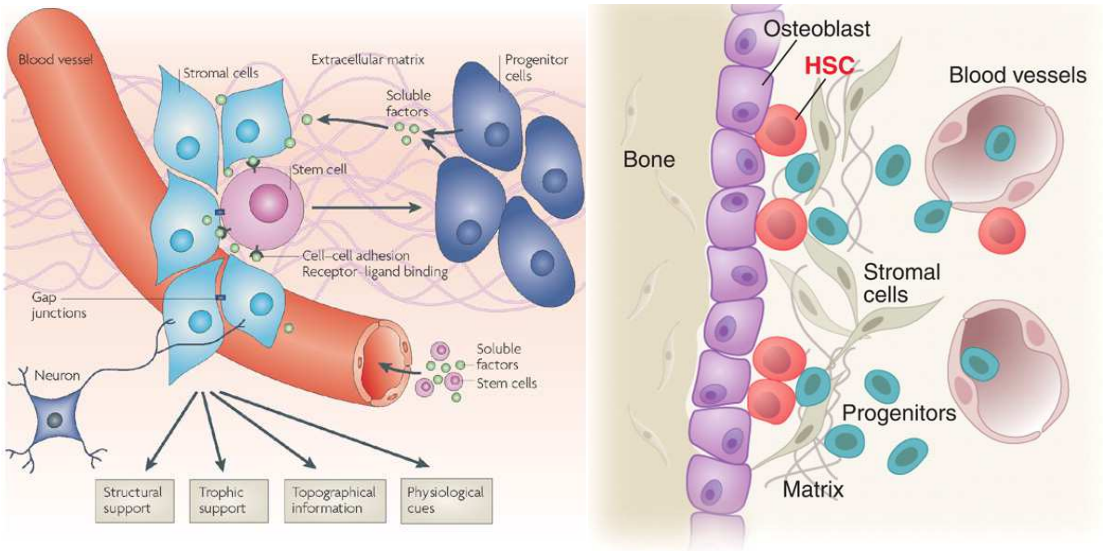


Figure 13. Scheme of the hypothetical structure of the stem cell niche; example of a stem cell niche: HSC niche (Jones and Wagers, 2008)**.**

The stem cell niche has been detected and characterized at the level of the intestinal cript, hair follicle, in the subventricular zone of the hippocampus and olfactory bulb (Jones and Wagers, 2008), and recently also at the vascular level (Zengin et al, 2006; Ergun et al, 2011). Indeed, the research of the last few years has reversed the idea of the vessel wall as passive and quiescent layer, introducing the new concept about a functional participation to tissue homeostasis and vascular processes, like post-natal angiogenesis. The vasculogenic niche has been identified at the adventitia level, along its border with the media and closely

to the vasa-vasorum network, which ensures the niche contact with the peripheral circulation. The vascular stem cell niche includes distinct cell subsets: MSCs, myofibroblasts, endothelial, hematopoietic and smooth muscle progenitors (Psaltis et al, 2011). Vascular progenitors participate to cell turnover and tissue homeostasis during physiological conditions and, on the other hand, may regulate or exacerbate pathological remodelling and neovascularisation.

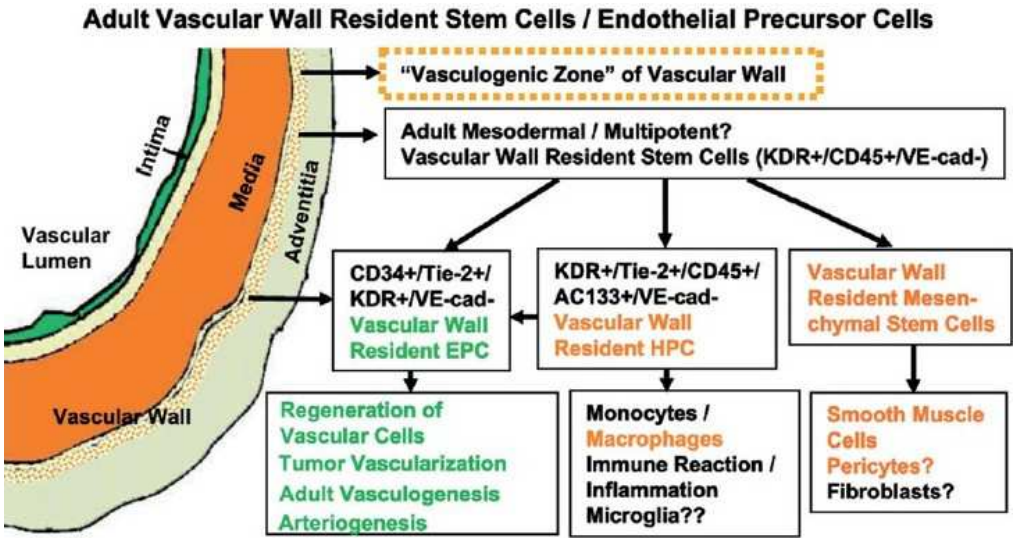


Figure 14. Hypothetical structure of the vasculogenic niche (Zengin et al, 2006).

Mesenchymal Stem Cells (MSCs)

Definition, in vitro phenotype and differentiation properties

Bone marrow has been considered as a source of stem cells belonging to the hematopoietic lineage for many years and the existence of non-hematopoietic stem cells, as stromal components of the HSC niche, was detected in 1970. Friedenstein and his co-workers, after plating whole bone marrow on plastic dishes, using growing media supplemented with 10% Fetal Calf Serum (FCS), observed the presence of plastic adherent cells able to proliferate and, after passaging in culture, assume a fibroblast-like morphology. Interestingly, these adherent cells were shown to form colonies of fibroblastoid cells, and were identified as Colony Forming Unit-fibroblasts (CFU-f); these CFU-f were also able to form bone and cartilage (Friedenstein et al, 1970; Friedenstein et al, 1976). The following workers by Castro-Malaspina (Castro-Malaspina et al, 1980) and Caplan (Caplan, 1991), confirmed the CFU-f ability to differentiate into the mesoderm lineage, giving origin to adipocytes, osteocytes, chondrocytes and myoblasts. Caplan in 1991 proposed the nomenclature Mesenchymal Stem Cells (MSCs), due to their differentiation ability into mesenchymal-type cells, and *stromal*, since they were considered as support cells to bone marrow HSC niche.

MSCs have been isolated from different species, including human and mice, as well as different tissues beside bone-marrow, including peripheral blood, umbilical cord blood (Erices et al, 2000), Wharton's jelly membrane (McElreavey et al, 1991), adipose tissue (Zuk et al, 2002), synovial membrane (De Bari, 2001), synovial fluid (Karystinou et al, 2009). The high proliferative rate and the multilineage potential that characterize MSCs make them promising candidates for clinical applications, such as cell-based therapies and organ transplantation. Meanwhile, MSCs constitute a cell population with high degree of heterogeneity, due to several aspects. First of all, the characteristics of the source tissue, including tissue type and age; another important element derives from the coexistence, in the same MSC population, of different subsets of cells at different development stages. Moreover, MSCs are heterogeneous in terms of proliferative and differentiation potential, thus representing an high complexity population; indeed, Pittenger et al showed that only one third of the whole bone marrow population that they plated, demonstrated the ability to

differentiate into the mesodermal lineages (Pittenger et al, 1999). As consequence, there is not a specific marker, able to unequivocally individuate MSCs.

Therefore, an important result in the field of the MSC research would be the standardization of isolation method and the identification of a specific signature for MSCs.

According to the International Society for Cell Therapy (ISCT), the minimal criteria for defining human MSCs are as follows (Dominici et al, 2006):

- Adherence to the tissue culture substrate;
- Specific surface antigen (Ag) expression;
- Multilineage differentiation potential.

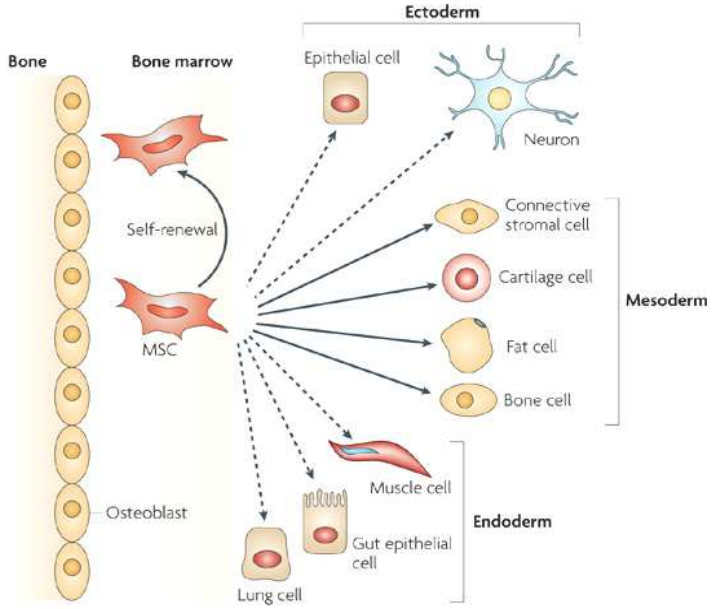


Figure 15. MSC properties: self-renewal and differentiation along the mesodermal lineage; potential plasticity of MSCs (Uccelli et al, 2008)**.**

MSC surface antigens

According to the ISCT, the expression of CD90, CD73 and CD105 Ag on cell surface, performed by flow cytometry, define the MSC population:

- **CD105**, or Endoglin, is a type I homodimeric transmembrane glycoprotein, belonging to the TGF-β receptor family and links with high affinity TGF-β1 and 3; it has been shown to be expressed on connective stromal cells and also on endothelial cells and monocytes.

- **CD90**, or Thy-1, a 25-37 kDa heavily glycosilated glycophosphatididynil (GPI)-anchored cell surface protein. It was identificated for the first time on thymocytes, which are the T cells precursors in the thymus. It is expressed on MSCs, HSCs, neurons, endothelium, myofibroblasts and its functions have not been clarified yet. It may regulate cell-matrix interactions and regulate apoptosis, fibrosis, inflammation and neuron regeneration processes.
- **CD73**, also known as ecto-5'-nucleotidase, a glycoprotein implied in AMP conversion to adenosine; it is involved in B lymphocytes differentiation and it is expressed on lymphocytes and monocytes.
- **CD44**, a cell surface glycoprotein, involved in intercellular interactions, cell adhesion and migration. It acts as receptor for hyaluronic acid, but also recognizes osteopontin, collagen and MMPs.

In addition, the expression of hematopoietic markers needs to be excluded: for this reason, antigens CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA class II may be included in the antibody panel to detect human MSCs.

- **CD34**, a glycoprotein expressed on many cell types, like HSCs, blood vessel ECs, endothelial progenitor cells (EPCs), mast cells; it acts as a cell-cell adhesion factor and is required for T cells entry into lymph nodes.
- **CD45**, also known as protein tyrosine phosphatases receptor type C. It is a transmembrane protein, exerting a regulatory role for many cell processes, including growth, cell cycle, differentiation and oncogenesis. It is expressed on differentiated hematopoietic cells and also in pathological conditions, such as lymphoma and B-cells chronic lymphocytic leukemia.
- **CD14**, mainly expressed by macrophages and neutrophils, acts as a receptor for bacterial lipopolysaccharide (LPS) detection.
- **CD31**, also known as platelet endothelial cell adhesion molecule (PECAM-1), is present on surface of platelets, monocytes, neutrophils, belongs to the immunoglobulin family and is involved in leucocyte transmigration, angiogenesis and integrin activation.

Recently, the lack of MSC selective markers and standardized isolation protocols has lead investigators to introduce additional and more specific methods to enrich cultures for MSCs. In this context, the use of monoclonal antibodies could be useful to identify a cell population with specific phenotype; the murine monoclonal antibody against Stro-1

recognizes non-hematopoietic bone marrow stromal precursors and it can be used as CFU-F marker, since it was demonstrated that Stro-1 negative cells were not able to form colonies (Simmons and Torok-Storb, 1991).

Stro-1 has a limited use as an universal MSC marker, so it is best to be associated with additional antigens, such as CD106, also known as Vascular Cell Adhesion Molecule-1 (VCAM-1). CD106 has been shown to be involved in many processes, like chemotaxis, cell adhesion and signal transduction. In addition, a study by Majumadar et al (2003) identified MSC surface molecules, such as integrins α1, α5 and β1, expressed at high levels by MSCs and mediate homing and cell binding processes.

MSC differentiation potential

The third aspect to identify human MSCs, as suggested by the ISCT in 2006 (Dominici et al, 2006) consists in the MSC *in vitro* differentiation into mesodermal lineages. Indeed, under the influence of certain culture conditions and growth factors, human MSCs isolated from multiple sources could differentiate into adipocytes, osteocytes and condhrocytes (Barry and Murphy, 2004; Chamberlain et al, 2007).

Osteogenic induction requires the addition of β-glycerophosphate, dexametasone, ascorbic acid and to FBS-supplemented growing media, resulting in the formation of calcium deposit and MSC expression oh phasphatese alkaline. Cartilage formation, evidenced by proteoglycans and collagen II expression, occurs under MSC exposure to TGF-β factor in serum-free conditions. TGF- β3 was shown to be the more potent condhrogenic induction factor, associated with the MSC synthesis of aggregan, fibromodulin, type II collagen and other typical components of articular cartilage matrix (Barry et al, 2001). The lipid-vacuoles indicative of adipogenic differentiation were observed after MSC exposure to FBS-supplmented media in presence of dexamethasone, insulin, indomethacin and isobutyl methyl xanthine. Culture conditions used for in vitro differentiation assay on human MSCs are summarized in Table 1.

Differentiation lineage	Culture conditions	Culture evidences
Osteogenic	FBS-media plus: β-glycerophosphate dexametasone ascorbic acid	Alkaline phosphatase up-regulation Calcium-rich matrix deposit (von Kossa staining)
Adipogenic	FBS-media plus: dexametasone Insulin Indomethacin isobutyl methyl xanthine	PPAR-γ Fatty acid sinthetase Lipid-vacuoles (Oil Red O staining)
Chondrogenic	FBS free media plus TGFβ in a three-dimensional culture format	Cartilage-specific ECM members: glycosaminoglycans (Alcian Blue staining) Type II collagen increase

Table 1. MSC differentiation culture conditions.

Beside the differentiation assay mentioned above, human MSCs displayed myogenic (Taylor and Jones, 1982), neurogenic (Deng et al, 2001) and angiogenic potential (Davani et al, 2003).

MSCs: immunomodulatory properties and clinical applications

One of the most intriguing aspect related to the therapeutic applications of human MSCs reside in their ability to evade immune recognition and suppress immune response (Barry and Murphy, 2004; Chamberlain et al, 2007). The low immunogenicity of MSCs is mainly due to the low expression rate of histocompatibility complex HLA-I and lack of the complex HLA-II, together with the absence of co-stimulatory molecules CD40, CD80 and CD86. On the other hand, the immunosuppressive function may be exerted at various levels, acting on many aspects of the immune system. Indeed, it has been reported that MSCs can suppress T cell proliferation, activating the fraction of regulatory T cells (Tregs); moreover, MSCs act on dendritic cells (DCs), impairing their differentiation and function, as well as on B cell and natural killer cells (NKs), limiting their proliferation and cytolytic function respectively (Le Blanc and Ringdén, 2007). These properties have been investigated on *in vitro* and *in vivo* experimental models, demonstrating an immunoregulatory activity associated both to direct cell-cell interactions and soluble factor secretion. Soluble factors include anti-inflammatory mediators, such as Transforming Growth Factor- β1 (TGF-β1), Inteurlekin-10, (IL-10), Indoleamine 2,3-dioxygenase (IDO), Hepatocyte Growth Factor (HGF), Prostaglandin E2 (PGE2). In most of cases, Interferon-γ enhances MSC immunosuppression, supporting the idea that the inflammatory environment contributes to MSC activation and following establishment of an immunosuppressive niche trough the release of soluble factors (Ryan et al, 2007).
A brief description of the immune cell system origin from HSCs and its functional organization are represented in Figure 16.

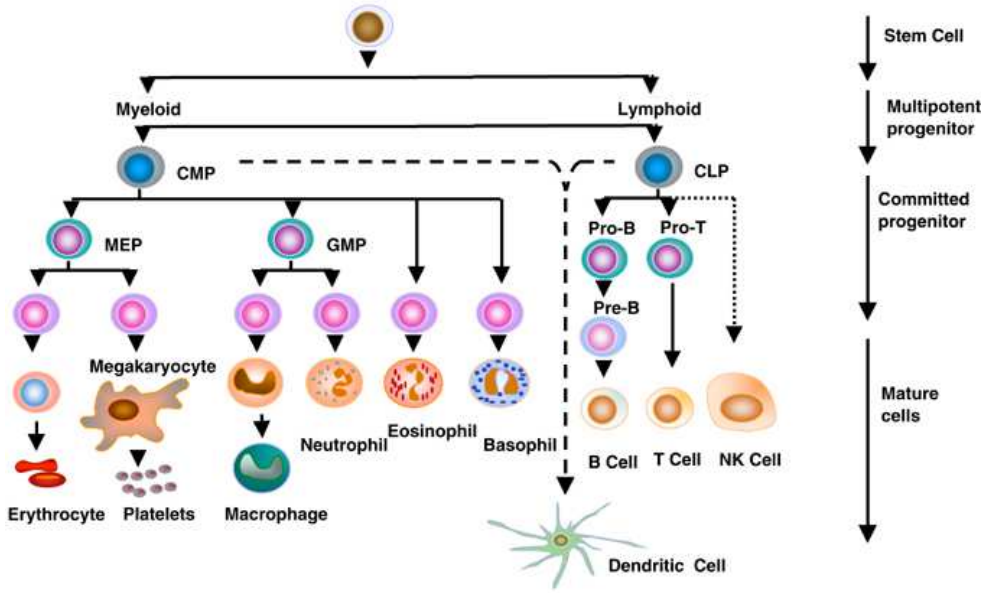


Figure 16. Immune cell system and hierarchical organization from stem cell precursor.

MSC-mediated immunosuppression: mechanisms and molecular mediators

Many evidences obtained from human, baboon and mouse models have showed a decreased T cell proliferation, under the influence of MSCs. More specifically, this process was induced by a cell cycle arrest during the quiescent phase G0/G1, together with reduced cyclin D2 and increased p27 expression levels (Glennie et al, 2005; Shi et al, 2011). Several molecular signalling may be involved in MSC-mediated T cell suppression. Di Nicola et al proposed the secretion of TGF-β1 and HGF, as active molecules against T cell proliferation (Di Nicola et al, 2002). This was confirmed by the use of neutralizing antibodies against TGF-β1 and HGF, able to restore T lymphocyte cell cycle. PGE_2 is a cytokine constitutively expressed by MSCs, undergoing an increased production in presence of peripheral blood mononuclear cells (PBMCs), and inhibits the T cell proliferation and the release of inflammatory cytokines T cells dependent. IDO represents a further mediator of MSC suppressive activity on T cells: its expression increased under the presence of IFN-γ, usually expressed during inflammatory process. Indeed, IDO promoter contains two IFN-stimulated response elements (ISREs) and an IFN-γ activated site (GAS) (Ryan et al, 2007). Increased IDO correspond to an enhanced tryptophan catabolism, resulting in the release of the kyneurine degradation intermediates that block lymphocyte proliferation. Nitric oxide (NO) signalling is also involved in

immunomodulatory mechanism, depending on STAT-5. STAT-5 is a transcription factor which is crucial for T cell activation and proliferation; the inhibition of STAT-5 phosphorylation, NO-dependent, mediates T cell cycle arrest (Sato et al, 2007).

Another mechanism by which MSCs regulate immune response consists in the activation of Tregs, designated to suppress T cell proliferation and cytokine release. Treg population comprises different subsets, the major part of which is defined on the basis on CD4 and CD25 positive profile, together with the expression of the transcriptional factor FoxP3 and the functional secretion of IL-10 and TGF-β (Singer and Caplan, 2011). The MSC-mediated release of TGF-β1 and HLA-G5, contribute to Treg induction.

HLA-G is a non-classical HLA class I, identified in cytothrophoblast (McMaster et al, 1995), where regulates the maternal-fetus tolerance (Rouas-Freiss et al, 1997), and results to be up regulated under specific conditions, such as tumorigenesis and organ transplatantation. It has been suggested as a crucial mediator to MSC immunosuppression, since it regulate many immune processes and beside Treg induction, HLA-G inhibits NK cytotoxicity and DC maturation (Selmani et al, 2007; LeRonde et al, 2006). The B cell inhibition mediated by MSCs was observed on murine models and later confirmed in human MSCs. B cells resulted affected on proliferative as well as functional aspects, including differentiation and chemotaxis.

A summary of the MSC-mediated immunosuppression is illustrated in the Figure 17, which underlines each mentioned molecular mechanism and the involved mediators.

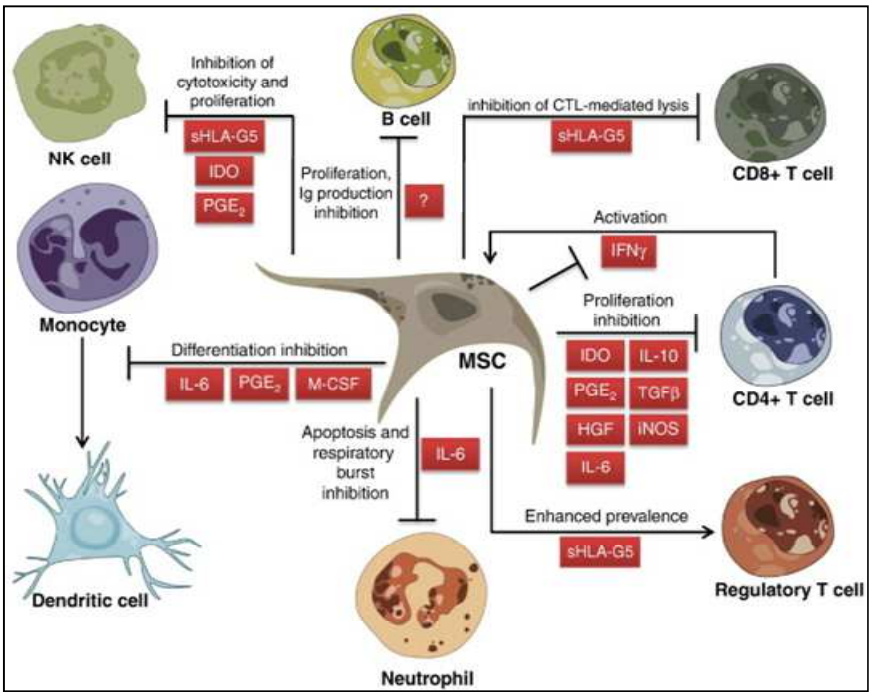


Figure 17. MSC-mediated immunosuppressive mechanisms.

The high proliferative and differentiation potential, together with the homing to damaged tissues, make MSCs really useful for therapeutic applications. The autologous or allogeneic MSC transplantation can be practiced through systemic or local delivery and it can be applied to several clinical fields, such as cardiovascular, neurodegenerative diseases, spinal cord injury or bone and cartilage affections, requiring tissue repair. For example, in 2001, bone marrow MSCs were shown to *de novo* regenerate myocardium and, in 2002, Sanchez-Ramow demonstrated the differentiation of bone marrow MSCs into neuronal lineage both in vitro and in vivo, using mice and rat models (Sanchez-Ramos, 2002).

The systemic delivery of cultured MSCs was performed to treat children affected by Osteogenesis Imperfecta, which is a defective type I collagen disease resulting in osteopenia, fractures, and bone malformations. After 3 months, surprising results were observed, highlighted by increase bone formation and reduced bone fracture frequency (Horwitz et al, 1999).

The hypoimmunogenic nature of MSCs and the above described immunoregulatory mechanisms allows MSC use to regulate immune reaction in the context of transplantation

and autoimmune diseases with an inflammatory origin, such as graft versus host disease (GVHD), Crohn's disease, systemic lupus erythematosus.

Stem cells in disease: a pathogenetic role?

Research on MSC clinical applications should also addresses the potential side effects deriving from MSC use, as well as the environment influence on MSC behaviour. Under specific tissue conditions, especially in presence of degenerative affections, stem cells can be altered or do not function in a proper manner. This impaired function may result in a worsening of the clinical disease conditions. An exemplificative situation is those represented by osteoarthritis, a disease caused by a progressive cartilage loss. Murphy et al (2002) demonstrated that MSCs isolated from patients had a lower proliferative potential, together with a reduced adipogenic and chondrogenic differentiation ability. Moreover, MSCs isolated from patients affected by idiopathic thrombocytopenia and systemic lupus erythematosus display an abnormal function.

Beside an impaired growth and differentiation potential, MSCs can undergo an alteration of the immunomodulatory function, thus creating or exacerbating the inflammatory process. Some evidences report that MSCs secrete also pro-inflammatory cytokines and it has been suggested the role of Toll-Like Receptors (TLR) as potential mediators of a MSC pro-inflammatory phenotype.

TLRs are a conserved family of receptors that after recognition of pathogen associated molecular patterns (PAMPs) activate immune cells. TLRs have been showed to be linked with the growth and differentiation of adipose-derived MSCs (ASCs), but current data are still controversial.

Bunnel et al suggested a mechanism involving TLR in MSC acquisition of a pro-inflammatory or an immunosuppressive phenotype: according to their model, a short-term and a low level exposure to TLR4 takes MSCs to a pro-inflammatory phenotype, contributing to tissue injury; on the other hand, TLR3 stimulates MSCs toward an anti-inflammatory, thus resolving the tissue damage (Bunnel et al, 2010). Moreover, this functional divergence corresponds to a differential cytokine and chemokine expression pattern, between the two MSC phenotypes (Waterman et al, 2010; Bunnel et al, 2010).

A study by Li et al demonstrated an impaired immunosuppressive activity for bone-marrow MSCs isolated by multiple myeloma (MM-MSCs) patients, reflected on an aberrant T cell function (Li et al, 2010). The alteration of MSC immunosuppressive property was also documented in a mouse model of collagen-induced arthritis (CIA). Indeed, MSCs exposed to an inflammatory environment, as in the CIA model, did not

result beneficial and *in vitro* experiments showed that consequently to TNF-α addition, MSCs lost the ability to regulate T cell proliferation (Djouad et al, 2005).

Based on these findings, it is clear that MSCs represent a great promise in the regenerative therapy field, and many other issues must be explored to completely elucidate their behaviour contextually to the tissue damage. Most importantly, the molecular mechanisms triggering MSC attitude toward disease progression, especially as regards inflammation and autoimmune disorders, needs to be investigated.

AIM OF THE THESIS

AIM OF THE THESIS AND EXPERIMENTAL DESIGN

The existence of the vascular stem cell niche has been proved in many laboratories (Zengin et al, 2006; Ergun et al, 2011; Psaltis et al, 2011; Gómez-Gaviro et al, 2012;). Our research team has a ten-year experience on MSC isolation from the vascular wall of different arterial segments obtained from healthy donors of various age and clinical characteristics (Pasquinelli et al, 2007; Pasquinelli et al, 2010; Valente et al, 2014). More specifically, a cell population expressing mesenchymal antigens, stemness transcriptional factors and able to differentiate into the adipogenic, osteogenic and chondrogenic lineage was isolated. Moreover, this cell population, named Vascular Wall MSCs (VW-MSCs), was shown to form capillary-like structures under the influence of VEGF soluble factor added to cell cultures. In addition to these phenotypical and functional characteristics, VW-MSCs were also tested for the immunomodulatory activity, which represents one of the most interesting MSC properties for their clinical and therapeutic use. Indeed, VW-MSCs, as the other tissue source human MSCs, were able to negatively modulate the immune response, through a decreasing effect on PBMC proliferation, acting on the cell cycle arrest in G0/G1 phase. On the other hand, VW-MSCs demonstrated a pronounced expression of HLA-G, a typical MSC non-classical antigen that inhibits immune reaction suppressing T cell and Natural Killer cell function and improving the expression of T regs.
In addition, we recently succeed to obtain a consistent cell number with a high proliferative capacity also from 5 years cryopreserved arteries, belonging to distinct vascular districts (common carotid, subclavian artery, aortic arch, thoracic aorta). These cells were shown to resist to nitrogen liquid temperature for long term (Valente et al, 2014).
Based on these findings, we planned to explore the presence as well as the functionality of the MSC niche in adverse pathological conditions, as occurs during abdominal aortic aneurysm (AAA) pathogenesis.

AAA is a focal aortic dilatation, necessary to be under monitoring since it has no sign or symptoms and the silent progressive enlargement of the aortic diameter can lead to rupture which can be fatal, carrying a 70-80% mortality risk. Currently, there is not an established pharmacological approach able to restore the normal vessel wall condition, and large aneurysms are treated only through surgical repair. AAA pathogenesis is associated with the arterial medial degradation, mediated by the proteolytic activity of enzymes secreted by

both vascular resident and inflammatory cells. In this context, MMPs represent the molecular mediators mainly involved in the ECM degradation. Meanwhile, inflammation invades the aortic wall and exacerbates the tissue remodelling, since inflammatory cells, such as T and B lymphocytes and macrophages migrate at the injured tissue, release cytokines (IL-1β, IL-6, TNF-α, etc) and MMPs.

Many investigators have supposed an immune-cell mediated origin for aneurysm initiation, based on the immune response mediated by T cells against an endogenous or exogenous antigen (like herpes simplex, CMV). Moreover, an autoimmune origin was also suggested.

Beyond inflammatory cells, MMPs are produced and released by MSCs, at different levels, as demonstrated by several research works (Silva et al, 2003; Mannello et al, 2006). Indeed, MMPs especially MMP-2 and MT-MMP1 are essential to MSC migration and invasive abilities (Ries et al, 2007).

These background considerations lead us to suppose a concrete contribution of vascular MSCs to pathological conditions involved in AAA disease. The aim of this study was so organized on different levels:

AIM 1: MSC isolation from aneurysm and characterization

- MSC isolation from vascular specimens collected by patients affected by AAA MSC.
- MSC characterization according to the classical criteria: morphology, mesenchymal antigens (Flow cytometry) and stemness transcriptional factors (RT-PCR).

AIM 2: MSC participation to pathological processes inside aortic wall

- MSC characterization for MMP expression: MMP-2, MMP-9, TIMP-1, TIMP-2 and EMMPRIN (mRNA, protein and activity evaluation).
- MSC immunomodulation: ability to regulate immune reaction, trough co-colture of MSC and Peripheral Blood Mononuclear Cells (PBMCs).
- Evaluation of MMP modulation mechanism, through an anti-inflammatory approach: use of healthy vascular MSCs; AAA-MSC exposure to IL-10.

The following diagram provides a schematic description of the experimental design followed in the present thesis.

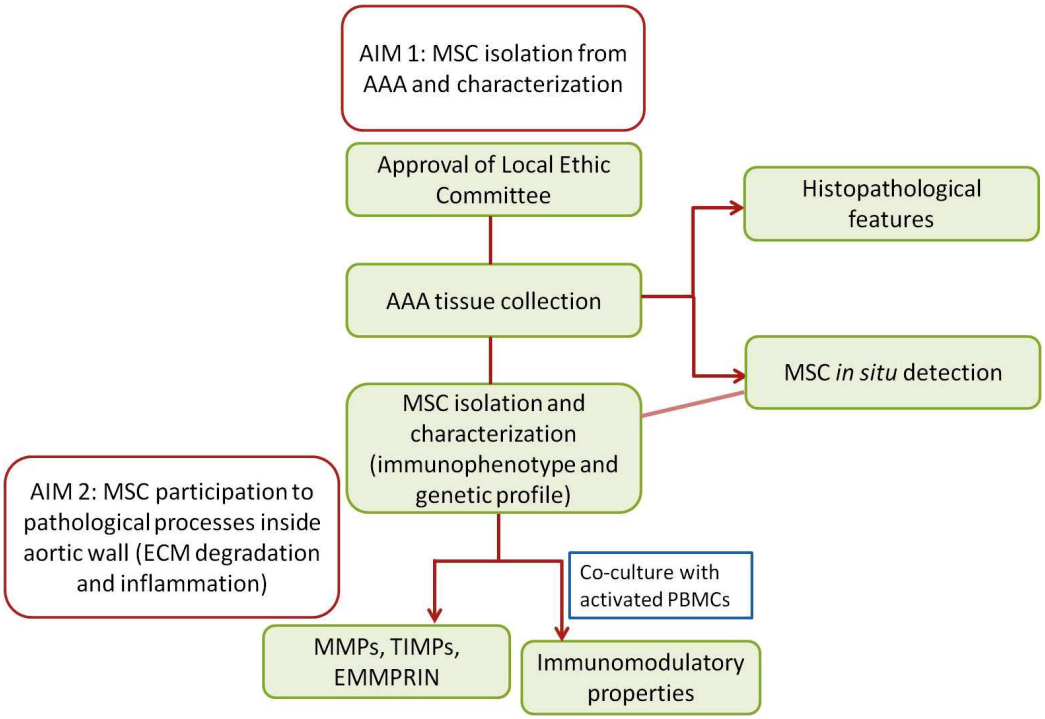


Figure 18. Aim of the thesis and experimental design.

MATERIALS AND METHODS

STUDY PROTOCOL AND TISSUE COLLECTION

The study protocol was approved by the Local Ethic Committee (ethic protocol number APP-13-01) and written informed consent was obtained from patients.

Aneurysm tissues of the abdominal aortic segment were provided by the Vascular Surgery Unit, University Hospital S. Orsola-Malpighi, Bologna. AAA tissues were collected from 12 patients (males, mean age= 69.2±5.0) subjected to surgical repair; demographical and clinical data were reported on a database.

Thoracic aortic wall tissues derived from 3 healthy donors (female:male=2:1, mean age=25±6.5) were provided from the Cardiovascular Tissue Bank, University Hospital S. Orsola-Malpighi, Bologna. The vascular tissues included in our study derive from the arterial segments discarded from the Bank and were used as healthy controls for our study, with the approval of the Local Ethic Committee (ethic protocol number APP-13-01). We selected the healthy aortic segments according to two specific criteria: age between 18 and 55 years, to exclude ageing process interference with our analysis; cause of death not associated with cardiovascular pre-existent affections, or brain hemorrhage, to compare patient group with healthy subjects in absence of cardiovascular risk.

This study was developed on different levels, evaluating both the histological and cellular analysis. For this purpose, pathological and healthy aortic samples were in part fixed for histological and immunostaining applications and in part treated with an enzymatic solution to allow cell isolation.

ANEURYSM-AFFECTED AORTA: HISTOPATHOLOGICAL FEATURES

Hematoxylin/Eosin staining

AAA tissues were in part reserved for histological exam, to characterize aortic wall and identify typical aneurysm features. Briefly, AAA tissues were fixed with a 10% formaldehyde solution, washed and dehydrated by passing the tissue through a series of increasing alcohol concentrations (70%-96%-100%) and embedded in paraffin to obtain four µm-thick sections. Each section was cut trough the microtome and stained with hematoxylin & eosin (H&E) for traditional histopathological analysis according to the histological procedures. After staining, slides were observed with light micsroscope (LM) and analyzed using the Image-Pro Plus® 6.

Immunohistochemical staining

Immunohistochemistry was performed on aneurysm and healthy specimens to characterize MSC population before enzymatic digestion, using a non-biotin amplified method (Novolink, Newcastle, UK). Mesenchymal antigens (CD44 and CD90) were used for the in situ detection of human MSCs. In addition, MMP-9 staining was performed to evaluate the contribution of the different cell types inside the vessel wall to pathological arterial remodeling.

Briefly, four µm-thick sections of formalin-fixed and paraffin embedded tissues were deparaffinized and rehydrated through a series of graded ethanol and rinsed in distilled water. Endogenous peroxidases activity was blocked in 0.3 % H_2O_2 in absolute methanol for 10 minutes at room temperature; antigen retrieval was performed using citrate buffer (pH 6) in microwave (750w) for 20 minutes and after cooling slides were washed with Tris Buffered Saline (TBS). Aortic sections were subsequently incubated with primary antibodies against CD44 (1:100, BD Biosciences) and CD90 (1:100, BD Biosciences) and MMP-9 (1:325, Cell Signaling) in a moist chamber at 4°C o/n, then they were incubated with NovoLink Polymer for 30 minutes at room temperature and exposed to the substrate/chromogen 3,3′-diaminobenzidine (DAB) prepared from Novocastra DAB Chromogen and NovoLink DAB buffer. Nuclei were counterstained with Mayer's hematoxylin. Samples were dehydrated, coverslipped and observed under light microscope using the Image-Pro Plus program.

ANALYSIS ON MSCs ISOLATED FROM ANEURYSM-AFFECTED AORTA

MSC isolation and culture

The protocol used for MSC isolation from vascular segments consists of an adjustment of the previously described technique (Pasquinelli et al, 2010).

Two cm^2 sections of healthy and aneurysm-affected aortic walls were digested in an enzymatic solution containing 0,3 mg/mL Liberase type II (Liberase TM Researche Grade, Roche) in serum-free Dulbecco's modified Eagle's medium (DMEM; SIGMA Aldrich) at 37°C o/n in a rotor apparatus. The resulting tissue homogenate was filtered using cell strainers at decreasing diameter (100-70-40µm) to eliminate not-digested tissue residuals and centrifuged at 1200 rpm. Cell viability was assessed by Trypan Blue exclusion. MSCs isolated from aneurysm tissue were identified with the acronym AAA-MSCs, whereas MSCs isolated from healthy aorta were defines as control MSCs (cMSCs). AAA-MSCs and cMSCs were cultured according to the standard culture conditions (37° C incubator,

5% CO2) in DMEM enriched with 20% Fetal Bovine Serum (FBS; SIGMA Aldrich) and *in-vitro* expanded.

MSC immunophenotype: flow cytometer analysis

Flow cytometer analysis was performed on AAA-MSCs and cMSCs at passage 3 to investigate the expression of typical mesenchymal markers, exploring eventual different expression patterns of the investigated antigens.

Briefly, cells grown on monolayer when arrived at passage 3 were trypsinized and, after centrifuge at 1200 rpm for 5 minutes, the pellet was fixed with the Fixation Kit (Beckman-Coulter). Then, samples were stored at 4°C until use. Fixed MSCs were incubated for 20 minutes with each antibody; for our purpose to identify MSC populations, an extensive conjugated panel of antibodies was used: anti-CD44-fluorescein isothiocyanate (FITC), anti-CD73-phycoerythrin (PE), anti-CD90-phycoerythrin-cyanine 5, anti-CD14-FITC, anti-CD34-FITC, anti-CD45-allophycocyanin, anti-CD146-PE, anti-platelet-derived growth factor receptor beta (PDGFR-β) (R&D Systems, Inc., Minneapolis, MN, USA).

Flow cytometry analysis also included the expression of HLA-G, associated with the MSC immunomodulatory activity. For this purpose, HLA-G detection was performed trough the incubation with anti-HLA-G-FITC antibody (Abcam, Cambridge, UK).

Negative controls were performed using appropriate conjugated irrelevant antibodies. Samples were analyzed using a Navios FC equipped with two lasers for data acquisition (Beckman-Coulter). Results were analyzed with Kaluza FC Analysis software (Beckman-Coulter).

MSC MOLECULAR PROFILE: STEMNESS MARKERS, AAA MEDIATORS AND IMMUNOMODULATORY FACTORS

Total RNA extraction and Reverse-Transcription

Total RNA was extracted from cMSCs and AAA-MSCs at passage 3, by using TRIreagent (TRIzol reagent, Invitrogen, Italy) according to the manufacturer's instructions.

Total RNA was extracted from cMSCs and AAA-MSCs at passage 3, by using TRI Reagent (TRIzol reagent, Invitrogen, Italy) according to the manufacturer's instructions.

Briefly, cultured cells were homogenized in TRI Reagent solution and incubated at room temperature for 5 minutes. After lysis, the chloroform was added to homogenate samples and left at room temperature for 10 minutes, to allow the phase separation: after centrifuge, RNA contained in the aqueous phase was collected and transferred into a new tube. A step

of RNA precipitation by addition of isopropyl alcohol, incubated at room temperature for 10 minutes, was performed; the visible RNA pellet was finally washed with 75% alcohol, centrifuged and air-dried for 10 minutes. RNA was dissolved in RNAse free water and stored at 20°C until use.

At the end of the extraction protocol, RNA integrity and concentration were evaluated by using ND-1000 spectrophotometer (NanoDrop, Fisher Thermo, Wilmington, DE, USA). Reverse transcriptase PCR was performed on all RNA samples with an absorbance (260/280) ratio between 1.8-2.2. One µg of total RNA was reverse transcribed in a 20 µL reaction volume using High Capacity Reverse Transcription Kit (Life Technologies).

Stemness marker evaluation by RT-PCR

RT-PCR was performed on AAA-MSCs to evaluate the stemness genes Nanog, Oct-4 and Sox-2 expression; primers sequences (SIGMA Aldrich) are listed in Table 2.

Gene	Primer sequence	Amplicone length (bp)	T annealing (°C)
GAPDH	FWD 5'-ACCACAGTCCATGCCATCAC-3' REV 5'-TCCACCACCCTGTTGCTGTA-3'	452	61
NANOG	FWD 5'- AAGGCCTCAGCACCTACCTA-3' REV 5'-ACATTAAGGCCTTCCCCAGC-3'	326	58
OCT-4	FWD a 5'- CTCCTGGAGGGCCAGGAATC-3' FWD b 5'- ATGCATGAGTCAGTGAACAG-3' REV 5'-CCACATCGGCCTGTGTATAT-3'	380 402	62
SOX-2	FWD 5'ACCGGCGGCAACCAGAAGAACAG-3' REV 5'-GCGCCGCGGCCGGTATTTAT-3'	208	62

Table 2. Primer sequences used to detect ESC transcriptional factors AAA-MSCs. *bp: base pair.*

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as housekeeping gene to value the cDNA quality. RT-PCR was performed according to the reaction program described in Table 3, using a Thermocycler with gradient function to allow the simultaneous amplification assay for each gene.

PCR Step	T (°C)	Time	N° cycles
Initial Denaturation	94	1 min	1
Denaturation	94	30 sec	39
Annealing	60±5	1 min	
Extenstion	72	30 sec	
Final Extenstion	72	10 min	1

Table 3. Scheme of PCR protocol reaction.

All samples were loaded on a 2% agarose gel with Tris-acetate-EDTA buffer 1X (TAE) and a 100 bp DNA ladder was used to allow amplicone size identification. The gel was subjected to electrophoresis at a constant 100 V and observed under ultraviolet light, through ethidium bromide incorporation.

Quantitative Real-Time Polymerase Chain Reaction (qPCR)

Semi-quantitative Real Time PCR was performed to evaluate stemness transcriptional factors, AAA molecular mediators and anti-inflammatory cytokines mRNA levels in human MSCs isolated from aneurysm and healthy aorta.

qPCR was carried out in a Gene Amp 7000 Sequence Detection System (Applied Biosystems) using the TaqMan approach for the gene β-glucoronidase (GUS) (Applied Biostystems) and the SYBR green approach for other genes, using specific couples of primers (SIGMA Aldrich; Table 4). Each assay was executed in triplicate and target gene expression was normalized to GUS. The final results were determined by the comparative 2^-ΔΔCt method (Livak and Schmittgen, 2001) where ΔΔCT = [CT Target − CT GUS] AAA-MSCs -[CT Target − CT GUS] cMSCs. Results were expressed as fold changes relative to cMSCs as controls.

Gene	Primer sequence
MMP-2	FWD 5′-CCCCAAAACGGACAAAGAG-3′ REV 5′-CTTCAGCACAAACAGGTTGC-3
MMP-9	FWD 5′-GAACCAATCTCACCGACCAG-3′ REV 5′-GCCACCCGAGTGTAACCAT-3′
TIMP-1	FWD 5′-GTGGCACTCATTGCTTGTCC-3′ REV 5′-CAAGGTGACGGGACTGGAAG-3′
TIMP-2	FWD 5′-TCTCGACATCGAGGACCCAT-3′ REV 5′-TGGACCAGTCGAAACCCTTG-3′
EMMPRIN	FWD 5′-GGGAGAGTACTCCTGCGTCTT-3′ REV 5′-CGACTTCACGGCCTTCAC-3′
IL-10	FWD 5′-GGGGCTTCCTAACTGCTACA-3′ REV 5′-TAGGGGAATCCCTCCGAGAC-3′
HLA-G	FWD 5′-GTCCTGGACTCACACGGAAA-3′ REV 5′-GTCTGGGGAGAATGAGTCCG-3′
NANOG	FWD 5′-ACCTACCCCAGCCTTTACTC-3′ REV 5′-GGACTGGATGTTCTGGGTCT-3′
OCT-4	FWD 5′-TGAGTAGTCCCTTCGCAAGC-3′ REV 5′- GAGAAGGCGAAATCCGAAGC-3′
SOX-2	FWD 5′-AGGATAAGTACACGCTGCCC-3′ REV 5′- TAACTGTCCATGCGCTGGTT-3′

Table 4. Primer sequences of genes measured trough Real Time PCR.

MMP-9 protein detection by Western Blot analysis

Western Blot was performed on AAA-MSCs and cMSCs to evaluate MMP-9 expression. Total cellular proteins were extracted by AAA-MSCs and cMSCs using lysys buffer (KH2PO4 0.1 M pH 7.5, NP-40 1%, 0.1 mM β-glycerolphosphate, supplemented with complete protease inhibitors cocktail, Roche Diagnostics) and quantified spectrophometrically with the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hempstead, UK). Thirty µg proteins were subjected to 8% SDS-PAGE and transferred to nitrocellulose membrane (GE Healthcare Life Sciences, Amersham) at 30 mA for 2.5 h. The membrane was blocked with 5% non-fat dry milk in TBS-tween (TBS-T) for 1h at room temperature and incubated with primary antibodies against MMP-9 (1:1000, Cell Signaling Technology, Beverly, MA, USA) and β-actin (clone AC-74, Sigma-Aldrich) at 4°C o/n. Incubation with secondary antibodies human anti rabbit/mouse horseradish peroxidise-conjugated (GE Healthcare Milan, Italy) was performed at room temperature for 1h. Protein signal was detected using Westar ηC chemiluminescent substrate (Cyanagen) and band intensities were quantified by densitometric analysis using ImageJ software (NIH, USA).

Metalloproteinase activity assay: gelatin zymography

Briefly, substrate-specific zymography is a simple technique used to analyze MMP activity trough the electrophoretic separation of samples on SDS-PAGE, containing the substrate specific for the MMP member on study; in our work, we used gelatin, as MMP-2 and MMP-9 substrate (Grzela et al, 2011).

MSCs isolated from healthy and AAA tissues at 80% confluence were cultured in serum-free DMEM; after 24h, conditioned media from both healthy and pathological cells were recovered and stored at -80°C until use. Equal aliquots of each sample were loaded on 10% SDS-PAGE containing 0,1% gelatin, in non-reducing conditions. Human colon cancer cell line (HCT116) was used as positive control. After electrophoresis, the gel was washed in Triton X-100, a detergent used to remove SDS and partially allow MMP reactivation, at room temperature for 1 h. Then, the gel was incubated at 37°C in zymography buffer, which contains Ca^{2+} ions necessary to reactivate MMPs (NaCl, $CaCl_2$*2H2O, Tris-HCl, pH=8). After 18h, the gel was stained with Coomassie blu and the gelatinase activity was detected in correspondence of unstained bands on dark area and quantified by densitometry using ImageJ software (NIH, USA). Figure 14 summarizes the main steps of this technique.

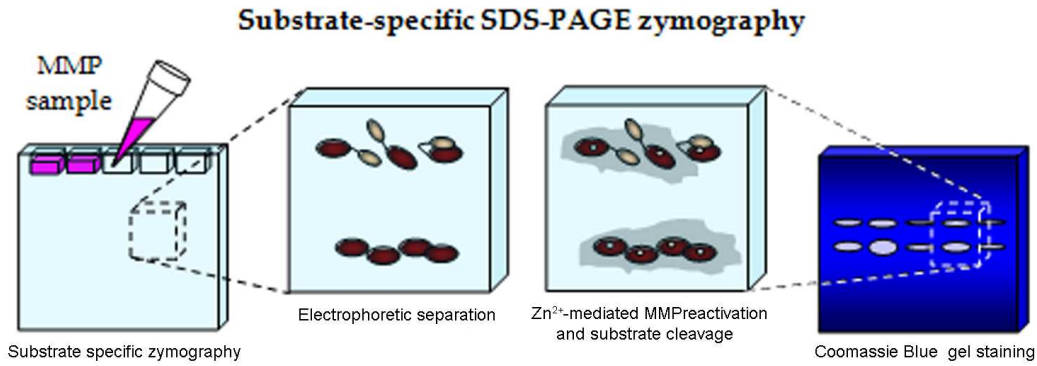


Figure 19. Gelatin zymography.

EVALUATION OF AAA-MSC IMMUNOMODULATORY ACTIVITY

Co-culture assay of Peripheral Blood Mononuclear Cells (PBMCs) and MSCs

Human AAA and healthy MSCs at passage 3 were seeded at $2.5x10^5$ cells in a six-well plate in DMEM 20% FBS. After 24h, peripheral blood mononuclear cells (PBMCs) were

isolated by discarded buffy coats of healthy donors and provided by the Service of Transfusional Medicine, University Hospital S. Orsola-Malpighi, Bologna. PBMCs were obtained using a Ficoll-Histopaque density gradient centrifugation. Diluted peripheral blood (1:3 dilution with PBS) was gently stratified on Ficoll-Histopaque solution and later centrifuged at 1500 rpm for 30' (Figure 15). Blood components resulted to be 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, after two washes with PBS, a step of adhesion to plastic flask at 37°C for 1h was performed to avoid monocytes contamination. PBMCs in suspension were recovered and plated at a density of 2.5×10^6 cells/well on MSC feeder layer in RPMI 1640 (Lonza, Walkersville, MD, USA). Phytohemagglutin (PHA; Sigma Aldrich) at the concentration of 5 μg/ml was added to cultures to activate PBMCs and stimulate their proliferation. Positive control was represented by activated PBMCs cultured in absence of MSCs. Negative control was represented by not-activated PBMCs, lacking of PHA. Schematic illustration of the PBMC/MSC co-culture system is represented in Figure 15.

After 72h co-culture, PBMCs were recovered and $1x10^5$ cells were transferred in a 96-well plate for the 5-bromo-2′-deoxyuridine (BrdU) incorporation assay. BrdU was added to each culture condition and after 4h evaluation of PBMC proliferation was executed using the BrdU-Assay kit (Roche Applied Science) and according to the manufacturer's protocol. The remaining part of PBMCs following the co-culture with MSCs, was fixed with 75% ethanol at 4°C, stained with Propidium Iodide (Beckman Coulter) at room temperature for 10 min for cell cycle analysis by flow cytometer.

Regarding MSC feeder layer, AAA-MSCs and cMSCs were trypsinized and one part was processed for HLA-G cytofluorimetric analysis, while the other one was designated to RNA extraction, cDNA reverse transcription and qPCR for IL-10 and HLA-G mRNA, as described above.

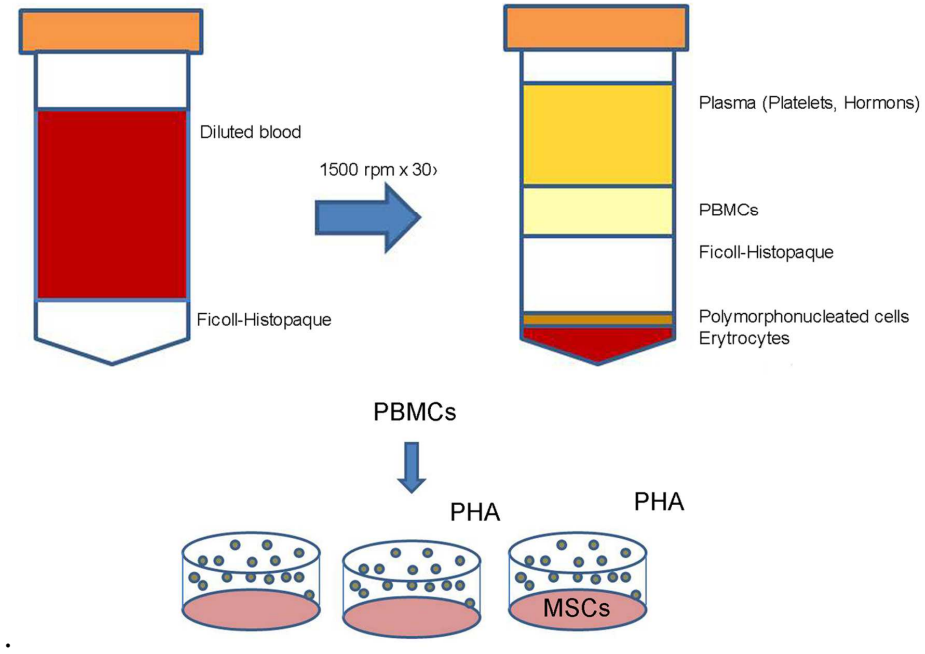


Figure 20. PBMC isolation based on Ficoll-Histopaque density gradient separation.

MMP TRANSCRIPTIONAL REGULATION IN AAA-MSC

In vitro co-cultures of AAA-MSCs and cMSCs

According to the reparative MSC property, we investigated on the potential contribution of MSCs derived from healthy aortic wall to MMP transcription by pathological AAA-MSCs. For this purpose we tested two culture conditions: 1) AAA-MSCs and cMSCs seeded at the density ratio 1:1/ well in a six-well plate; 2) AAA-MSCs cultured using conditioned media recovered by cMSC cultures, evaluating also a paracrine mechanism. After 72h, total RNA extraction, reverse-transcription were performed from each culture conditions, as described above, and qPCR was assessed to measure MMP-2 and MMP-9 mRNA levels.

MMP-9 regulation by IL-10 addition to AAA-MSC cultures

IL-10 is an anti-inflammatory cytokine and has been demonstrated to down-regulate MMP-9 (Roth and Fisher, 1999). For this reason, we investigated IL-10 effects also on human AAA-MSCs.

AAA-MSCs belonging to three different samples were seeded at a density of 60000/well in a 6-well plate and after 24h they were exposed to soluble IL-10 (SIGMA Aldrich, Italy) at 0-10-20 ng/mL, using serum-free DMEM. After 24h total RNA extraction, cDNA synthesis and qPCR were performed to quantify MMP-9 transcription.

STATISTICAL ANALYSIS

Data analysis and graphical representation were performed by using GraphPad Prism 5 statistical software (GraphPad Software Inc). Results are expressed as mean ± standard deviation and statistical analysis was assessed using t test (for comparison between two groups), one-way and two-way ANOVA test followed by Bonferroni post hoc test (for comparison between more than two groups). Results were considered statistically significant at the 95% confidence level (p value $< 0,05$).

RESULTS

STUDY SUBJECTS: AAA PATIENTS AND CONTROL GROUP

The analysis of our study involved two main groups, represented by 1) aortic tissues derived from twelve-male patients underwent surgical repair for abdominal aortic aneurysm and provided by the Vascular Surgery Unit of S. Orsola-Malpighi Hospital (Bologna); 2) aortic tissues derived from three-healthy donors collected from the Cardiovascular Tissue Bank of S. Orsola-Malpighi Hospital (Bologna). Clinical characteristics of AAA patients were reported on a database, including demographical data, cardiovascular risk, therapies and pre-existing diseases. Table 5 summarizes patient clinical data.

Clinical characteristics of AAA patients (n=12)	
Age (mean ± S.D.)	69.2 ± 5
Sex	Males
DAAA (mean ± S.D.)	73 ± 3 mm
Colesterol (mean ± S.D.)	158 ± 25 mg/dl
LDL(mean ± S.D.)	90 ± 24 mg/dl
HDL(mean ± S.D.)	49 ± 8 mg/dl
Triglycerids (mean ± S.D.)	100 ± 31 mg/dl
Smoking (%)	42
Hypertension (%)	100
CAD (%)	25
COPD (%)	25
CVD (%)	17
PAOD (%)	33
CRF (%)	25
Statins (%)	75

Table 5. Clinical data of AAA patient group.

Abbreviations: DAAA, abdominal aortic aneurysm diameter; LDL, low density lipoprotein; HDL, high density lipoprotein; CAD, coronary artery disease; COPD, chronic obstructive pulmonary disease; CVD, cerebro-vascular chronic disease; PAOD, peripheral arterial obstructive disease; CRF, chronic renal failure.

Regarding the control group, we selected aortic tissues deriving from healthy donors of arterial segments and valves, under discard by the Cardiovascular Tissue Bank. Selection

criteria excluded vascular segments affected by calcifications and included cause of death not associated with cardiovascular affections. Table 6 summarizes donor data.

Basic characteristics of healthy donors (control group)	
Age (mean ± S.D.)	25 ± 6.5
Sex	F:M=2:1
Clinical data	No cardiovascular risk, absence of aortic dilation, *Main death cause: traumatic brain injury (excluded cerebral hemorragy)

Table 6. Basic characteristics of healthy donors of vascular segments, selected as control group.

*Donors dead following haemorrhage were excluded since it can involve MMPs and interfere with our analysis.

ABDOMINAL AORTIC ANEURYSM: HISTOLOGICAL CHARACTERISTICS OF THE INJURED AORTA

Histological features of aneurysm-affected abdominal aorta

Hematoxilin/Eosin staining was performed on 4 µm sections of aortic tissues, collected from patients subjected to open repair for aneurysm, and highlighted the typical histological features of the diseased aorta.

Distinctive trait of aneurysm aorta is represented by the complete loss of the vessel wall architecture, so that the three wall layers could not be distinguished; this condition is due to the loss of muscular tone and the exaggerate proteolytic activity, which promotes an extensive medial degradation. Tunica media destruction is another typical histological feature of AAA, as it can be observed in Figure 22. The triad of AAA histo-pathological characteristics is completed with the high degree inflammatory infiltrate: this aspect can be revealed by the presence of many immune cells, including lymphocytes and macrophages. In addition, the lipid plaque affecting the AAA wall can be identified and this feature is common among aneurysms associated with atherosclerotic lesions.

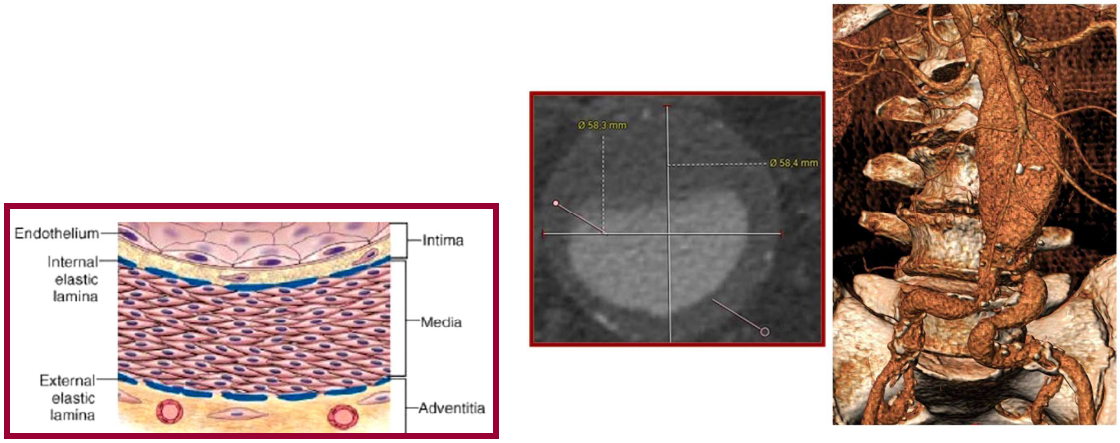


Figure 21. Schematic representation of healthy aortic wall and imaging of AAA.

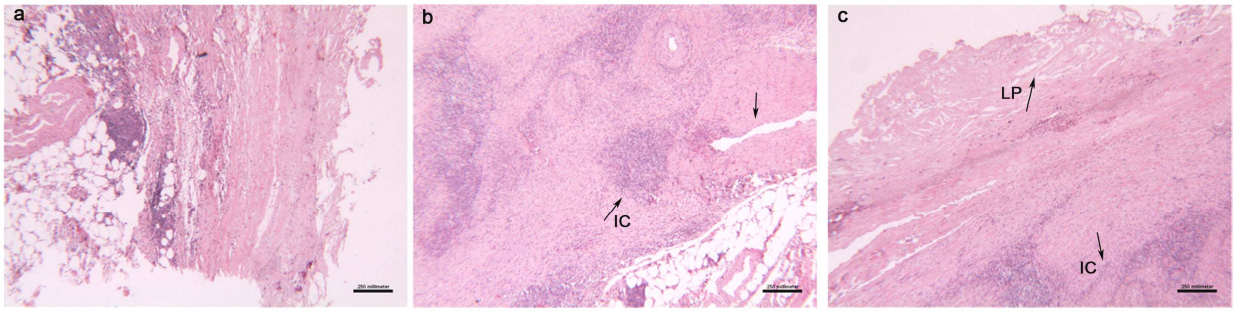


Figure 22. Representative H/E staining on aneurysm aortic sections.

Referring to the illustration shown in Figure 21, as an exemplified illustration of ordinary arterial wall, H/E performed on AAA tissues highlights the main distinctive traits of aneurysm aorta: (a) disorganized vascular layers and medial degradation; (b) inflammatory infiltrate and neovessel formation (arrow); (c) presence of lipid plaque within intima layer. *IC: Inflammatory Cells; LP: Lipid Plaque.*

MSCs AND VASCULAR DISEASES: PRIMARY IDENTIFICATION AT THE LEVEL OF ANEURYSM-AFFECTED ABDOMINAL AORTA

In situ detection of MSCs positive to CD44 and CD90 antigens

Previously to the enzymatic digestion and cell isolation from aortic tissues, we evaluated MSC distribution within the diseased arterial wall: for this purpose, we performed an immunohistochemical staining for typical mesenchymal markers on 4µm aortic tissues, collected from AAA patients as described in the above section. CD44 and CD90 positive cells were identified in correspondence of vasa vasorum, thus indicating the existence of a cell population with mesenchymal origin and its localization in the perivascular niche.

CD44 also stained inflammatory cells. Slides representing CD44 and CD90 immunostaining are represented in Figure 23.

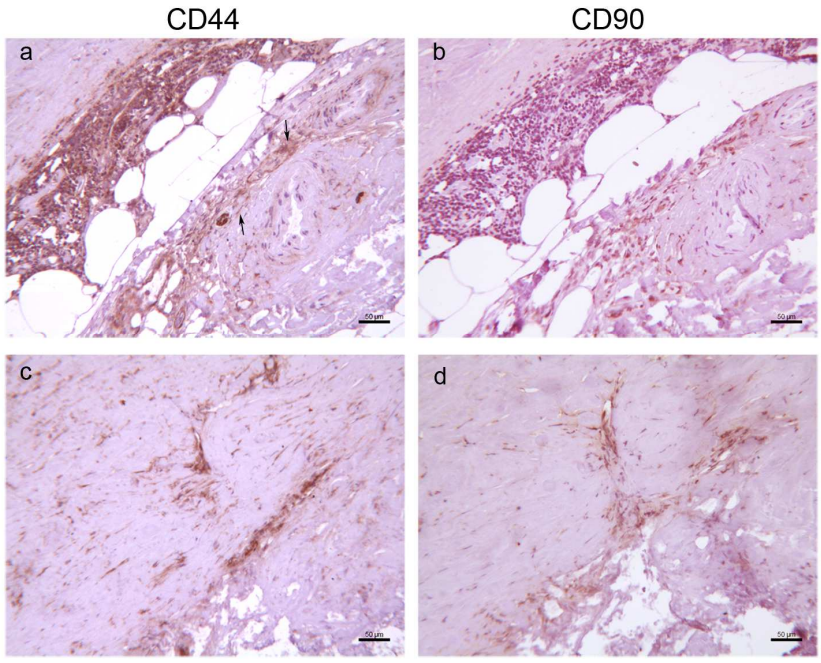


Figure 23. MSC in situ detection in aneurysm-affected aorta.
CD44+ (a, c) and CD90+ (b, d) cell populations can be observed in AAA tissue; beside inflammatory mononuclear cells, CD44 is expressed by spindle cells of the perivascular niche that also express CD90 molecule. (Scale bars indicates 50 μm).

MMP-9 expression within healthy and AAA wall

MMP-9 involvement in pathological aortic remodeling contextually to AAA, was demonstrated by mRNA quantification by Real Time qPCR. As shown in Figure 24e, MMP-9 transcription was increased in AAA tissue, compared with healthy aorta (33.2 ± 27 fold increased in AAA aorta, in comparison to healthy sample) even if not significant, possibly related to the high variability rate existing between different samples and their clinical characteristics (Table 5).

Aortic wall affected by aneurysm, as we know from literature and histological analysis, contains many cell types as possible sources of MMP-9. For this reason, we performed immunostaining on 4 μm aortic tissues, collected from patient and control groups, using antibody against MMP-9 protein to identify vascular cell contribute to the expression of this MMP member.

Consistent with literature data, an extensive vascular wall positivity to MMP-9 protein could be observed on AAA sections, whereas healthy aortic tissue resulted negative and no signal was detected. In more detail, MMP-9 could be easily identified in correspondence of inflammatory infiltrate, as seen in Figure 24b. The high concentration of inflammatory cells is due to the transmural migration of circulating cells in response to chemoattractive factors; moreover, vascular resident cells greatly contribute to MMP secretion, and MMP-9 too. Indeed, we could notice this aspect in Figure 24b-c, where positive signal was detected at the level of the perivascular niche located at the vasa vasorum; furthermore, spindle-shaped stromal were also shown to be positive to MMP-9 labeling, as demonstrated by Figure 24d.

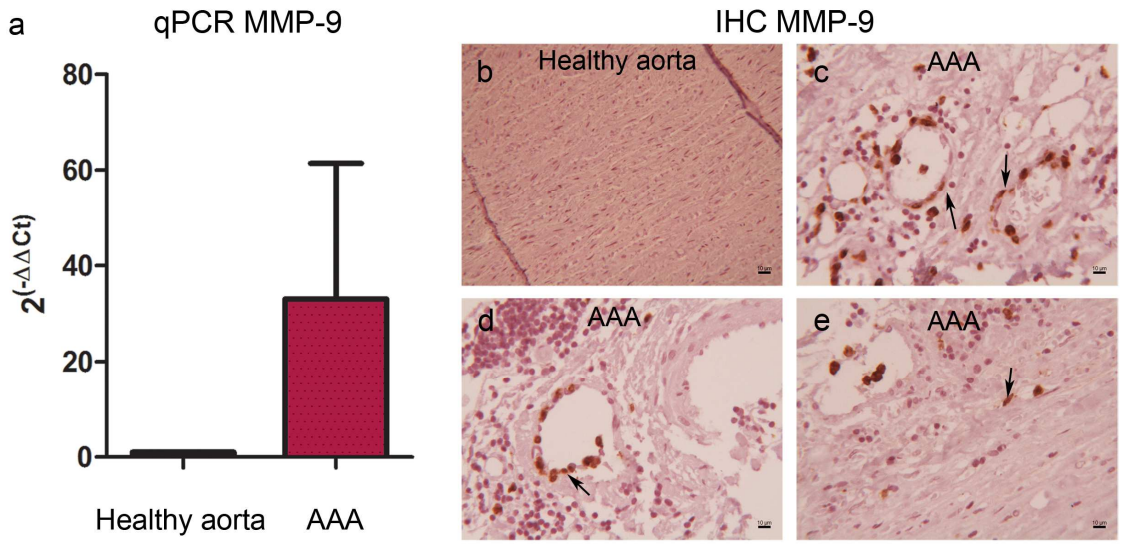


Figure 24. MMP-9 detection within AAA wall: mRNA and protein expression. (a) MMP-9 mRNA transcription by healthy and AAA tissue, performed by using qPCR. Results are shown as fold changes compared to healthy aortic tissue. GUS gene was used as housekeeping. MMP-9 protein expression at the level of aortic wall: (b) healthy aortic tissue; (c), (d), (e) AAA tissue (Scale bar indicates 10 μm)

The perivascular localization of MMP-9, together with the detection of fusiform cells belonging to the stromal tissue of the aortic wall, constitute a clear premise regarding the existence of vascular wall MSCs (more evidenced by CD44 and CD90 expression) and their hypothetical participation to the parietal disease processes.

MSC ISOLATION FROM HUMAN HEALTHY AND ANEURYSM-AFFECTED AORTA: CELL RECOVERY, CHARACTERIZATION AND FUNCTION ASSAYS

MSC isolation from human healthy and aneurismal aorta

As described in the Material and Method section, aortic tissue was subjected to an enzymatic digestion, resulting in an homogenous tissue compound, through the loss of gap and adherent junctions that join together vessel wall cells. An amount of about $8x10^5$ cells were recovered from the enzymatic digestion of 2 cm^2 sections of aneurismal aorta. This cell recovery was successfully obtained from the tissues of all the patients enrolled. Cells were seeded in DMEM 20% FBS and after 24-48 h the appearance of cells adherent to the plastic culture flask was observed. From this point we will refer to this cell population as Abdominal Aortic Aneurysm-derived Mesenchymal Stromal/Stem Cells (MSCs). AAA-MSCs in culture displayed a typical fibroblast-like morphology and exhibited a growing kinetic similar to what we previously observed in MSCs isolated from fresh and cryopreserved aortas belonging to healthy volunteers (Valente et al, 2014). MSCs isolated from cryopreserved aorta have been used as healthy controls in this study and we define them as control MSCs (cMSCs). AAA-MSCs at seven days from seeding reached confluence and they have been *in vitro* expanded for at least 12-13 passages. Moreover AAA-MSCs, as cMSCs, showed a heterogeneous aspect at p0, consisting of different subpopulations, and after 1-2 passages in culture they started to assume a more homogenous phenotype. These morphological features are shown in Figure 25.

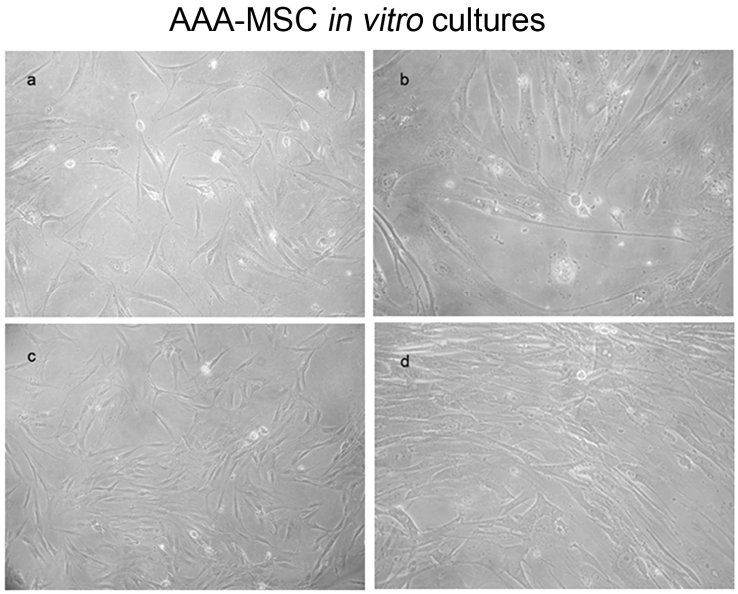


Figure 25. AAA-MSC *in vitro* cultures.

(a), (b) Heterogeneous primary cultures of AAA-MSCs displaying a typical fibroblast-like morphology and a with long cytoplasmatic pseudopodia (a, 100x magnification; b, 200x magnification); (c), (d) AAA-MSCs nearly confluence at passage 2 showed an homogenous aspect (c, 100x magnification; d, 200x magnification).

MSC immunophenotype and molecular profile

AAA-MSCs were grown on a monolayer and when they reached the third culture passage, they were processed for phenotypic and molecular characterization. Immunophenotyping was evaluated by using an antibody panel addressed against specific surface antigens: CD44, CD73, CD90, CD146 and PDGFR-β (mesenchymal origin markers); CD14, CD34 and CD45 (endothelial and hematopoietic markers).

AAA-MSC immunophenotype resulted specular to our previous observation on healthy cMSCs (Valente et al, 2014); indeed, as revealed by flow cytometry analysis, AAA-MSCs expressed a typical mesenchymal signature, with a positive profile to CD44, CD73, CD90, CD146 and PDGFR-β. On the other hand, AAA-MSCs resulted negative to CD14, CD34 and CD45, thus excluding the vascular MSC belonging to the hematopoietic and endothelial class. Elaboration of flow cytometry results are shown in Figure 26.

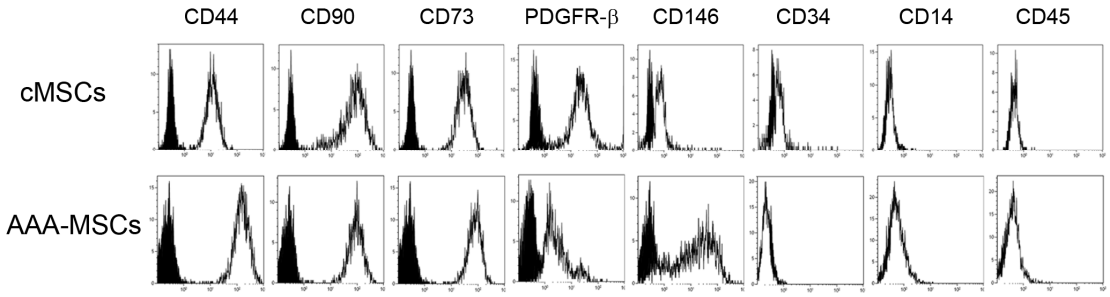


Figure 26. Representative flow cytometry assay on cMSCs (a) and AAA-MSCs (b).
The panels representing flow cytometry elaborations evidence a shared surface antigen expression between healthy and pathological MSCs, suggesting a common origin for the two cell populations.

AAA-MSCs were also characterized for Nanog, Oct-4 and Sox-2 genes. These are peculiar ES transcriptional factors and, as documented by literature works, constitute a transcriptional unit which regulates genes associated with cell development, activating self-renewal and pluripotency pathways. Figure 27a reports a representative electrophoresis gel image following ES gene amplification by RT-PCR performed on our AAA cell model: AAS-MSCs expressed all the investigated genes, especially Nanog and Oct-4, while a weaker signal was detected in correspondence of Sox-2 band.
In addition, to best evaluate the effect of vascular disease on MSCs we isolated from patient tissues, a semi-quantitative analysis of ES genes was performed through qPCR on healthy and AAA-MSCs. As observed in Figure 27b, ES gene expression pattern was quite similar between healthy and AAA MSCs, since semi-quantitative analysis did not reveal significant differences.

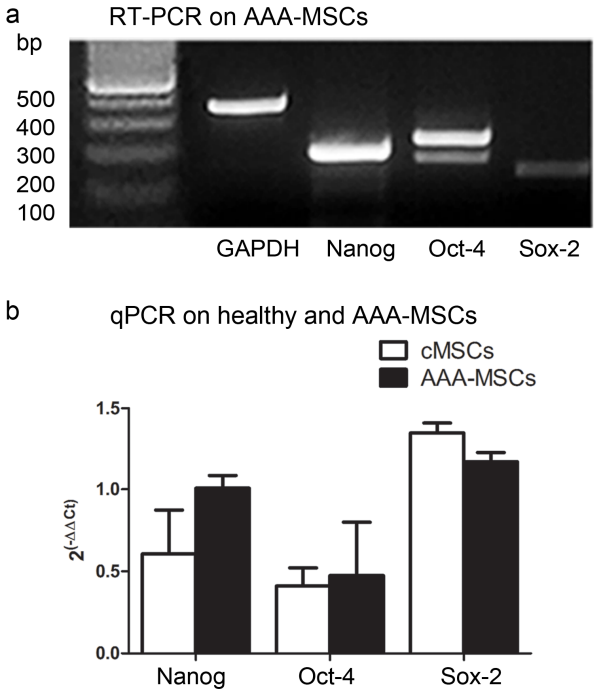


Figure 27. Genetic profile of AAA-MSCs.

(a) Nanog (326 bp), Oct-4 (380-402 bp) and Sox-2 (208 bp) expression in AAA-MSCs by RT-PCR. (b) Semi-quantitative analysis of stemness genes AAA-MSCs compared to cMSCs. Results are expressed as fold change relative to the control group. Values are represented as mean ± standard deviation and are representative of at least three independent experiments carried out in triplicate (*$p<0.05$, unpaired t test).

The preliminary analysis on AAA-MSC phenotype indicates the maintenance of the basic characteristics (morphology, growth dynamic under *in vitro* cultures, immunophenotype and genetic profile) in respect to MSCs isolated from healthy aortic samples, thus suggesting that the arterial degradation and the high degree inflammation did not affect the MSC phenotype.

Expression of AAA molecular players: MMPs, TIMPS and EMMPRIN

MMP-2 and MMP-9 are known to be up-regulated contextually to pathologic vascular remodeling, so we explored this aspect in our cell model directly isolated from aneurysm tissue. MMP-2, MMP-9 and their respective tissue inhibitors TIMP-2 and TIMP-1 were analyzed in AAA-MSCs compared to cMSCs (Figure 28a). Semi-quantitative analysis of MMP-2 and MMP-9 mRNA by qPCR showed that MMP-9 was really hyper-expressed in AAA-MSCs, in comparison to cMSCs (449.2 fold higher, compared to healthy aortic

MSCs, p=0.0155, unpaired t test), whereas MMP-2 was slightly increased (2.4 fold higher in AAA-MSCs, compared to cMSCs; p=0.0458, unpaired t test). Moreover, TIMP transcript levels were measured: a not significant increase of TIMP-1 mRNA was recorded in AAA MSCs (3.4-fold higher than cMSCs), whereas TIMP-2 mRNA resulted decreased (0.55-fold lower in AAA MSCs than cMSCs, p=0.02, unpaired t test). In addition, we observed a higher MMPs/TIMPs ratio in AAA-MSCs compared with cMSCs (Figure 8b), representing a demonstrative feature of abnormal MMP production and consequent enhanced proteolysis. The analysis of AAA players was completed with the quantification of a MMP inducer EMMPRIN, also knows as CD147 (Extracellular Matrix Metalloproteinases Inducer), recently associated with cardiovascular diseases: a 2.6-fold increase of EMMPRIN mRNA was shown in AAA-MSCs, compared to cMSCs (Figure 8a) (p=0.035, unpaired t test).

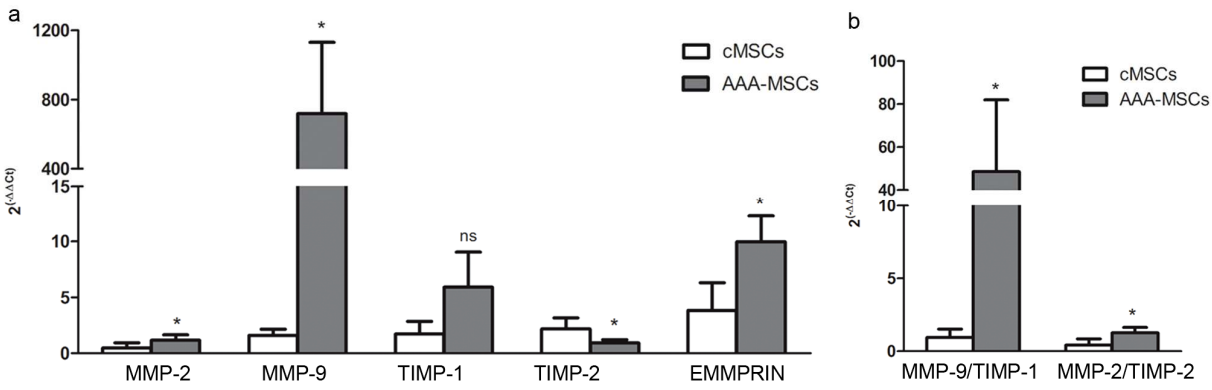


Figure 28. Expression of AAA molecular mediators in AAA-MSCs.

(a) Relative mRNA expression of MMP-2, MMP-9, EMMPRIN, TIMP-1 and TIMP-2 in AAA-MSCs compared to cMSCs. (b) MMP-9/TIMP-1 and MMP-2/TIMP-2 ratios. Results are expressed as fold change relative to cMSCs. Values are represented as mean ± standard deviation and are representative of at least three independent experiments carried out in triplicate (*p<0.05, unpaired t test).

Western Blot performed on whole cell lysates of MSCs isolated from the aortic wall, demonstrated an increased MMP-9 protein in AAA-MSCs compared to cMSCs. More specifically, an intensive signal in correspondence of MMP-9 bands at 92 kDa and 82 kDa could be detected in all the lanes representing AAA-MSCs, as shown in Figure 29a; conversely, a weak signal was detected in cMSC samples. Band intensities quantified by

densitometry were normalized to cMSCs, considered as reference: MMP-9 expression by AAA-MSCs resulted 149 times higher than cMSCs (Figure 29b).

These results on protein expression by AAA-MSCs are consistent with the above described analysis on AAA tissue revealing MMP-9 positive signal in correspondence of perivascular and stromal cells.

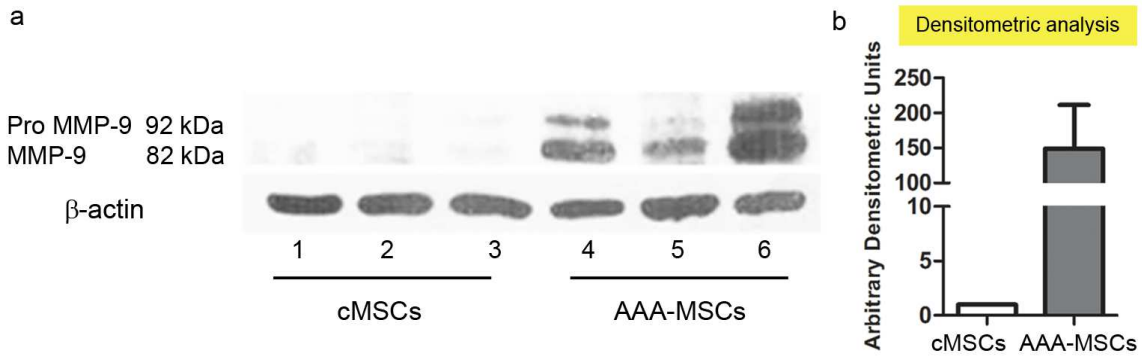


Figure 29. MMP-9 protein expression in AAA-MSCs.

(a) Immunoblot on AAA-MSCs and cMSCs cell lysates and bands quantification by ImageJ densitometric software.

Figure 30a is a representative gelatin zymography performed on cMSC and AAA-MSC conditioned media, to test MMP-2 and MMP-9 activity. MMP-9 secretion was mainly detected in AAA-MSCs (lanes 1-4), as reflected by the digestive activity in correspondence of 92 kDa, even if lane 4 displayed a weaker signal. Densitometry analysis indicated a significant increase of MMP-9 activity in AAA-MSC conditioned media, in comparison to cMSC samples (Figure 30a). Regarding MMP-2 secretion, we did not observe relevant differences between healthy and AAA samples; indeed, the degradative activity of MMP-2 could be broadly observed in AAA-MSCs and cMSCs. Band intensities quantified by densitometry were normalized to cMSCs (Figure 30b).

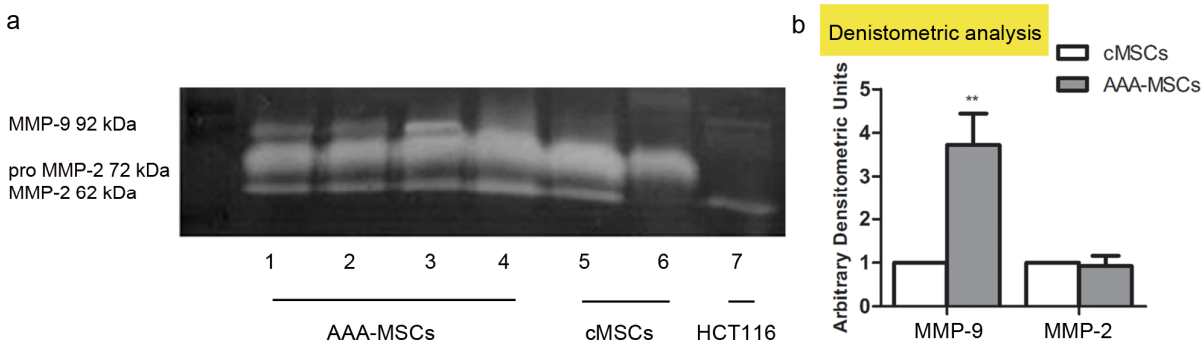


Figure 30. MMP-2 and MMP-9 activity assay on MSCs.

(a) Gelatin zymography performed on AAA-MSC and cMSC conditioned media: MMP-9 at 92 kDa could be detected only in AAA-MSC lanes (Lanes 1-4), whereas MMP-2 was broadly identified in correspondence of 72 and 62 kDa, in all the lanes, included cMSCs (Lanes 5-6); Lane 7 refers to HCT116, used as reference to identify MMP bands.

IMMUNOMODULATORY ACTIVITY OF AAA-MSCs: IN VITRO CO-CULTURE WITH PHA-ACTIVATED PBMCs

HLA-G and IL-10 expression following MSC cultured under inflammatory conditions

MSC immunosuppressive function was performed under inflammatory conditions, created trough a co-colture of MSCs seeded on culture flask as feeder layer and PHA-activated PBMCs. Real Time PCR performed on MSCs after 72 h co-cultured with PHA-PBMCs revelead a reduced immunomodulatory cytokine expression in AAA-MSCs, compared to cMSCs.

At first, we analyzed IL-10 mRNA, which resulted 3-fold decreased in AAA-MSCs ($p=0.0386$, unpaired t test) (Figure 31).

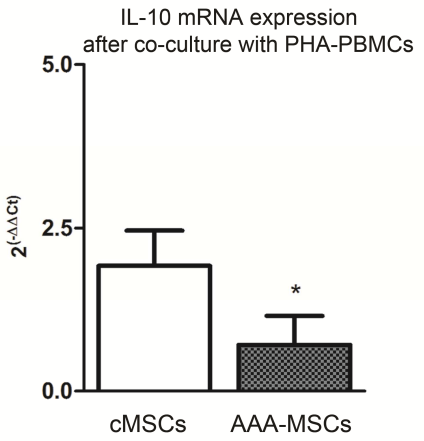


Figure 31. IL-10 mRNA in AAA-MSCs after co-culture with PHA-PBMCs, normalized to cMSCs.

Results are expressed as fold change relative to controls (cMSCs co-cultured with activated PBMCs). Values are represented as mean ± standard deviation and are representative of at least three independent experiments (*$p<0.05$; unpaired t test).

Beside IL-10, flow cytometer analysis was assayed to detect HLA-G expression on MSC surface. Figure 32a that confirmed that HLA-G was expressed on cMSC surface, as expected; interestingly, its expression resulted even before co-colture with inflammatory cells. Conversely, HLA-G was not detected on AAA-MSC surface (Figure 32b). Accordingly with this analysis, HLA-G was evaluated also through qPCR, resulting in a decreased, even if not significant, expression of HLA-G mRNA in AAA-MSCs (4-fold decreased in AAA-MSCs; no significant differences were observed), in comparison to cMSCs exposed to the same inflammatory condition (Figure 32c).

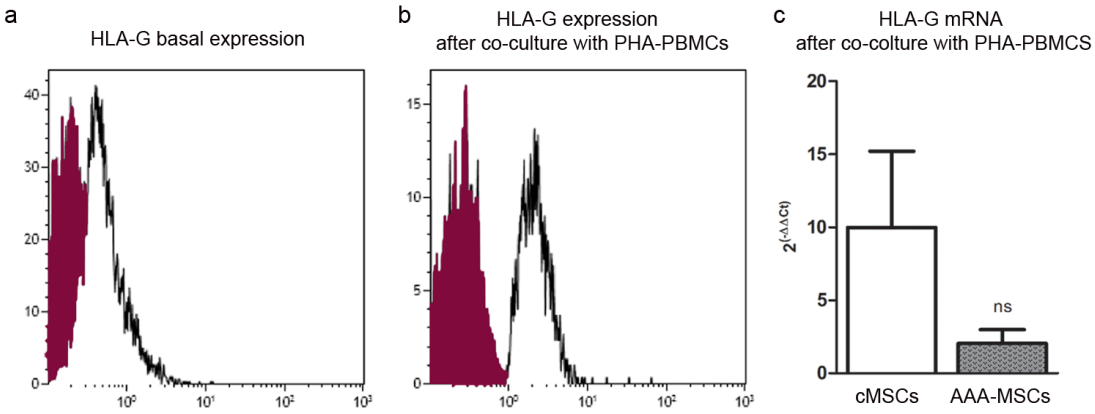


Figure 32. HLA-G expression in AAA-MSCs versus cMSCs.

HLA-G expression on AAA-MSCs and cMSCs, in basal condition, (a) without and (b) after 72 h co-culture with activated PBMCs. Purple histograms refer to AAA-MSCs, while white histograms are referred to cMSCs. Results are shown as a representative example of at least three independent experiments. (c) HLA-G mRNA expression in AAA-MSCs compared with cMSCs.

MSC effects on PBMC cell cycle

Among MSC clinical applications, many evidences support an effective immunomodulatory activity and contextually to the aneurysm disease, as object of our study, we tested this ability also on our cell model. As expected, cMSCs were able to inhibit PHA-PBMC DNA synthesis, conversely to AAA-MSCs. As observed in BrDU assay PHA-stimulated PBMCs cultured on cMSC feeder layer showed a lower proliferation rate ($p<0.05$, one-way ANOVA test), whereas the proliferative rate measured on PHA-PBMCs grown on AAA-MSCs was higher ($p<0.05$, one-way ANOVA test) and quite comparable to PHA-PBMCs cultured alone (Figure 33).

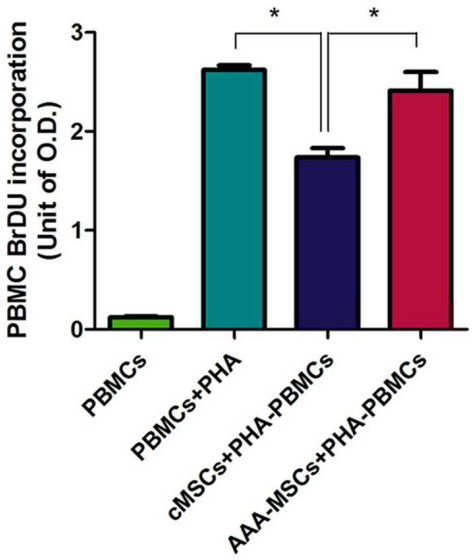
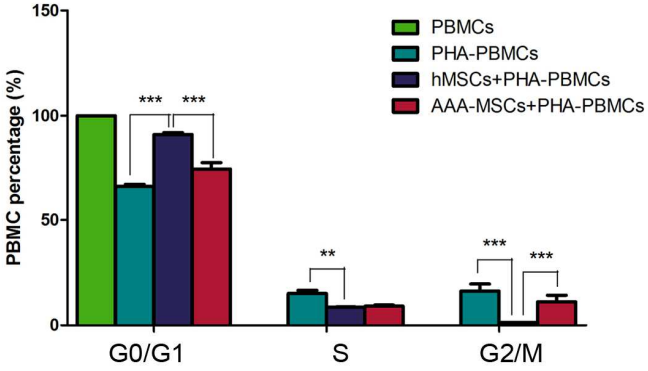


Figure 33. BrDU incorporation assay in PBMCs cultured with healthy and AAA MSCs.

BrDU is incorporated into DNA helix and it is an index of cell proliferative status. Data are expressed as mean of optical density units. (*$p<0.05$, one way-ANOVA, followed by Bonferroni post hoc test).

Data obtained from immunomodulatory assay were enriched with PBMC cell cycle analysis. Figure 34 is a representative cell cycle analysis and shows the PBMC percentage, measured by flow cytometer, for each culture condition, in presence of healthy or pathological MSCs. Not activated PBMCs were set as a reference control, indicated by 100% concentration during the G0/G1 quiescent phase (100%), whereas the PHA addition to PBMC cultures stimulated their progression along cell cycle and the following percentages were observed for each cell cycle phase: 65.9% ±1.6 in G0-G1; 15.2% ±0.9 in S phase and 18.9% ±4.8 in G2/M phase. The presence of cMSCs in PHA-PBMC cultures strongly reduced their percentage during active cell cycle stages (S phase: 8.6% ± 0.2 and G2/M phase: 1.3% ± 0.2) and enhanced their stabilization during G0-G1 (91.2% ± 1.4). PHA-PBMCs cultured in presence of AAA-MSCs did not show significant differences when compared to PHA-PBMCs cultured alone; indeed, they resulted mainly concentrated during G2/M phase (14.2% ±1.5) than G0/G1 (74.4% ± 5.4), whereas the cell amount in S phase was slightly higher than PBMCs grown with cMSCs (10.5 % ± 0.9).

a



b

PBMC Percentage

Culture condition	% G0/G1	% S	% G2/M
PBMCs	100	0	0
PHA-PBMCs	65.9 ± 1.6	15.1 ± 0.9	18.9 ± 4.8
PHA-PBMCs + cMSCs	91.2 ± 1.4	8.6 ± 0.2	1.3 ± 0.2
PHA-PBMCs + AAA-MSCs	74.4 ± 5.4	10.5 ± 0.9	14.2 ± 1.5

Figure 34. PBMC cell cycle analysis following co-culture with PHA-PBMCs.
(a) PBMC distribution along cell cycle phases; (b) summary of cell percentage in each cell cycle phase, calculated for all the culture conditions performed (**p<0.01, ***p<0.001; one way-ANOVA and two way-ANOVA test, followed by Bonferroni post hoc test).

Taken together, the results obtained from immunomodulatory assays, revealed a compromised function in AAA-MSCs, that lost the property to regulate the immune response as it can be observed for cMSCs, suggesting that inflammation may be more than a histopathological feature in AAA pathogenesis and it may constitute a key process influencing vascular resident MSC ordinary activities.

MMP-9 HYPER EXPRESSION BY MSCs UNDER PATHOLOGICAL CONDITIONS: POTENTIAL MECHANISMS TO MODULATE ITS EXPRESSION AT THE TRANSCRIPTIONAL LEVEL

cMSC influence on MMP transcription

MMPs are hyper expressed under an inflammatory context and many anti-inflammatory approach can be applied to regulate their expression. For this purpose, we investigated on the healthy cMSC potential to regulate MMP transcription by AAA-MSCs, appealing to the secretion of immunomodulatory molecules. MMP transcription was evaluated by qPCR and MMP-9 was shown to be reduced following co-culture of healthy and AAA MSCs (a 80% reduction observed in comparison to AAA-MSCs control; p=0.004, paired t test) and, in a less evident measure, after AAA-MSC culture in presence of cMSC conditioned media (0.8 fold lower than AAA-MSCs grown in conventional DMEM; p=0.0043, paired t test)

(Figure 35b). Conversely, MMP-2 mRNA was not modulated by cMSCs (Figure 354a), supporting the constitutive MMP-2 expression by cells of mesenchymal nature.

Inhibitory effect of anti-inflammatory cytokines on MMP-9 expression: the example of IL-10

IL-10 is an anti-inflammatory cytokine, ordinarily expressed by human MSCs and involved into immunomodulatory mechanism. We observed that the addition of soluble IL-10 to AAA-MSC cultures, at the concentration of 20 ng/ml, for 24 h, resulted in the reduced MMP-9 transcription (0.2 fold decreased, relative to untreated control, p=0.04, paired t test) (Figure 35c).

Therefore, an anti-inflammatory approach, addressed toward vascular MSCs, could be developed to restore their impaired function.

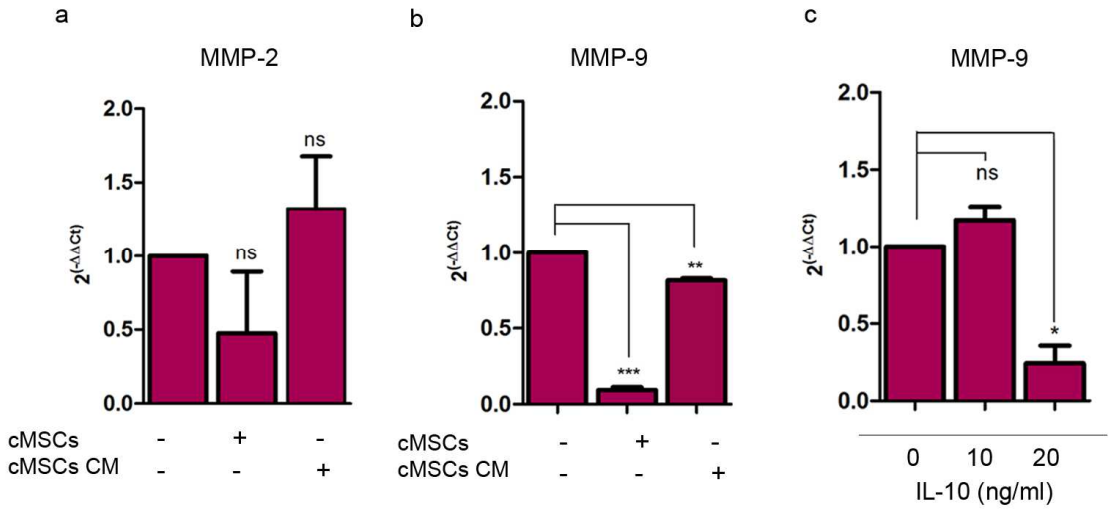


Figure 35. MMP modulation at the transcriptional level.

(a) MMP-2 and (b) MMP-9 transcription following 1) AAA-MSCs co-cultured with cMSCs and 2) AAA-MSCs cultured in presence of cMSC conditioned media. (c) MMP-9 transcript decrease in AAA-MSCs exposed to anti-inflammatory cytokine IL-10. Results are expressed as fold change relative to untreated AAA-MSCs. Values are represented as mean ± standard deviation and are representative of three indipendent experiments (*p<0.05, **p<0.01, ***p<0.001; paired t test). *CM: conditioned media.*

DISCUSSION

The human vessel wall represents a dynamic component of the blood vessel system, being made of distinct cell types, each of one contribute in a different manner to tissue homeostasis. An altered working of specific cellular and molecular functions can occur, in some cases as consequence to genetic defects or environmental factors, leading to vascular disorders.

Blood vessel diseases display a heterogeneous localization along the arterial tree, according to the structural characteristics of the vascular wall, that in turn depends on the heterogeneity of the resident cells and their reactions to pathological alterations. Some vascular segments are more susceptible to develop specific diseases and the large-diameter vessel aorta represents an example of how structural heterogeneity influences disease localization. Human aorta is composed of three segments: aortic arch, ascending aorta, descending thoracic aorta and descending abdominal aorta. Abdominal aortic media has peculiar morphological characteristics, consisting in a decreased elastin content correspondent to a reduced number of lamellar units (28-32), in comparison to the thoracic segment (55-60). This aspect, together with a poorer nutrient to the SMCs, makes abdominal aorta more susceptible to aneurysm occurrence (Ruddy et al, 2008).

Abdominal aortic aneurysms (AAAs) are degenerative aortic affections, ascribed to an extensive ECM proteolysis, which leads to the decrease of elastin and collagen proteins, responsible of vessel wall tensile strength. The ECM turnover is a physiologic process, necessary for the maintenance of tissue homeostasis and mediated by proteolytic enzymes, among which MMPs are the most well characterized and studied (Delay et al, 2007). MMP hyper expression and hyper activity can result into an exaggerate ECM degradation that, together with the SMC depletion, reduce the vessel wall tone. The presence of an inflammatory infiltrate, composed of T cells, B cells and macrophages, has been reported in aneurysm-affected tissues. Generally, AAAs are distinguished between atherosclerotic and inflammatory, basing on the different etiologic factor. On the basis of this classification, IAAAs are primarily associated with a more extensive inflammatory process and more evident signs and symptoms accompany their natural history. Non-inflammatory aneurysms are mostly related to atherosclerotic complications, even if current researches are investigating upon the nature of this relationship (Tonar et al, 2010). According to the most validated theory, these aneurysms arise as consequence to intimal atherosclerotic lesions. Beside these differences, the inflammatory infiltrate is extensively represented among all the aneurysm forms and recent investigators raised the possibility of an immune-

mediate origin for aneurysm initiation. One of the mechanisms supporting this view is the so-called molecular mimicry, that is the sharing of common epitopes between foreign and self antigens, taking to T cell activation also against self antigen (Ozsvath et al, 1996; Oleszak et al, 2004). The same vessel wall components, such elastin, collagen or the AAAP-40, have been proposed as antigen capable to induce T cell activation. In this regard, an antigen-driven T cell response has been suggested as leading cause to aneurysm development; moreover, aneurysm has been proposed as autoimmune disease (Hirose and Tilson, 2001).

In this view, targeting the inflammatory disease affecting the vessel wall, could represent a promising strategy to monitor aneurysm expansion and limit rupture cases, since wall rupture following the aortic progression is responsible of the 65-85% death cases (Sakalihasan and Limet, 2005).

Scientific research addressing the behaviour of human MSCs and their properties has gained great interest and important findings, overcoming the ethical question regarding the use of embryonic tissues as source of stem cells able to regenerate damaged tissues (ESCs) (Reubinoff et al, 2000). Indeed, MSCs can be easily isolated from different source tissues, including bone-marrow (Friednstein et al, 1970; Castro-Malaspina et al, 1980), fat (Zuk et al, 2002), umbilical cord blood (Erices et al, 2000), vascular wall (Ergün et al, 2011) and others. MSCs can be easily from human tissues and to expanded in *in vitro* cell cultures. The well-known MSC properties of multilineage differentiation and proliferative potential, can be exploited to treat many human pathological states, including cardiovascular diseases, brain and spinal cord injury, connective tissue disorders (Barry and Murphy, 2004). Moreover, MSCs are hypoimmunogenic, representing an important resource for transplantation medicine applications, such as GVHD additional therapeutic applications of human MSCs can be extended to the affections with an inflammatory origin, (i.e. Chron's disease, autoimmune pathologies, etc) since MSCs possess the capacity to negatively modulate immune reaction, exerting an anti-inflammatory function (Barry and Murphy, 2004; Chamberlain et al, 2007). This property is performed through different mechanisms, mediated by the release of anti-inflammatory cytokines (TGF, IL-10), and enzymes (IDO, NO), leading to the anergy and cell cycle blocking of T and B cells, inhibition of DC maturation, inhibition of NK cell cytotoxicity (Le Blanc and Ringdén, 2007; Ryan et al, 2007).

It is clear that MSCs could be effectively employed for many clinical applications, even if optimization and standardization of isolation protocols have not been established yet. Different tissue sources as well as different donor age determine a high heterogeneity degree, so that MSC preparations contain distinct subsets of cells in terms of differentiation and proliferative status (Hart, 2014). This may influence the outcome of MSC administration as therapeutic tools.

Less is known about the potential side effects or, more particularly, the MSC participation to disease initiation and progression. Some literature works have raised this issue, suggesting that MSCs, under specific conditions, can undergo an impairment of their basic functions, referring to their proliferative potential, differentiation and immunomodulation. In these conditions, MSC behaviour can evolve into a pathologic direction, leading to the initiation or the exacerbation of tissue damage. For example, MSCs isolated from patients affected by osteoarthritis, determined by a progressive cartilage deficiency, demonstrated low proliferative potential and reduced adipogenic and chondrogenic differentiation (Murphy et al, 2002). Evidences on an ineffective immunomodulation have been reported in a mouse model of collagen-induced arthritis (Li et al, 2010) and also in multiple myeloma patients (Djouad et al, 2005). Few works addressing this topic suggest that a defective MSC activity and immunosuppression could exert a crucial role in inflammatory affections.

Given that many arterial diseases have an inflammatory basis, we found interesting the study of the MSCs resident within the vascular wall. It has been discovered that endothelial progenitors and cell populations with mesenchymal properties localize at the adventitia level, occupying the boundary with media tunica (Zengin et al, 2006). Our research group achieved an experience in this field, isolating MSC populations from vessel wall of healthy donors (Pasquinelli et al, 2007; Pasquinelli et al, 2010). Interestingly, these cells displayed high resistance to stressful conditions, indeed in our previous work (Valente et al, 2014) the MSC isolation from cryopreserved arteries was successfully performed. Arteries belonging to healthy donors were collected after 5-years store in liquid nitrogen; cells were cultured according to standard conditions (37 °C; 5% CO_2) and did not show signs of sufferance, conversely they were characterized, expressing typical mesenchymal markers. These Vascular-Wall MSCs (VW-MSCs) displayed adipogenic, osteogenic, chondrogenic and angiogenic potential, clonogenic property and immunoregulatory activity. Exploring

the MSC resistance and management of vascular diseased condition represents the next step of our studies.

In this regard, AAA constitutes an ideal pathological model to perform vascular MSC evaluation of their basic characteristics and functionality. We therefore collected twelve aortic tissues from patients subjected to open repair for abdominal aortic aneurysm, provided by the Vascular Surgery Unit of S. Orsola-Malpighi Hospital (Bologna). The first observation derives from the *in situ* detection of MSCs, evaluated trough an immunostaining for mesenchymal antigens on AAA sections. Beside the great amount of inflammatory cells invading the AAA wall, cells positive to CD44 and CD90 could be localized at the level of the perivascular niche.

Later, we focused on the MSC component of the aortic wall. Healthy aortic MSCs (cMSCs) and whereas aneurysm-derived MSCs (AAA-MSCs) were compared on different levels: in vitro morphological features, immunophenotype and molecular profile. A consistent number of AAA-MSCs could be obtained from all the tissues we collected and all the cell samples revealed basic characteristics comparable to our previous observations on cMSCs.

AAA-MSCs displayed a typical fibroblastic shape and adherence to the plastic culture substrate, representing primary peculiarity of MSC identification. Then, AAA-MSCs showed a typical mesenchymal antigen expression pattern on their surface, as demonstrated by flow cytometry: mesenchymal and perycite markers CD44, CD90, CD73, CD146 and PDGFR-beta were broadly expressed on AAA-MSC surface, whereas hematopoietic and endothelial antigens CD34, CD14 and CD44 were not detected. AAA-MSC immunophenotype was totally specular to cMSC, as we observed for stemness markers. We analyzed typical embryonic stem cell marker, represented by Nanog, Oct.4 and Sox-2, which posses a regulatory role in determining stem cell properties: these genes compose a transcriptional core unit that activates downstream genes involved in pluripotency and self-renewal of stem cells and, on the other hand, repress genes regulating cell developmental processes (Chambers et al, 2003). This group of stemness markers resulted to be expressed by AAA-MSCs, and the semi-quantitative analysis by Real Time qPCR showed that no significant differences existed between healthy and pathological MSCs.

Definitely, the pathological arterial remodeling and the inflammatory process affecting the aortic wall, did not influence the basic properties of vascular resident MSCs.

We therefore explored AAA-MSCs from a functional point of view, investigating on the MMP expression and the ability to counteract the immune reaction. MSC mobilization from bone marrow and their homing to diseased tissues, are mediated by MMPs that, cleaving specific ECM substrates, allow MSC migration. In particular, among MMP members, MMP-2 and MT-MMP-1 are constitutively expressed by human bone marrow MSCs; a work by Ries et al (2007) showed the different MMP expression pattern in bone marrow MSCs and how their expression and activity are influenced under specific stimulations. In this regard, MMP-9 is known to be an inducible protein, indeed its expression usually depends on external factors, like TNF-α. Contextually to aneurysm initiation and progression, MMP-2 and MMP-9 have been demonstrated to play a decisive role into ECM degradation (Tamarina et al, 1997; Longo et al, 2002). Based on these premises, we evaluated expression levels of MMP-2, MMP-9 and their respective tissue inhibitors, TIMP-1 and TIMP-2, in AAA-MSCs compared to healthy cMSCs. The most relevant results were observed for MMP-9 expression, both at the mRNA and protein levels: a prominent mRNA transcription (400-fold higher than cMSCs) and protein production were observed in AAA-MSCs; MMP-2 and TIMP levels were quite similar between the two groups of our study, whereas EMMPRIN, or CD147, was shown to be up-regulated at the mRNA level in AAA-MSCs. According to literature studies, EMMPRIN have been associated with atherosclerotic plaque (Yoon et al, 2005) and aneurysm pathogenesis (Chen et al, 2009), as inducer of MMPs. Interestingly, EMMPRIN is produced by stromal cells, so its upregulation supports the view of a MSC defective functionality under pathological conditions. Further confirmation of data obtained on vascular MSCs, derived from the tissue analysis of MMP-9 expression. An increased MMP-9 mRNA was recorded in AAA tissue, which may contain different cell sources of MMPs, especially inflammatory cells, like macrophages. Effectively, immunostaining for MMP-9 labeled many inflammatory cells, but MSC-like populations positive to MMP-9 were also detected between perivascular cells of vasa vasorum district and stromal cells.

In order to elucidate the mechanism leading to this MSC degradative and pathological phenotype, we tested AAA-MSCs for their immunomodulatory activity. The premise to the second phase of the present study, derives from the extensive inflammatory infiltrate observed inside aortic wall and the hypothesis that AAA-MSCs could have lost their innate capacity to modulate the immune reaction. BrdU incorporation and cell cycle assays on PHA-stimulated PBMCs cultured in presence of AAA-MSCs, indicated inflammatory cell

ongoing along the cell cycle; PHA-PBMCs did not arrest at the Go/G1 stage, as occur in presence of cMSCs.

The continuous PHA-PBMC proliferation could mean that AAA-MSCs behave like quiescent cells, unable to arrest the proliferation of PHA-PBMCs, possibly explained with the reduced expression of IL-10 and HLA-G, well-documented cytokines that mediate some of the MSC-dependent immunosuppressive mechanisms.

The defective MSC immunomodulation could represent a critical step into AAA evolution; MMPs can be regulated at many levels, including transcription, so we evaluated possible regulatory mechanisms for MMP-9 transcript levels. Co-culture experiments of healthy MSCs and pathological MSCs took to a significant reduction of MMP-9 transcription, following direct contact between healthy and AAA-MSCs; a decrease, even if less evident, was observed in AAA-MSCs cultured with cMSC conditioned media, suggesting that cMSCs may regulate pathological mediators at the mRNA level, also in a paracrine manner. Thus we supposed the involvement of IL-10, hat has been shown to inhibit MMP-9 transcription in human placental trophoblast (Roth and Fischer, 1999). In fact, preliminary data showed a negative MMP-9 transcriptional regulation in AAA-MSC exposed to IL-10, indicating that IL-10 administration could represent a key strategy of MMP-9 regulation, in addition to restore MSC immunomodulation (Rizzo et al, 2011).

Conclusions

The present study explored the basic and functional characteristics of a MSC population isolated from aortic well affected by aneurysm. The molecular mechanisms involved in arterial remodeling and chronic inflammation, characteristic of AAA disease, do not affect the survival/self-renewal properties of the vascular MSC niche. Meanwhile, AAA-MSCs displayed defective functions, in terms of ECM turnover and immunomodulation. Indeed, AAA-MSCs showed to hyper express MMP-9 and its inducer EMMPRIN, in comparison to cMSCs isolated from healthy aortic wall. Moreover, AAA-MSCs behaved in a weak manner with respect to activated immune cells, which in turn show to proceed along the cell cycle. This is opposed to what has been documented among MSCs isolated from healthy aorta, suggesting a clear MSC dysfunction under pathological context. On the other hand, both healthy MSCs that soluble factors associated with anti-inflammatory abilities, showed to negatively modulate MMP-9 at the transcriptional level.

These preliminary data suggest that AAA-MSC dysfunction can be resolved through an anti-inflammatory approach; based on these observations, it would be of great importance exploring the mechanisms and the molecular targets able to restore and improve MSC immunomodulation. An approach focused on MSC function, aimed at potentiating their therapeutic abilities could represent novel promising strategies to monitor aneurysm progression, as well as other degenerative diseases in the cardiovascular medicine field.

Limits of our study

Limitations of our study reside in the selection of the control subjects, specifically the limited number (3 healthy controls vs 12 AAA patients) and the absence of sex and age-matching with patients. Regarding the age gap, we cannot exclude that our data on MMP-9 mRNA hyper-expression and activity could be a result of the physiologic vascular aging process. Preliminary data on total RNA extracted from aortic tissue of two male subjects, aged between 56 and 70, age-matched with AAA patients collected, showed a 9-fold increased MMP-9 mRNA transcription, in comparison to healthy aortic tissues used in our study (data not shown). Conversely, the MSCs isolated through enzymatic digestion behaved like cMSCs from young donors, as revealed by results on MMP-9 mRNA and immunomodulatory ability (data not shown). They were not used to extend the control group in our study because their clinical data (i.e. cause of death associated with cerebral hemorrhage, presence of vascular calcifications) were not in accordance with the inclusion criteria. Thus, the age of control subjects does not significantly influence the differences between our study subject groups, as the presence of the aortic dilation does. Our controls can be considered as healthy subjects with no cardiovascular risk and as negative controls for AAA molecular mediators expression in human perivascular MSCs.

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