Charles University

Faculty of Pharmacy in Hradec Kralove

Department of Biological and Medical Sciences

Soluble Endoglin effects on endothelial dysfunction markers in mice

(Master Thesis)

Supervisor: prof. PharmDr. Petr Nachtigal, Ph.D

Hradec Králové 2018 Konstantinos Bountalis

AUTHOR'S DECLARATION

I declare that this thesis is my original work. All literature and other resources which were used during the preparation of this review are listed in bibliography and properly cited.

Date: *Signature:*

AKNOWLEDGEMENT

Firstly, I would like to express my sincere gratitude to my supervisor Professor. PharmDr. Petr Nachtigal, Ph.D for his continuous and valuable support of my Master thesis, for his patience, motivation and immense knowledge. His guidance helped me in all time of understanding and writing of this thesis. I could not have imagined having a better mentor for my undergraduate study.

My sincere thanks also belong to Mgr. Barbora Vitverová who participates in my experiment and gave me valuable insights and its member of the Department of Biological and Medical Sciences who provided me the courage and equipment i needed in order to complete this thesis.

Last but not least, I would like to thank my family and friends: my parents, my sister, my friend Dimitris for supporting and advising me spiritually throughout writing this thesis and my life in general.

ABSTRAKT

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Název diplomové práce: Vliv dlouhodobého působení solubilního endoglinu na markery endotelové dysfunkce u myší

Endotelová dysfunkce je spojena s modifikací množství endotelových buněk, poruchou cévní homeostázy a vede k prozánětlivému stavu, stejně jako k progresi vaskulárního poškození. Je charakterizována vysokou expresí adhezních molekul, sníženou vazodilatací a zvýšenou cévní permeabilitou.

Cílem této diplomové práce bylo zjistit vliv solubilního endoglinu na expresi VCAM-1 a ICAM-1 u myší. Experiment byl proveden za použití imunohistochemických metod ve světelné a fluorescenční mikroskopii.

Podávání vysokutové diety vedlo k rozvoji mírné hypercholesterolemie u zvířat v obou skupinách. Výsledky imunohistochemické analýzy ukázaly, že exprese VCAM-1 a ICAM-1 v aortě se významně neměnila u myší s vysokými hladinami solubilního endoglinu ve srovnání se skupinou kde byly hladiny solubilního endoglinu nízké.

Výsledky této studie tedy neprokázaly zvýšení zánětlivé reakce na cévním endotelu aorty u myší s vysokými hladinami solubilního endoglinu.

ABSTRACT

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Endothelial dysfunction is associated with modification of endothelial cells amount failing to maintain vascular homeostasis and result in variable pathological inflammatory states as well as in progression of vascular damage. Particularly its state is characterized by high expression of cell adhesion molecules, reduction of vasodilation and accumulation of cholesterol molecules.

The purpose of this study is to detect soluble endoglin effects on the expression of VCAM-1 and ICAM-1 in aorta in mice. The experiment was accomplished utilizing specific immunohistochemical methods for the detection of expression of selected adhesion molecules. High fat diet administration resulted in development of mild hypercholesterolemia in mice in both groups. High levels of soluble endoglin however did not significantly affect the expression of VCAM-1 and ICAM-1 in aortic endothelium when compared to mice with low levels of soluble endoglin.

The results of this study did not show pro-inflammatory effects of soluble endoglin in aorta in mice.

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.List of abbreviations

- Activitin receptor like kinases - ALK
- Acute coronary syndrome – ACS
- Apolipoprotein E – apoE
- Avidin/Biotin complex – ABC
- Calmodulin – CaM
- Cell adhesion molecules – CAMs
- Cyclic guanosine monophosphate – cGMP
- Endoglin – CD105, Eng
- Endothelial cells – ECs
- Endothelial nitric oxide synthase – eNOS
- Endothelial protein C receptor – EPCR
- Glucose transporters 1,4 – GLUT-1,4
- Hereditary hemorrhagic telangiectasia – HHT
- Horseradish peroxidase – HRP
- Human umbilical vein endothelial cells – HUVECs
- Hyalunorate – HA
- Immunoglobulin superfamily – IgSF
- Insulin receptor substrate 1 – IRS-1
- Intercellular adhesion molecule 1 – ICAM-1
- Liver X receptor – LXR
- Low density lipoproteins – LDL
- Low density lipoprotein receptor – LDLR
- Lymphocyte function associated antigen – LFA
- Matrix metalloproteinase 14 – MMP-14
- Mucosal addresin cell adhesion molecule – MAdCAM
- Neural cell adhesion molecule – N-CAM
- Nicotine amide adenine dinucleotide phosphate – NADPH
- Nitric oxide – NO

- Nuclear factor-κB – NFκB
- Peroxidase/Antiperoxidase – PAP
- Phosphoinositide-3 kinase – P13 kinase/ Akt system
- Platelet activating factor – PAF
- Prostacyclin – PGI2
- Protease activating receptor 1 – PAR-1
- Protein tyrosine phosphatase receptor – PTPRT
- Reactive oxygen species – ROS
- Soluble endoglin – sEng
- Transforming growth factor beta – TGF-β
- Vascular cell adhesion molecule 1 – VCAM-1
- Vascular endothelial growth factor – VEGF
- Very late antigen 4 – VLA4
- Von Willebrand factor – VWF
- Zona pellucida – ZP

1. Introduction

Endoglin is a type of integral membrane 180-kDa homodimeric glycoprotein that binds to TGF-β superfamily acting as a co-receptor that interacts with TGF-β type I, II signaling receptors and facilitates cellular responses to TGF-β (1). Due to its expression on smooth muscle and endothelial cells of variable blood vessels and overexpression upon a pathological condition (e.g. inflammation), it has an essential role in cardiovascular disease progression, vascular remodeling, tumor angiogenesis, hereditary hemorrhagic telangiectasia (HHT) while it contributes, among other factors, in development of diabetes mellitus, hypertension, preeclampsia and possibly to atherosclerosis (2).

Soluble form of endoglin (sEng) is defined as an extracellular domain released of the homodimeric transmembrane glycoprotein (Eng) by proteolytic cleavage and detected in blood of patients diagnosed with numerous disorders, including atherosclerosis, preeclampsia, HHT and cancer (3). Until now its role is not definite but since recent studies took part, it was observed that during the progression of pathogenesis related with endothelial damage or inflammation sEng levels were raised, thus it was assumed it may have a potential effect in endothelial alteration and cardiovascular disorders (4).

Endothelial dysfunction refers to its decline to modulate the normal regulation of vascular tone, cellular adhesion, vascular smooth muscle migration and protection against thrombosis, often as nonadoptive response to pathological trigger. It mainly concerns impairment of vasodilator substances process including nitric oxide (NO) and prostacyclin among with the raised production of vasoconstrictor substances and decrease protection to thrombosis (5). A common feature of its state is the increased amount of oxidative stress leading to enormous production of super-oxide anions, consequently breakdown of NO which is catalyzed by endothelial nitric oxide synthase (eNOS) and is one of the most dynamic vasodilators.

Due to its functional relevance, such decrease of its activity may conduce to the induction and development of atherosclerotic lesions and further to promote atherosclerosis (6). However, it has been recognized that Eng limits eNOS expression in endothelium through Smad2/3 transcription factors without presence of TGF-β whereas it was declared

that sEng has a dynamic opposite role on vascular endothelium leading to the blockage of eNOS dependent-vasodilation as well as overexpression of cellular adhesion molecules (7).

Nevertheless, further elucidation is needed in order to evaluate if a combination of pathological disorders that promote endothelial dysfunction contributes to the endothelial function in response to high levels of sEng. Due to the lack of sufficient knowledge we aimed to evaluate the effects of sEng in mice afflicted with endothelial dysfunction, using as biomarkers the pro-inflammatory VCAM-1 and ICAM-1 molecules in order to observe a possible immunological response in endothelial cells of aortic sections. Therefore, this study was conducted by several immunohistochemical methods and quantification of mice proceeded through western blot analysis. Specifically, we used transgenic mouse model expressing human sEng (Sol-Eng^{+}) and divided them into two groups. Application of biomarkers took place on microscope slides containing aortic sections, utilizing various immunohistochemical techniques while visualization of results was accomplished under the light and fluorescence microscope.

2. Theoretical Part

2.1 Histological structure of blood vessels

Blood vessels system, as widely known vascular system, acts as passage of blood flow. This system is responsible for the transfer of different metabolites, specific hormones, a variety of nutrients and waste products. In fact, it occurs within the blood and tissues in the whole body, as well as in immune mechanisms. Nonetheless, it is also pledged with maintenance of body temperature (8). Vascular system consisted though by five dominant types of blood vessels. All of them are close related with blood circulation. Arteries carry the blood away from the heart while arterioles, which are smaller branches of arteries, ensure blood encountering, capillaries are involved in gas exchange process and veins/venules transfer the blood back to the heart (9).

However, its histological structure determines how they are able to control this specific circulation process. Blood vessels are consisted of one to three main layers; tunica intima, tunica media and tunica adventitia. Specifically, tunica intima (inner coat) is composed by a monolayer of endothelial cells, an elemental basal lamina and "sticking" molecules (fibronectin, laminin) (8). In any case, intima of arteries, are mainly consisted of endothelial cells, smooth muscle cells and sometimes white blood cells (lymphocytes or monocytes). On the other hand, endothelial cells facilitate vascular homeostasis by specific interaction between circulating cells and cells which present in the vascular wall (e.g. smooth muscle cells). They've also a unique ability of forming an interface among blood and tissue, therefore because of its ability endothelial cells could be vulnerable to endothelial changes in composition and blood flow (10). Tunica media (middle coat), is dwelled of muscle fibers, arranged in lamellae according the size of the vessel while it is further characterized by the wall thickness, with respect to arterial issue. Tunica adventitia (external coat) is linked with a perivascular tissue existing in small pieces of fibroblasts that serves as partial sticking material of the matrix as well as a protective coat against over development of the media. Moreover, it involves elastic fibers, nerves and the small

vasculature which is called “vasa vasorum” which facilitates blood flow to the vessel wall and is developed from adventitia to media of large arteries and veins (8).

Arteries from histological point of view consisted of three basic layers of all cardiovascular tissues and are divided into two basic groups. Hence, principally, the first layer is tunica intima which consists of a constant endothelium regulated by a flexible, collagenous coat of non-specific thickness. Second layer is tunica media, a layer of various ratios of muscular and flexible components. Finally, adventitia is the third layer, which is a relatively thick collagenous layer that contains vasa vasorum.

Arteries are categorized into elastic and muscular. Elastic arteries obtain the blood from the heart and spread it in large volumes during systole. Further, it crunches the blood amount without a loss of energy. This type is relatively flexible containing collagen and elastin fragments. Thus, every significant artery of this group has to show predominant stretch with respect to each pulse. Aorta, carotid and subclavian arteries, are great examples of this group. Histologically, elastic arteries consisted of three layers; tunica intima, media and adventitia. Intima contains an endothelial layer, embedding with a collagenous tissue. The thickest part of the tissue it belongs to media though, involving elastin, collagen and smooth muscle additionally. Adventitia is comprised by a big collagenous coat, being occupied with vasa vasorum providing constant “fund” to the external vessels (11).

Muscular arteries are medium sized, they receive blood from elastic arteries and split it in different vessels (e.g. arterioles) and are used as energy storage area caused by contraction of the heart. Cerebral, coronary and radial arteries are examples belonging to this group. Muscular arteries consist of three histological layers like elastic arteries, but with functional differences. Tunica intima contains an endothelial coat embedded with a collagenous layer, having an external unique tissue, which is frequently named as “elastic lamina”. Tunica media exists outside of the elastic lamina, involving a fine smooth muscle layer, which is circulated by another light elastic tissue, which is called “external elastic lamina”. Adventitia consisted of another collagenous layer, having various elastin fibers (11).

Moving forward, arterioles or small arteries are located in the arterial side of the vascular system. Principally, arterioles facilitate the arterial pressure due to their distinctive ability of supplying resistance to blood flow. Histologically, the wall of arterioles is comprised by three layers. Intima contains an endothelial layer on the top of a collagenous membrane. Media is consisted of an internal elastic lamina, surrounded by one or two smooth muscle layers, while adventitia is made of collagen fragments, nervous completion and fibroblasts (12).

Capillaries have a unique structure, enabling transportation of water, nutrients, gases and waste products between blood and tissues. Also, they are responsible for the development and separation of these transporting vessels (13). They don't work freely; besides, they are regulated by a capillary bed, which is a strong network of respectful capillary mesh. Capillary bed is composed by two groups of vessels, firstly capillaries that connects arterioles and promotes transportation between cells and circulation and secondly the vascular shunts, which are small vessels that branches arterioles and venules, enabling blood to flow. They are three major classes of capillaries; continuous, fenestrated and sinusoidal. Continuous is made of an endothelial layer, activating light molecules to distribute through solid and compact junctions. Non-distributed fragments though, are known as "intercellular clefts". Fenestrated capillaries contain small holes which partially "filters" any non-welcome material, permitting small molecules and proteins to flow. Sinusoidal, shows a unique structure due to their pored capillary cleft in the endothelial layer. Their distinctive shape corresponds to their special ability, permitting erythrocytes and lymphocytes to enter by using basal lamina. This type of capillaries is found in the bone marrow, lymphatic nodes and adrenal glands. Some others are located in the spleen and the liver, where do not present any compact fragments (11).

Venules are located in the continuous ending of capillaries of microcirculation. They are tiny vessels which enable the restore of deoxygenated blood from the capillary bed to greater blood vessels, known as veins. Their wall is consisted of an internal endothelium layer attaching a basement membrane, a middle coat which is made of elastic and smooth muscle tissue and an external layer composed mainly by a connective tissue. Furthermore, their structure is characterized by various fenestrations, allowing the blood to diffuse

rapidly from the bloodstream in their walls. Venules allows movement of white blood cells in the circulation directly, introducing to the lymphatic node are called “high endothelial venules”. This kind is being occupied particularly in post-capillary venous system (11).

Veins, return the blood from the capillaries, all the way through the venules of various parts of the body, back to the heart (14). They are classified as pulmonary, portal, systemic, deep, superficial and communicating veins. Pulmonary veins, transfer oxygenated blood from the lungs to the heart, while systemic form channels which the blood can run off and drop deoxygenated blood to the heart. Systemic veins are subdivided into deep which are located deep enough in the body, matching with complementary arteries, and superficial that are found closely to the external tissue without having any complementary artery. Communicating veins, branch both deep and superficial together, having a key role in venous system (11). Portal veins, are positioned in the abdomen which delivers the venous blood from the spleen to the liver and further is transmitted to the hepatic veins and then up to inferior vena cava (14). With respect to the arteries, veins ensure the proper direction of blood flow and they include valves, which guarantee specific blood flow within the heart, avoiding backward direction of venous blood (11). Histologically, they are enclosed by three layers; intima contains an endothelial coat seating with a collagenous basement membrane, while superiorly venous valves are formed. Media is made of a massive smooth muscle layer which usually contains elastic fibers and adventitia includes a collagenous membrane, which is tightly connected with the surrounding tissue (15).

2.2 Vascular endothelium and its function

Vascular endothelium is defined as a layer composed of endothelial cells (ECs) covering entirely all blood vessels in the vascular system conclusively. Endothelium structure varies, but usually in a fully-grown person it weights roughly 1 kg filled nearly with ten trillion (1.6×10^{13}) cells. Despite of its functional importance, endothelial layer is still motionless featured with a movable coat, forming a strong, semipermeable barrier responsible for the proper transportation of short and large molecules. Further is characterized as a dynamic multi-functional body chargeable for the development or protection of thrombosis by keeping the non-thrombogenic blood tissue combination balance and for thrombolysis, vascular tone, platelet adherence and major blood flow additionally (16,17).

Endothelium acts as a barrier allowing transportation of cells and/or molecules in the blood circulation and thus completing the positive conclusion of metabolic demands in the organism. Therefore, ECs are responsible for the conveyance of glucose, amino acids and caveolae throughout narrow pores, enabling the access of molecules from the circulation. Emphasizing in glucose transport, it is a fact that GLUT-1,4, which belong to GLUT supergene and glucose transporters group, are expressed through ECs. GLUT-1 is very rich in EC number, while expression of GLUT-4 is very important for the achievement of glucose transport. However, most GLUT transporters are expressed through the endothelial tissue of blood brain barrier. Transportation of amino acids is mainly accomplished by a cationic amino acid transporter (y^+). There are multiple options of amino acids delivery, but this cationic system is preferred, because it allows transportation of L-arginine. Success of this transportation is so crucial considering that L-arginine is a substrate of nitric oxide essential for the vascular tone balance. Caveolae is described as formed like pockets in the cell membrane modulating the transcellular delivery of ECs and act as principal cell carriers for this delivery system. Furthermore, they are involved in nitric oxide signalization by caveolin-1, which is a protein introduced in cytoplasm of plasma membrane in order to facilitate the caveolar process. In all, endothelium transportation vascular system occurs through compact pores allowing molecules which are welcome and

inhibits the entrance to those that should be avoided. Great example of this process is the blood brain barrier, which controls the entrance of ions, large molecules and various harm cells (17).

On the other hand, endothelium controls vascular outflow and vasomotor tone, by producing vasoconstrictive (endothelins, platelet activating factors) and vasodilated (nitric oxide, prostacyclin) substances keeping the blood pressure balance. However, production of the above mentioned substances is very crucial for the proper strategy against inflammatory cells and maintenance of thrombosis (17).

Nitric oxide is the most dominant vasodilator. Genesis of nitric oxide by endothelium helps to maintain vascular tone of vasodilation in normal levels, protecting elevation of blood pressure by forcing vascular smooth muscles to rest by binding to guanyl cyclase. Also, it is major contributor in prevention of thrombosis, by inhibiting platelet adhesion, improving platelet separation and deactivation of phospatidyl-inositol 3 kinase, which is essential for any conformational change in glycoproteins IIb-IIIa. Nitric oxide is produced in endothelial cells through the oxidation of amino acids L-arginine to nitric oxide, catalyzed by nitric oxide synthases (NOS) (18).

Prostacyclin (PGI2) belongs to eicosanoid family while it is only synthesized when a negative imbalance in the endothelial hemodynamic function occurs. It is freed from endothelial cells and bind to platelet and/or smooth muscle cell receptors, in order to relieve any vasoconstrictive state and desegregation of platelets (18).

Endothelins are great vasoconstrictors, produced by endothelial cells and contribute in the maintenance of vascular tone. There are three types of endothelins, but only one is released from endothelial cells (ET-1). As it follows it causes vasoconstriction only in non-vascular tissue. Vasoconstrictive effect though, starts immediately from the time of triggering ET_A receptors, located in vascular smooth muscles to the time of irritating ET_B receptors which are found in endothelial cells and causing vasodilation. Moreover, ET-1 is responsible for cell propagation, developing of various genes (prostaglandin, collagenase) and imitative platelet growth factor (16).

Platelet activating factor (PAF) is classified in phospholipid group, which is closely connected with endothelium surface. It has minor influence in vascular tone, while it ensures proper functioning of white blood cells, bounding to its receptor found on the surface of endothelium (16,17).

Apart of regulating the vascular tone, endothelium is responsible for thrombosis blockage and promoting of thrombolysis (diminishing of thrombus). Thus, it has a key role, keeping hemostatic balance accurate by the continuous supply of a pseudo-thrombogenic vascular internal layer, which assures the proper blood flow. Upon a vascular injury or any hemostatic disturbance, endothelial cells are demanded to "build up" again the vascular purity, avoiding any probable clot formation at any part of the organism. Consequently any affection in this strict balance could result in vascular bleeding and/or thrombosis (19). Besides its characteristics endothelium contributes in the proper function of coagulation assuring normal coagulation while at the same time it initiates protein C and S pathway. Triggering Protease-Activated Receptor 1(PAR-1) by thrombin with the activated endothelial protein C receptor (EPCR) complex result in precise anti-coagulant and anti-inflammatory effects (18).

During an inflammation, an infection or any other morphological damage, immediately a platelet-leukocyte interaction takes part. It is a complicated, rapid procedure occurring on the endothelium and is clarified by the endothelium its self. The whole process involves Weibel-Parade bodies having large components, such as von Willebrand factor and P-selectin, which are essential for the stimulation of platelet leukocyte adhesion. Von Willebrand factor (VWF) is a glycoprotein having various polypeptide chains and plays a critical role during a vascular damage, triggering platelet adhesion molecules and promoting platelet aggregation (20). P-selectin is saved in Weibel-Parade bodies, which exist as secretory channels, and in case of morphological damage it bridges cell surface mucin on leukocytes (16).

Furthermore, endothelium is a great contributor for the genesis of blood vessels from the pre-existed ones. This process is known as "Angiogenesis" and is mediated by Vascular endothelial growth factor (VEGF) formed by endothelial cells and some others, binding to

specific receptors on the surface of endothelium. Also, VEGF takes part during an inflammatory response by promoting cell adhesion molecules (21).

2.3 Endothelial dysfunction

Noticeable is the fact that a presumed negative imbalance in the well-organized system known as endothelium, could result in hazard and/or either fatal consequences. Thus, endothelial dysfunction mainly affects cardiovascular system and formation of atheromatous plaque has a serious influence in pathogenesis of hypertension and thrombosis while it contributes in a false disturbance of diabetes and it is partially responsible for metastatic cancer issue as well as the connection with some other diseases (18).

Regarding the cardiac system it is reported that a potential change in the properties of endothelial vascular wall can lead to acute coronary syndrome (ACS) or formation of atheromatous plaque and further genesis of atherosclerosis. Specifically, based on the importance of endothelial function, there are four main pre-stages which endothelium is involved for the occurrence of ACS; plaque rupturing or destabilization, vasoconstriction, increase of oxidative stress, vasa vasorum and bleeding (18,22)

Plaque fracture is a fundamental process resulted by an inflammatory action that "employees" several cell units and various pro-inflammatory factors including cytokines, chemokines and lymphokines. This important procedure is driven by nitric oxide decreasing the expression of inflammatory factors and adhesion molecules take part during an inflammation. Consequently, a progressive plaque susceptibility occurs (23). Plaque vulnerability is facilitated by transcription factor nuclear factor-κB found in many inflammatory proteins and take part in atherosclerosis. Hence, endothelial dysfunction is related with ACS due to the reduction of anti-inflammatory expression (24).

Vasoconstriction linked with endothelial dysfunction contributes to stimulation of coronary plaque separation. On the other hand, a great adequate amount of dynamic

vasoconstrictor endothelin-1, is liberated at the site of formed unstable plaque, which consequently worsen the plaque and further involved in myocardial ischemia (25). Thus, precipitation of ACS provides a major factor for the development of endothelial dysfunction (22).

Endothelial impairment, is closely linked with an increase of Oxidative stress (24). Reactive oxygen species (ROS) are activated reasonably during an inflammation or a vascular damage. Their function is divided according their number. At high number they can harm cells and produce an endothelial injury, while when the amount is negligible they participate in cell growth and cell adaption. Hence, an increase of oxidative stress could easily promote vascular diseases and affect the normal function of endothelium, by increasing accessibility of macromolecules (from blood to tissue) and stimulating inflammatory process (22).

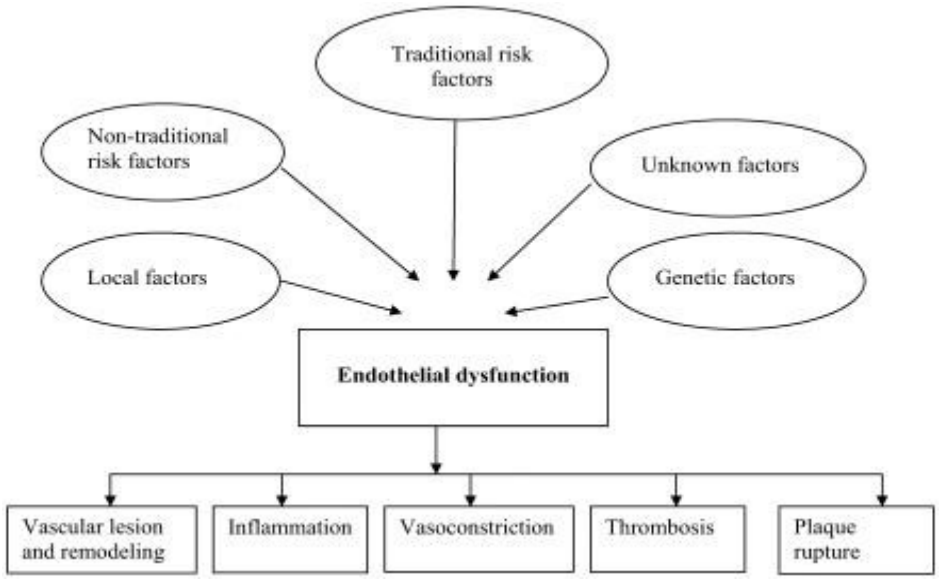
Vasa vasorum, as explained in the above, circulates the extracellular adventitia layer found in great arteries and veins, including coronary artery (26). In fact, this type of blood vessel is composed of smooth muscle cells boundary with endothelial cells and form a single endothelial coat. Acts independently to control vascular tone from the anchor vessel and hence, to develop endothelial dysfunction (27). Principally, a sudden change in the vascular wall has to occur in order for an endothelial alteration to happen. Impairment edges to ischemia of vascular wall and further in the formation of atheromatous plaque and/or in the internal plaque bleeding, corresponding to ACS (24).

Certain volume of evidence declares that endothelial dysfunction can be recognized as the first stage of atherosclerosis. Atherosclerosis is associated with a lack of the dynamic vasodilator nitric oxide which blocks oxidation of low-density lipoprotein (LDL cholesterol), forcing it to accumulate in the wall of arteries (28). Moreover is characterized by high secretion of ROS which under pathogenesis it promotes ACS and formation of atheromatous plaque (29). It must be stressed, that atherosclerosis exists as a disease of arteries and not of veins while it occupies macromolecules, cytokines, lipids, enzymes and growth factors to accumulate in the endothelial vascular walls and lead in a potential thrombosis. Endothelial cells are necessary for leukocyte adhesion which is further chemoattracted by endothelin. Additionally, endothelium facilitates the oxidation of LDL

where the newly oxidized molecules of LDL trigger the production of endothelin and E-selectin (definition is mentioned above, 3.2 vascular endothelium and its function). A slight decrease of nitric oxide (NO) is observed, represented and caused by various lipids and overproduction of oxidative stress. Reduction of nitric oxide immediately stimulates the bracing of certain pro inflammatory paths, including activation of transcription factor nuclear factor-κB (NFκB). Thus in total, LDL cholesterol, oxidative stress and shear stress acquired by blood flow commotion contribute to the development of inflammation and prolongation of endothelial dysfunction as well as the generation of pro-atherogenic genes and atherosclerosis (30).

Figure 1: The factors that affect endothelium and consequences of endothelial dysfunction (22).

Adopted from: Endothelial dysfunction: cardiovascular risk factors, therapy and outcome. Vascular and risk management. Hadi, H.A.R., Carr, C.S. and Al Suwaidi, J. 2005.



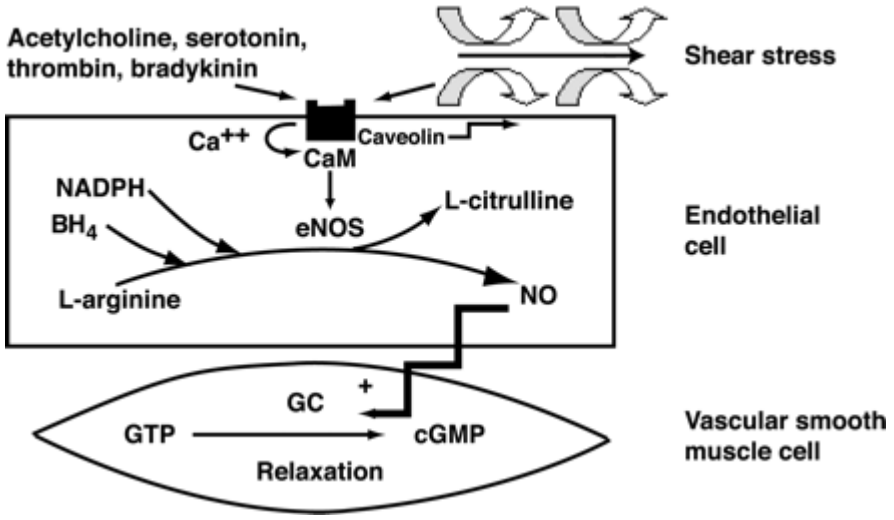
Further, endothelial dysfunction occurring in diabetes mellitus it negatively influences several pathways for the activation off endothelial nitric oxide synthase (eNOS). In fact,

nitric oxide secretion is strictly facilitated by enzymatic transformation of amino acid L-arginine to nitric oxide and Citruline, mediated by eNOS. Hence, affecting this pathway would only decrease the vasodilated nitric oxide production presenting several consequences. ENOS is signified in endothelial cells which are found in caveolae. They are characterized by a " flask-shaped" structure of the plasma membranes and composed of proteins and lipids (31). Important to mention though is the fact that insulin hormone triggers eNOS activation, by binding of insulin to endothelial cell receptors. As a result, insulin receptor substrate-1 (IRS-1) is phosphorylated initiating eNOS production through phosphoinositide-3 kinase (P13 kinase or Akt system). Inhibiting IRS-1 would lower insulin-triggered eNOS phosphorylation and expression in endothelial cells (32) . A disturbance of signaling eNOS and P13 kinase/Arkt system would increase the risk of vascular endothelial impermanent and complications of it. Additionally based on certain studies, it is observed that high abnormal levels of insulin could increase the amount of vasoconstrictor endothelin-1, which consequently improves insulin resistance (33). In all, diabetes mellitus is associated with multiple functional impaired pathways, thus an imbalance (e.g. signaling of cells and promoting of eNOS inhibitors) leads to inability of a physiologic trigger to produce nitric oxide (34).

On the other hand, oxidative stress contributes to endothelial alteration in diabetes mellitus. High amount of oxidative stress affects bioactivity of nitric oxide due to the reaction of superoxide anion with nitric oxide. As a result peroxynitrite molecule is formed limiting bioactivity of nitric oxide (28). In regard, peroxynitrite influences action of eNOS and further guanylyl cyclase in endothelial and vascular smooth muscle cells similarly, leading to the decrease of nitric oxide (35). Moreover, high secretion of ROS affects eNOS co-factors e.g. tetrahydrobiopterin, that expanses production of superoxide and its (eNOS) activation while it eliminates the amount of nitric oxide and shortens the access in the target tissues (36,37).

Figure 2: Production of nitric oxide (NO) by endothelial cells. NO is produced by the action of endothelial nitric oxide synthase (eNOS) on L-Arginine. This reaction requires a number of cofactors, including tetrahydrobiopterin (BH4) and nicotinamide adenine dinucleotide phosphate (NADPH). Increased intercellular Ca^{++} in response to vasodilator agonists or shear stress displaces the inhibitor caveolin from calmodulin (CaM), activating eNOS. NO diffuses to vascular smooth muscle and causes relaxation by activating guanylate cyclase (GC), therey increasing intracellular cyclic guanosine monophosphate (cGMP) (28).

Adopted from: Role of endothelial dysfunction in atherosclerosis. Davignon and Ganz, 2004.

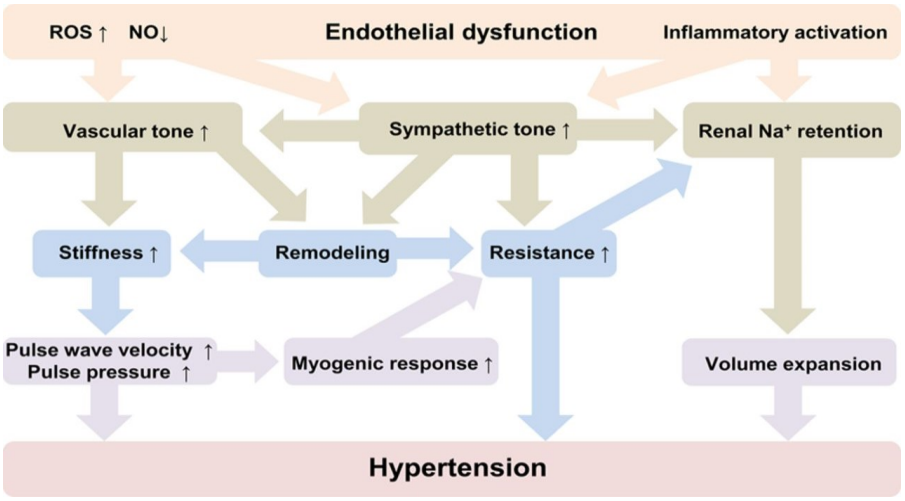


Interestingly, despite the various numbers of certain diseases caused by endothelial dysfunction, another clinical aspect appears to have a fundamental contribution in this pathological state (endothelial dysfunction). Arterial hypertension is a condition in which endothelial impairment has been accepted, but it is not clear whether if dysfunction is a predictor or a consequence of hypertension. That is why its process is not clear and its conflict has been on trenches (38). In fact, there is a negative imbalance between the substances of which endothelial cells release, producing contraction and vasodilator factors that maintain sympathetic and vascular tone, important for arterial hypertension. Endothelial predictor of contractive factors includes vasoconstrictive prostanoids, superoxide anions, angiotensin II, and endothelin-1. Endothelial vascular dilators involve

mainly nitric oxide and prostacyclin (39). Therefore, a sudden change in physiological balance could be hazard while dysfunction of nitric oxide synthesis or inactivation by massive production of superoxide radicals could increase sympathetic tone. In the same manner, high production of endothelin-1 or sensitivity to smooth muscles could result in gradual increment of vascular tone (40).

Figure 3: Pathways of endothelial dysfunction related with hypertension. Reactive oxidative species (ROS) (41).

Adopted from: Endothelial dysfunction and hypertension. Ralf P. Brandes 2014.



Important function of endothelium is to maintain body homeostasis promoting blood clotting, vascular restoration and stimulation of platelets in case of vascular injury. Additionally, healthy endothelium releases antiplatelet and anticoagulant factors preventing the growth of fibrin and thrombus eventually. It provides a variety of inhibitors forbidding enormous clot development. Therefore upon endothelial impairment endothelium stimulates massive fibrin production among with promotion of platelet cohesion and aggregation (42). Accumulative evidence states that a thrombus might occur because of deficiency of regulated proteins C and S. Lack of regulated proteins leads to a decrease of synthesis and secretion of tissue plasminogen as well as mutations of factor V that contributes in development of thrombosis (43).

It is declared that endothelial cells participate in case of tumor condition featured as great target of tumors, resulting in metastasis while rapid secretion of chemokines and vascular growth endothelial factors (VGEF) during metastasis is another important characteristic (43). Chemokines are produced by cancer cells and induce a great number of adhesion molecules on the surface of ECs necessary for the detection of tumors whilst VGEF are equally released by cancer cells trigger angiogenesis process. As a result, neoplastic blood vessels are formed making a trafficking pathway for tumor cells to pass through it initiating metastasis (44).

2.4 Cell adhesion molecules

Cell adhesion molecules (CAMs) is a fundamental area of biomedical science, which over the last decade has been incredibly advanced (45). Nowadays it is clear that CAMs are defined as transmembrane glycoprotein receptors with extracellular binding domains and cytoplasmic functional territories. They facilitate specific interactions between cell-cell and tissue matrices acting as a membrane cell system that forms connections between internal and external environment of cells (46). Moreover, selective interactions mentioned in the above, which are driven by CAMs, have a serious concern in cellular facilities involving hematopoiesis, angiogenesis, cellular growth, migration, differentiation, invasion, embryogenesis, tumorigenesis and tumor metastasis (47).

Currently we classify CAMs according their protein structure, chemical and functional affinity. Four of them participate on interactions between proteins while the remaining two on interactions between proteins and carbohydrates (48). The protein-protein adhesion molecules are; 1) Integrins 2) Cadherins 3) CAMs correlating to the superfamily of immunoglobulins 4) Protein tyrosine phosphatases receptors. Molecules taking part in protein-carbohydrate interaction include Hyalunorate and Selectin receptors (Described in the above, Unit 2.2). Particularly hyalunorate receptors applied in cell-cell adhesion system, while integrins and hyalunorate perform in cell-matrices interaction. These

interactions have been grouped according the presence of Ca^{2+} into Ca^{2+} dependent and independent (49).

Cell adhesion molecules guiding selective interactions, including cell-cell, cell-pathogen and cell-extracellular matrices, have been known as Integrins. They contribute in the proper function of immune system by consulting leukocyte movement (migration or trafficking), stimulation during pathogen induction, immunological synapses creation and phagocytosis. Furthermore, they are responsible for signaling during co-stimulation and cell irregularity. Integrins took their name because they integrate the extracellular environment with the intracellular environment by connecting ligands outside of cell and various cytoskeletal units with signaling molecules inside the cell. As cell surface adhesion molecules, they provide a heterodimeric structure having 24 groups of αβ subunits and comprised of 18 α and 8 β subunits requiring the presence of bivalent cations, usually Ca^{2+} and Mg^{2+}, to succeed their adhesive function. Thus their structural configuration (subunits) is important for identification of ligands as well as the connection to cytoskeletal units and formation of signaling pathways (50). Despite its connection with various different ligands, integrins are highly selective due to their ligand specificity. The majority of them are connected with arginine, glycine, and aspartate amino acid sequences which is further found in macromolecules (e.g. collagen, laminin), in blood (e.g. fibrinogen, von Willebrand factor) and cell surface proteins (51). There are three main classes of Integrins including β1, β2 and β7. Usually β2 and β7 are strictly occupied on white blood cells while the remaining β1 group functions throughout the cells of the body while the rest are less selective (52).

Adhesiveness of Integrins is accomplished by a mechanism known as “inside-out signaling“ initiating its function when a trigger irritates the cell surface receptors from cytokines or chemokines or other pathogens, immediately begins to influence the Integrin cytoplasmic domain, facilitating adhesiveness to external cellular ligands. As it follows the already bound ligand spread a new signal from the extracellular environment to the intracellular cytoplasmic one with “outside-in” signaling direction. This process plays a key role in the development of legitimate work of the immune system (53).

On the other hand, Cadherins belong to calcium dependent hemophilic contact adhesion molecules due to their continuous high demand of calcium binding to its specific protein sequence. Usually they are provided with one or several transmembrane glycoproteins, sitting on the exterior part of cellular domains and form newly subdomain units. Their mechanism of action believed it process as a result of a bond formation between adhesion molecules (cadherin) and the surrounding cells by repetitive conformational change. Therefore, Cadherins have the ability to link the cytoskeleton components and some transitional filaments through protein sequences on the cytoplasmic side of plasma membrane. Yet they have been established three vital groups; E-cadherin, N-cadherin and P-cadherin (54).

E-cadherin was firstly identified, and it ensures epithelial cell coherence having a great impact in embryonic characteristics. It is usually located in kidney tubules, in the skin and in the abdominal epithelium (55). N-cadherin exists as a vital adhesion protein component of neuronal and lens cells whilst it contributes in behoove function and purity of muscle fibers as well as the proper intracellular binding of heart muscle fibers. P-cadherin is another important protein component joining placental thromboplastic cells. It is located in the skin (epidermis), the lungs, the heart and the abdominal epithelium (48).

The superfamily of immunoglobulins is characterized by a diversity of cell surface and soluble proteins, included in a great number of vital processes (49). Adhesion molecules of immunoglobulin superfamily (IgSF CAM) take part in antigen recognition and cohesion to specific lymphocytes (CD3, CD4, CD8) which together form a complex of antigen-peptide essential for the proper function of the immune system and they are implicated with cell communication, connection and integrity. However, the most important feature is the number of immunoglobulin domains consisting of several members that lay on immunoglobulin domains as spreading from the membrane allowing the formation of bonds between cells. In total, there are two calcium independent types of binding; homotypic and heterotypic as they can bind to other cell surface molecules additionally (56).

Neural cell adhesion molecule (N-CAM) is one of the oldest members of immunoglobulin superfamily and is characterized by a certain number of molecules that

are encoded by the same gene resulting, according their size, in a large variety of protein products (48). Furthermore, they are involved in learning and memory thus can be found primarily in neurons, in glial cells and striated muscle fibers. Another important adhesion molecule that belongs in this group is Intercellular adhesion molecule (ICAM) which is expressed on leukocytes as well as on epithelial cells while is responsible for the formation of a heterophilic bond acting as a crucial integrin component known as $\alpha_L\beta_2$, already expressed only in leukocytes. ICAM facilities a large series of action, including T-lymphocytes proliferation and antigen presentation, which are further discussed in the following units. There are many types of adhesion molecules that belong in this family laying in endothelial cell surface. One of them is vascular cell adhesion protein (VCAM), important for the behavior of immune system. It promotes white blood cells adhesion and exist as another ligand component of leukocytes in a similar way as ICAM (45).

Protein tyrosine phosphatase receptor (PTPRT) is a crucial member of CAMs and has been involved in cell-cell adhesion, signal transduction and neurons extension (57). Their main duty is formation of synapses between two neurons as a result of the interaction of cell adhesion molecules and FYN protein tyrosine kinase. The specific interaction takes place in central nervous system of which PTPRTs are uniquely expressed. Accumulative evidence declares that PTPRTs have not only initiated the creation of synapses between neurons, but also perform in the maturation and integrity of synaptic formation. There are various types belonging to this group and one of the most dynamic PTPRT type is O which facilitates the formation of synaptic imitation (58).

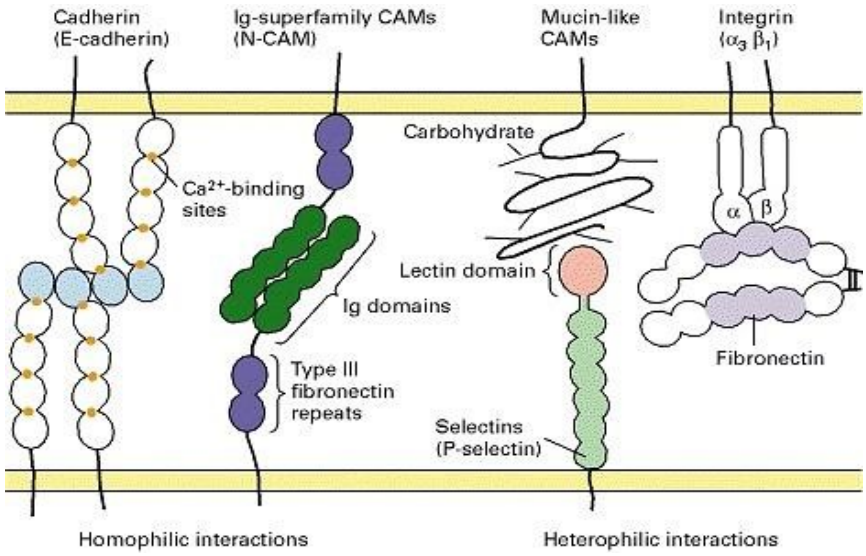
The fifth member regarding the large group CAMs is defined as a fundamental guideline in cell growth, tumor development and cell differentiation. Specifically, hyaluronate (HA) is an exclusive sugar adornment of external cellular matrix that participates in various crucial cellular operations, such as inflammation, tumorigenesis, tissue regeneration and formation of cellular breaches. Despite its vital processes, HA is further bound to certain cell surface proteins, including CD44, RHAMM and thus is associated with lymphocyte activation and cell migration (59). As it turns CD44 proteins are totally operate as CAMs and their main job is to keep the organ-tissue structure by

improving cell-cell and cell-matrices adhesion as well as initiating stimulation of lymphocytes and formation of hormones and other chemical units (60).

Figure 4: Schematic demonstration of CAMs

Integral membrane proteins are built of multiple domains. Cadherin and the immunoglobulin superfamily of CAMs mediate hemophilic cell-cell adhesion. For cadherin, calcium binding to sites (orange) between the five domains in the extracellular segment is necessary for cell adhesion; the N-terminal domain (blue) causes cadherin to dimerize and to bind cadherin dimers from the opposite membrane. The immunoglobulin superfamily contains multiple domains (green) and frequently contains type III fibronectin repeats (purple). In a heterophilic interaction, the lectin domain of selectins binds carbohydrate chains in mucin-like CAMs on adjacent cells in the presence of calcium. The lectin domain is separated from the membrane by a series of repeated domains. The major cell-matrix adhesion molecule, integrin, is a heterodimer of α and β subunits. They bind to the cell-binding domain of fibronectin, laminin or other matrix molecules (61)

Adopted from: Molecular Cell Biology 4th edition. Lodish H, Berk A, Zipursky SL, et al. New York: W.H.Freeman; 2000.



2.4.1 Structure and function

Immunoglobulin superfamily (IgSF) is responsible for the proper function of the immune system, by improving cell to cell recognition during inflammation and enhancing cell signaling within the immune response. Principally is constructed by vascular adhesion molecules (VCAM), intercellular adhesion molecules (ICAM) and mucosal addressin cell adhesion molecules (MAdCAM) complementary. Although after a high-resolution X-ray crystal examination it was observed clearly two domain N-terminal parts of four precise molecules including ICAM-1, ICAM-2, VCAM-1 and MAdCAM-1. All of them are highly expressed in endothelial cells and are associated in leukocytes normal function, especially in the wall of blood vessels and promotion of an adequate cell signal to the inflammatory sites. MAdCAM-1 is predominately expressed in mucosal endothelial tissues and contains two IgSF domains. Also, it collaborates with leukocytes that have bound to selective antigens in mucosa. ICAM-2 has an equal amount of IgSF domains as the previous member, but unlikely is expressed mainly in platelets, lymphocytes and monocyte. Usually the first domain is necessary for the binding of integrin-cell surface while the second has a great impact in integrin recognition and "approval" of the first domain instantly (56).

2.4.2 VCAM-1 and ICAM-1

Vascular adhesion molecule one and Intercellular adhesion molecule one are special types of adhesion molecules and important segments of immunoglobulin superfamily, facilitating variable interactions throughout the human organism whilst are contribute to the improvement of the immune and inflammatory response (62). Specifically, in this chapter we'll explain the functional and structural definition of those mentioned adhesion molecules as well as the way of their expression in human organism.

Functionally VCAM-1 is involved in cell adhesion, initiating the linkage to leukocytes and melanoma cells, and signal transduction as a result of selective interaction with integrin

very late antigen-4 (VLA4) presenting a crucial role during the inflammatory process. VCAM-1/VLA4 selective interaction is further required throughout the time of an immune response and to inflammatory sites by firstly, triggering and secondly trafficking T cell lymphocytes to their receptor (63). Structurally is constituted by extracellular immunoglobulin like domains large sequence, containing strong chemical disulfide bonds, a 19 amino acid cytoplasmic domain with carboxylic terminal and a single transmembrane domain (64). Worth mentioning is the fact that this large amino acid sequence is purely similar between humans and animals (e.g. mice, rabbits) (65). In humans there are two models of VCAM-1 usually provide seven immunoglobulin-like domains, excepting the case of lacking the forth domain and provide six domains additionally. In mice VCAM-1 is consisted of seven immunoglobulin-like domains form or a shorten form with only three domains (66). Interestingly is the part outside of the adhesion molecule, which is surrounded by seven immunoglobulin-like domains presenting high affinity to each other. Regarding the way and location of VCAM-1 expression it has been reported that is fully expressed on cytokine stimulated endothelium and on inflamed endothelial cells among with dendritic type and macrophage-like cells in inflamed and non-inflamed tissue (62). Thus it starts with the secretion of cytokines in tissues, increased levels of ROS, low density lipoproteins, further elevation of shear stress, high glucose and activation of microbial endothelial cell tall-like receptors (67,68). Initiation of this process is driven mainly by NFκB and interferon regulatory factor one (66). On the contrary, recent studies have stated that there is a great linkage between the expression of VCAM-1 and cardiovascular diseases declaring that high level of ROS could significantly stimulate NFκB and eventually force VCAM-1 to expressed in aortic endothelial cells and promote monocyte aggregation to arterial intima. Importantly, this unique feature might be a strong tool for the early detection of a cardiac abnormality in conjunction with atherogenesis (69).

ICAM-1 acts as an important mediator for cell adhesion on leukocytes and endothelial cells. Thus, on endothelium, which in related manner is covered by the cell surface and located in plasma is entitled as sICAM-1, it functions principally in the stimulated white blood cells trafficking, especially in their migration to the inflammatory sites. Various scientific studies reported that sICAM-1 level is increased under any pathological circumstance and/or a negative influence on the proper function of the immune system,

including malignancies, clinical pathogenic rhinovirus and large amount of inflammatory diseases as atherosclerosis, ischemia and organ transplantation (70). Respecting its structure is characterized by five immunoglobulin-like domains together with three-dimensional atomic structure of a circulated N-terminal domain, which upon a pathogenic invasion is totally expressed on white blood cells, epithelial and endothelial cells. Moreover it exists as an individual protein ligand providing the lymphocyte function associated antigen (LFA) and "offering" several binding sites for the proper attachment of integrins CD11a and CD18 (71,72).

2.5 Mouse models of atherosclerosis

A chronic pathological condition encounters with medium and large size of arteries being involved in inflammatory cardiovascular disorders usually leading in stroke, myocardial infraction and peripheral vascular damage, is referred as atherosclerosis (73). It must be stressed it is globally related with morbidity and mortality in developed and developing countries around the world. Important to understand is not how atherosclerosis is defined, but how it proceeds. Therefore, atherogenesis is a progressive condition which the internal space of arteries shortens by chemical and organic substances resulting to arterial obstruction through three basic stages. At the very beginning inside the sub-endothelial space is filled with lipids-macrophages known as foam cells, starting to form a fatty streak canker whereas a complete atheromatous fibrous plaque is developed composing of foam cells, lipids, collagen and smooth muscles. As a result a clear evidence of a thrombotic lesion filled with platelets and fibrin arises at the last stage as atherosclerosis has been progressed (74).

Over the past decades, most of atherosclerotic studies have accomplished by using experimental animals including pigs, rabbits and monkeys. However, these experimental large animals have not been successful providing a series of inaccurate scientific results. Additionally, a high number of accumulative evidence declares that mice have become progressively useful in biomedical research, especially in vascular pathology while

currently these mouse models are particularly used to study the atherogenic precept. Mice provide beneficially absolute genetic information which is further genetically manipulated in order to achieve rapid and continuous monitoring of atherogenesis in a significant time (73). Manipulation of a single gene or a number of genes allows the elucidation of variable methods on molecular level that take place on the formation and evolution of atheromatous plaque, helping to consider how immune cells affect the disease. Genetic manipulation is facilitated either by interbreeding providing a variety of inbred strains or by knockout method for elucidation of the particular genes (75). As it turns, genes have the ability to interact with the environment and each other causing a sudden change in atherosclerotic phenotype and progression of the disease itself.

Their small size and low cost enable the use of a great portion to study the atherogenic mechanism while the fact that mice lesions seems to be inherited to next generation allowing better statistical evaluation of data (76,77). Additionally, their size contributes practically to evaluate maximum efficacy of novel synthesized drugs and antibodies. In fact, newly synthesized drugs cannot produced massively in large scales and thus with its small size make the process susceptible (78).

Oppositely a crucial restriction of mouse models is their physical resistance to atheromatous plaque formation because of the quite different lipoprotein and cholesterol metabolism than humans. Most of the times mice have a lower blood cholesterol level (approximately 60- 100 mmol/L) which without a certain increased level of LDL there is a low potential of atherosclerosis development. Therefore, a genetically modified mouse with induced hypercholesterolemia should take part to continue the experiment. Despite the benefits of mice size, practically it is found that based on their morphological structure several difficulties could be found. Firstly, their coronary arteries are so small for observation; besides the arterial tunica intima is comprised of endothelium which is laid to the elastic lamina missing of smooth muscle cells and connective tissue, comparing with humans. Secondly, there is a lack of vasa vasorum while the size of tunica media is slightly thinner (79). Most importantly the structure of atherosclerotic lesions is totally different presenting a principal defection of the bulk fibrous cap (79–81).

The first mouse model was handled by Wissler and his employees at the end of 1960s. They fed the mouse a special fat diet rich in cholesterol, lipids and cholic acid resulting in high expression of hypercholesterolemia and subsequently the formation of various fatty streak lesions around the vascular area. Nevertheless, the highly fat diet which had followed was equally toxic leading to loss of weight and improving sensitivity of the mouse to infections (82). Another study was initiated, fed this time the mouse less fat diet assuming that the toxicity would be lower. After three to nine months of fat feeding, toxicity issue was fulfilled but the formatted lesions were not alike as in humans (83). Thus, scientists understood by using wild-type mice models they could face either toxicity problems or morphological issues. To avoid the problematic behaviors they begun the development of genetic-modified mice models which was at the core (84). The most frequently used of mouse atherosclerosis are Apolipoprotein E (apoE) and LDL receptor (LDLR) deficient model differing on fed diet. ApoE is located on the surface of lipoproteins except LDL and facilitated a high number of lipoprotein interactions with many receptors, including LDL and related LDL receptor (76). It is further present a particular hypercholesterolemia without the consumption of highly toxic diet and the same time it functions for the rapid development of atherosclerotic lesions in the aortic root, aortic arch and various different points around the aorta totally similar to human. Also, it is believed that the rapid formation of lesions could be owed to the western type of diet in which apoE mouse models were fed to observe such a significant level of blood lipid levels (85). However, apoE knockout model present its limitations based on data obtained from humans. In fact, it was found that apoE knockout models might work independently of blood lipid levels as its expression decrease the asperity of atherosclerosis without influencing the blood lipid amount (86,87).

In order to overwhelm the above complications a new mouse model was established, which comparing to apoE it shows several advantages. At the beginning, LDLR deficient model authorize fewer functions, making the effect of lipoprotein homeostasis more efficient and less sensitive while the plasma lipid level shows a minor increase due to the higher LDL amount carried by cholesterol-blood protein as a result of lipoprotein uptake and clearance, driven obviously by LDLR (88,89). Missing of LDL in human population leads to familial hypercholesterolemia following with a high risk of cardiovascular event,

exactly the same way as in mice. The ability to provide common features in familiar human hypercholesterolemia, including the formation of lesion in aortic valve and aortic root among them is the most valuable characteristic. This model is correlated with diabetes which has particularly used for the detection of a linkage with atherosclerosis (90).

2.6 Immunohistochemical methods

2.6.1 Basic principles of immunohistochemistry

Immunohistochemistry (IHC) is an antigen spotting method in tissue fragments respectively to specific antibodies. Principally it is related with the detection of an antigen in functional and pathological tissue or a cell, as well as the characterization of cell function and formation of fibrous lesions. Clearly it is understandable that this method has the ability to combine Immunology, histology and chemistry, however in order to succeed this process acquires a primary antibody gently bind to a specific antigen, a secondary enzyme-conjugated antibody and finally a substrate with a chromogen to catalyze the enzyme reaction of antibody-antigen binding (91). Despite the definition of IHC, practically to achieve the detection goal of antigens several methods were established including the direct, indirect, peroxidase-antiperoxidase (PAP), polymeric and avidin-biotin complex (ABC) methods, fully described below.

As its name indicates, it is a one-step direct staining method involving a labeled antibody which reacts immediately with an antigen in tissue fragment. This method is characterized from the usage of a single antibody and the required time which is fast and short (92).

Additionally, indirect method has been known as a double layer method. The first layer contains an unlabeled primary antibody reacting with a tissue antigen, while the second layer is composed of a labeled secondary antibody that reacts with the primary antibody. Comparing with the previous method, it is more sensitive due to the high signal amplification with secondary antibody reactions and it is longer, having less financial cost

because the second layer can be utilized with alternative first layer antibodies to different antigens (93).

Evolution of the indirect method is PAP which involves a third layer usually comes from a rabbit antibody to peroxidase and couple with it, forming a rigid peroxidase-antiperoxidase complex. This complex is catalyzed by horseradish peroxidase (HRP) enzyme and makes a molar ratio of two antibodies to three enzyme molecules. HRP has the ability to activate the immune response and thus it improves the immunolocalization signal. Unique feature of PAP and difference comparing with other methods is the precise dependence on the immunological binding and the sensitivity that presents the three step signal amplification (94).

2.6.2 EnVision system

EnVision system was developed primarily to improve the immunohistochemical antigen detection system. In principal is a two-step staining method in which the polymeric conjugate is attached to the primary antibody while the molecules of a secondary antibody tend to bound instantly to the activated dextran backbone. Besides, this method was developed to be guide by dextran technology giving the permission to a high amount of enzyme molecules in order to bind to the secondary antibody (91). Though, it has reported that the protocol is quite simple using a primary antibody, an enzyme labeled polymer and the suitable substrate chromogen, thus it makes it beneficial regarding the total number of steps comparing with other methods and the significant reduction of no-specific staining (95).

2.6.3 ImmPRESS technique method

Concerning the polymeric immunohistochemical group another important system was recognized. ImmPRESS polymerized enzyme staining system is described as a functional technique that utilizes polymerized enzymes strictly connected to antibodies. As a result

there is a clear formation of enzyme micropolymers complex preventing them from the internal shortcomings or other macromolecules (e.g. dextran, backbones). By connecting the uniquely made enzyme micropolymer complex with a high density of the powerful enzymes to a secondary antibody it introduces a reagent that provides a valuable accessibility to its target, leading at the same time in a background staining reduction and outstanding signal intensity (96). ImmPRESS technique method was used in our experiments, thus further information is provided in the chapter of experimental part additionally.

2.6.4 Avidin-Biotin Complex (ABC) method

ABC method is an optimal IHC technique widely regular used as a method of immunohistochemical staining. It works simply based on the abilities of avidin and biotin constructing three main layers. The first layer is an unlabeled primary antibody lying with a biotinylated secondary antibody (second layer) which follows an avidin-biotin peroxidase complex (third layer). This technology enhances the detection of a larger amount of enzyme molecules at the antigen sites and thus increases sensitivity whilst the affinity between the two molecules is so incredibly high making the biding practically irreversible and simultaneously avoiding errors regarding the assay (97,98).

2.7 Endoglin

Current evidence regarding antibody-based diagnostic and therapeutic strategies in tumors and atherosclerosis has progressed in a certain level in which more potential antigens have been studied. Endoglin, frequently known as CD105 belongs to this group acquiring the attraction for further research. The following chapter highlights the functional

and structural traits of endoglin as well as its expression and relationship with eNOS function and endothelium.

Endoglin is a cell membrane homodimeric glycoprotein that binds to assorted transforming growth factors-beta (TGF-β) superfamily, which is a pleiotropic cytokine responsible for cellular migration, proliferation and differentiation. TGF-β1 builds up a complex with activitin receptor-like kinases (ALK-1) type one and a transmembrane second type of serine-threonine kinase receptors. Smad proteins are intracellular signal inducers taking part in atherogenesis and are triggered by the activation of those receptors (3). Reasonably TGF-β defined as an important dynamic motivator of proteoglycan biosynthesis in human smooth muscle cells essential for the production of lipoprotein-trapping proteoglycans leading to their adherence in the blood vessel wall, in such a way that perform in the occurrence of atherosclerosis and is confirmed by the elevated levels of TGF-β1 detected in the lesions of vessel wall. (99). However, it has been reported that endoglin participates in various pathological conditions, including cancer angiogenesis, hereditary hemorrhagic telangiectasia (HHT) and contribute (among other factors) in the development of diabetes mellitus, hypertension and preeclampsia. Although is not accurate yet that endoglin regulates the progression of atherogenesis and atherosclerosis, but due to the expression and the behavior of cells against atherosclerosis seems to be connected with its pathogenesis (3).

2.7.1 Endoglin structure

Human endoglin is a 633 amino acids with 180 kDa homodimeric disulfide bond inducible hypoxia transmembrane glycoprotein. It is mainly composed of three domains, including a bulky extracellular, a hydrophobic transmembrane and a tiny intracellular domain. The extracellular domain provides a tripeptide amino acid while it is observed that the intracellular domain contains several phosphorylated serine-threonine slags (100,101). In a more closer look, structurally there is no doubt that endoglin belongs to zona pellucida (ZP) group of extracellular proteins which a single domain of ZP is comprised by 260

amino acids and 8 cysteine slags nearly attached to the transmembrane fragment (102). Until now two isoforms of endoglin have been established differing in the phosphorylation degree, tissue distribution and length of intracellular domain. Both of them though, are phosphorylated considering the continuous activity of TGF-β1 serine-threonine kinases type one and two receptors (99). The primer L-isoform cytosolic domain contains 47 amino acids featured with the presence of proangiogenic effects while the secondary S-isoform has 14 amino acids expressing antiangiogenic effects. Moreover, comparing to L-isoform is recognized that S-isoform has more influence in eNOS expression by decreasing its signal. Nevertheless, both of them interact with ALK-1 and ALK-5 showing different affinity to each receptor (3). Though, interesting was the fact that after isolation of CD105 in mice and pigs there was a commercial identity (70%) in comparison with human sequence (103).

2.7.2 Endoglin expression

CD105 as cellular membrane glycoprotein is principally expressed on normal tissue within the vascular system, over-expressed on vessels during a pathological condition (inflammation) or vascular injury and increasingly on proliferating endothelial cells that take part in angiogenesis (2). Despite its functional relevance, large volume of endoglin was recognized in both smooth muscle and endothelial cells of variable blood vessels regarding the progression of atherosclerosis. Although a high ratio of tissue angiogenesis and its reconstruction (blood vessels) was successfully detected in experimental animals and humans (104). Several lines of evidence suggested further that CD105 was identified on endothelial cells of intra- and peri – tumoral cells, while its expression is short or vanished on cancer cells leading to a promising diagnostic, prognostic and therapeutic tool against neoplastic disorders (2).

2.7.3 Endoglin and its relation to eNOS function and function of the endothelium

Endothelial nitric oxide synthase (eNOS) modulates the construction of nitric oxide (NO) by endothelium, which is necessary for preservation of normal function of vascular tone (3). Therefore, upon a reduction of eNOS would immediately decrease NO bioavailability, raise oxygen production and influence vasomotor tone resulting in endothelial dysfunction, which is an essential step for a cardiovascular disease pathogenesis (atherogenesis). After several studies utilizing resistance arteries of $Eng^{+/-}$ mice, was recognized a gradual reduction of eNOS in endoglin deficient endothelial cells considering its (eNOS) decrease protein half-life. Thus, it was clear that endoglin contributes in maintenance of its effect for eNOS. At the same time any mice described with lack of endoglin would have a significant decrease of NO and consequently abnormal low levels of eNOS (105).

2.8 Soluble endoglin

Soluble form of endoglin (sEng) is described as an extracellular domain of the homodimeric transmembrane glycoprotein CD105, detected in blood of hale population and/or pathogenic patients being diagnosed with a certain number of disorders, including atherosclerosis, preeclampsia, cancer and HHT (3). Yet its role is not accurate, but due to recent studies and observation of high levels during the development of pathogenesis associated with vascular endothelium damage, it was assumed that sEng may have a potential effect in endothelial impermanent (4).

2.8.1 Generation of soluble endoglin

It is observed that CD105 soluble form (sEng) provides two oligomeric parts, thus exists as a dimer and a tetramer. Interestingly the dimeric part seems to improve TGF-β response forming a promising potential for future therapeutic applications (106). SEng was established as a result of several experiments with variable metalloproteinases (MMPs) on human umbilical endothelial cells (HUVECs). Specifically, metalloproteinase-14 (MPP-14) as the most dominant metalloproteinase expressed in vascular cell linages was involved in this test, and by isolating at position 586 the disconnected product of N-terminal endoglin was formed. MMP-14 effect was performed primarily when sEng plasma levels have been enhanced due to the overexpression of MMP-14 in mice and secondary after oxysterol treatment, triggering liver X receptor (LXR) transcription factor and expression of MMP-14 in placental explants and jar cells (107). However the results were contradicted by another study claiming that sEng levels were solid without any change from its (MMP-14) secretion, summing up due to the failed response of MMP-14 in HUVECs that it might not be the most abundant cleavage protease (108). Additionally, large volume of evidence proposed that chipping alternatively at position 586 of a single 80kDa sEng molecule from HUVECs surface would indicate the release of extracellular domain Eng1-586 (109). The specific extracellular domain was further exploited to initiate the generation of sEng. Despite the accumulative studies, there is not concrete evidence carried out for atherosclerosis leading in lower amount of data concerning the structure of its molecule which is related with the disease (atherosclerosis) whether it is not clear where the sEng chipping position is located for other pathologic disorders (107).

2.8.2 Soluble endoglin and Hypercholesterolemia

Familial hypercholesterolemia is a frequent congenital disorder of cholesterol metabolism characterized by elevated levels of cholesterol in the body, resulting to early cardiovascular morbidity and mortality. It is further defined as the most richly studied risk factor that leads to endothelial dysfunction, tissue angiogenesis and atherosclerosis (110).

Successfully was observed a continuous elevation of sEng serum levels in patients suffered with atherosclerosis due to only the enhancement of total cholesterol levels while rest biomarkers participating in endothelial dysfunction or vascular injury were stable (111). Another study showed that sEng levels were raised only in the initial stage of atherosclerosis as a result of a possible damage on endothelial cells, but since the enlarged production of endoglin-TGF-β1 complexes its levels were dropped in further stages of the disease (112). Therefore, was summed that sEng acts as a marker heightened with elevated total cholesterol levels in patients suffering by familial hypercholesterolemia (113).

In earlier studies, utilizing apoE-LDL receptor mouse models facilitated with double knockout technique method, after consumption of a rich cholesterol diet an increase in sEng and cholesterol levels was appeared as well as an enlargement of atheromatous plaque (114). Accumulative evidence of the continuous study occupied with a variable number of mouse models suffered by atherosclerosis, outlined that a gradual raise of sEng level was observed in apoE-LDL deficient mouse models fed with cholesterol diet opposed with apoE deficient mice which have fed with normal food diet. Worth mentioning though was the fact that both apoE-LDL receptor and apoE deficient mice of atherosclerosis showed a progressive hypercholesterolemia, atherosclerosis and endothelial dysfunction (115). Hence, a bright connection between the elevation of total cholesterol and sEng levels was obviously arose (107). Although, in another field of experiments was proposed that upon a change of blood sEng levels, it might was possible to associated with a change in blood vessels due to the development of familial hypercholesterolemia (111).

It was summarized that familial hypercholesterinemia could change blood sEng levels by disconnecting CD105 in various blood vessels only from vascular cell linages whereas tissue endoglin expression was detected only in mice endothelium (116).

2.8.3 Soluble endoglin and possible Indication of endothelial dysfunction

Cardiac risk factors are responsible causing negative aspects (e.g. oxidative stress) that modify the amount of endothelial cells resulting to the so called "endothelial dysfunction" decreasing its amount it fails to keep vascular homeostasis, leading in several pathological inflammatory stages and in progression of vascular damage (117). Precisely this state is characterized by high expression of adhesion molecules, sudden reduction in special substances that promotes vasodilation and raise vascular permeability enabling white blood cells to start an inflammatory response leading to gradual aggregation of cholesterol molecules as atherogenesis progression begins. Yet there is not a clear or even a partial connection between the biomarker sEng causing endothelial damage and development of endothelial dysfunction, thus the correlation of it is doubtful (28). However, further investigation and studies are on trenches.

The first attempt was made presenting dynamic effects of sEng on endothelium and/or vascular cell linages based on its antiangiogenic properties, blocking tube formation. In the same study, an obstruction of eNOS dependent vasodilatation in chipped rat renal microvessels among with mesenteric vessels was observed after recombinant sEng application to rat featured with TGF-β1 attached to TGF-β receptor II and reduction of Smad protein stimulation. It was proposed then a possible relation with preeclampsia disorder due to its relation with hypertensive effects (7). Another study took part stating that in contrast with membrane endoglin response sEng restricts immune cells adhesion in venules, however current results came to our attention proposing an alternative role of sEng (118). Particularly this experiment was utilized by transgenic mice that overexpress human sEng and MMP-14. Overexpression of MMP-14 leads to elevation of plasma sEng levels and consequently in systolic blood pressure. In the same manner regarding experimental

mice, overexpression of MPP-14 would increase levels of sEng in blood and raise systolic blood pressure in comparison with the controlled group of mice. In fact, concrete evidence of which elevated sEng levels could increase blood pressure was not appeared, but researchers believed that its effect might disturb the TGF-β1/ TGF-β receptor II pathway resulting in limitation of eNOS dependent vasodilation and increase blood pressure comparing with the control group of mice.

It is essential to be stressed that based on recent data it is described that sEng might have a correlation with endothelial dysfunction, yet there is no linkage yet of sEng effects in atherosclerotic arteries. Therefore, sEng appears to have a potential influence across the vascular endothelium uniquely affecting muscular arteries, aorta and veins-venules. Besides its characteristic importance though it needs further exploration to figure out if a combination of variable aspects (e.g. pathological state) that promote endothelial dysfunction have a cooperating role affecting endothelial function/dysfunction with respect to expanded sEng levels (107).

3. Aim of Thesis

In our research project, we aimed to monitor the effects of sEng in mice suffering by endothelial dysfunction applying the pro-inflammatory biomarkers VCAM-1 and ICAM-1 molecules, to detect the inflammatory state in endothelial cells located in aortic sections.

4. Experimental Part

4.1 Materials and Methods

Our experiments were divided into two basic immunohistochemical techniques. The first was driven by VCAM-1 while the second was accomplished using ICAM-1 antibody staining. Tested materials were visualized under light and fluorescence microscope additionally, whereas the materials were microscopic slides containing aorta's endothelium sections obtained from mice.

4.1.1 Chemicals

- VCAM-1
- Primary antibody: anti VCAM-1 Rat Anti-Mouse (Purified Rat Anti-Mouse CD 106, BD Pharmingen^{TM}, USA), dilution 1:100
- Secondary antibody: ImmPRESSTM Reagent Anti-Rat Ig (mouse adsorbed) Peroxidase (Vetor Laboratories, USA)
- ICAM-1
- Primary antibody: anti ICAM-1 Hamster Anti-Mouse (Purified Hamster Anti-Mouse CD54, BD Pharmingen^{TM}, USA), dilution 1:200
- Secondary antibody: Goat Anti-Hamster Ig (Vector Laboratories, USA)
- Primary antibody Fluorescence ICAM-1: anti ICAM-1 Hamster Anti-Mouse (Purified Hamster Anti-Mouse CD54, BD PharmingenTM, USA), dilution 1:200
- Secondary antibody Fluorescence ICAM-1: Goat Anti-Hamster dilution 1:400
- PBS ("phosphate buffered saline") prepared by dilution of concentrated PBS with water in ration 1:10
- PBS:

NaCL	80,0

KCL	2,0
Na2HPO4·2H2O	11,5
KH2PO4	2,0
Aqua bidestilata	add 1000,0

- Milk used to block non-specific binding (Bio-Rad Laboratories, USA)
- Mouse serum (DAKO, USA)
- DAB (DAKO, USA)
- Avidin/Biotin Blocking Kit (Vector Laboratories, USA)
- ABC complex elite (Vector Laboratories, USA)
- Eukitt (Sigma-Aldrich, Germany)
- Extravidin CY3 (Sigma-Aldrich, Germany)
- DAPI (4,6-diamidino-2-phenylindole) (Sigma-Aldrich, Germany)
- DABCO diazabicyclo(2,2,2)octane, (Sigma-Aldrich, Germany)
- Microscope cover glasses (Bamed, Czech Republic)

4.1.2 Animals and Experimental Design

Transgenic mice overexpressing human sEng (Sol-Eng+) on the CBAxC57BL/6J background were generated at the Genetically Modified Organisms Generation Unit (University of Salamanca, Spain). Six-month-old female mice with low levels of sEng fed a chow rodent diet (Sol-Eng+ low chow) were used as control mice for biochemical analysis only. All other experiments were performed with three-month-old female mice with high plasma levels of sEng (Sol-Eng+ high) (mice with sEng levels higher than 100 nanogramms per milliliters (ng/ml) were considered as Sol-Eng+ high) and their age matched female transgenic littermates with low levels of sEng (Sol-Eng+ low) fed a high fat rodent diet containing 1.25% of cholesterol and 40% of fat (Research Diets, Inc., USA) for the following 6 months. The animals were kept in controlled ambient conditions in a temperature-controlled room with a 12h-12h light-dark cycle with constant humidity and

had access to tap water *ad libitum*. No weight differences were observed among experimental groups.

All experiments were carried out in accordance with the standards established in the directive of the EU (2010/63/EU) and all procedures were approved by the Ethical Committee for the protection of animals against cruelty at Faculty of Pharmacy, Charles University (Permit Number: 21558/2013-2), and the Bioethics Committee of the University of Salamanca (Permit Number: 006-201400038812). Mice were anesthetized with ketamine/xylazine, and all efforts were made to minimize the suffering of the animals.

4.2 VCAM-1 ImmPRESS reagent technique method

In the first method, we had to test a certain number of microscopic slides. Primary step was to put the slides in a specific cup, made of glass and fill it with acetone carefully placing it in the freezer for twenty minutes. Once the time had finished we brought out the cup of the freezer, isolated the slides and let them dry for 2-3minutes. Further we moved our slides in another cup filled with PBS resting them for 5 minutes. PBS was used mainly for washing up the slides containing cells. Later on, a mixture of 0,5g (grams) of powdered milk and 10 milliliters (ml) of PBS was added immediately with an Eppendorf pipette (50 microliters) while the slides had to remain with the applied mixture for 30 minutes. During the incubation of milk solution, we started to prepare the primary antibody taking 5, 5 microliters (μl) of VCAM-1 and 550 microliters (μl) of BSA (bovine serum albumin). Once we apply the primary antibody to the slides, the incubation time was one hour. Vascular cell adhesion molecule-1 is primarily used in vascular endothelial cells for the purpose of early prediction in the pathogenesis of atherosclerosis and other inflammatory diseases (119). Bovine serum albumin (BSA) is employed mainly in immunohistochemistry as a blocking buffer. In fact, albumin serum binds to non-specific protein binding sites of our samples preventing non-specific binding of any other source (e.g. antibody) taking part in our procedure (120).

Moving forward, we had to ‘’wash’’ our slides twice with PBS keeping five minutes intervals between each wash. Once the cleaning phase had finished, we prepared a solution of 3% H2O2 (hydrogen peroxide) 8 milliliters (ml) and 70 milliliters (ml) of PBS putting the slides inside a glass type cup and allow to rest for 15 minutes. By the time we moved the slides away of the mixture, PBS was further used two times and a mixture of ImmPRESS reagent (550 microliters (μl)) and mouse serum (11 microliters (μl)) was distributed to them. ImmPRESS reagent is an enzymatic, non-biotic one step detection kit associates a novel conjunction technology that allows the entry to the target, binding specificity and signal intensity with low background staining (Vector Laboratories, INC 2015). Normal mouse serum on the other hand, is literally used for continuous inhibiting and/or dunking specific binding interactions for immunodetection methods (Normal mouse serum control, Thermo Fisher Scientific, catalog #31881, RRID AB_2532173).

As the experiment was running we put the slides in PBS (two times for 5 minutes) whilst a new solution of 10 microliters (μl) DAB buffer solution (3,3-Diaminobenzidine) in 500 microliters (μl) of PBS was made applying to each slide solution for 25 seconds and briefly rinsed in PBS solution placing them in a specific cup filled with distilled water. DAB is an organic compound frequently used for staining of proteins and nucleic acids. Our tested protein is targeted by VCAM-1 which is coupled with a peroxidase system and in presence of hydrogen peroxide (H2O2), DAB is catalyzed to its oxidized form giving a brown precipitate (Protocols Online,2015).

Approaching the end of our experiment, we took the cup of slides filled with distilled water and put it in a natural occurring chemical Hematoxylin for 5 seconds. Hematoxylin is used regularly as the basis of laboratory die (121). Quickly, we turned on the water fount and settled down the cup of microscopic slides so that the normal water would pass inside the cup for 1 minute. Thereafter we turned off the water, took the slides, and placed them firstly in pure acetone for several seconds, secondly in a solution of acetone-xylen (10:1) and in acetone-xylen (1:10) for 3 minutes additionally. Close at the end the slides were inserted in three containers with only xylen which they had to remain in each one for two minutes. Finally, we took out the slides, topically dry them and apply Eukit which is the intermediate sticking material between our aortic endothelium slides and the microscopic

glass. Thus, when we finished every slide we let them stand for 5 minutes and immediately after visualized them under the microscope (4x and 10x magnification). The microscope was linked with a digital camera and attached to a computer.

4.2.1 ICAM-1 Fluorescence staining method

Third technique occurred by inserting the slides in acetone solution remaining inside the freezer for 30 minutes. PBS buffer was used in the incoming step for 5 minutes whilst a mixture of 0,5 g dry Milk with 10 milliliters (ml) diluted PBS was applied immediately after following with 30 minutes incubation time. Though, during incubation phase we prepared the primary antibody which was consisted of 4 microliters (μl) ICAM-1 diluted in 200 microliters (μl) of BSA and since the previous step was fulfilled, we inserted the solution to the slides which had to rest for one hour and placed in PBS for another 10 minutes whereas the formation of secondary antibody was taking place. In fact secondary antibody was constructed by 1 microliters (μl) of Goat Anti-Hamster diluted in 400 microliters (μl) of BSA and applied to the slides with incubation period was 30 minutes. As the procedure continued we transferred the slides to PBS buffer for 10 minutes by the time Extravidin CY3-BSA mixture with dilution 1:300 microliters (μl) had been prepared. It must be stated that due to the fluorescence microscopic observation of the slides we had to execute the remaining steps without the presence of any light source; hence the mixture Extravidin CY3-BSA was inserted to the slides for the rest 30 minutes. Since the time had ended DAPI solution was the next formulation, comprised by 40 microliters (μl) DAPI in 200 microliters (μl) of PBS and enforced immediately after PBS buffer (10 minutes) for a half hour additionally. In all, PBS was utilized twice for 5 minutes taking at the same time DABCO adhesive material to stick the microscopic glasses with the aortic endothelial sections of the slides. Once we placed the cover glasses in the slides our experiment was completed and visualization part under fluorescence microscope was initiated.

Photo documentation and image digitizing were performed by using a digital firewire camera Pixelink PL-A642 (Vitana Corporation, Ottawa, Canada) and a VDS Vosskuehler

CD-1300QB monochromatic camera (VDS Vosskühler GmbH, Germany) for fluorescence with image analysis software NIS (Laboratory Imaging, Czech Republic).

4.3 ELISA analysis of human soluble endoglin in mice

Blood samples were obtained from vena cava inferior and plasma levels of human sEng were determined by means of Human Endoglin/CD105 Quantikine ELISA Kit (R&D Systems, MN, and USA) according to the manufacturer's instructions. Due to the specificity of the transgenic mouse model, the methodology of human sEng evaluation in plasma had to be adjusted for our studies (300-fold dilution of murine samples compared to typical 4-fold dilution in standard human studies). Concentrations were reported as nanograms per milliliter (ng/ ml) for human sEng.

4.4 Biochemical analysis

Total concentration of plasma cholesterol was measured enzymatically by conventional enzymatic diagnostic kits (Lachema, Brno, Czech Republic) and spectrophotometric analysis (cholesterol at 510 nm, ULTROSPECT III, Pharmacia LKB Biotechnology, Uppsala, Sweden). Concentrations were reported as millimolar (mmol/L).

4.5 Statistical analysis

The statistical analysis was performed by GraphPad Prism 7.0 software (GraphPad Software, Inc., CA, and USA). All data are presented as mean ± S.E.M. All multiple comparison data were analyzed using ANOVA with Tukey's multiple comparisons test. Direct group-group comparisons were carried out using non-parametric Mann-Whitney test because D'Agostino & Pearson omnibus normality test failed. P values of 0.05 or less were considered statistically significant.

5. Results

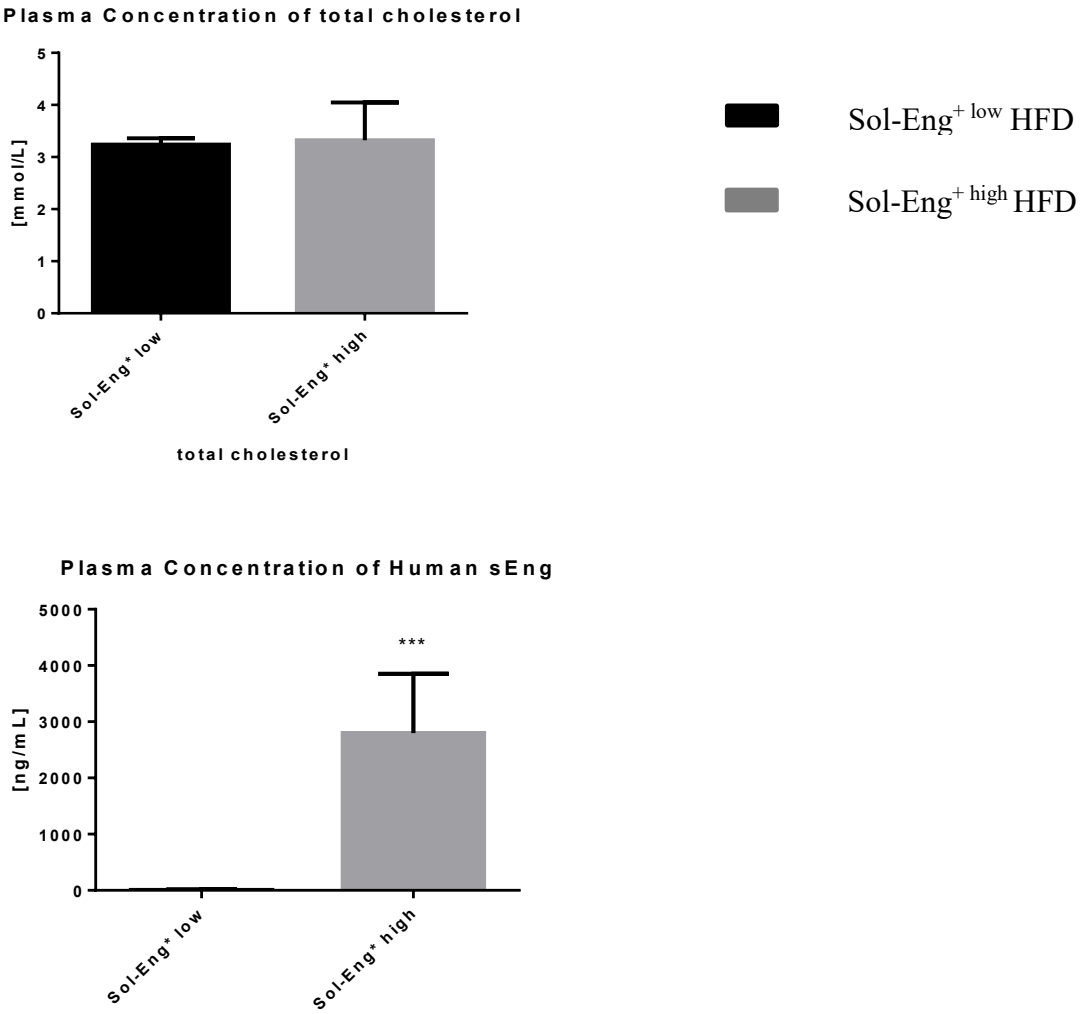
Mice with high and low levels of soluble endoglin were fed for six months with high fat diet (HFD). We evaluated cholesterol levels, soluble endoglin levels. Mainly we aimed to evaluate sEng effects in mice suffered with endothelial impairment on the expression of the pro-inflammatory biomarkers VCAM-1 and ICAM-1 to observe the inflammatory state in aortic endothelial cells by means of immunohistochemical techniques. Immunohistochemical staining with VCAM-1 was visualized under light microscope, whilst staining with ICAM-1 was evaluated under fluorescence microscope.

5.1 Plasma concentration of total cholesterol and human sEng in Sol-Eng^{+} mice

Mice were fed for six months with high fat diet (HFD) and later plasma samples were collected. Six-month old mice Sol-Eng$^{+\ low}$ were used as control group and subsequently biochemical analysis of plasma samples showed no significant differences in total cholesterol concentration (4.42 ± 3.04 mmol/L) vs (4.56 ± 2.87 mmol/L) between Sol-Eng$^{+\ low}$ HFD and Sol-Eng$^{+\ high}$ HFD mice (Figure 5A).

On the contrary, since ELISA measurements took place was observed that human sEng plasma concentrations were significantly higher in Sol-Eng$^{+\ high}$ (4604.22 ± 547.66 ng/mL) as to Sol-Eng$^{+\ low}$ (0.82 ± 48.6 ng/mL) mice (Figure 5B).

Figure 5: Total cholesterol and human sEng concentration in plasma. Plasma concentration of total cholesterol (A) and human sEng (B) in Sol-Eng$^{+\,high}$ and Sol-Eng$^{+\,low}$ mice fed with high fat diet for six months. Data are shown as mean ± S.E.M. Data are shown as mean ± S.E.M. Following data are analyzed by Mann-Whitney test, ***$p \leq 0.001$. n = 8 mice per.



5.2 Immunohistochemical analysis of VCAM-1 and ICAM-1 in aortic sections

Sixty aortic sections in thirty slides were evaluated per each experimental group. Mice aortic section was used as a positive control of VCAM-1 and ICAM-1 staining in aorta. The expression of both VCAM-1 and ICAM-1 was detected in both experimental groups. The expression was strictly localized in endothelium in aortic section. The results did not reveal significantly visible changes in the either area or intensity of immunohistochemical staining for both ICAM-1 and VCAM-1 in mice with high levels of soluble endoglin when compared to mice with low levels of soluble endoglin.

Figure 6: Representative sections of VCAM-1 immPRESS technique method staining in aorta of Sol-Eng+ low mice. Positive staining for VCAM-1 (arrows). 100 µm.

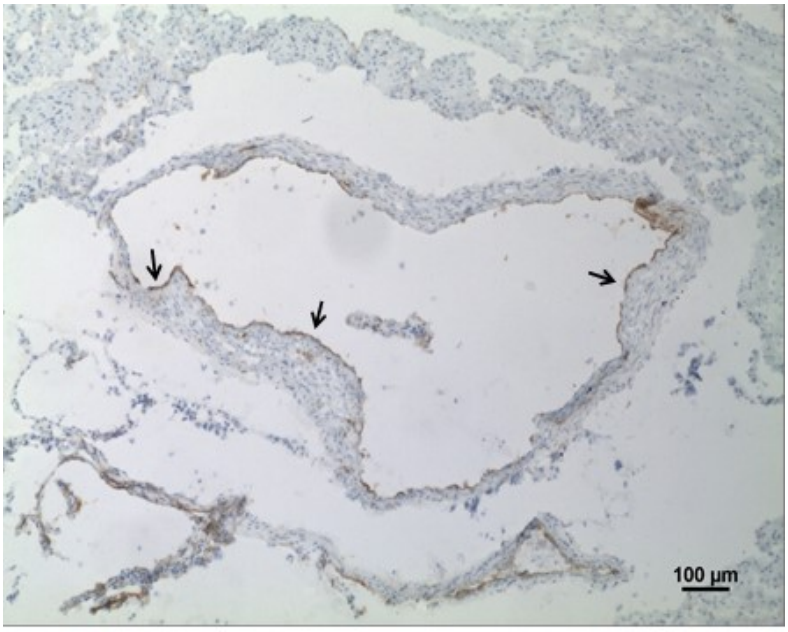


Figure 7: Representative sections of VCAM-1 immPRESS technique method staining in aorta of Sol-Eng+ low mice. Positive staining for VCAM-1 (arrows). 100 µm.

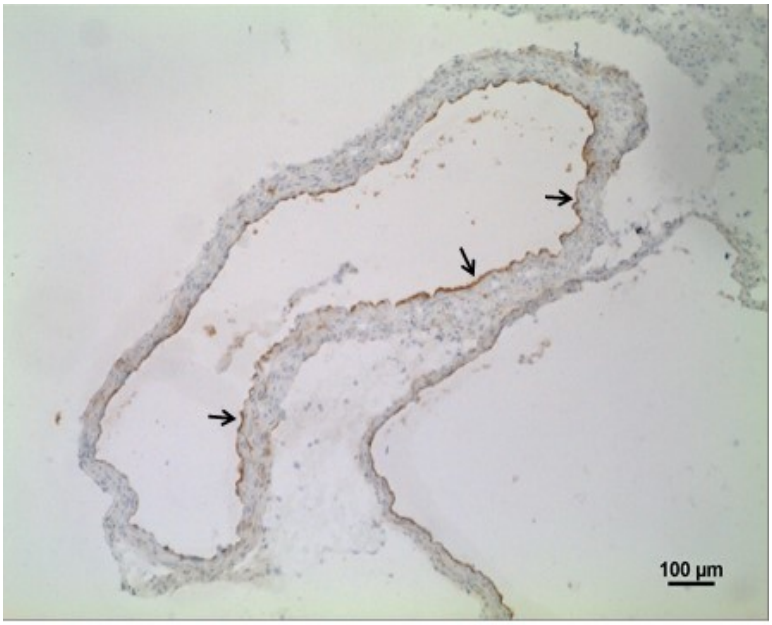


Figure 8: Representative sections of VCAM-1 immPRESS technique method staining in aorta of Sol-Eng+ low mice. Positive staining for VCAM-1 (arrows). 100 µm.

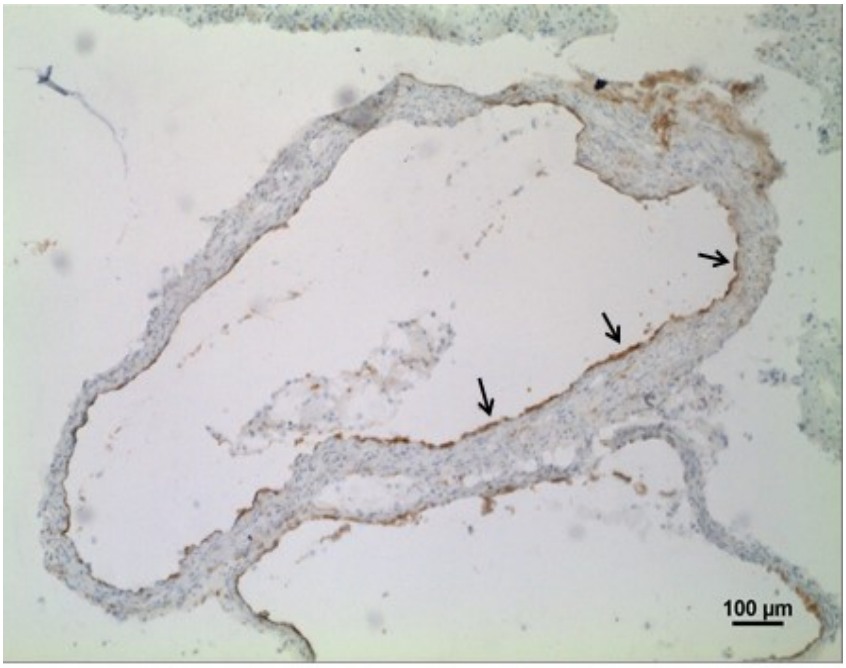


Figure 9: Representative sections of VCAM-1 immPRESS technique method staining in aorta of Sol-Eng+ low mice. Positive staining for VCAM-1 (arrows). 100 µm.

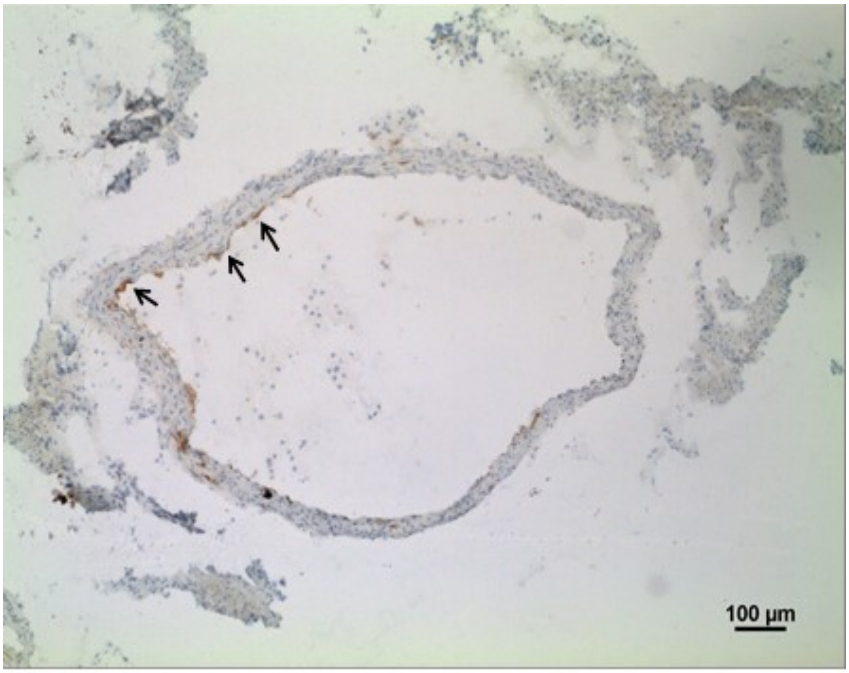


Figure 10: Representative sections of fluorescence ICAM-1 staining in aorta of Sol-Eng+ low mice. Positive staining (arrows) of ICAM-1, elastic fibers (Green) and nucleus (Blue) were detected additionally. 50 µm.

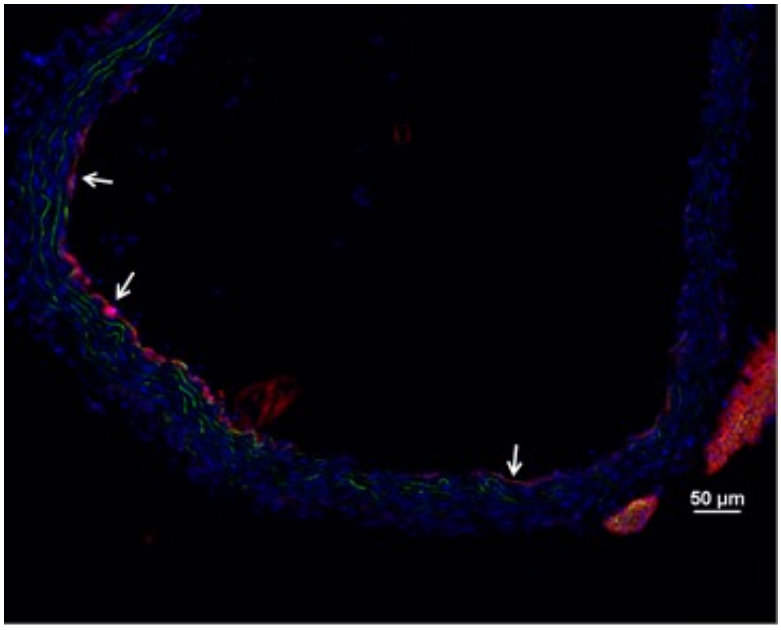


Figure 11: Representative sections of fluorescence ICAM-1 staining in aorta of Sol-Eng+ low mice. Positive staining (arrows) of ICAM-1, elastic fibers (Green) and nucleus (Blue) were detected additionally. 50 µm.

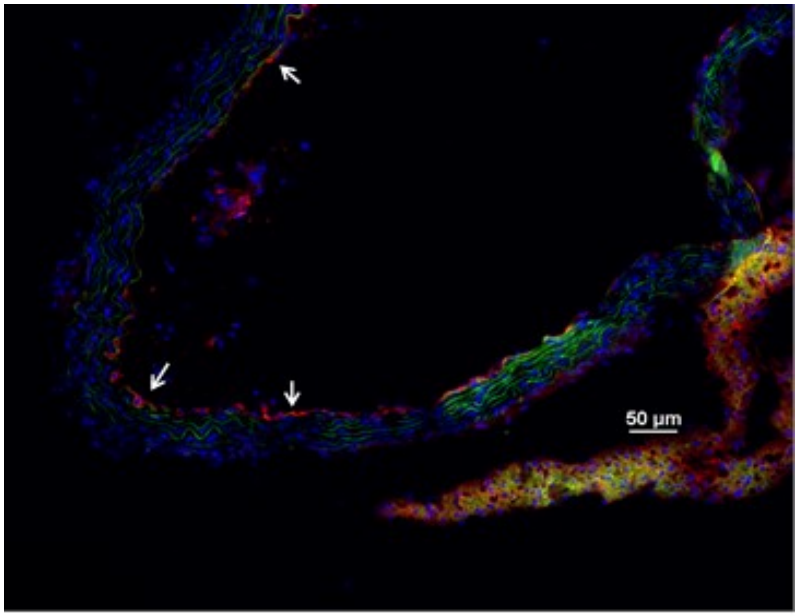


Figure 12: Representative sections of fluorescence ICAM-1 staining in aorta of Sol-Eng+ low mice. Positive staining (arrows) of ICAM-1, elastic fibers (Green) and nucleus (Blue) were detected additionally. 50 µm.

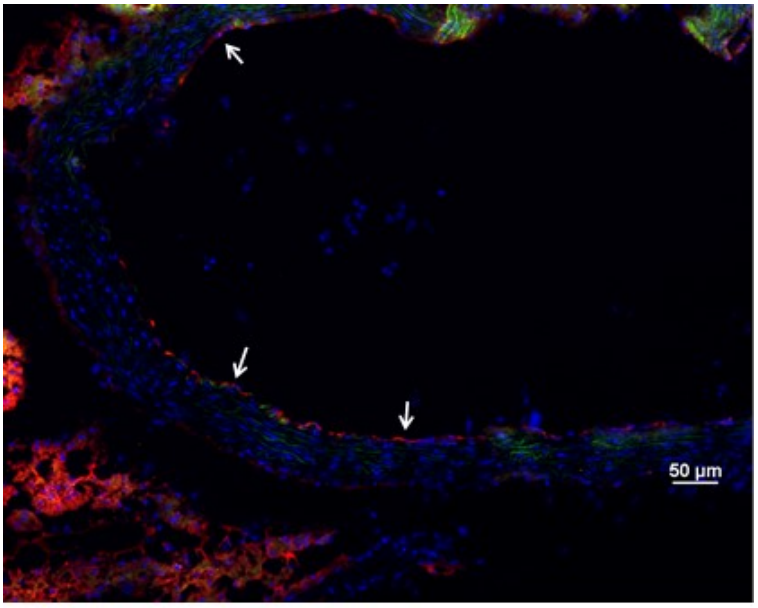


Figure 13: Representative sections of fluorescence ICAM-1 staining in aorta of Sol-Eng+ low mice. Positive staining (arrows) of ICAM-1, elastic fibers (Green) and nucleus (Blue) were detected additionally. 50 µm.

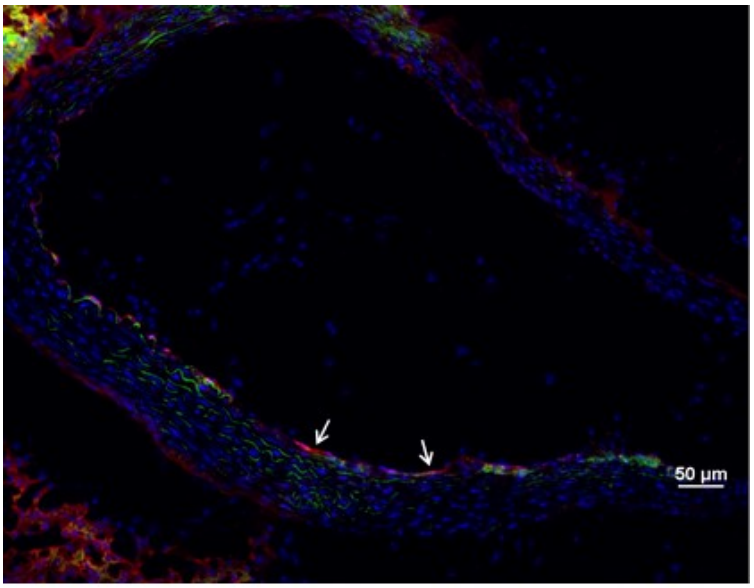


Figure 14: Representative sections of VCAM-1 immPRESS technique method staining in aorta of Sol-Eng+ high mice. Positive staining for VCAM-1 (arrows). 100 µm.

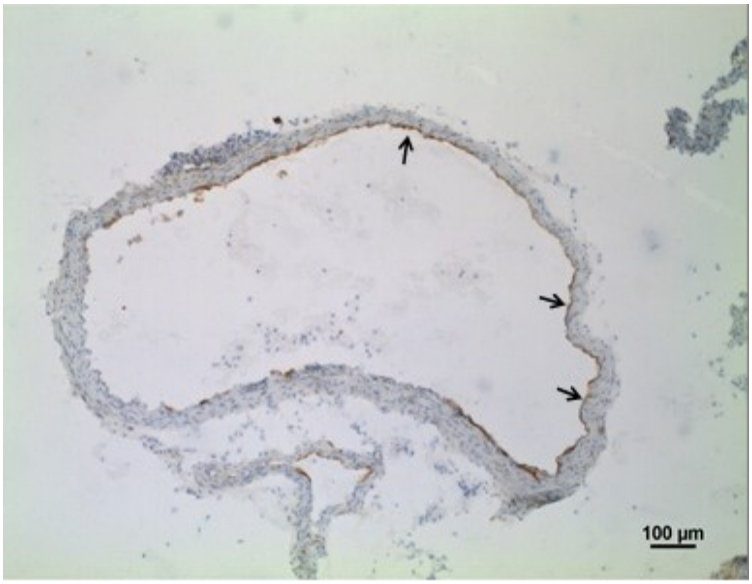


Figure 15: Representative sections of VCAM-1 immPRESS technique method staining in aorta of Sol-Eng+ high mice. Positive staining for VCAM-1 (arrows). 100 µm.

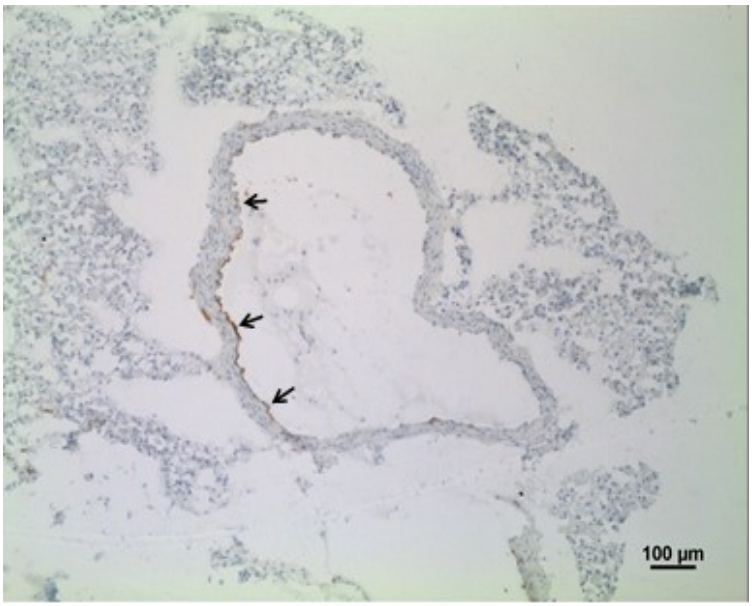


Figure 16: Representative sections of VCAM-1 immPRESS technique method staining in aorta of Sol-Eng+ high mice. Positive staining for VCAM-1 (arrows). 100 µm.

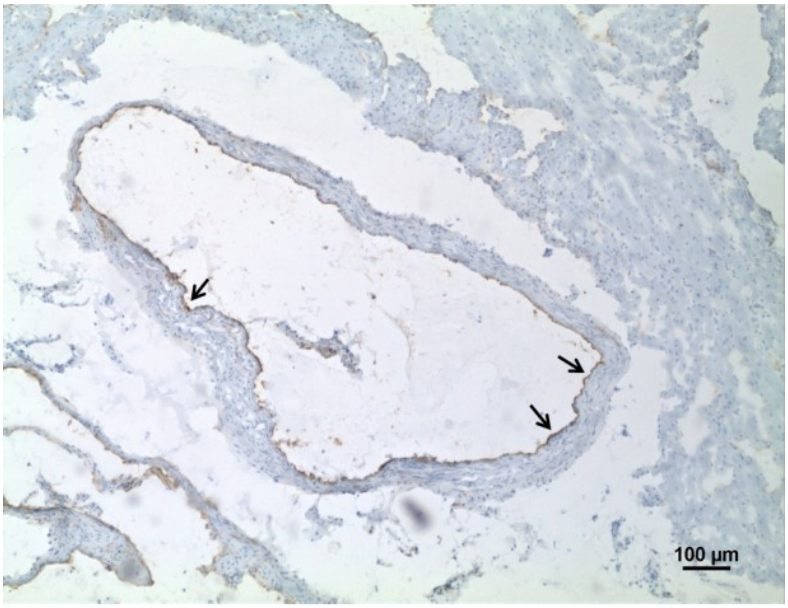


Figure 17: Representative sections of VCAM-1 immPRESS technique method staining in aorta of Sol-Eng+ high mice. Positive staining for VCAM-1 (arrows). 100 µm.

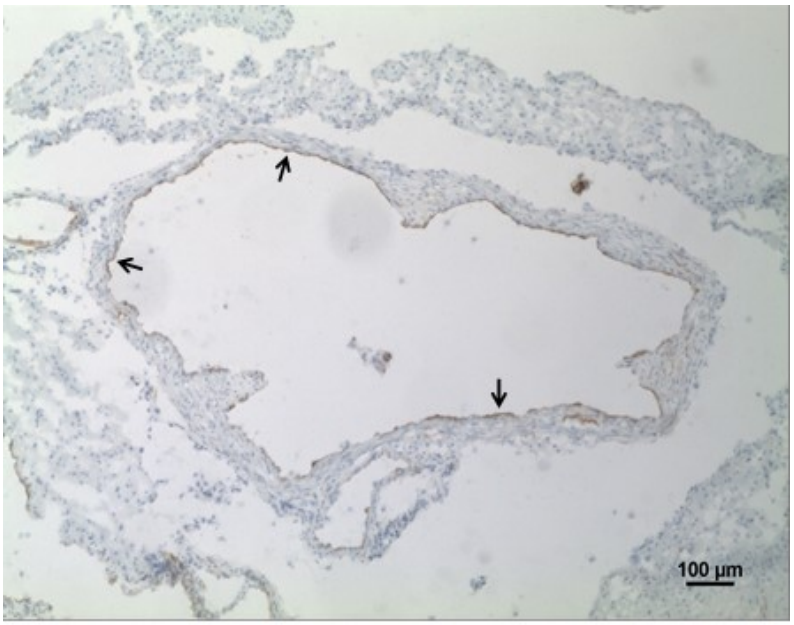


Figure 18: Representative sections of fluorescence ICAM-1 staining in aorta of Sol-Eng+ high mice. Positive staining (arrows) of ICAM-1. Elastic fibers (Green) and nucleus (Blue) were detected additionally. 50 µm.

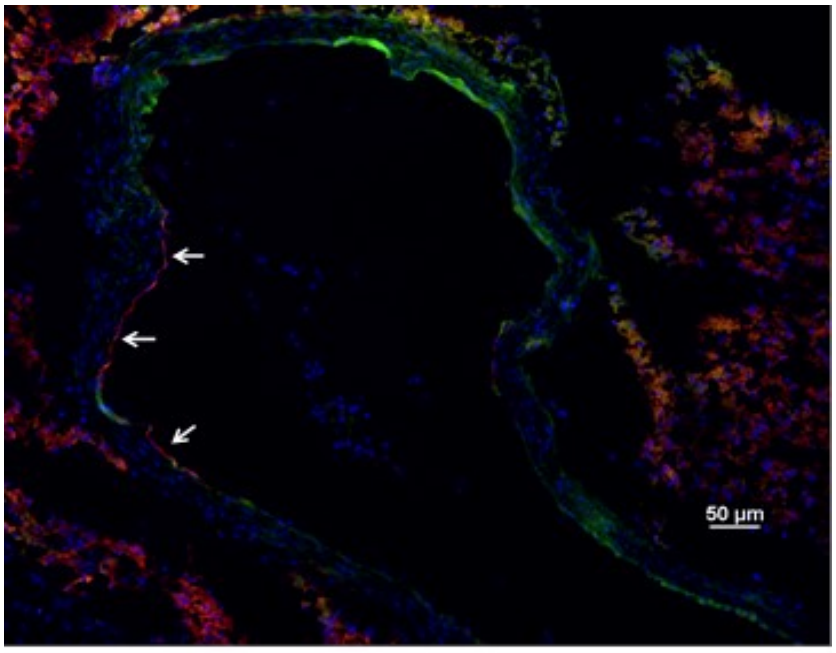


Figure 19: Representative sections of fluorescence ICAM-1 staining in aorta of Sol-Eng+ high mice. Positive staining (arrows) of ICAM-1. Elastic fibers (Green) and nucleus (Blue) were detected additionally. 50 µm.

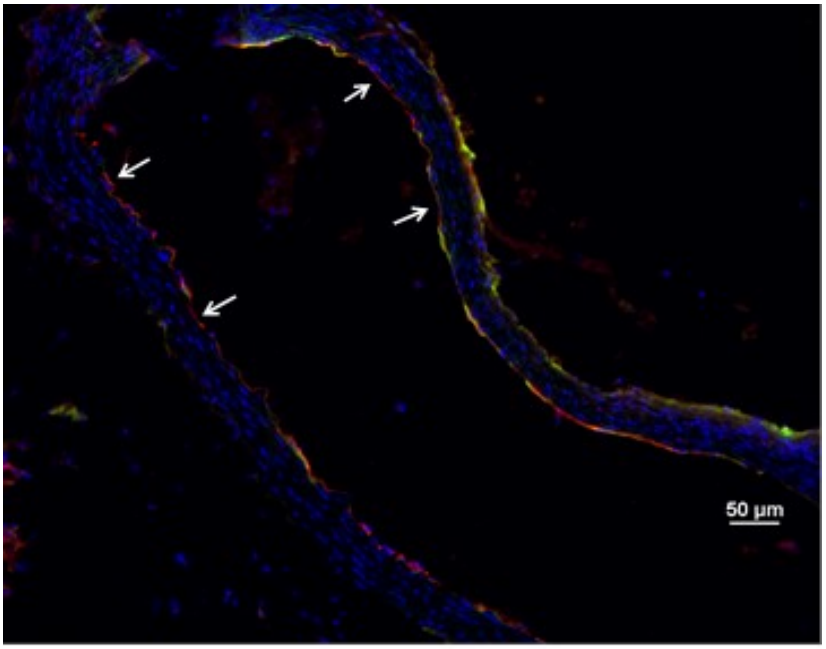


Figure 20: Representative sections of fluorescence ICAM-1 staining in aorta of Sol-Eng+ high mice. Positive staining (arrows) of ICAM-1. Elastic fibers (Green) and nucleus (Blue) were detected additionally. 50 µm.

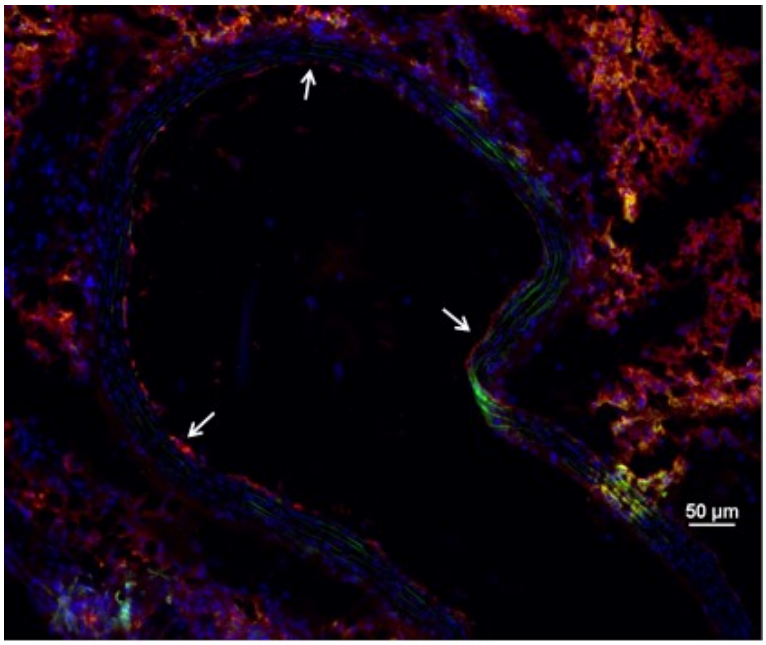
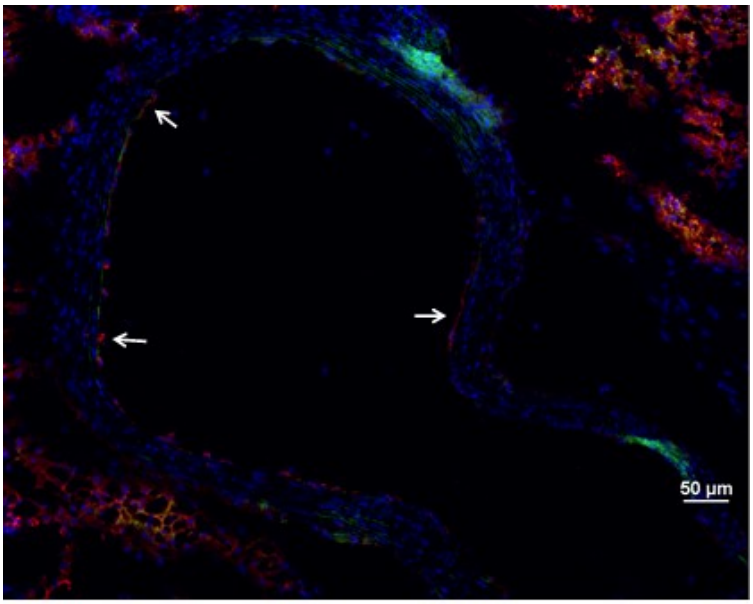


Figure 21: Representative sections of fluorescence ICAM-1 staining in aorta of Sol-Eng+ high mice. Positive staining (arrows) of ICAM-1. Elastic fibers (Green) and nucleus (Blue) were detected additionally. 50 µm.



6. Discussion

Accumulative evidence declared that high levels of sEng could be associated with various pathological illnesses, including diabetes mellitus type II, atherosclerosis, hypercholesterolemia and hypertension, proposing at the same time that these abnormal levels could be responsible for endothelial dysfunction which is a common characteristic for the above mentioned disorders (107). Nevertheless, the unresolved issue is if sEng could be affect endothelial function of those morbid disorders usually followed by hypercholesterolemia.

In this diploma thesis we aimed to evaluate whether high levels of soluble endoglin combined with mild hypercholesterolemia affect endothelial expression of cell adhesion molecules ICAM-1 and VCAM-1 in aorta.

Several papers indicated possible effects of soluble endoglin on vascular endothelium. Hence, it has been illustrated that application of recombinant sEng in rats result to alteration of TGF-β1 binding to its receptor followed by inhibition of eNOS dependent vasodilation in its microvessels and mesenteric vessels, leading to the development of hypertension (7). Several papers also mentioned that insertion of adenovirus in mice expressing sEng had a tendency to endothelial impairment in mesenteric endothelial venules, which were recognized by increase amount of cell adhesion molecules and damaged vasodilation (122). It must be stressed that we carried out the study in order to shed light the possible effects of sEng in aorta, since the previous studies performed were not linked to atherosclerosis blood vessels (107).

In fact we used transgenic mouse models that express human sEng (Sol-Eng^{+}) presenting numerous symptoms of preeclampsia disorder, including renal damage and hypertension, but on the contrary we did not observe any morbid effect of sEng regarding the function of aorta and expression of biomarkers connected with endothelial alteration (123). Hence, for this reason we induce in the experiment a combination of high fat diet and high level of sEng in order to provide the same "characteristics" as in patients suffered with atherosclerosis and diabetes mellitus type

II, where high cholesterol level disorder is a typical feature. Previous study revealed that the above mentioned combination leads to a pro-inflammatory tendency of aorta together with a strong expression of NF-κB and CAMs; however a negative effect of high sEng plasma levels concerning the proper function of aorta was not detected (124). Thus, we hypothesized that a combination of hypercholesterolemia with high sEng levels in plasma is an issue of relevant up-to-date study with longer exposure to high fat diet and soluble endoglin.

In this thesis, Sol-Eng+ high and Sol-Eng+ low mice we did not show any significant differences with respect to cholesterol levels, but on the other hand mice exposed to high fat diet express mild hypercholesterolemia than mice on normal diet. It is also necessary to note that the only difference observed between those two groups was in human sEng levels (125).

Moreover, immunohistochemical staining showed the expression of both VCAM-1 and ICAM-1 in aorta in low and high soluble endoglin mice suggesting at least partial development of endothelial dysfunction after high fat diet feeding in both groups. On the hand we did not detected any significantly visible differences in the expression of both VCAM-1 and ICAM-1 between mice Sol-Eng+ high and Sol-Eng+ low mice. These results were confirmed also by Vitverova et al paper by Western blot analysis (126).

Despite of this, Vitverova et al showed numerous results after six months consumption of high fat diet, including an alteration of endothelium dependent vasodilation triggered by acetylcholine, an inhibition of endothelium independent vasodilation triggered by SNP, a gradual limited contraction effected by KCL and a restricted phosphorylation of myosin light chain of Sol-Eng^{+} high mice. Hence, we suggest that a combination of significant raised plasma levels of sEng and hypercholesterolemia complicates the presence of aortic endothelial and vascular impairment without significant effects on the expression of cell adhesion molecules.

7. Conclusion

In conclusion, high levels of soluble endoglin did not induce inflammation and higher expression of cell adhesion molecules VCAM-1 and ICAM-1 in moue aorta despite the fact it resulted in the aggravation of endothelial dysfunction.

8. References

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