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Challenging the mystique connection

Deciphering cell-matrix interactions role
in atherosclerosis and restenosis

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EXPERIMENTAL MEDICAL SCIENCE | FACULTY OF MEDICINE | LUND UNIVERSITY



Challenging the mystique connection

Deciphering cell-matrix interactions role in
atherosclerosis and restenosis

Vignesh Murugesan



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DOCTORAL DISSERTATION

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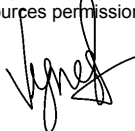
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| Title and subtitle Challenging the mystique connection – Deciphering cell-matrix interactions role in atherosclerosis and restenosis | | |
| <p>Abstract</p> <p>Atherosclerosis is the underlying cause for myocardial infarction, stroke and peripheral arterial disease, collectively referred to as cardiovascular diseases. These represent the major cause of death globally. Phenotypic modulation of vascular smooth muscle cells form a contractile to a synthetic state and subsequent migration into the intima, is one of the prime events that marks the genesis of atherosclerosis. To effectuate, cells are required to weaken the connection with their own basement membrane, a network of extracellular matrix molecules. This thesis explores the importance of Cell-ECM connections in atherosclerotic and restenotic settings using mice as a model. In detail, this thesis discusses the role of dystrophin and beta-sarcoglycan, two important components of the Dystrophin-glycoprotein complex — one important way cells use to communicate with basement membranes.</p> <p>In paper I & II, we investigated whether beta-sarcoglycan has a role in the development of atherosclerotic plaques and vascular injury induced neointima formation. Much to our surprise, we found that the absence of beta-sarcoglycan reduced advanced atherosclerotic plaque development, and in contrast, stimulated neo-intimal hyperplasia under conditions involving the presence or absence of an ApoE-deficient background, respectively. Under an atherosclerotic setting, the relative area of differentiated smooth muscle cells in the plaques was increased in beta-sarcoglycan deficient mice. Further, deficiency of beta-sarcoglycan decreased the weight of visceral adipose tissue and reduced the size of both brown and white adipocytes.</p> <p>In paper III, we utilized <i>mdx</i> mice to determine if there is also a role for dystrophin in the process of atherosclerotic plaque development. We obtained similar results as in the study with sarcoglycan deficient mice; the <i>mdx</i> mice exhibited a significant attenuation of advanced atherosclerotic plaque growth with an increase in the relative area of differentiated smooth muscle cells in their plaques. A reduction in T-cell content in these plaques and an overall reduction in systemic inflammation was also observed.</p> <p>In paper IV, we observed that actin dynamics influence genes important for regulation of VSMC phenotypes and found dystrophin to be significantly increased and decreased by actin polymerization and depolymerization, respectively. A significant downregulation of dystrophin was observed under the influence of vascular injury.</p> <p>To summarize, this thesis uncovers the important influence of a functional CELL-ECM linkage mediated by dystrophin and beta-sarcoglycan on vascular lesion development.</p> | | |
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Challenging the mystique connection

Deciphering cell-matrix interactions role in
atherosclerosis and restenosis

Vignesh Murugesan



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Coverphoto An advanced lesion from ApoE^{-/-} deficient mice displaying beta-sarcoglycan immunoreactivity in red and auto-fluorescence in green.

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I hope this thesis gives you a better appreciation and different perspective on the subject Atherosclerosis; not to forget that we once believed the earth is flat.

The above image is the widely known “*The pale blue dot*” by Carl Sagan, Image courtesy: Wikimedia commons

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Preface

As we know CVD remains the paramount cause of mortality in the developed world, with Atherosclerosis being the underlying cause. It's astonishing that the direct and indirect cost associated with CVD for the European Union is a colossal 210 billion € a year. In 2013 estimate by WHO, the number of death's caused by CVD is 17.3 million. To put things in perspective, it's nearly as twice the population size of Sweden. Hence, wouldn't it be good to live in a society free of CVD, instantaneously claiming lives of our near and dear one's?

In the course of my time as a PhD student, my aim has been to explore the cell-matrix interaction with respect to atherosclerosis. Mechanisms concealing vascular smooth muscle cell (VSMC) phenotypic modulation are still a holy grail! Questions are such as: are VSMCs a friend or a foe? What the ideal proportion of VSMCs for plaque stability may be, is still obscured and a subject of debate. To boil down, my study is focused on characterizing Dystrophin and beta-sarcoglycan, parts of the dystrophin-glycoprotein complex (DGC), one of the possible mechanisms, by which the VSMCs connect to the basement membrane. Upon the weakened interaction of this connection, cells may modulate their phenotype to migrate and proliferate differently, for instance during vessel injury and stress, exhibiting altered plasticity. Consequently, the aim of my thesis is to check whether Dystrophin and beta-sarcoglycan has any role in the development of atherosclerotic plaques and restenosis.

In the time ahead, drugs that can alter the expression of Dystrophin and beta-sarcoglycan expression may help achieve an ideal composition of VSMCs in order to have an atherosclerotic plaque with a good stability, and at the same time hamper uncontrolled proliferation. I hope my project bestows a better understanding and appreciation about the atherosclerotic process. This is *my* outlook to address atherosclerosis!

“The scientific man does not aim at an immediate result. He does not expect that his advanced ideas will be readily taken up. His work is like that of the planter - for the future. His duty is to lay the foundation for those who are to come, and point the way” - **Nikola tesla**

Original papers

This thesis is based on the following original papers and manuscripts

- I. **Vignesh Murugesan**, Eva Degerman, Ann-Kristin Holmen-Pålbrink, Pontus Duner, Anki Knutsson, Anna Hultgårdh-Nilsson, Uwe Rauch. *Beta-sarcoglycan deficiency reduces atherosclerotic plaque development in ApoE-null mice.* Accepted, DOI: 10.1159/000478014, Journal of vascular research. 2017
- II. **Vignesh Murugesan**, Anki Knutsson, Sabrina Hsiung, Uwe Rauch, and Anna Hultgårdh Nilsson. *Beta-sarcoglycan deficient mice display increased injury-induced carotid fibrotic lesion formation.* Manuscript.
- III. Annelie Shami, Anki Knutsson, Pontus Dunér, Uwe Rauch, Eva Bengtsson, Christoffer Tengryd, **Vignesh Murugesan**, Madeleine Durbeej, Isabel Gonçalves, Jan Nilsson, Anna Hultgårdh-Nilsson. *Dystrophin deficiency reduces atherosclerotic plaque development in ApoE-null mice.* Sci Rep. 2015 Sep 8;5:13904. doi: 10.1038/srep13904.
- IV. Karolina M. Turczyńska, Karl Swärd, Tran Thi Hien, Johan Wohlfahrt, Ingrid Yao Mattisson, Mari Ekman, Johan Nilsson, Johan Sjögren, **Vignesh Murugesan**, Anna Hultgårdh-Nilsson, Pilar Ciudad, Per Hellstrand, M.Teresa Pérez-García and Sebastian Albinsson. *Regulation of Smooth Muscle Dystrophin and Synaptopodin 2 Expression by Actin Polymerization and Vascular Injury.* Arteriosclerosis, Thrombosis and Vascular Biology,35(6),1489-1497. DOI:10.1161/ATVBAHA.114.305065

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Abbreviations

| | |
|---------------|---------------------------------------|
| CVD | Cardiovascular disease |
| CHD | Coronary heart disease |
| LDL | Low density lipoprotein |
| ECM | Extracellular matrix protein |
| ECs | Endothelial cells |
| VSMCs | Vascular smooth muscle cells |
| PDGF | Platelet derived growth factor |
| VCAM | Vascular cell adhesion molecule |
| ICAM | Intracellular cell adhesion molecule |
| MCP-1 | Macrophage chemo attract protein -1 |
| M-CSF | Macrophage colony stimulating factors |
| ET-1 | Endothelin-1 |
| FGF | Fibroblast growth factor |
| IL-1 | Interleukin-1 |
| CD36 | Cluster of differentiation |
| MMP | Matrix metalloproteinases |
| SM MHC | Smooth muscle myosin heavy chain |
| α -SMA | Smooth muscle α -actin |
| CAMs | Cell-adhesion molecules |
| DGC | Dystrophin glycoprotein complex |
| DAP | Dystrophin associated protein complex |
| DMD | Duchenne muscular dystrophy |
| LGMD | Limb girdle muscular dystrophy |

| | |
|-----------------|-------------------------------------|
| SGC | Sarcoglycan complex |
| bSG | β -Sarcoglycan |
| <i>dmd</i> | Dystrophin |
| WAT | White adipose tissue |
| BAT | Brown adipose tissue |
| TG | Triglyceride |
| FFA | Free fatty acids |
| SAT | Subcutaneous adipose tissue |
| VAT | Visceral adipose tissue |
| T2DM | Type 2 diabetes mellitus |
| PVAT | Perivascular adipose tissue |
| ATM | Adipose tissue macrophage |
| NO | Nitric oxide |
| eNOS | endothelium nitric oxide synthase |
| RAS | Renin–angiotensin system |
| F/G actin ratio | Filamentous to Globular actin ratio |
| ApoE | Apo-lipoprotein E |
| <i>mdx</i> | Dystrophin mutant mice |
| Jasp | Jasplakinolide |
| Lmod1 | Leiomodin 1 |

Background

The following chapter provides a brief introduction about atherosclerosis and progression of the disease, an overview of the dystrophin glycoprotein complex, and the cell-extracellular connection.

What is atherosclerosis; how far are we?

In 2013, cardiovascular disease (CVD) accounted for 30.8% of all the death i.e. 1 in 3 people die of CVD. That being said—the approximate annual cost for CVD and stroke in 2011-2012 were colossal with \$316.6 billion—by comparison to cancer \$88.7 billion [1]. Atherosclerosis is a chronic inflammatory disease, the root cause for cardiovascular disease (CVD), which includes coronary heart disease (CHD), ischemic stroke, and peripheral arterial disease [2, 3]. The disease is thought to be a modern one, a conception due to contemporary lifestyle in modern human beings. Despite that the 1900's were marked as the period for the detection of heart diseases, one study found that already Egyptian mummies had heart disease, in which arteries plagued by atherosclerosis were observed [4]. Today, we have technically evolved to treat coronary artery disease, and narrowed arteries. However we are still a long way from completely erasing the disease.

Our cardiovascular system comprises both muscles (heart) and blood flow system (vessels). Blood is transported through two distinct channels, namely the arteries and the veins, where the arteries carry oxygenated blood from the heart to other peripheral parts, and veins doing the opposite with the un-oxygenated blood. Atherosclerosis is, in part, a progressive inflammatory disease of the artery, marked by accumulation of lipids and cellular fractions on the vessel wall [5]. In the early days, the disease is conceived as an outcome of lipid storage on the walls of the vessel. Recent research has found that inflammation is the outcome of the response by the tissue to lipid storage; a driving factor of the disease from its very inception to its long clinically silent phase of progression, up to including the most dramatic consequence of plaque rupture and thrombosis [6]. This led to questions, which are still debatable: Can atherosclerosis be considered as being totally a measure of the immune system response? And if so, how far contributes it to the

pathology? Prior to explaining how atherosclerosis develops, one should have a clear view of the basic morphology of a healthy artery.

The healthy artery

Not merely a hollow pipe that carry fluid, but a high-pressure complex pipeline.

The arteries, system of tubes that transport oxygenated blood around the body are tri-laminated tubes, each layer separated by a thin elastic membrane.

The inner side of the artery called the “**intima**” comprises a dynamic interface with the blood flow, and possess a thin single sheet of endothelial cells (ECs), which sits upon a basement membrane rich in Laminin, Collagen type IV, heparan-sulphate proteoglycans (HSPGs) and nidogens [7]. The endothelial cells perform mechano-sensing and transduction processes to maintain vascular homeostasis and are normally seen as a vasodilator, an anticoagulant, pro-fibrinolytic and anti-inflammatory in nature [8]. The intima is like a delicate epidermis for the artery, so delicate that it’s prone to injury from circulating factors in the system.

The middle layer referred to as the “**media**” is substantially made up of layers of VSMCs embedded in basement membrane molecules, supported by additional connective tissues such as: proteoglycans, Collagen type I/III/V, fibronectin and elastin elements. The layer handles changing blood pressure, through vasoconstriction, and relaxation to maintain constant blood pressure.

The “**adventitia**” (or tunica externa), the outermost layer is tough and comprises collagen I&III rich connective tissue (provides tensile strength) with interspersed fibroblasts, and also other cell types such as macrophages, T-cells, and mast cells, and also nerves and small blood vessels (vasa vasorum) are present.

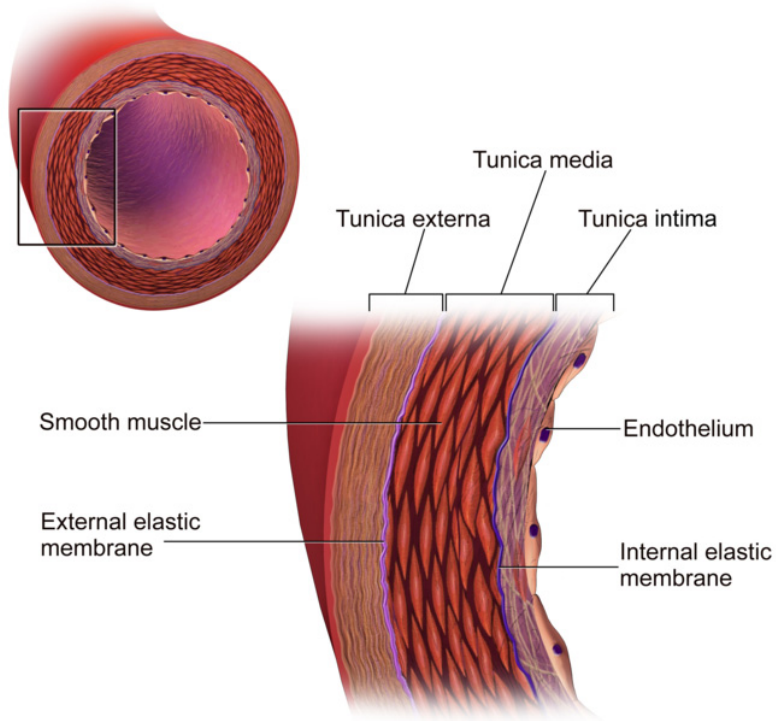


Figure 1:
A normal healthy artery with the three distinct layers. Image courtesy from Blausen 2014 [9].

Theories on atherosclerosis

Research into the disease has led to many possible hypotheses on the development of atherosclerotic plaques.

- Response to retention (sub endothelial retention of lipoproteins)
- Response to injury (Endothelial injury)
- Lipid oxidation hypothesis (oxidation of lipids)

In this thesis, the description of the process of plaque development follows the retention theory, as it appears more relevant.

Sites prone to atherosclerotic plaque development

The circulation display's different types of blood flow, which can be, for example, pulsatile, oscillatory, laminar or turbulent. Turbulent flow occurs usually at branch points and bifurcations, so do atherosclerotic plaques. Plaques are not randomly distributed but usually form in inner curvatures and at bifurcations, where the normal laminar flow is disturbed and EC's exposed to low or oscillatory shear stress resulting from flow obstruction [10] [8].

The shear stress acting on the ECs is among the important physical forces that influence EC's morphology. Generally, ECs display an athero-resistant phenotype in a normal state of the vessel.

Process of atherosclerotic plaque development

A schematic outline, on the development of an atherosclerotic plaque is depicted in (figure 2). To simplify the steps involved in atherosclerotic plaque development, I will classify the process based on influential stages.

A fully-grown advanced inflammatory plaque undergoes a series of steps summarized as follows: 1) *Sub endothelial retention of LDL particles and foam cell formation.* 2) *Inflammation cascades and recruitment of other immune cells.* 3) *Migration and proliferation of vascular smooth muscle cells (VSMCs) into lesion area.* 4) *Production of ECM and formation of fibrous cap by VSMCs.* 5) *Apoptosis of lesion cells and formation of necrotic core.* 6) *Thrombosis (an event occurring, after the plaque has grown substantially)*

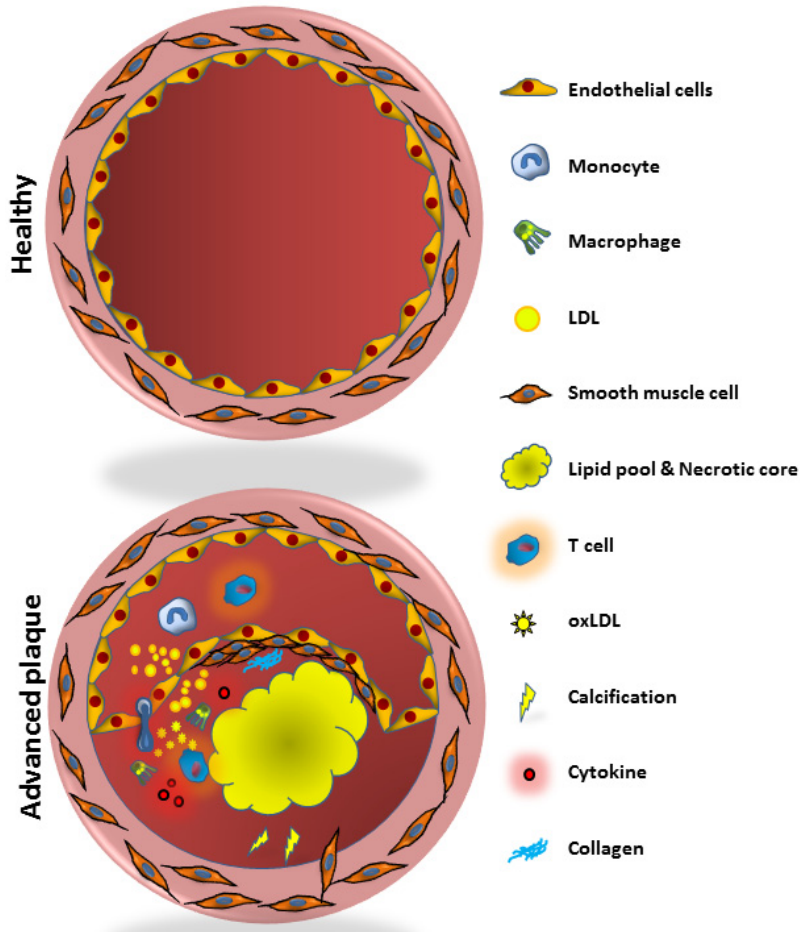


Figure 2: Schematic illustration of a healthy artery and an advanced plaque in the artery. Drawing by Murugesan.V

Sub endothelial retention of Lipoproteins and Infiltration of monocytes—A nascent atherosclerotic lesion

The disease starts with sub endothelial retention and accumulation of LDL particles in a mesh of proteoglycans by passive diffusion through endothelial junctions; later ECs are activated, and a multistep cascade of events is initiated that leads to recruitment of leukocyte to the site of inflammation [5, 11, 12]. Normally, the ECs with the help of their intercellular tight junctions, resist firm and prolonged adhesion of monocytes and lymphocytes that pass along the

endothelium millions per moment and function as a selective barrier between blood and tissue[5]. Under pathological conditions, these cells are in an activated state and, instead of resisting contact with the leukocytes, express adhesion molecules that attract them onto their surface. The trapped native LDL undergoes upon residing in the sub-endothelial space for an extended time chemical modifications—oxidation, lipolysis and proteolysis. The oxidized LDL stimulates the overlying EC's to expose various adhesion molecules like VCAM, ICAM, and growth factors such as macrophage colony stimulating factors (M-CSF) leading to inflammation and foam cell formation explained below[5].

In detail, the monocytes perform “tethering and rolling”, along the activated endothelial surface. Adhesion of circulating monocytes onto the endothelium is mediated on the endothelial side by selectin's—P & E, and adhesion molecules such as VCAM and ICAM [5, 13, 14]. The adhered monocytes transmigrate and differentiate into macrophages based on chemo attractants and stimulants such as macrophage chemo attractant protein-1 (MCP-1) and macrophage colony stimulating factors (M-CSF). The resulting macrophages start to internalize the modified LDL particles using specialized high capacity low affinity receptors—the scavenger receptors, ultimately evolving the cell into a so called “foam cell”. This stage is recognized as a precursor for an advanced lesion [14, 15].

Inflammation cascades and recruitment of other immune cells

Fatty streak into a complicated atheroma

The foam cells now existing as a professional inflammatory cell secretes pro-inflammatory cytokines, can amplify and sustain the inflammation along with activated endothelium to recruit T-cells and more monocytes, leading to a vicious cycle of inflammation. These activated leukocytes then release cytokines and proteolytic enzymes degrading matrix proteins, stimulating smooth muscle cell proliferation and migration from the media into the intima, a process dealt with under the next subheading [14].

Migration and proliferation of vascular smooth muscle cells (VSMCs) into the lesional area

The inflammatory cells, inside the intima mediate the stimulation of VSMCs through production of growth promoters and cytokines. In a normal artery, VSMCs reside in the media, and remain in a contractile form, resisting growth factors [16]. Under pathological conditions, they migrate into the intima and start to proliferate, due to mitogens (mentioned above), by changing from a quiescent

‘contractile’ phenotype to an active ‘synthetic’ phenotype. This is a prerequisite for progression of the disease. Moreover, this facilitates the cells to lay out altered ECM in the intima[17]. Some of the critical growth promoters for VSMCs are: platelet derived growth factor (PDGF), endothelin-1 (ET-1), thrombin, fibroblast growth factor (FGF) and interleukin-1 (IL-1). The MMP’s could also play a pivotal role in SMC migration by virtue of degrading the basement membrane surrounding the SMC, facilitating contacts with interstitial matrix [17]. Much research exhibits the occurrence of a spectrum of phenotypes amidst contractile and synthetic states manifested by VSMCs, which raises concerns on the unique function of these cells in the plaque.

Production of ECM and formation of fibrous caps by VSMCs

Once inside the intima, synthetic VSMCs generate a bulk of ECM proteins, for instance: collagen, elastin and proteoglycans, leading to a “**fibro-fatty**” lesion. The VSMCs typically produce collagen I, and to a lesser extent collagen III & proteoglycans, providing structural stability to the plaque. Fibrous tissue remains as a major component of atherosclerotic lesions upon progression. That being the case, collagen in the fibrous cap is therefore considered as a guardian for the stability of the plaque. The fibrous cap of a stable lesion is enriched in versican, biglycan and collagen type I, whilst, in fibrous caps of unstable lesion it is predominantly collagen type III and hyaluronan, and versican in erosion sites [18]. It has been estimated that, collagen has a half-life of 60-70 days in the vessel wall! [19]

Apoptosis of lesional cells and formation of a necrotic core

Macrophages and smooth muscle cells are often the cell types that undergo necrosis. As a result, plaque cells unleash their cytoplasmic components due to lost membrane integrity, into a pool of lipid—better referred as the “**necrotic core**”. The core is devoid of collagen and nuclei; however, it contains cellular debris and a mixture of lipids, largely cholesterol and cholesterol esters, derived from lipids that have infiltrated the wall of the artery, besides lipids from dead foam cells[13].

The core forms early in the lesion development during the transition from a fatty streak to a fibrous plaque. Weak, soft and highly thrombogenic, is what the central core is best described. An area of acellularity, containing small cholesterol clefts and proteoglycans, characterizes early necrotic cores. Contrastively, late necrotic cores are defined by an absence of extracellular matrix, numerous cholesterol

clefts and cellular debris. Intriguing, is what creates a necrotic core: The continuous influx of mononuclear cells and inefficient apoptotic cell clearance by phagocytosis from infiltrating macrophages (a process known as efferocytosis) are the major factors for the formation of advanced central necrotic cores [20, 21]. The resulting core instigates further cell necrosis due to a vicious cycle of inflammation, owing to secretion of cytokines, and growth factors by macrophages and T-cells, further activating VSMCs to migrate and proliferate. As a consequence, the VSMCs take-up cholesterol by means of expressing a variety of receptors such as: VLDL, CD36, Type I and II scavenger receptors [22]. High portions of tissue factors are released by necrotic cells in the core, ultimately stimulating thrombus formation; this is the moment when thin fibrous caps rupture [20].

Rupture and Thrombosis

Since ruptures constitute the majority of cases in patients, the emphasis will be laid to a greater extent on rupture (55-65%) over plaque erosion (30-35%) and calcified nodules (2-7%) [23]. The rupture of a plaque and formation of thrombosis, is the ultimate and critical event in the process of atherosclerosis; it is a trigger to myocardial infarction or stroke. The vulnerable, or unstable plaques (discussed later) can exhibit relatively minor stenosis, but are in most cases the culprit for rupture and thrombotic event, comparable with plaques manifesting severe luminal stenosis [24]. Succinctly, during a rupture, the plaque fibrous cap breaks apart and plaque components are exposed to the blood stream. Interactions with platelet receptors and coagulation components can occur. Aggregation and formation of a superimposed thrombus are consequences of activated platelets [23]. Eventually, a blood clot can get big enough to completely occlude the vessel, and all the downstream tissue deprived of oxygen and nutrients will start to die eventually. On the other hand, the thrombus does not have to form in the plaque region; rather a blood clot formed elsewhere can break apart and get pumped into a narrow vessel, which it can occlude completely. Also a portion of plaque can detach and cause a blockage elsewhere. Such occluding objects are referred to as “emboli” and the process is called embolism. Often, an eccentric distribution of lipid cores leads to an increased vulnerability for rupture in plaque shoulder regions. It has been estimated that around 60% of rupture occurs in shoulder regions [25, 26]. Usually, rupture is a ramification of fibrous cap thinning promoted by death of VSMC’s and breakdown of ECM [27].

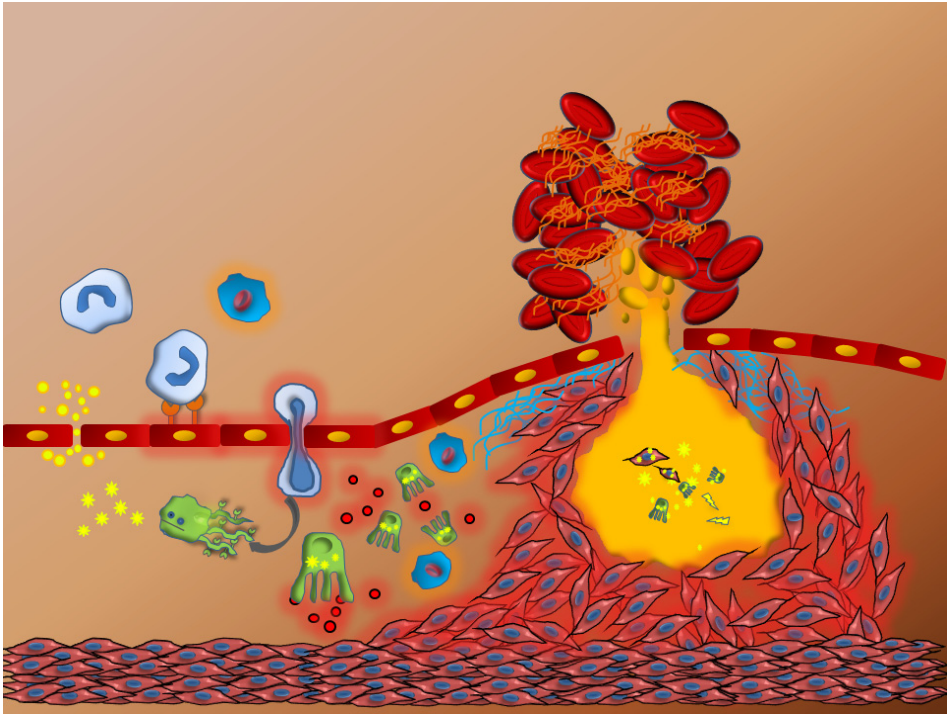


Figure3: Schematic illustration of sequences involved in the development of atherosclerotic plaques and rupture. Drawing by Murugesan.V

A dynamic atherosclerotic plaque: what matters composition or volume?

Apparently, a plaque makeup includes: cellular elements and extracellular matrix. Matrix constitutes >50% of the plaque volume [14]. The extracellular matrix comprises: glycosaminoglycans, proteoglycans, laminin, collagen, elastin, vitronectin, fibronectin and thrombospondin; it is largely produced by activated SMCs [14]. The cellular fraction consists largely of macrophages and smooth muscle cells; it has lesser proportions of T-cells and dendritic cells. Cell migration, division, death, matrix synthesis and degradation, and loss or accumulation of lipids, are the influential biological processes that perpetuate the plaque. Each of these processes increases, or decreases the relative stability of plaques. For example, macrophage accumulation increases matrix degrading proteinases which directly relates to chances of plaque rupture, whilst loss of vascular smooth muscle cells by apoptosis, leads to thin fibrous caps, increased necrotic cores and

increased inflammation which promotes plaque instability. This reinforces the notion that rather plaque composition than plaque volume is important for plaque stability/instability.

Characteristics associated with a symptomatic plaque include:

- Active inflammation
- Large lipid core ($\geq 40\%$ plaque volume) with a thin fibrous cap depleted of VSMCs and collagen
- Paucity of SMC
- Fissure of plaque surface
- Thrombus
- Stenosis with a narrowed luminal diameter of 10% or less
- Presence of calcified nodules
- Intra-plaque hemorrhage
- Outward remodeling
- Increased adventitial and intimal neo-vascularity

Table 1. Characteristics associated with Stable plaque vs. Unstable plaques

| Stable | Unstable |
|--|-------------------------------------|
| Often relatively small volume | Often relatively large volume |
| Small lipid core | Large lipid core |
| Lower number of inflammatory cells | Higher number of inflammatory cells |
| Decreased neo-vascularity | Increased neo-vascularity |
| Thick fibrous cap (higher SMC density) | Thin fibrous cap (low SMC density) |
| In ward or negative remodeling | Outward or positive remodeling |

Composition of an atherosclerotic plaque

The biological components of an atherosclerotic plaque include extracellular matrix, lipids and a cellular fraction

Lipids

Lipids greatly play a role in initiation and progression of atherosclerosis.

Lipoproteins contain triglycerides (TG) and cholesterol, which are important in delivering peripheral tissues with fatty acids from triacylglycerol (energy production), and cholesterol for synthesis of cellular membranes and steroid hormones. To understand how atherosclerosis is initiated, it's important to comprehend, the body's lipoprotein transport system. Lipids are transported in the circulatory system via special carrier proteins, called the lipoproteins. A lipoprotein is made up of cholesterol esters and TGs at its core. A monolayer of phospholipids surrounds the cholesterol and TGs. Embedded on it are amphipathic proteins called the apo-lipoproteins (figure 4). The apo-lipoproteins through their various functions, such as enzyme activation and receptor binding, choreograph the transport of lipids from sites of absorption or synthesis to sites of utilization. This system circulates TGs to muscles for energy use or to adipose tissue for storage. Besides, it also cycles cholesterol for distribution throughout the body for the synthesis of cell membranes, bile acids, and steroid hormones. ApoB containing lipoproteins are otherwise called non-HDL lipoproteins; they are involved in the lipid delivery pathway, whilst, Apo A containing lipoproteins (HDL) are associated with the reverse cholesterol transport.

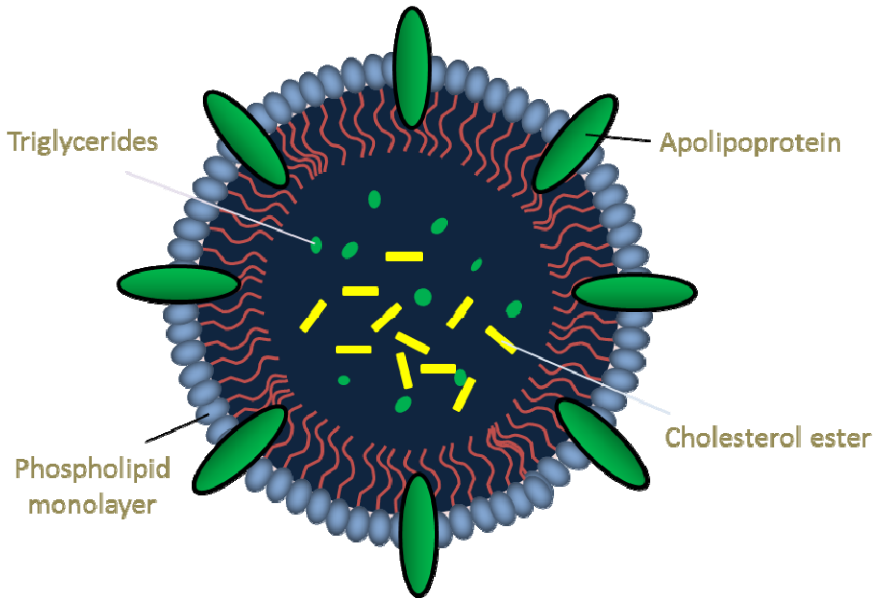


Figure 4:
General structure of a lipoprotein. Drawing by Murugesan.V

In general, lipoproteins can be broadly divided into five types, based on their density and type of apolipoprotein they contain. They are: chylomicrons, very low density lipoprotein (**VLDL**), Intermediate dense lipoproteins (**IDL**), Low density lipoproteins (**LDL**) and High density lipoproteins (**HDL**) [28]. The chylomicrons and VLDL are triglyceride rich lipoproteins. These structures function to deliver energy rich triacylglycerol to the cells. On the other hand, LDL delivers cholesterol to the cells, used in membranes, or to synthesize steroid hormones. Lastly, HDL as mentioned is involved in reverse cholesterol transportation, where it collects excess cholesterol from cells and delivers it back to the liver. Despite the fact that, HDL can counteract a high content of LDL, in certain instances HDL can be pro-inflammatory. Therefore, in general HDL can be regarded as a chameleon. Since, LDL is recognized as a major atherogenic lipoprotein, I will briefly touch on, how LDL is formed and retained in sub-endothelial spaces.

During lipoprotein remodeling processes, the lipoprotein's core TGs are hydrolyzed into free fatty acids via activation of lipoprotein lipase. As fatty acids

exit the lipoproteins, these becomes smaller and smaller until they reach a stage of transforming into LDL. The formed native LDL is continually exposed to endothelial cells in the circulation. It has been shown that receptor mediated transcytosis driven by plasmalemmal vesicles and alternatively leaky junctions of the endothelium (for example in states of cell turnover or death) are the processes by which LDL crosses the endothelial barrier [29-32]. Once in the sub-endothelial space, binding of proteoglycans evoke changes in the configuration of apoB-100 and in the composition of lipid, making it susceptible to free radical damage causing oxidation. The sub-endothelial accumulation of LDL vastly depends on the balance at which the LDL enters and leaves the arterial wall.

Table 2. Functions of different lipoproteins and their major respective Apo-lipoproteins

| Lipoproteins | Major apo-lipoproteins | Functions |
|--------------|------------------------|---|
| Chylomicrons | ApoB-48 | Transports TG & Cholesterol |
| VLDL and IDL | ApoB-100 | Transports TG & Cholesterol |
| LDL | ApoB-100 | Transports TG & Cholesterol |
| HDL | Apo A-I, A-II | Collects TG & Cholesterol back to the liver |

Smooth muscle cells, role and phenotypic modulation

Smooth muscle cells are spindle shaped cells, which are important functional units of the vessel; they are critically regulating the vascular tone—vasoconstriction and relaxation—that influences homeostasis [3]. The VSMCs show a remarkable phenotypic plasticity in the course of blood vessel development, and repair, due to the fact that these cells are unique compared to other muscle cells by being not terminally differentiable [33]. Adult VSMCs are normally quiescent and continue to be in a highly differentiated contractile phenotype regulating lumen caliber. In a contractile state, the cytoplasm contains contractile filaments consisting of contractile proteins; smooth muscle α -actin (α -SMA) and actin associated/binding proteins such as smooth muscle myosin heavy chain (SM MHC), caldesmon, SM22 α , calponin and smoothelin [3, 33, 34]. Of note, less specific markers such as α -SMA may also be transiently expressed in other cells as myo-fibroblasts [35].

During pathological condition, for instance, in response to vascular injury, VSMCs exhibit considerable plasticity and can modulate from a differentiated contractile to a dedifferentiated synthetic phenotype, increasing their migration and proliferation ability. The synthetic VSMCs work like a factory for producing large amounts of ECM by increasing their matrix synthesizing machines such as rough

endoplasmic reticulum and Golgi apparatus, while reducing their myo-filaments in the cytoplasm, as shown in (figure 5). Chemotactic and mitogenic factors such as interleukins, FGF, PDGF and angiotensin II stimulate synthetic VSMCs and promote their migration and proliferation [10]. So, from where does this synthetic VSMCs originate? The contemporary paradigm is that, a large proportion comes from contractile VSMCs in the media, however some evidences point to the fact that, synthetic VSMCs can originate from bone marrow derived stem cells and resident vascular stem cells [34, 36, 37].

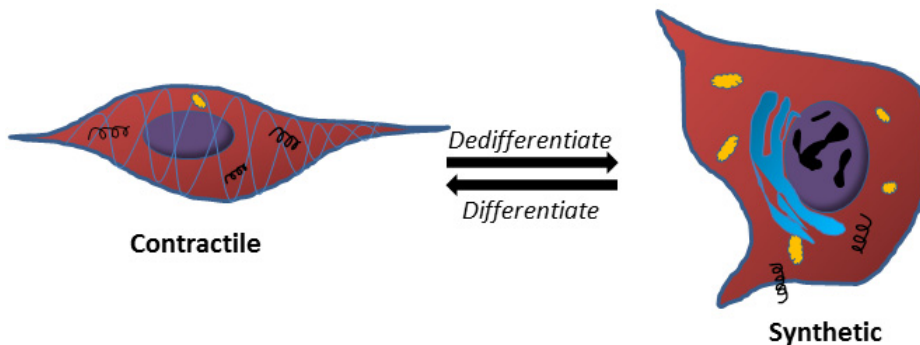


Figure 5:
Phenotypic modulation of VSMC, Drawing by Murugesan.V

VSMCs → friend or foe?

Defining VSMCs as good or bad is a complex question to address. Given the fact that, in an advanced lesion VSMCs can be atheroprotective, and at the same time detrimental in developing lesions. Studies suggest that, VSMCs undergo multiple processes, concurrently at different stages and regions of the plaque. The phenotypic switching, cell proliferation, migration, cell death and senescence are all factors that determine the consequences of plaque morphology. Research suggests that, VSMCs can be transformed into a macrophage like cell, based on environmental cues. The lipid accumulation in the plaque may lead to down regulation of VSMC markers, activation of pro-inflammatory genes and macrophage markers, leading to phagocytic activity [38]; however, the exact mechanism is still obscured. It has been suggested that these cells have a potential to express scavenger and VLDL receptors [39, 40]. Even macrophage like VSMCs are observed, although studies show that the genes expressed from those are

different from the classical macrophages [41]. Furthermore, macrophage like VSMCs have reduced phagocytic capabilities compared to a regular macrophage and can lead to reduced clearance of dead cells and subsequent increase in necrotic core in advanced lesions [42]. On the other hand, one has to consider the fact that, VSMCs are beneficial in protecting the fibrous cap from rupture [43, 44].

Multiple ruptures and repair events, leading to luminal stenosis, often define complicated plaques. Successful repair processes necessitate VSMCs to proliferate and synthesize ECM, which can be greatly altered by cell death and senescence. Thus an optimal balance of the appropriate proportions of VSMC populations determinates the success of repair processes and their significance for plaque stability. As already discussed, signals regulating VSMCs phenotype are of great importance, as it has been shown that an intact ECM protects the cells from being activated by pro-inflammatory factors and retains the ability of the cells to proliferate and migrate appropriately [45-47].

Smooth muscle cell actin filament formation

The phenotypic fate of VSMCs depends upon the amount of contractile filaments the cell has. Actin polymerization has been shown to play a dynamic and influential role, in increasing the amount of contractile filaments [33]. This is through increased transcription of genes that encode actin and actin binding proteins. It has been demonstrated that, synthetic VSMCs have reduced F/G actin ratio (Filament to Globular actin ratio) compared to contractile VSMCs; this is signifying that, perhaps reduced actin polymerization can be an underlying mechanism for phenotypic switching [33]. Moreover, a reduced F/G actin ratio down regulates the expression of dystrophin [33].

Actin polymerization

The polymerization of actin filaments occurs in three stages as shown in (figure 6), starting with a pool of an ATP bound monomeric globular form of actin called G-actin (basic unit of actin filament.)

Nucleation

Nucleation is when new actin filaments are produced and it's considered a rate-limiting step for actin polymerization. The process starts when G-actin monomers start to aggregate until a stable nucleus is formed. This is when three G-actin monomers form a trimer.

Elongation

The formed nucleus rapidly elongates by addition of monomers at either ends. The plus (barbed end) end elongates faster than the minus end (Pointed end).

Steady state

At steady state, the concentration of the unassembled pool of G-actin has reached a critical concentration. At monomer concentrations below the critical concentration the F-actin depolymerizes. At steady state the barbed end has still a tendency to grow, while the pointed end has a tendency to shrink. This process of continuous polymerization and de-polymerization without changing the concentration of G-actin is termed tread milling.

There are a number of proteins that modifies the existing actin filaments, and play a prominent role in the dynamic function of actin. This includes cofilin and profilin. Cofilin exerts its function by binding to the actin filament and enhancing the rate of dissociation at the pointed end. Profilin does the opposite by binding to the barbed end for polymerization of G-actin monomers in the increasing chain of F-actin [48].

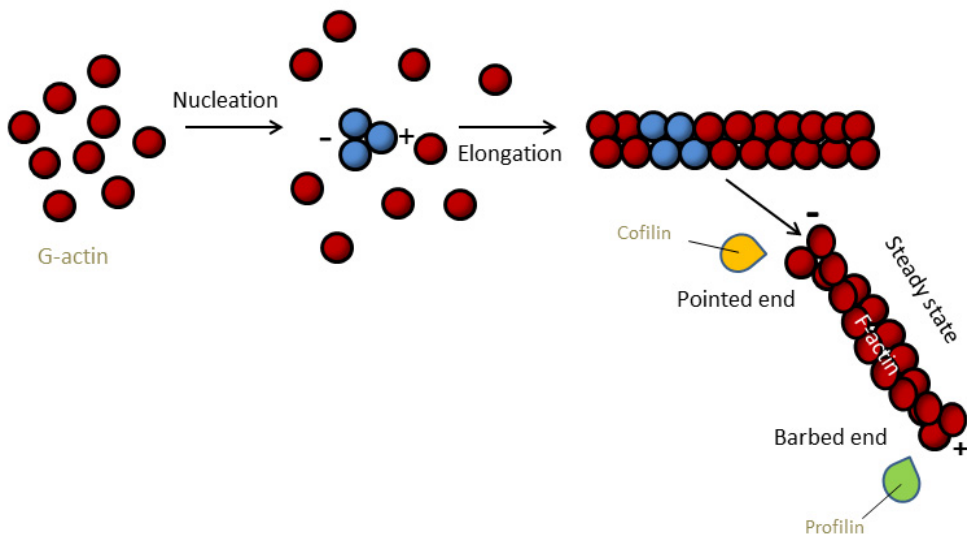


Figure 6: Schematic Illustration of steps involved in actin polymerization. Drawing by Murugesan.V

The Cell-ECM linkage

Extracellular matrix (ECM)

In brief, the ECM is composed of glycoproteins, collagen and proteoglycans [49] and cells modulate their communication with these molecules via the cell adhesion molecules (CAMs)—cell surface receptors. The ECM includes

- ❖ Interstitial matrix
- ❖ Basement membrane

The interstitial matrix is found between cells, whilst, the basement membrane surrounds (VSMCs) or underlies (in ECs) cells comprising a thin sheet of ECM.

The basement membrane (BM)

The basement membrane provides structural support, by means of providing a solid scaffold and by extending binding sites for cell adhesion molecules (CAMs); it is influencing cell signaling, proliferation, differentiation and migration. The BM also regulates tissue organization and functions as a growth factor reservoir [49]

A typical BM is composed of:

- Laminins
- Collagen type IV
- Nidogens
- Perlecan

The collagen IV and laminin are critical for BM stability, although, the nidogens and perlecan function to link laminin and collagen IV networks resulting in a BM with a better structural integrity [49].

The basement membrane greatly influences the VSMCs growth, migration and apoptosis [50]. In an activated state, VSMCs produce new transitional ECM consisting of versican, osteopontin, hyaluronan, fibronectin, collagen VIII, biglycans and lumicans.

In a normal vessel wall, the collagen IV and laminin reinforce the contractile state of VSMCs, however during injury, the synthetic cells respond by remodeling their microenvironment through secreting transitional ECM which binds and retains lipids. This is an instrumental step in early plaque formation [49].

Cell adhesion molecules (CAMs)

The fate of a cell is largely influenced by intricate interactions with its surrounding ECM, largely influencing, for example cell migration, adhesion and signaling. The connection between the cell and the ECM plays a major role in positioning the cell and also to be able to sense the microenvironment [40]. The cells communication with the ECM is delivered through its surface receptors, known as cell adhesion molecules (CAMs), and problems in CAM–ECM signaling lead to pathological conditions for example muscular dystrophies and atherosclerosis. Most CAMs are transmembrane receptors consisting of three domains: an intracellular domain (cytoskeleton interaction), a transmembrane domain and an extracellular domain (interacts with ECM), helping in transmitting the extracellular cues across the cell membrane and facilitating cell migration. The most important cell surface receptors are the integrins and dystroglycan (part of Dystrophin glycoprotein complex). The integrins consists of α and β subunits and binds to the ECM proteins such as fibronectin, laminins and collagen [49]. In this thesis mainly dystroglycan will be discussed. Dystroglycan consists of α and β dystroglycan, which are part of a bigger complex called the Dystrophin glycoprotein complex (DGC), also essential in bridging the cell to the ECM. Dystroglycan connects the ECM with the intracellular cytoskeleton [49].

The Dystrophin glycoprotein complex (DGC)

The DGC found in the membrane of cardiac and skeletal muscle is a large membrane associated multicomponent complex [51]. The DGC is important for both mechanical stability and signaling; it is one of the mechanism by which the cell cytoskeleton connects to the ECM to maintain the cells integrity. The interaction is mediated between the cytoskeleton, the plasma membrane and the ECM [52]. In skeletal muscle cells the DGC is thought to protect myofibers from contraction induced damage [53, 54].

The DCG consists of Dystrophin, an intracellular protein and a complex of cytoplasmic and transmembrane proteins collectively called the dystrophin associated protein complex (DAP's)

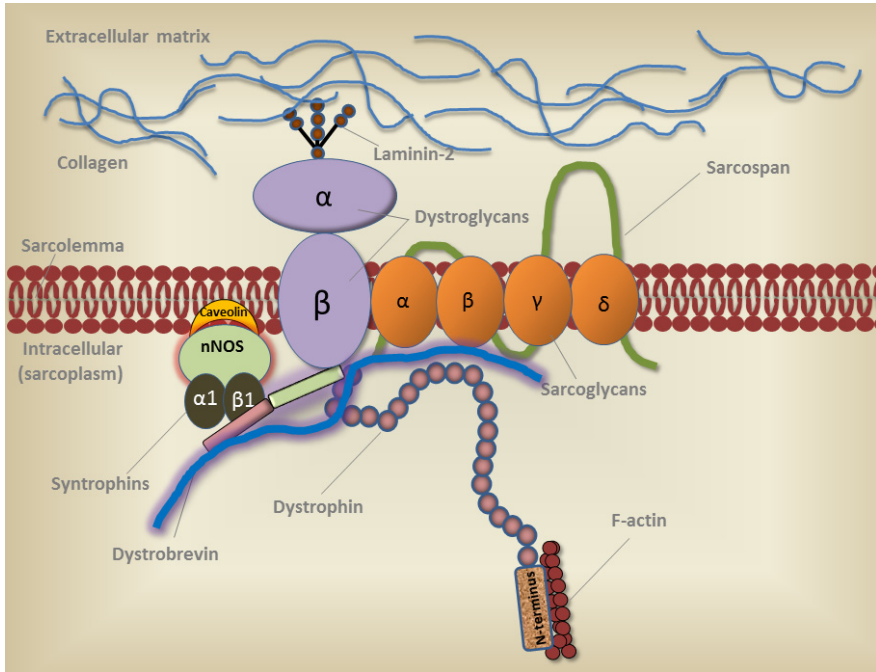


Figure 7:
Organization of dystrophin-glycoprotein complex in skeletal muscle. Drawing by Murugesan.V

Dystrophin

Dystrophin located in the cytoplasmic region of the sarcolemma (cell-membrane of a muscle cell) [55, 56] is a 427KDa intracellular protein, consisting of four functional domains. Its encoded by the largest gene in the human genome consisting of 2.5mb of sequence located on the X chromosome [57]. Dystrophin function is to link intracellular actin to the DAPs and in skeletal muscles it prevents sarcolemmal damage during contraction and relaxation [58].

The four functional domains in brief:

N-terminal region – Consists of cytoskeletal filamentous actin (F-actin) binding domain [59, 60].

Central domain – incorporates 24 spectrin-like repeats [59, 60].

Cysteine rich domain – Pivotal for interacting with intracellular portion of the transmembrane protein β -dystroglycan, anchoring dystrophin to sarcolemma [59, 60].

Carboxyl terminal region – Important to mediate interactions with other components of DGC, such as syntrophins [60].

The dystrophin has five isoforms, the full length Dp427, Dp260 – in the retina, Dp140 – in the CNS, Dp116 – in peripheral nerves and Dp71 – the most predominant isoform, almost present in every cell.

Mutations in the *dmd* gene, resulting in a loss of the expression of Dp427 cause severe muscle wasting called Duchenne muscular dystrophy (DMD) essentially due to sarcolemmal instability. On the other hand, missense mutations that allow for a production of a truncated protein (e.g. a protein lacking dystroglycan binding ability) can lead to a milder forms of DMD —the Becker muscular dystrophy [58, 61]. In patients with muscular dystrophy, every time the muscle contracts, small spaces appear in the membrane leading to a diffusion of calcium ions into the cell and an activation of calcium dependent proteases and also a loss of creatine kinase [58]. DMD affects mostly males, as a X-linked recessive disease. The prevalence is approximately 1 in 3,500 males (onset around 3 years of age). DMD usually leads to premature death around mid-20s, as a result of respiratory and cardiac failure [58].

The differentiated contractile VSMCs express high levels of dystrophin and any absence or decrease in dystrophin, support the cell's phenotypic modulation to their synthetic form [33]. Interestingly, loading the same amount of protein Western blots of smooth muscle tissues show higher expression than striated muscle tissue [33]. Dystrophin can also be considered as a contractile marker due to its close connection with actin. For instance, polymerization and depolymerization of F-actin has been shown to modulate dystrophin expression [33]. Thus, low expression of dystrophin can be correlated to a synthetic smooth muscle phenotype, which influences atherosclerotic plaque development.

Interestingly, dystrophin expression is reduced during a vascular injury [33]. In *mdx* mice, the normal contraction and relaxation machinery was affected in VSMCs, indicating considerable abnormalities in vascular responses [33]. Intriguingly, VSMCs in *mdx* mice are less sensitive to NO, conceivably contributing indirectly to plaque development. Moreover the force development in dystrophin mutant VSMCs is compromised despite an unaltered amount of F-actin, pointing to the possibility that, the function of F-actin might have been altered [33]. This raises the possibility, that DGC mediated mechanosensing (responsivity to mechanical stimuli) is affected in *mdx* mice, since dystrophin is a part of the DGC.

Dystrophin associated protein complex (DAP's) —A cluster of cytoplasmic and cell membrane proteins.

The DAPs are a group of proteins that span the cell membrane and/or complex with dystrophin. A defect in any of the components leads to different types of muscular dystrophy, as a result of compromised sarcolemmal integrity. Dystrophin and DAPs are interdependent, as mutations in the dystrophin gene also affect the levels of DAPs [62, 63]. DAPs are organized into three different sub-complexes.

- I. *Dystroglycan subcomplex*
- II. *Sarcoglycan-sarcospan sub complex*
- III. *Cytoplasmic subcomplex*

I. *Dystroglycan subcomplex* – Connects the intracellular cytoskeleton (via dystrophin) extracellularly to basement membrane components such as Laminin; aiding Cell-ECM connections.

- *α-dystroglycan (Extra-cellular)*
- *β-dystroglycan (Transmembrane)*

The dystroglycans are the core component of the DAPs. The α - and β -dystroglycan constitutes a heteromeric complex and physically links the basal lamina to the cytoskeleton. α -dystroglycan is a large (156 KDa) highly glycosylated extracellular protein, that binds to laminin, agrin and perlecan [64-66], whilst β -dystroglycan (43 KDa) is a transmembrane glycoprotein; it is binding to dystrophin with its cytoplasmic domain [61].

II. *Sarcoglycan-sarcospan subcomplex (SG-SSPN)*

- *α-, β-, γ-, and δ sarcoglycans (Transmembrane)*
- *Sarcospan (Transmembrane)*

Sarcoglycan complex (SGC)

The complex consists in skeletal muscle of four glycosylated transmembrane proteins, α -(50KDa), β -(43KDa), γ (35Kda)-, and δ (35Kda) sarcoglycans which are important in maintaining cell membrane integrity [67-69]. Mutations in the genes of different sarcoglycans lead to Sarcoglycanopathies—a group of autosomal recessive limb girdle muscular dystrophy (LGMD) types LGMD2D,

LGMD2E, LGMD2C, and LGMD2F respectively, a heterogeneous group of diseases [51, 70-76]. Enabling and maintaining a strong intra-extracellular link is achieved through the help of the membrane spanning sarcoglycan complex in stabilizing the interaction of α and β dystroglycans, and further by supporting the interaction of β -dystroglycan with dystrophin through binding to α -dystrobrevin [77-79]. Thus, the sarcoglycan complex interacts with dystrophin indirectly by its interaction with α -dystrobrevin. The β - together with δ -sarcoglycans are found to be crucial in formation and stabilization of the sarcoglycan complex and their deletion has been found to disrupt the whole sarcoglycan complex including sarcospan [80]. In β sarcoglycan null mice, which lack the sarcoglycan complex in all tissues, this deficiency results in whole body insulin resistance. Impaired insulin stimulated glucose uptake was demonstrated in skeletal muscle tissue. [80].

β - sarcoglycan (bSG)

β -sarcoglycan is a 43-KDa transmembrane protein consisting of 318 aminoacids (228aa extracellular, 90aa transmembrane and 63aa intracellular). bSG is important for SGC formation, and its loss leads to the absence of entire sarcoglycan complex and sarcospan [81]. Additionally, the loss of β -sarcoglycan renders the entire DGC complex unstable [76, 81]. Specifically, deficiency in bSG weakens the binding of both dystrophin to β -dystroglycan and β -dystroglycan to α -dystroglycan, respectively [82]. bSG-deficient mice have been shown to exhibit excess fibrosis and progressive muscular dystrophy, compared to *mdx* mice and relatively higher muscular hypertrophy [83]. The assembly of SGC is initiated by initial binding of bSG to δ -sarcoglycan then, in skeletal muscle, to γ -sarcoglycan and ultimately to α -sarcoglycan [67].

Sarcoglycan in vascular smooth muscle cells

The DGC of VSMCs consists of cytosolic and transmembrane proteins including dystrophin, α and β dystroglycans (dystroglycan complex), β , ζ , δ , ϵ sarcoglycans and sarcospan (sarcoglycan complex), syntrophins [84, 85]. Since α & γ sarcoglycans are absent in VSMCs, and ϵ is not a part of skeletal muscles, the association between α -dystroglycan and the SGC is likely either mediated by β or δ sarcoglycan [85]. Therefore, the striated muscle contains α , β , γ and δ and the vascular smooth muscle cells contain β , ζ , δ , ϵ sarcoglycans as shown in (figure 8).

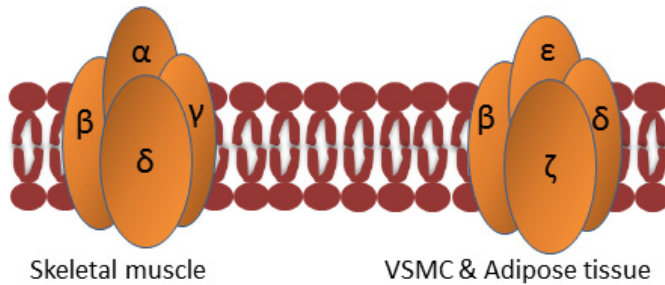


Figure8:
Expression of Sarcoglycan complexes in different tissues and cells. Drawing by Murugesan.V

III. Cytosolic subcomplex

Dystrobrevin

Dystrobrevin exists as α - and β -dystrobrevin. α - dystrobrevin is predominantly expressed in muscles, β -dystrobrevin in non-muscle tissues. Dystrobrevin binds to dystrophin and is rather than a homologue considered as a distant cousin to dystrophin by sharing some homologous sequence with the carboxy-terminal part of dystrophin [86]. It also binds to the SGC with its N-terminal region. Intermediate filament proteins like syncoilin and β -synemin bind to α -dystrobrevin [87] thereby supporting the mechanical stability of the cell.

Syntrophins

Intracellular proteins that interact with the carboxyl terminus of dystrophin [52]. Syntrophins function as modular adaptors to facilitate recruitment of signaling proteins to the membrane [88], for instance nitric oxide synthase (nNOS) thereby regulating local blood flow [89].

So, how does the DGC anchor now the cytoskeleton of the cell to the basement membrane?

The connection with the ECM is achieved through several interactions, starting with Dystrophin that binds to filamentous actin and also to the intracellular portion of the transmembrane protein β -dystroglycan. The extracellular portion of β dystroglycan interacts with α dystroglycan and finally, with basement membrane component laminin $\alpha 2$ -chain and perlecan, by means of extracellular α -dystroglycan [60].

Adipose tissue (AT)

Vacuum seal storage bags much more than a block of lard

Adipose tissue, which is well vascularized, is made up of functional cellular components such as adipocytes and non-fat cells (fibroblasts, pre-adipocytes, macrophages and other immune cells), and contains in addition connective and neural tissues [90]. AT is an active endocrine organ that regulates energy homeostasis, and functions as a central nexus in thermoregulation and metabolic communication. Moreover, it has a potential to undergo active remodeling through expansion in a fed state and, to shrink when there is a chronic cold stress [91, 92]. Being the largest storage site for triglycerides (TGs), it also secretes hormones, cytokines and other factors collectively referred as adipokines, influencing the fate of mobilization and storage of TGs and thereby largely determining the fate of the systemic physiology [93]. Imbalances in the levels of adipokines could lead to dyslipidemia and certainly have an influential role in the pathogenesis of atherosclerosis and metabolic disorders, such as insulin resistance and type 2 diabetes mellitus (T2DM) [94].

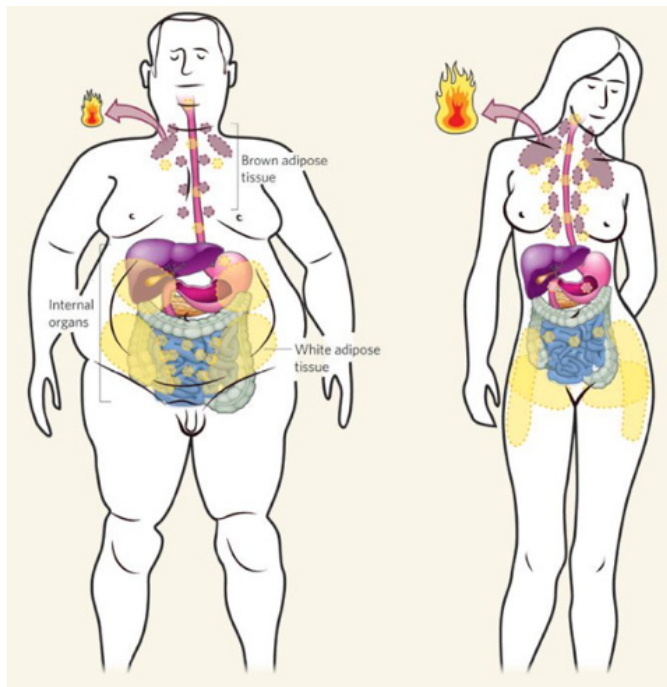


Figure 9: Distribution of WAT and BAT, Picture adapted from Kewlfit.com [95]

The AT can be generally classified into two types each having distinct biological function.

They are,

- White adipose tissue (WAT)
- Brown adipose tissue (BAT)

How does our body regulate the energy supply through fat?

In brief, our body stores excess energy through lipogenesis. This occurs, when glycerol and acyl CoA are used as ingredients to produce TGs, which are stored in adipocytes. During lipolysis, TGs break down into free fatty acids (FFAs) and get released in the blood stream or utilized directly as energy by β -oxidation in the mitochondria.

Adipocyte

The biological battery

Adipocytes are the functional unit and the primary component of AT, remaining as a cell type full of surprises as we increasingly appreciate it as an endocrine cell. Adipocytes are generally classified into white and brown; they are distributed over many fat depots located in various regions of the body.

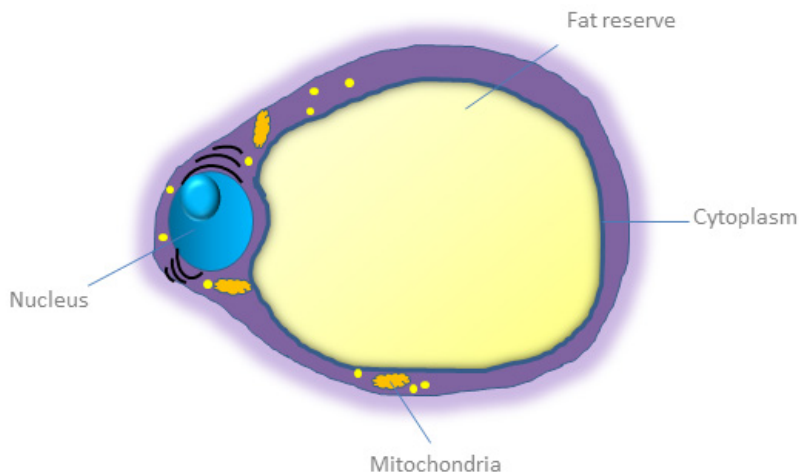


Figure 10:
The anatomical structure of an adipocyte. Drawing by Murugesan.V

White adipose tissue (WAT)

WAT is primarily located in subcutaneous and considerably in visceral regions. The key function of WAT is to help buffer excess nutrients, by storing and readily making them available during demand, thereby preventing toxic non-adipose tissue fat accumulation [91]. Apart from storage, WAT is metabolically active in communicating with other organs and secretes adipokines, regulating nutrient supply to other peripheral tissues [96]. Thus, WAT plays a key role in determining a healthy metabolic state.

WAT comprises white adipocytes representing the classical fat cells which are large, unilocular (TG stored in single locus) spherical cells of a cell size range between 25-200 μm , filled with a large single lipid droplet (90 % of cell volume) comprising TGs at its core [97] as illustrated in (figure10). Due to its large lipid droplet these cells are normally eccentric on account of its cytoplasm, and nuclei and other components are pushed towards the boundary [97]. White adipocytes are the most common fat type comprising 5-50% of the total human body weight [98]. As mentioned WAT can be found mainly in subcutaneous and visceral depot; hence it can be divided into:

- Subcutaneous adipose tissue (SAT)
- Visceral adipose tissue (VAT)

This thesis focal point will be visceral fat, since VAT is metabolically more active than SAT and contributes relatively more to atherosclerosis.

Visceral adipose tissue (Active fat)

VAT includes mesenteric and omental AT [94]. A protruding belly with large waist is a clear sign of an accumulation of dangerous VAT. It has been shown that a higher amount of visceral fat positively correlates with insulin resistance and T2DM [99-101]. The VAT adipocytes are relatively bigger and their TGs undergo more lipolysis to contribute to FFA levels than SAT adipocytes; VAT is also relatively more vascularized containing a large number of immune cells [90, 99, 102, 103].

Brown adipose tissue (BAT)

BAT is contemplated as a thermogenic tissue and is mainly located in visceral areas. Brown adipocytes are the primary cell types of BAT, described, as polygonal and multilocular cells of a size of about 60 μm with lipid droplets

reaching 25µm that are numerous. Brown adipocytes contain centrally located nuclei, large amounts of cytoplasm and are packed with abundant mitochondria, giving the cell its brown color. In contrast to white, brown adipocytes are smaller in size, and show in addition a limited distribution; they express high levels of thermogenic genes that, when the tissue is exposed to cold, dissipate chemical energy in form of heat.

This is achieved by producing energy from glucose and lipid, required to maintain body temperature during cold [97, 104-106]. Some classical experiments have proven that BAT gets activated when rodents are over fed, defined as diet induced thermogenesis as a compensatory mechanism to limit excess accumulation of fat [105]. In terms of the vasculature, many brown adipocytes are also located in perivascular adipose tissue (PVAT, discussed below), which is regarded as a tissue that needs more scientific exploration apart from its heat producing abilities.

Perivascular adipose tissue (PVAT) — the fat - vessel connection.

PVAT can be found around the majority of blood vessels providing mechanical support, and can have similarities to both BAT and WAT [107] as shown in (figure 11). In general the amount of PVAT increases with adiposity with compositions varying amidst vessel types. A typical PVAT consists of pre-adipocytes, fibroblasts, capillaries and immune cells. PVAT influences vascular functions through secretion of adipokines, which can modulate endothelium dependent vasodilation, inflammation and remodeling processes. Under normal conditions, PVAT has the ability to unleash a range of biologically active molecules, that can act both in autocrine and paracrine fashion; it can exert, for example, anti-contractile effects by releasing vasorelaxants such as adiponectin and angiotensin 1-7 [107, 108]. Besides, it also influences VSMC contraction, proliferation and migration [109].

At times of pathological conditions, such as obesity, PVAT has the potential to lose its BAT like property and become highly inflamed to secrete vasoconstrictive factors and pro-inflammatory cytokines such as TNF- α , IL-6 and MCP-1 along with a reduced adiponectin secretion [110]. Consequently, it is transformed into a ground for aberrant secretion of adipokines, leading to impaired vasodilation and amplified vasoconstriction. Thus, a highly inflamed PVAT seems to be a major risk factor for a dysfunction of ECs and vascular inflammation [107]. Furthermore, PVAT secretes visfatin, an important biomolecules acting as a VSMC growth factor, influencing proliferative and migrative properties of these cells [111]. However on the positive side, healthy PVAT with the ability to stimulate thermogenesis also decreases the risk of atherosclerosis by metabolizing fatty acids [107].

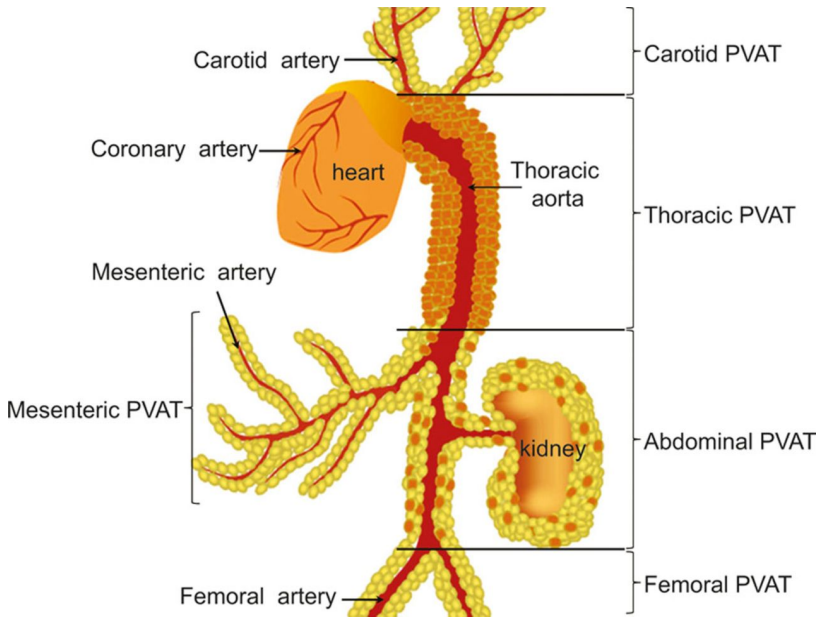


Figure 11: Distribution of PVAT comprising BAT and WAT, Image courtesy: Brown, N.K., et al, 2014 [107]

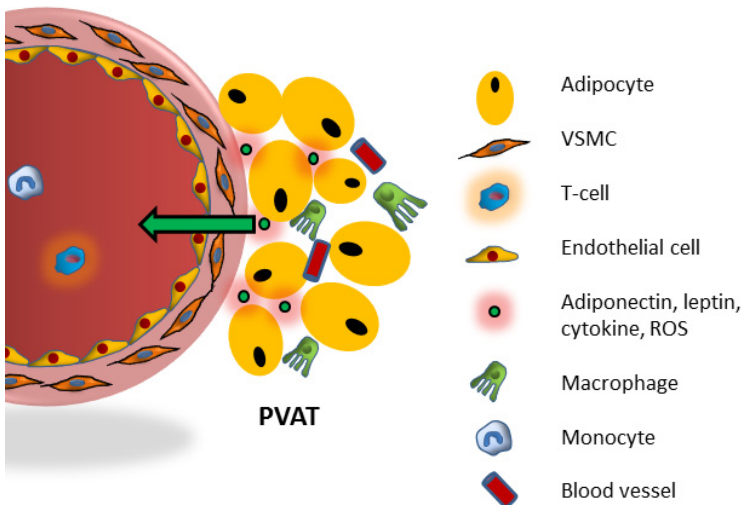


Figure 12: A typical cross-talk between the vessel wall and PVAT, Drawing by Murugesan.V

The changing color of fat

Shades of a cell

Under dietary or environmental changes such as hypothermic condition, WAT can be phenotypically converted into BAT for heat production (Browning) and in contrast, more WAT can be generated during an increased demand for lipid storage (Whitening) [97]. Within WAT immature cells can be incorporated, that can change into brown adipocytes when exposed to cold. These transformed cells are called “**beige fat cells**”. They have similar functions and express comparable genes with brown adipocytes; however, they are not strictly considered as brown adipocytes [104, 112].

Is fat an hero or a villain?

Deprived of adipocytes, lipids accumulate in other tissues, for instance muscles and liver. This is toxic and detrimental, contributing to lipotoxicity. Therefore, as a safe storage bag and a gate keeper of high energy reserves, they release energy into the blood stream, when required; simultaneously they keep up with high concentrations of lipids without experiencing lipotoxicity, which is conspicuously not the case with other cell types [113]. Retaining a healthy operating adipose tissue hampers metabolic disease, minimizing to a great extent the risk of atherosclerosis [94, 104].

Complications arise, when adipocytes, become incompetent of handling increasing amounts of lipids. They eventually start to accommodate more by getting bigger in size (hypertrophy), besides proliferating and expanding (hyperplasia), similar to a solid tumor. As the tissue cannot keep up the oxygen demand by an unaltered blood supply, these processes lead eventually to a state of “*tissue turning bad*”. The condition of cells in a hypoxic environment and lack of space to expand initiates their death, thereby releasing their engulfed lipids. The macrophages attempting to clean up the lipid mess find it difficult to swiftly clean up; this can lead to a spillover of lipids to tissues such as liver and pancreas. This creates an ambiance of ectopic lipid deposition. As these tissues are unequipped to handle so many lipids, this consequently leads to a low-grade inflammation, which eventually results in metabolic diseases.

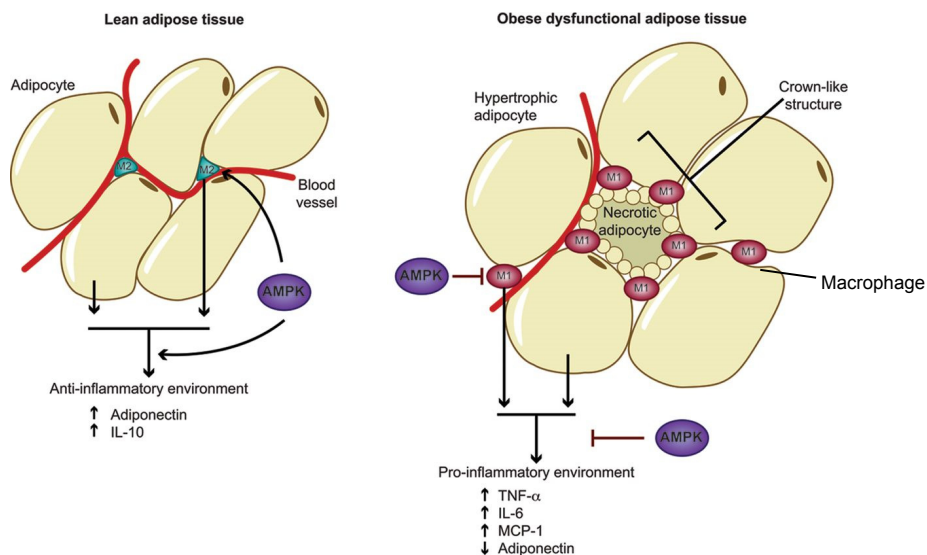


Figure 13: Adipose tissue in Lean and obese. Image courtesy: Bijland, S, 2013 [114]

In the light of the statements above, altered adiposity and dysfunction seem to exert an effect on the balance between pro and anti-inflammatory adipokines, which ultimately leads to changes in immune responses and metabolic dysfunction. The two prime factors leading to adipokine imbalances are AT inflammation and hypertrophy. In cases of inflammation, primarily due to activation of adipose tissue macrophages (ATM), the adipokine levels are altered, leading to an uncontrolled release of FFA and pro-inflammatory cytokines. This has a substantial effect on the lipoprotein metabolism and insulin sensitivity. Adiponectin is one of the prime adipokines (discussed below). The adiponectin dependent increase in fatty acid oxidation is mediated through up-regulation of several genes in lipid metabolism, for instance acyl-CoA oxidase and mitochondria uncoupling protein 2 (UCP2). Pro-inflammatory cytokines such as TNF- α , IL-6 and IL-8 released due to alterations in adiponectin levels, have been shown to reduce insulin sensitivity [115, 116]. On the other hand, the inability of adipocytes to store excess fat, even at times of hypertrophy and hyperplasia, redirects the FFAs to organs such as the liver and is leading to dyslipidemia.

As mentioned, similar to other cells, adipocytes secrete a collection of bioactive substances (Adipokines) such as hormones and cytokines, which either have pro or anti-inflammatory activity. Some of the important molecules are briefly discussed below.

Adiponectin (APN)

Adiponectin is secreted exclusively by adipose tissue. APN regulates lipid and glucose metabolism. A decreased level of APN strongly correlates with increased amount of VAT and also with development of insulin resistance and T2DM. The Adiponectin exerts its action through receptors such as AdipoR1 (skeletal muscle), AdipoR2 (Liver) and T-cadherin (Artery). Primarily, it exerts beneficial action by increasing the oxidation of fatty acids, thereby lowering the FFAs in the circulation. Thus, increased APN leads to decreases in atherosclerotic plaques, insulin resistance and dyslipidemia. In the vasculature, APN has a bigger role in inhibiting macrophage activation and foam cell accumulation and helps to activate eNOS.

Various studies have shown the beneficial effect by the hormone, in increasing NO production and supporting NO-mediated and potassium channel mediated vasorelaxation [117-119]. The SMCs express adiponectin receptors, which have the capability to activate potassium channels, and nitric oxide production. Together with ECs, SMCs mediate the anti-contractile activity of APN through NO dependent mechanism. Furthermore, APN has the property to reduce platelet aggregation and helps to protect the vasculature. During the course of obesity, the anti-contractile effect is reduced, which is mediated through reduced PVAT dependent release of APN [120]. It is believed that APN in VSMCs blocks numerous atherogenic growth factors resulting in inhibition of cell proliferation and migration[120]. It has not been proven, how APN crosses the vascular wall; however, some studies have shown, that exogenous application of APN to a pre-constricted artery segment deprived of PVAT led to vasorelaxation [117].

Leptin

Leptin is mainly secreted by WAT and its levels positively correlate with body fat. In fact, circulating leptin levels may reflect the amount of stored energy as fat. Leptin also plays a role as an influencer during vasodilation. Leptin exerts its effect through its receptor ObRb that are expressed in brain and other peripheral tissues. The expression of ObRb receptors in VSMCs has been detected in rats [121]. Leptin is predominantly released in response to high degrees of TG uptake. The significant roles of leptin are: Regulation of energy homeostasis, stimulation of thermogenesis, increased fatty acid oxidation, decreased glucose level and reduced body weight. Under normal conditions, leptin acts on ECs and activates protein kinase B (AKT) signaling, which induces enhanced vasorelaxation. On the contrary, leptin can induce vasoconstriction by activating the sympathetic nerve system in the brain. This makes leptin an important player in modulating the vascular tone. In addition, leptin also modulates vascular tone by directly acting on

VSMCs to inhibit an angiotensin II dependent intracellular calcium release, thereby blocking the vasoconstrictor activity of angiotensin II. Further, it has been shown that leptin affects VSMCs proliferation and growth [122, 123].

Main methods

Mouse models of atherosclerosis

A mouse is miracle enough to stagger sextillions of infidels - Walt Whitman

Mice under normal conditions are highly resistant to atherosclerosis [124] and hold myriads of benefits in using them as a model over other animal models. Moreover, this thesis is entirely based on tissues from mice and with that in mind, this section is dedicated for those unrecognized Nobel Prize winning souls. In brief some of the benefits of using the mouse as a model are: small in size, short time frame for lesion formation, cost effective for acquiring and maintaining, short gestation time (19-21 days) and having their whole genome sequenced, making it easier to be genetically modified for different pathological conditions.

However, there are limitations concerning use of mice, which are discussed below.

Limitations:

Table 3. Factors that contribute to difference in experimental lesions over human lesions

| Mouse | Humans |
|---|---|
| Surgical scale: Small size, not impossible but hard to perform surgery. | Not the case. |
| Size of plaque small, restriction for the use in western-blotting and other analysis. | Not the case. |
| Lipid profile: Cholesterol and triglycerides transportation through HDL. | Cholesterol and triglycerides transportation through LDL. |
| Lipoproteins mostly contain ApoE | Lipoproteins contain ApoB-100. |
| Plaque development may be as a result of inflammation | It could be several other factors |
| Lipid retention through Perlecan | Lipid retention through Versican |

Thus, anatomical and physiological conditions are not the same between mice and humans. For instance, the media of a mouse may just contain 2-3 layers of smooth muscle cells and the endothelial layer directly sits on the internal elastic lamina.

Hence, it's good to keep in mind those facts when extrapolating data from mouse to human equivalents.

ApoE-deficient mice

Presented in 1992, is the first genetically engineered mouse model for atherosclerosis referred as the “Apo-lipoprotein E knockout mice”. ApoE is a 34KDa glycoprotein, present on the surface of all lipoproteins except LDL. ApoE functions as a high affinity ligand that binds to apoB, LDL and chylomicron remnant receptors, required for uptake of apoE-containing molecules by the liver [125]. This results in removal and absorption of triglycerides from the circulation into the liver preventing hyperlipidemia. ApoE-deficient mice, as the name suggests lack Apo-E protein on the surface of lipoprotein. These mice are hypercholesterolemic and spontaneously develop atherosclerotic lesion under normal chow diet. With lesions well characterized in ApoE deficient mice, it makes them the best choice to study pathogenesis of atherosclerotic lesions with evidence for plaque rupture.

Looking at the plasma profile, the ApoE knockout mice have a 5-fold increase in total plasma cholesterol with a higher cholesterol and triglyceride levels, when put under normal diet, with a marked decrease in HDL level. This is apparently due to a shift of lipid transport from HDL to LDL and chylomicron remnants, pointing to a more atherogenic plasma lipid level. In addition these mice lack cholesterol ester transfer proteins, which transfers the cholesterol from HDL to VLDL and LDL.

Diet - A key experimental condition

Though these mice can develop atherosclerosis under normal diet at several vascular branch points, an increased lesion size together with an advanced plaque phenotype can be achieved by exacerbating plasma cholesterol (15 fold elevation) under a fatty diet, widely known as western type diet (21% fat, 0.15% cholesterol and no cholic acid) [126, 127]. The western, as the name implies has similar components as those of an average American diet. The diet is a modified version of the earlier used Piagen diet (15% fat, 1.25% cholesterol, 0.5% Cholic acid), which has been discontinued due to toxic effect and, moreover the lesions apart from fatty streak phase don't progress to intermediate lesions. Thus, using western type diet markedly increased the plasma cholesterol and triglycerides level with a decrease in HDL level, resulting in a shift of triglyceride transportation from HDL to LDL. This facilitates development of complex lesions (advanced) in ApoE knockout mice, having characteristics of fibro proliferative stages with a well-

defined fibrous cap, necrotic core besides plaque ruptures, rendering it an optimal and desirable model system to study complications in human atherosclerotic lesions [125].

Localized induction of plaque formation

Arterial manipulations were originally studied in rabbits, and later optimized for mice [128]. One such method is the use of perivascular collars on experimental animal models, leading to either modification of shear or mechanical stress, to generate plaques of different phenotypes in a site controlled manner [129]. A perivascular collar is either made up of polyethylene, silastic or tygon tubing. In this thesis two types of perivascular collars are used. A constrictive collar was used to induce an atherosclerotic plaque in ApoE knock out mice, as a consequence of shear stress manipulation (Paper I) and a non-constrictive one was used to create intimal hyperplasia by inducing mechanical stress (Paper II).

What is a shear stress and how does it influence atherosclerotic plaque formation?

The shear stress is the frictional drag force exerted by the flowing blood on endothelial cells [130, 131], which largely depends on blood viscosity and flow velocity [132]. The vascular wall adapts its luminal diameter to the changing mechanical force and the changing vessel diameter has a large influence on wall shear stress. Under normal conditions, the shear stress in the artery wall ranges from 10 to 70 dyne/cm² maintaining an athero-protective endothelial cell phenotype [133]. In areas of low shear stress, as in vessel bifurcations, fluid shear stress is significantly lowered with values around ± 4 dyne/cm²; these are the regions where atherosclerotic plaques appear. On the other hand, higher shear stress is normally atheroprotective. For instance, it biologically promotes increased production of eNOS, which attenuates smooth muscle cell proliferation by a decreased production of growth factors such as PDGF [134, 135]. Likewise, shear stress also takes part in modulating endothelial cell proliferation; an increased shear stress promotes higher proliferation of endothelial cells, and the opposite presents itself with lowered shear stress, marked by loss of endothelial cells and desquamation. Besides altered morphology of the endothelial cell, there is an increased endothelial cell turn over, decreased antioxidants, and increased susceptibility to adherence and migration of monocytes across the layer [136].

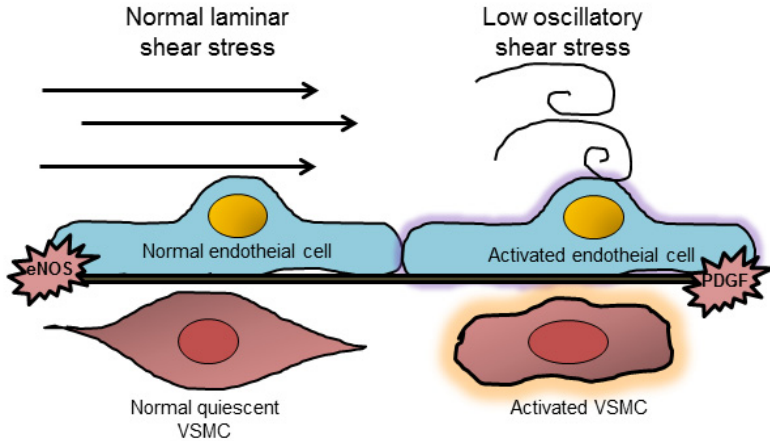


Figure 14:
Types of shear stress acting on endothelial cells and their subsequent effect. Drawing by Murugesan. V

The endothelium is constantly exposed to the hemodynamic shear stress and hence responds rapidly and sensitively to the mechanical force. This connection is the basic mechanism behind altered shear stress induced plaque formation [137]. Consequently, mechanotransducers like G-proteins, integrin-matrix interactions and ion channels convert mechanical forces into biochemical signals. In the course of a lower shear stress, the endothelial cells become pro-thrombotic and pro-migrative, regulating different processes like coagulation, growth of underlying smooth muscle cells, leukocyte adhesion and transmigration across the vessel, and allow lipoprotein accumulation and modification [138, 139]. Hence, for our study, using perivascular collars to alter hemodynamics provides the frameworks to study the influence of beta-sarcoglycan and dystrophin on intimal hyperplasia and atherosclerotic plaque development in a site controlled manner.

Shear stress modifying perivascular cuff (referred simply as cast)

The function of shear stress has been studied comprehensively in-vitro on endothelial cells and tissue explants to study the flow dynamics and their physiological effects. Research utilizing ligations of the common carotid artery resulted in a creation of devices producing regions of pre-determined flow characteristics. The use of a cast as a tool to study atherogenesis in ApoE knockout mice was first developed by Cheng et al [129]. Its cone shaped structure modifies the inner lumen of the carotid artery to produce defined regions of low, high and oscillatory shear stress. In a normal scenario, the carotid artery is under high pulsatile shear stress devoid of atherosclerotic plaques [140].

In paper 1, at approximately 4-5 months ApoE deficient mice with or without beta-sarcoglycan deficiency, fed a high fat diet were used to produce phenotypically different atherosclerotic plaques, proximal and distal from the cast. Proximal to the cast, a low shear stress produces an extensive plaque development, showing an advanced phenotype characterized by increased inflammation. Distal to the cast a bottleneck broadening of the artery leads to an oscillatory pattern of flow, inducing a less extended plaque development of fibrous nature. A higher laminar flow inside the cast, due to a conical inner lumen, generates high shear stress resulting in no plaque deposition. In preparation for the experiments, at 16 weeks of age, mice started on a western diet (21 % fat, 0.15% cholesterol). Two weeks later, the cast is placed around the right carotid artery with the left carotid artery remaining as a control — uninjured. At 30 weeks of age, 12 weeks later, tissues such as: the heart, carotid arteries, descending aorta, ovary fat pads, perivascular adipose tissue and blood were saved for experiments.

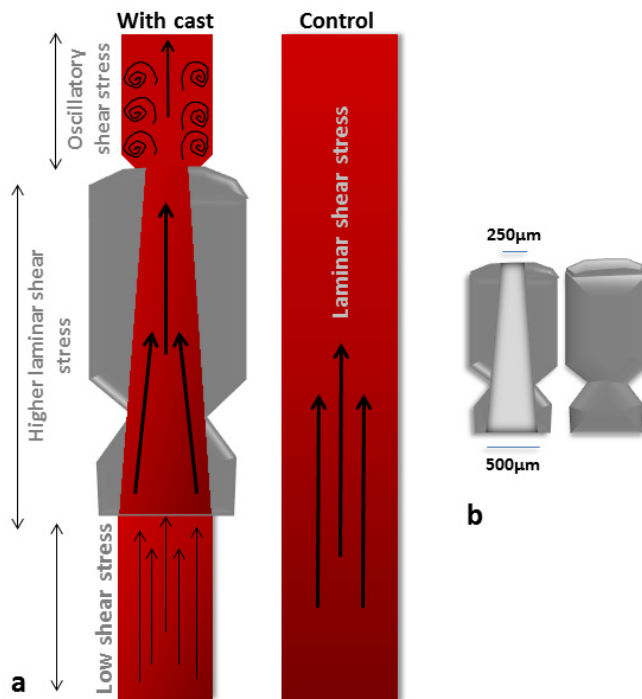


Figure 15: Cast placement around the common carotid artery: Low shear stress upstream of the cast, a high shear stress region inside the cast area and Oscillatory shear stress downstream of the cast (a). Adjacent is a normal vessel (control) with laminar flow pattern. (b) represents illustration of Interior (cone shaped groove) and outside of the cast. Drawing by Murugesan.V

Non-occlusive perivascular collar induced intimal hyperplasia

In paper II, we studied the neo-intima formation using a non-occlusive plastic collar having an internal diameter of 0.51mm and 3mm in length. It should be noted, that the average outside diameter of a common carotid artery is 0.36mm at 80mm Hg perfusion pressure. This method has similarities with balloon angioplasty procedures, involving stripping of endothelial cells to induce intimal hyperplasia and vascular remodeling in rats [141]. The collar is placed around the right common carotid artery (the left uninjured, used as a control) in beta-sarcoglycan deficient mice and wild type mice, fed a normal chow diet, for 3 weeks. The mice were 4-5months old. This resulted in formation and thickening of a neo-intima, which was rich in VSMCs and ECM with no lipid deposition, after cast placement. Thus, during vascular repair, for instance after using a balloon angioplasty as mentioned above, there exists an excessive proliferation of VSMCs. Hallmark of this kind of neo-intima formation are the smooth muscle cells proliferation and extracellular matrix deposition.

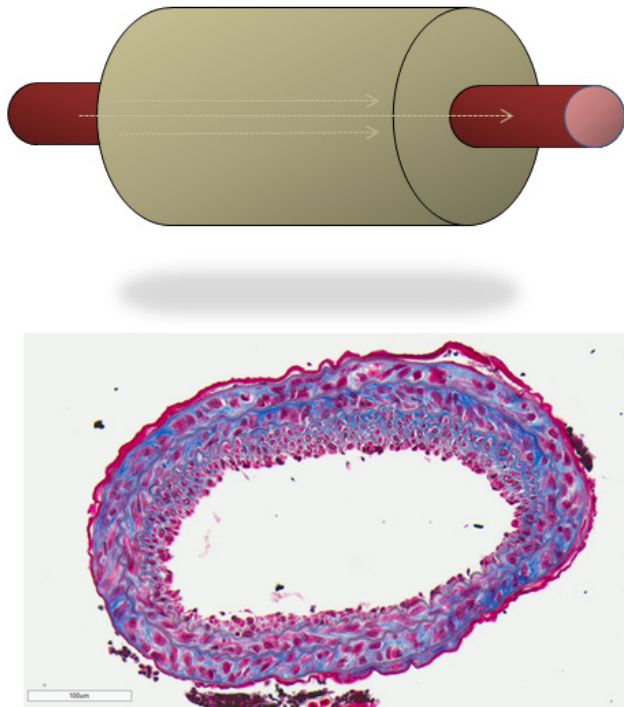


Figure 16: The Non-occlusive collar and the resulting plaque, visualized using Masson's trichrome stain. Drawing by Murugesan.V

Aims and key findings of the present studies

General aim of the thesis

I tried to explore how the connection of vascular smooth muscle cells to the basement membrane affects plaque development. In short, the goal is to better understand the role of dystrophin and beta-sarcoglycan in the process of vascular smooth muscle cell migration and proliferation under the influence of an injury. On that account, to shed some light, the thesis explores how destabilization of dystrophin and dystroglycan mediated connections of VSMCs to the basement membrane via deletion of beta-sarcoglycan influences restenotic and atherosclerotic plaque development.

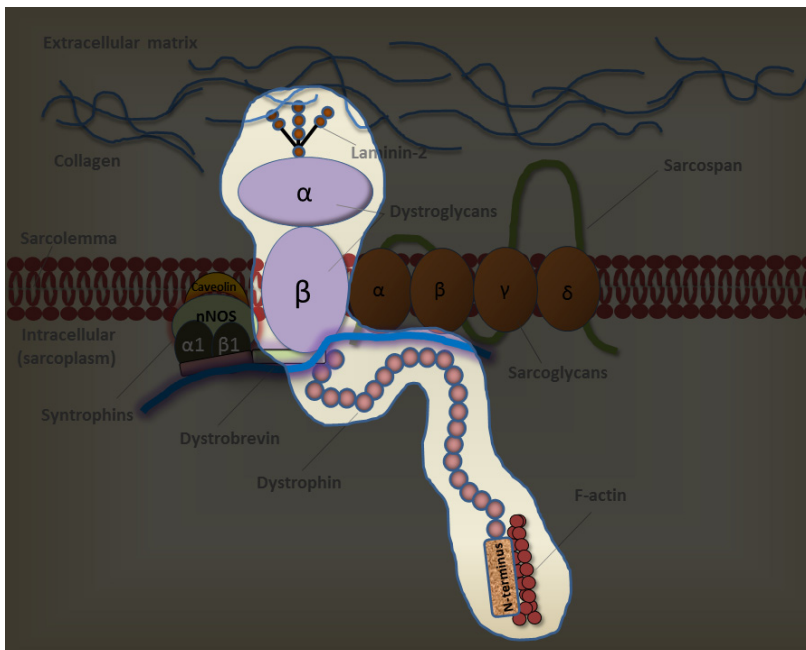
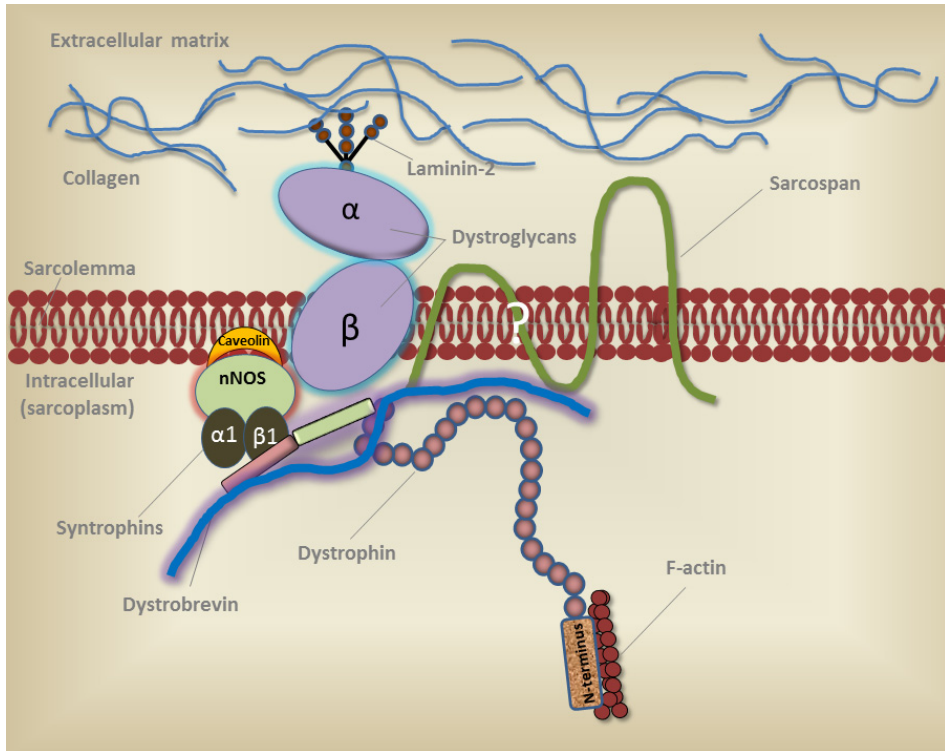


Figure 17. Illustration of the cell connection with the basement membrane via DGC. The highlighted chain of connection involves cell's cytoskeleton, dystrophin, beta-dystroglycan, alpha dystroglycan and Laminin-2.

Paper I

Aim

To investigate the role of beta-sarcoglycan in atherosclerotic plaque development.



Key findings

We found a decrease in atherosclerotic plaque size in ApoE/bSG^{-/-} mice compared to ApoE^{-/-} mice in advanced plaque development in the lower shear stress region. Moreover there was a decrease in total plaque burden in the ApoE/bSG^{-/-} mice. When stained for the differentiated smooth muscle cell marker myosin heavy chain 11 we found an increase in the content of differentiated smooth muscle cells in advanced plaque of ApoE/bSG^{-/-} mice. The cells expressing the marker were distributed exclusively in the plaque regions close to the lumen. We also found in ApoE/bSG^{-/-} mice a higher phosphorylation of AMPK in visceral adipose tissue accompanied by less of this tissue and smaller adipocytes. The levels of plasma leptin, adiponectin, triglycerides and cholesterol were significantly decreased.

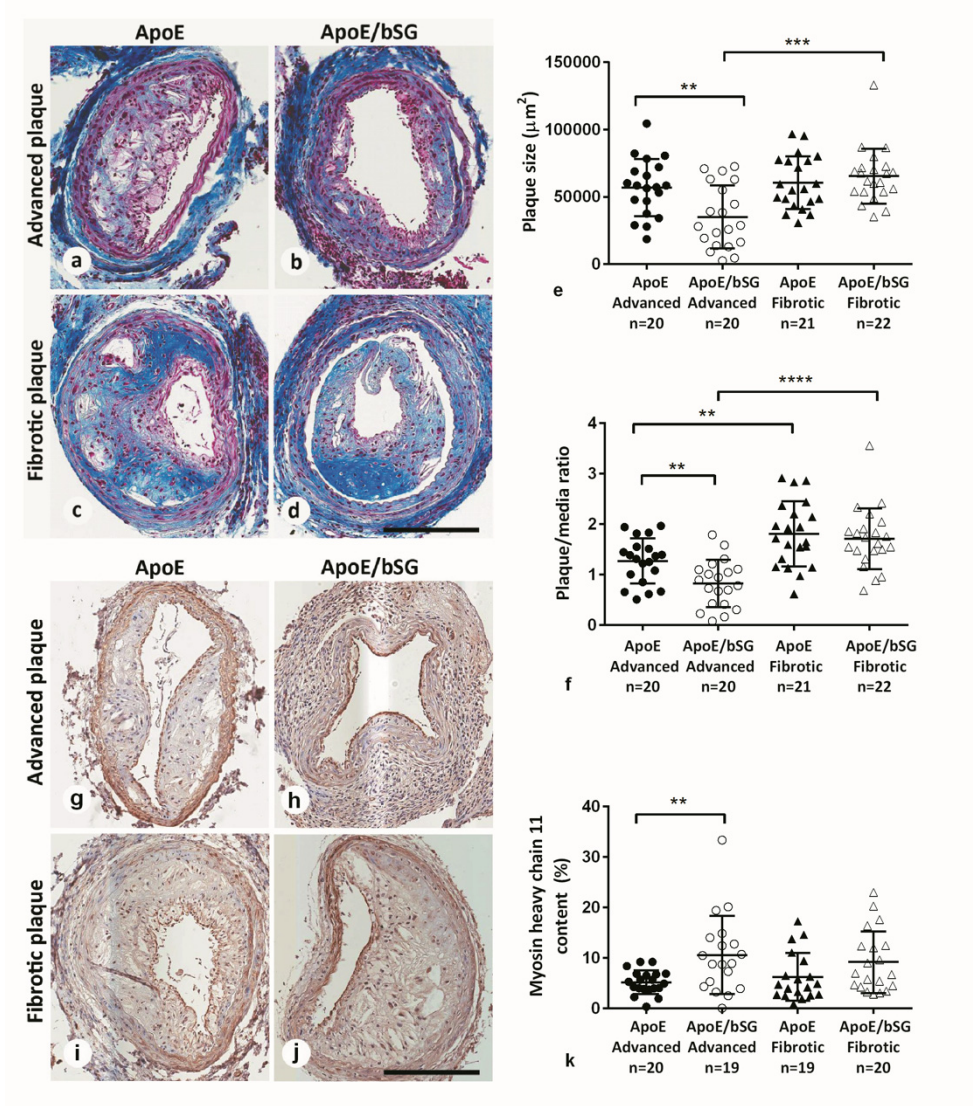


Figure 18. Decreased size and increased content of differentiated smooth muscle cells of advanced lesions in bSG-deficient mice.

Representative sections stained with Masson trichrome (a-d) and for myosin heavy chain 11 (g-j) are shown. Scale bars represent 200 μm . Graphs show the total plaque area (e), intima/media ratio (f) and the myosin heavy chain 11-positive fraction of the plaque (k).

Paper II

Aim

To study the role of beta-sarcoglycan in injury induced intimal hyperplasia.

Key findings

Beta-sarcoglycan deficient mice exhibited a significant increase in neo-intimal formation.

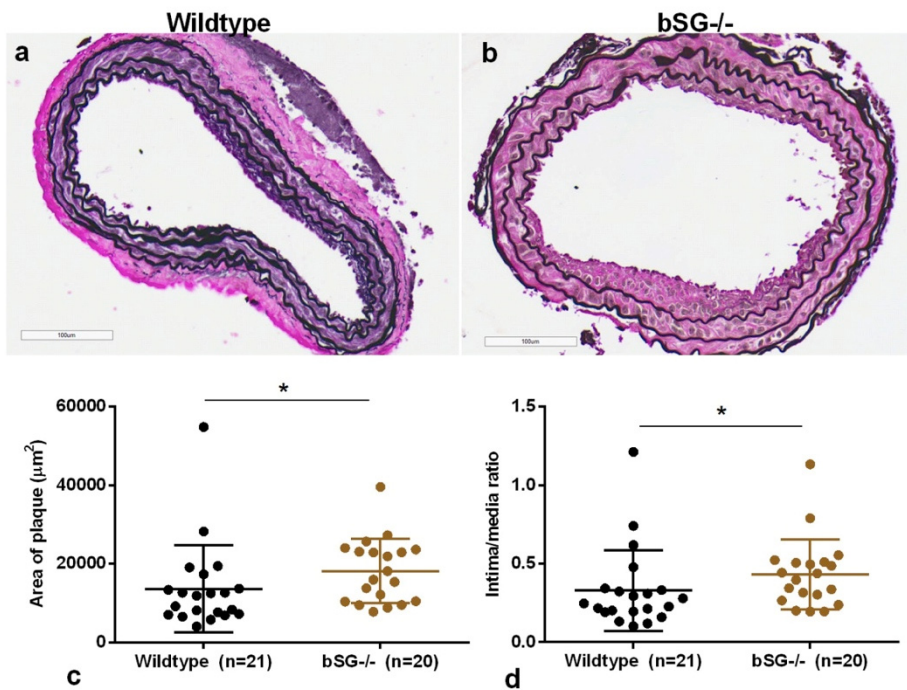


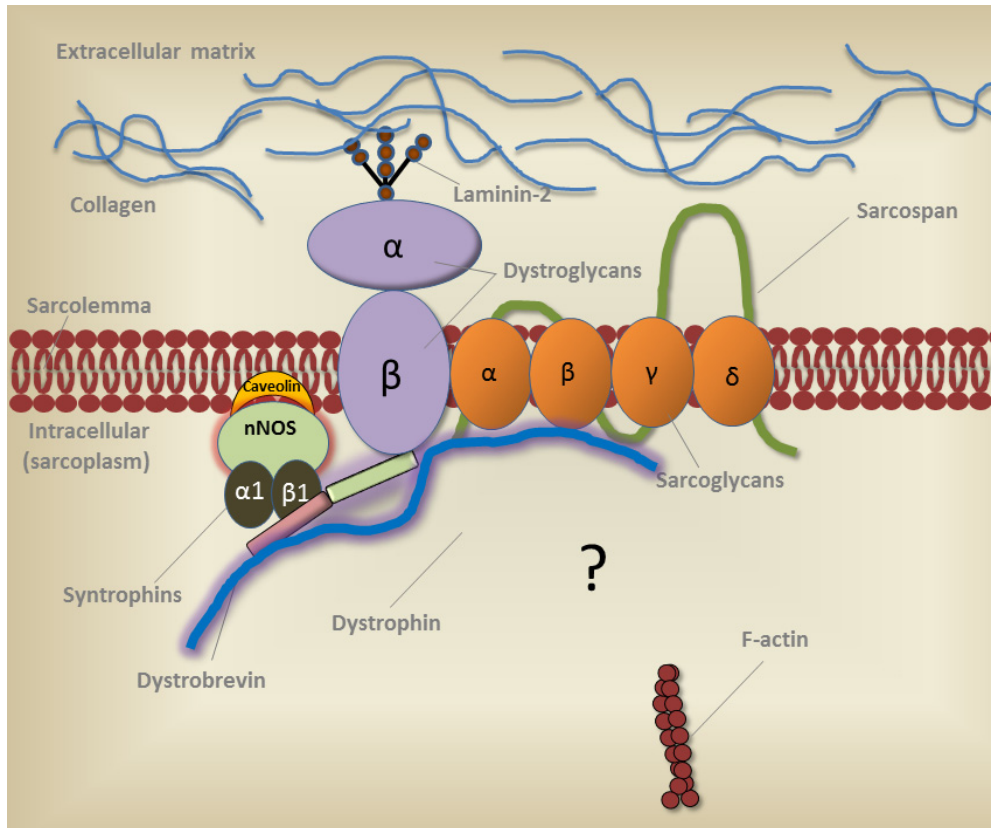
Figure 19. Increased neo-intima formation in bSG-/- mice.

Representative carotid artery sections of elastin staining from wildtype (a) and bSG-/- (b). Plaque size represented as total plaque size (c) and intima/media ratio (d). Scale bars represent 100 μm.

Paper III

Aim

To investigate the role of dystrophin in atherosclerotic plaque formation as a structural linker between the actin cytoskeleton and the ECM.



Key findings

The dystrophin deficient *mdx*/ApoE^{-/-} mice had a significant decrease in atherosclerotic plaque size compared to the control ApoE^{-/-} mice. The overall inflammatory profile (plasma levels of most cytokines) of *mdx* mice is reduced compared to ApoE^{-/-} mice and with a significant decrease in T-cell density in these plaques.

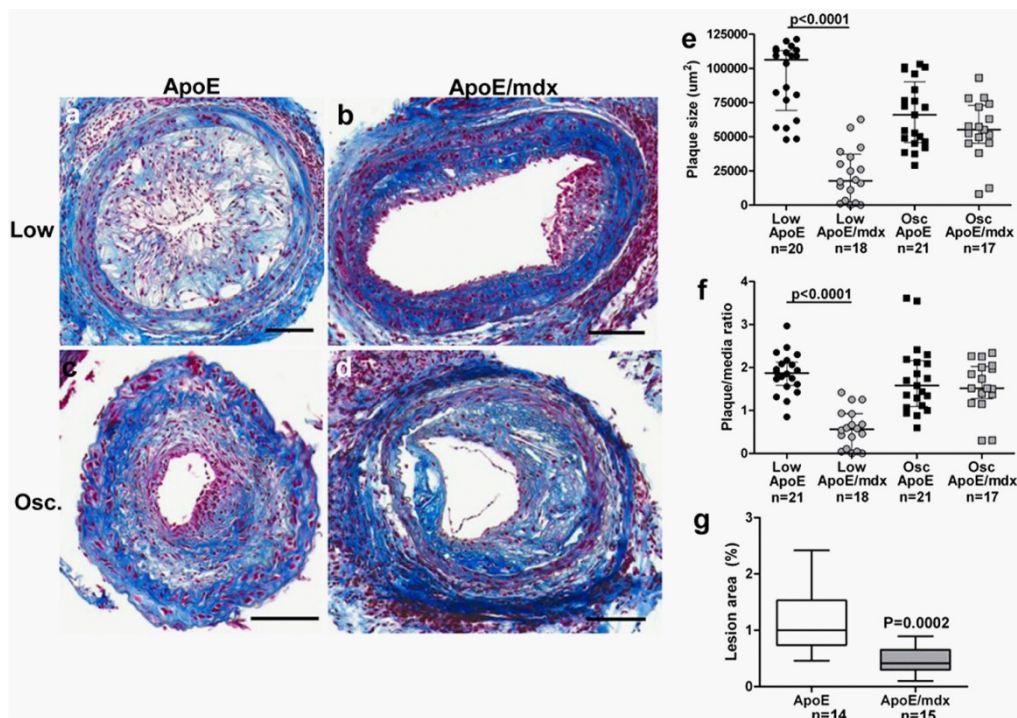


Figure 20. Reduced atherosclerotic plaque size in Low shear stress region (a–b) and no difference in oscillatory shear stress (c–d) plaques in ApoE/mdx mice compared to ApoE^{-/-} mice. Representative graphs of plaque/media ratio (e) and total plaque size (f). Overall plaque burden represented in (g) assessed by Oil Red O-staining of aortas from ApoE and ApoE/mdx mice (g). Scale bars represent 100 μm

Paper IV

Aim

To find genes that are regulated by actin polymerization and to analyze the effect of their alteration in vascular functions and vice versa.

Key findings

Actin polymerization increased the expression of dystrophin and other markers for a differentiated smooth muscle cell phenotype, while down-regulation of these molecules was observed with actin de-polymerization. Balloon injured human arteries showed a decrease in the expression of dystrophin. Tail artery rings from

mdx mice, subjected to contraction and relaxation paradigms exhibited decreased response, indicating a defect in structural contractile machinery in SMC's of *mdx* mice despite of unchanged F/G actin ratios.

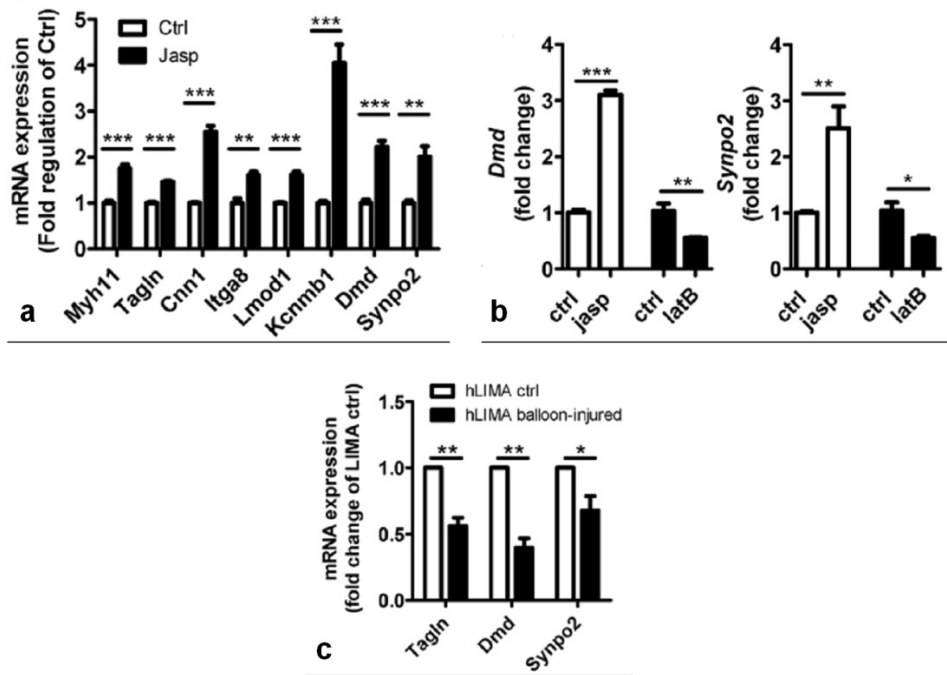


Figure 21: Graph shows result of (a) SMCs from mouse aorta stimulated with 100 nmol/L jasplakinolide (Jasp) for 24 hours (n=4) shows expression of selected genes from an Affymetrix gene array. (b) Verification of the array results for *dmd* and *synpo2* using individual quantitative polymerase chain reaction (white bars). Mouse aorta treated with actin depolymerizing agent, latrunculin B (latB, 250 nmol/L) for 24 hours (n=3–5) is represented by black bars, ***P<0.05, **P<0.01, and *P<0.05. (c) Balloon-injured injury of human left internal mammary arteries (hLIMA) ex vivo and subsequent organ culture for 48 hours.

Discussion

A mind that is stretched by new experience will never go back to its old dimensions." - Oliver Wendell Holmes, Jr.

How important is a cell-matrix connection in atherosclerosis? A tug of war!

To bind to the basement membrane muscle cells can use integrins for protein-protein interactions, and dystroglycan for carbohydrate mediated interactions. These alternatives might be comparable with those of extravasating leucocytes using first carbohydrate mediated interactions for temporary interactions, which can quickly be build up and released (rolling), and protein interactions for a firm attachment to the endothelium.

The DGC can be considered as a regulator of cell behavior such as differentiation, proliferation, migration and apoptosis. As an example, the migration of the smooth muscle cells is a pivotal function and prime process in atherosclerotic plaque development; it is regulated through actin dynamics, which in turn is a product of the Cell-ECM interaction. According to the molecular clutch hypothesis, force transmission and transduction are regulated through an *actin-talin-integrin clutch*. One question lies in what happens with the weakened connection of the chain interaction of *actin-dystrophin-dystroglycan* clutches, when either dystrophin or beta-sarcoglycan is deficient, as we know that, dystrophin could act as an alternative molecular clutch in connecting the actin cytoskeleton with the basement membrane. In a previous study, with collar induced mechanical injury to the carotid artery deficient of dystrophin, we found an increased neo-intima formation [142]. In that study, when cells were cultured in-vitro, devoid of their natural environment, dramatically reduced dystrophin and beta-sarcoglycan expression during their modulation from a contractile to a synthetic state was observed. In one study by Wang et al., 2014 [143], it was found that, dystrophin inhibits myogenic sarcoma cell migration, acting has a tumor suppressor and anti-metastatic factor in human cancers.

What is the role of Beta-sarcoglycan in the atherosclerotic process? Does it influence atherosclerotic plaque development and restenosis?

DGC components, as sarcoglycans are necessary for the stabilization of dystroglycan and their deletion, or improper function leads to muscular dystrophies [79]. We have previously demonstrated that smooth muscle hyperplasia as a response to carotid injury is increased in *mdx* mice as compared to wild type mice [142]

In papers I and II, we addressed this question by examining the function of beta-sarcoglycan, a protein, which is needed to stabilize the DGC, in an atherosclerotic and restenotic setting, respectively. This indirectly points to the importance of the DGC connection as such, impaired through deficiency of beta-sarcoglycan. In paper 1, in an atherosclerotic setup using mice with an ApoE deficient background, and on a high fat diet we found that the advanced inflammatory plaques are reduced in the mice deficient for beta-sarcoglycan. The reduction in plaque size of these mice is in line with our study in paper III, where we encountered a reduction of atherosclerotic plaques in the ApoE^{-/-}/*mdx* compared to ApoE^{-/-} mice. According to findings in the *mdx* study in paper III, the reduced atherosclerotic plaque could be a result of a less active immune system. In contrast, the plasma level of pro-inflammatory cytokines, like TNF- α was increased in beta-sarcoglycan deficient mice. In high fat diet fed ApoE-null mice, the *mdx* mutation resulted in reduced plasma levels of most analyzed cytokines and a reduced density of T-cells in the plaques, pointing to a less activated immune system that could be the cause for the observed alterations in plaque development. On the same fat-fed ApoE-null background a less activated immune system could definitively not be observed in beta-sarcoglycan deficient mice. All cytokines, which were significantly reduced in *mdx* mice, were at least not significantly altered, and IL-6 was even increased in beta-sarcoglycan deficient mice. Concomitant with IL-6 also TNF α and IL-17 were increased, the latter explainable by the ability of IL-6 to induce Th17 cells.

IL-6 is strongly associated with atherogenesis, and has been identified locally, as well as in the circulation as a marker of coronary plaque inflammation. However, the categorization of IL-6 as a pro-inflammatory cytokine has been challenged by the observation, that exercise of skeletal muscles leads to an acute increase in circulating levels of IL-6, which was associated with enhancement of fat oxidation, improvement of insulin stimulated glucose uptake and general anti-inflammatory effects, like inducing an upregulation of IL-10 [144].

One of the possible explanations for reduced plaque size in the beta-sarcoglycan deficient mice is dysregulation of the adipocyte lipid metabolism. The loss of beta-

sarcoglycan in adipose tissue led to metabolic changes, which could lead to a decreased atherosclerotic plaque development. We found an increased phosphorylation of AMPK in the adipocytes of beta-sarcoglycan deficient mice, a sign of a healthy metabolic state of these cells. A reduced size of adipocytes has in various studies been associated with an improved insulin response of adipocytes and attenuation of metabolic imbalances [145-148]

A common observation in atherosclerotic plaques of dystrophin-deficient *mdx* mice and sarcoglycan deficient mice is an increased expression of smooth muscle cell differentiation markers, pointing to the presence of smooth muscle cells lining up along the luminal border. The staining by myosin heavy chain 11 was more close to the lumen, which may be a sign, that the cells could have regained their contractile elements due to the pulsatile blood pressure. This could indicate the formation of a layer of tightly associated cells forming an endothelial like cellular barrier structure. Such a cellular barrier could impair the influx of lipoproteins into the plaque, reducing its growth.

It has been shown by Hien et al., 2016 [149], that the expression of contractile markers are increased in SMCs cultured under high glucose conditions as a result of an activation of protein kinase C and Rho/Rho-kinase signaling pathways, thereby stimulating actin polymerization. As sarcoglycan deficient mice are in general considered as glucose intolerant, a further conceivable possibility being that an elevated glucose level, according to Hien et al., 2016 [149], could have promoted the contractile elements in the smooth muscle cells, bordering the lumen, where they are more exposed to glucose, compared with cells in the lesion distant from the lumen. Although we unfortunately didn't measure the blood glucose levels in these mice directly (glucose cannot be reliably measured in the collected plasma samples at later time points), we could presume that these mice should have a higher glucose level as a result of lower insulin levels in their plasma.

Beta-sarcoglycan in restenosis and therapeutic implications

Restenosis still represents a major problem, though drug-eluting stents had a significant effect in reducing its occurrence. Target of most of the contemporary studies is developing agents, which are capable of delivering an antiproliferative and antifibrogenic outcome. The three main mechanisms, which lead to development of restenosis are elastic recoil, negative vascular remodeling and neo-intimal proliferation [150, 151]. In our study using beta-sarcoglycan and wild type mice under normal diet, we induced neo-intimal hyperplasia, which is usually a result of the migration and proliferation of VSMCs, with a non-occlusive collar and found an increased neo-intima in beta-sarcoglycan deficient mice. According

to De Meyer GR et al., 1997 [152], it has been shown that vascular injury and obstruction of transmural flow are the main consequences, which can be achieved by placing a non-occlusive collar around the carotid artery. Our previous work under collar induced mechanical injury to the carotid artery with deficiency of dystrophin has also resulted in increased neo-intima formation [142]. To test the importance of dystrophin with respect to an intact basement membrane, in that study, when cells were cultured in-vitro, devoid of their natural environment, dramatically reduced dystrophin and beta-sarcoglycan expression during their modulation from a contractile to a synthetic state was noted. Therefore it's evident, that under a threat, such as vascular injury, VSMCs potentially weaken their DGC-mediated interaction with the basement membrane by down regulating the expression of major proteins such as beta-sarcoglycan and dystrophin.

What causes increased intimal hyperplasia in beta-sarcoglycan mice?

Previous studies have shown that, under the influence of a mechanical injury on the carotid artery, the cells migrate from the media into the intima and proliferate subsequently. According to the review by Hedin et al., 2004 [50] as the neo-intima matures, the cells in the intima regain their contractile features by regaining basement membrane. Hence, in paper II, it's obvious from the staining for contractile elements, such as Myosin heavy chain 11, that we found an area in the intima with an increased MHC11-positivity compared to the media in both of the genotypes, possibly due to the fact that in the media the cells down regulated contractile elements. Cells, which have migrated into the intima, may have regained their basement membrane, evident by virtue of co-localization of MHC11-positive areas with laminin1-positive areas in the intimal region of these mice. Even more surprising was the observation that the media in injured arteries of most analyzed bSG^{-/-} mice displayed very limited to no staining for MHC11 and greatly reduced staining for laminin1 compared to media's of injured arteries of wild type mice.

The possible rationale behind these observations could be that, beta-sarcoglycan deficiency supports VSMCs in weakening their connection with the basement membrane, which is enhancing their phenotypic switch, by an amplified down regulation of their contractile proteins. This could facilitate advanced migration from the media to the intima in response to injury, which is contributing to the increased plaque size in these mice. Interestingly, looking back on alpha smooth muscle actin staining's of injured arteries of dystrophin-deficient (*mdx*) mice, also in these experiments the media of injured arteries displaying neo-intima formation showed very limited staining for this contractile protein [142]. One has to consider that the migrations of VSMCs are not merely the product of a weakened cell-matrix connection. All aspects of VSMC migration are a coordinated result of

growth factors, cell-matrix interaction and cell-cell interaction. As one example of many showing that the migration of VSMC's is not solely affected by the DGC per se, a study by Kallenbach et al., 2009 [153] found that, the migration of human aortic smooth muscle cells were reduced, when these cells were transduced with adenoviral vectors encoding human MMP-3 leading to inhibition of neo-intimal formation in arterialized vein graft.

One has to consider the fact that the vascular response to heal doesn't solely depend on the cells within the vessel, but also circulating bone marrow derived cells. Another perspective would be how efficient cells with a weakened interaction via the DGC can be triggered/stimulated by growth factors and cytokines. The surrounding basement membrane is supposed to usually maintain the contractile state of the cell by keeping the cell in a growth factor resistant state and preventing cell cycle entry [50]. Changes in composition of ECM components, such as an increase in fibronectin, can promote the synthetic state, and an increase in laminin can support the contractile state of VSMCs. The paradigm that, monomeric Collagen I which can promote cell proliferation can however in its fibrillar state prevent proliferation of cells [50].

Another conceivable argument could be that, the activated or injured cells can produce proteases that can cleave ECM for migration. The subsequent changes in ECM could promote new interactions with integrins, and facilitate cytoskeletal reorganization, promoting migration and proliferation under specific stimulations. It would have been interesting to check the ECM turnover in these beta-sarcoglycan animals to investigate, if there is any altered ECM, which could have facilitated migration of these cells, pointing to the fact, that a normal vessel ECM is highly adhesive. In some previous research, it has been shown that, some of the intimal cells could also come from adventitial fibroblasts later differentiating into myo-fibroblasts [154, 155].

In support of our finding of an increased plaque size, we observed a tendency for an increased intimal proliferation of cells in bSG^{-/-} mice compared to wild-type mice, as detected by staining for Ki-67. That we couldn't observe a difference, which was statistically significant, was mainly due to the fact that some of the plaques from bSG^{-/-} mice (plaques from wild type mice as well) showed no positive nuclear staining for Ki-67 at all. It has been previously shown that in a mechanically induced lesion at a time point of 21 days, most of the proliferating cells are vascular smooth muscle cells [142, 156].

To check and exclude other possibilities aside from the potential effect of the impairment of the DGC in VSMCs due to deficiency of beta-sarcoglycan, we analyzed the concentration of the important cytokine IL-6. For *mdx* mice increased levels of IL-6 have been shown, which positively correlate with the exacerbation of muscular dystrophy [157]. IL-6 is considered to be an important cytokine in

restenosis [158], and in the activation of VSMCs from a contractile to a synthetic state [159]. In our study II, we didn't find a significant difference in plasma IL-6 levels between the genotypes after three weeks of collar placement. This renders a major responsibility of IL-6 for the observed differences in neo-intima development unlikely. Thus, the beta-sarcoglycan deficient VSMCs have more likely been differentially activated by other stimulatory factors, possibly an impaired interaction with basement membranes.

When investigating restenotic processes, apart from typical smooth muscle cell proliferation, also, factors like constrictive remodeling, a process producing a smaller lumen due to adventitial scarring, have to be taken into consideration [160]. A more common response to neo-intima formation is outward remodeling, a compensatory mechanism by the vessel to accommodate increasing plaque size, which is independent of diet and atherosclerotic prone genetic background [128]. In general, a chronic increase in outward remodeling due to changes in blood flow is a sign of normal functioning of the vessel via NO dependent dilation [161]. When *mdx* mouse mesenteric resistant arteries were subjected to high flow by the process of arterial ligation, due to defects in NO dependent dilation, the arteries were shown to be unresponsive to flow induced dilation [162]. To check whether beta-sarcoglycan deficiency has an influence on outward remodeling, we analyzed the outward remodeling by measuring the area inside of the external elastic lamina and found in accordance with an increased plaque size an increased outward remodeling in *bSG*^{-/-} mice comparable with wild-type mice, implying that, beta-sarcoglycan deficiency doesn't influence the remodeling process.

To sum it up, the dogmatic cause for the observed increase in neo-intima formation in our *bSG*^{-/-} mice could be a result of reduced expression of important components of DGC leading to impaired or weakened connections of VSMCs with the basement membrane. Not to forget, the potential research that could target ECM-cell signaling elements in VSMCs as a therapeutic option.

Adipose tissue functions in atherosclerosis, a correlative or a causative effect? Beta-sarcoglycan in adipose tissue, Does altering adipose tissue structure and function effects atherosclerotic plaque development?

In our beta-sarcoglycan deficient mice, we found reduced plasma levels of TG and cholesterol, and a reduced size of visceral adipocytes. In general, an excess accumulation of fat causes dyslipidemia and inflammation. Excess accumulation of TGs, in the ectopic fat (deposition of TGs within non-adipose tissue cells, which otherwise contain small amount of fat) is deemed, the most likely cause for

abnormal function of adipose tissue. The proposition is that, unused excess FFA released into the circulation, can lead to storage/accumulation in the liver in the form of TG depots causing hepatic steatosis [163] [164]. In order to counteract the TG accumulation, hepatocytes increase their production of VLDL to accelerate their intracellular TG transportation. This is ultimately leading to an increase secretion into the circulation and moreover, over a sustained period of time pro-atherogenic [165]. The reduced plasma TG levels in beta-sarcoglycan deficient mice could point to that a healthy adipose tissue function per se, can be one of the prime causes for reduced atherosclerotic plaque development in these mice. Moreover, the increasing contemporary research on PVAT, points out its importance in influencing arterial wall physiology. In our beta-sarcoglycan deficient mice we found in addition to the reported alteration in ovarian fat pads also a reduced size of brown adipocytes in the PVAT of the aorta. This could indicate that the FFAs released upon lipase activity by adipocytes in general could be minimal compared to non-beta-sarcoglycan deficient cells. In such a situation, minimal FFAs can subsequently lead to a lower prevalence of endothelial dysfunction. Moreover, PVAT is mostly comprised of BAT and according to Bartelt et al., 2011 [166], the normal functioning healthy BAT burns fatty acids for heat production. PVAT could control the vascular lipoprotein homeostasis, by initiating a metabolic program to increase TG turnover and divert lipids into BAT, which can further be burned off by thermogenesis. With reduced TGs in the circulation and a reduced PVAT in beta-sarcoglycan deficient mice, one could argue that a healthy activated state of adipocytes could be the cause for a reduced atherosclerotic plaque. Therefore as mentioned, it could be possible that the activities of BAT in these mice are normal and prevent excess circulation of TGs in the circulation. Having said that, it is still ambiguous whether the alteration in the atherosclerotic plaque development and adipose tissue function in beta-sarcoglycan deficient mice is correlative and causative.

Well beyond the adventitia, the perivascular adipose tissue (PVAT) — conventionally underrated and less overlooked

PVAT or adventitial fat has grown to be a hot topic these days. Thus, we analyzed the PVAT from aorta and found smaller adipocytes in beta-sarcoglycan deficient mice compared to Wildtype (unpublished observation). In general, PVAT secretes cytokines such as TNF- α and IL-6, hormones such as leptin, visfatin and adiponectin. This indicates that in spite of a high fat diet PVAT in beta-sarcoglycan deficient mice could have been healthy with favorable effects. PVAT has also shown to be involved in proliferative and migratory functions of VSMCs

by secreting visfatin (an adipocyte hormone), which can act as a VSMC growth factor stimulating proliferation. In our study, we found a decreased level of plasma adiponectin in beta-sarcoglycan deficient mice. Though adiponectin is considered as a good hormone, it has been shown that increased levels of adiponectin have an inhibitory effect on vascular smooth muscle cell proliferation and migration by hindering the interaction of diverse atherogenic growth factors with their receptors [109]. In our study, we found that blood of beta-sarcoglycan deficient mice carries less adiponectin. This paucity of adiponectin is not reflected in the decreased atherosclerotic plaques and favorable adipose tissue constitution of the beta-sarcoglycan deficient mice and the reduced positive influence of adiponectin might have been overridden by even more reduced negative influences of other adipokines such as leptin.

Leptin is another important hormone secreted by PVAT. Leptin can impair vascular functions indirectly by acting in the brain to activate sympathetic nervous system, exerting the function of VSMCs [109]. As a healthy PVAT provides resistance to HFD, one could argue that in the absence of beta-sarcoglycan, the adipocytes were smaller and have a healthier profile compared to wildtype mice, and potentially could possess more resistance to HFD. Several studies have shown the accumulation of inflammatory cells in the adventitia of atherosclerotic arteries [167-169]. A study by Guzik et al., 2007 [170] in mice, reported that the resident T cells in the adventitia are key mediators in angiotensin II induced hypertension and that cytokines produced by these cells resulted in adventitial collagen production, wall stiffening and onset of hypertension. A study by Wu et al., 2014 using mice deficient in production of T-cell derived cytokine IL-17a resulted in protection against angiotensin II induced aortic wall stiffening and adventitial fibrosis [171]. From these observations it's obvious that to have a healthy PVAT is advantageous, as hypertension, which can be caused by adventitial inflammation, is one of the main contributors for atherosclerosis.

The mechanism by which these adipokines enter the vessel is still ambiguous. Some recent studies have found that, the adipokines produced by PVAT determine the functions of endothelial and smooth muscle cells, which could indirectly have an influence on vascular tone, which can be something to ponder on! Not to be forgotten, that the positive effect of adipose tissue on the metabolic balance is more dependent on the normal size and function of the adipocytes, than on the total volume of the adipose tissue.

To check for the role of beta-sarcoglycan in a restenotic process, we found in paper II that beta-sarcoglycan deficiency increased neo-intimal hyperplasia. In this study, we found in the beta-sarcoglycan deficient mice a tendency for reduced levels of insulin, which almost reached significance. This could mean that in these mice the plasma glucose levels are altered. Considering this aspect, another

hypothesis could be that due to lower glucose levels there could have been a reduced differentiation of adipose and muscle derived stem cells into adipocytes, since usually their differentiation is positively correlated to higher glucose levels [172]. In retrospective, it might have been worthwhile to check the muscles of beta-sarcoglycan deficient mice for fat infiltration to examine the severity of muscular dystrophy that could perhaps influence the plaque development indirectly. In general, as a fact, the beta-sarcoglycan deficient mice are found to have more fibrosis and inflammation in muscles compared to *mdx* mice [83]

Is targeting DGC in adipocytes, a therapeutic strategy to decrease atherosclerotic plaque progression?

Adipocytes are in constant contact with their ECM [173]. Studies show that the synthesis and remodeling of ECM is a major event during adipogenesis [174, 175]. It has been shown that, in obese patients, several components of basement membranes are potentially augmented and disorganized, resulting in an extensive fibrosis. Additionally, these obese patients, have increased amounts of Col IV in BMs [176]. During adipogenesis, the pre-adipocytes commit themselves for the storage of TGs. At this stage, the laminar collagens and other components of basement membranes are increased, in a milieu, where fibrillar collagen around the cell remains in a constant concentration. Further, the developing cell then embeds itself into a basement membrane. From here, the cell accumulates a huge amount of triglycerides by modulating their shape and growth. This is mediated by the traction force between the cell and the ECM, which is important for this process. At this point the fibrillar collagen helps the cell to be protected from mechanical disruption by acting as an outer skeleton, aiding to increase the accumulation of TGs [177].

It could be possible from our study I, (beta-sarcoglycan deficiency with ApoE and HFD background) that the weakened connection between the DGC and the BM, might have aided the developing adipocytes to less firmly embed themselves with BM, leading to less accumulation of TGs due to difficulties in changing their shape and growth, which could have led to an observed healthier version. It would be interesting to possibly tweak different components of the DGC to check for phenotypic changes. The laminin $\alpha 4$ chain is present in BMs of fully differentiated adipocytes and studies using laminin $\alpha 4$ chain KO mice, have displayed reduced depot specific adipose tissue mass [173]. This shows that the connection between the cell and BM could be crucial for adipocyte function. Thus, alterations of the DGC complex in adipocytes could potentially represent molecular mechanisms

that can change adiposity and fat distribution, thereby, modulating metabolic disorders via altering cell-cell/or cell-matrix interaction in adipose tissue.

What is Dystrophins effect on atherosclerotic plaque formation; does it have the same effect as sarcoglycan?

In our paper III, we discussed the role of dystrophin in atherosclerotic plaque development and found a reduction, similar to our study using beta-sarcoglycan deficient mice (paper 1). Dystrophin has its effects intracellularly, whilst sarcoglycan exerts its function as well and probably mainly extracellularly. The deficiency in dystrophin leads to a decreased activation of immune cells, especially T-cells. Study by Cascabulho et al., 2012 [178], found that migration of T-cells is affected in *mdx* mice due to alterations in adhesion molecules, which makes the cells less competent to adhere to vessel wall. Well, though the decreased staining of differentiated VSMCs is comparable with Paper I (beta-sarcoglycan deficient mice), the immune system is not under-activated in beta-sarcoglycan deficient mice. As we know that deficiency in dystrophin or sarcoglycan de-stabilizes dystroglycans, the central molecule, which communicates with the basement membrane directly, it has to be kept in mind that the deficiency of sarcoglycan can also influences indirectly the interaction of β dystroglycans with dystrophin through lack of α -dystrobrevins cross-linking ability.

How actin polymerization influences phenotype of SMCs? Is targeting actin polymerization a therapy for stabilizing atherosclerotic plaques?

The actin polymerization and de-polymerization during contraction and relaxation of VSMCs is dependent on the stretch and relaxation of the vascular wall [179] and the pathological migration is deemed as the prime factor in restenosis. The force transmission between the contractile elements and ECM is achieved through linker or adapter proteins. As we know apart from the actin-talin-integrin link, the other way the cell communicates with the ECM is through the DGC. Here, dystrophin is considered as the linker or adaptor protein that links actin and dystroglycan. As phenotypic modulation of SMCs from contractile to synthetic is crucial for the migration of VSMCs, which ultimately determines the size of restenotic and stability of atherosclerotic plaques, in paper IV, we investigated the effect of a modulation of actin polymerization and vascular injury on the

expression of the linker protein dystrophin. Loss of dystrophin in smooth muscle cells of dystrophin-deficient *mdx* mice resulted in dysregulation of the structural contractile machinery, without affecting the F/G actin ratio, suggesting that the function rather than amount of actin filaments is affected. That being said; how far this dysregulated F-actin affects migration is still a debatable topic and something to ponder on. The expression of dystrophin in our study is promoted using actin polymerizing agents and reduced with de-polymerizing agents, respectively. This clearly shows that dystrophin is highly expressed in differentiated smooth muscle cells and that actin polymerization is vital for smooth muscle cell differentiation. During a balloon dilation of human left mammary artery vessels, there was a substantial reduction in the expression of dystrophin. Dystrophin is crucial for endothelial mechanosensing; it has been demonstrated, that the flow induced vascular dilation is altered in *mdx* mice, due to deficiency in ECs and SMCs [180]. Therefore in *mdx* mice, altered perception of shear stress can lead to a dysfunctional/abnormal mechanosensing of ECs, which can impose a direct influence on the biology of SMCs and their function. A study by Dardik et al., 2005 [181] shows that, subjection of ECs to arterial shear stress in-vitro led to secretion of SMC chemo-attractants such as PDGF that induced migration of VSMCs via mitogen activated protein kinase (MAPK) pathways. This can be a consequence of an unregulated transmission of signals through VSMCs, promoting activation of certain genes as consequences of cellular signaling events ultimately regulating the SMC function. Accordingly, due to a disruption in signaling between ECM and cells, and improper sensing of the local environment in dystrophin and beta-sarcoglycan deficient mice, the SMCs could have modulated their phenotype. As a result of it, they may have reduced their actin filaments. In our beta-sarcoglycan and dystrophin deficient mice, we found an increased differentiated SMC content through staining with antibodies that stain for contractile elements. The staining's are often and mostly in the plaque region bordering the lumen, and sparse in other parts of the plaque. This could point out that the cells after migration would have regained their contractile nature due to possibilities such as pulsatile blood flow and high glucose, for which the appropriate mechanisms have been discussed previously in this thesis. Thus, actin polymerization can affect the mechanosignalling system of the cell by modulating the expression of linker proteins such as dystrophin, and likewise possibly through beta-sarcoglycan, since beta-sarcoglycan is important for stabilization of dystroglycans.

Strength and limitations

Although 99% of all mouse genes are homologous to human genes, extrapolating data from animal models are difficult, due to a different range of response in different individuals. It is important to note that, with an ApoE background the results are not exactly comparable to humans, as the compensatory pathways, which are expressed in the ApoE mouse, need not necessarily to be the same in humans. One way to address this is to use pharmacological inhibition apart from knocking out the gene, as this combination will address the concern raised due to possible compensatory pathways with ApoE background. Another limitation could be that performing surgical manipulation induces variability and it's hard to have the exact consistent degree of inflammation produced during the surgery as isolating carotid arteries from adjacent connective tissue can vary to some extent from animal to animal. However, using large number of animals per group can help to compensate for variability to some extent. Not to forget, the extent of similarities of experimental lesions and lipoprotein metabolism to humans is an important factor to consider when extrapolating data. The advantage and disadvantage of using ApoE as a background is that, lesions progress to a fibroproliferative phase, though plaque thrombosis is absent.

From paper III, and I, although there is a reduced plaque development, due to deficiency of the respective genes, the results should be crosschecked with an alternate strategy in order to hold a therapeutic significance.

Conclusion

The main conclusions from the four studies are as follows:

- Beta-sarcoglycan deficiency attenuates advanced atherosclerotic plaque development, however stimulates neo-intimal hyperplasia in a restenotic setting.
- The deficiency of beta-sarcoglycan in adipose tissue decreases the weight of visceral fat with reduction in size of both brown (PVAT) and white adipocytes and reduced plasma cholesterol and triglyceride levels.
- The deficiency of dystrophin demonstrated by the *mdx* mice attenuates advanced atherosclerotic plaque development with a reduction in T-cell content in these plaques and an overall reduction in systemic inflammation.
- The relative area of differentiated smooth muscle cells in advanced atherosclerotic plaques is increased under the influence of dystrophin and beta-sarcoglycan deficiency.
- Actin polymerization influences the expression of the actin binding protein dystrophin, which is greatly reduced during deregulation of actin polymerization, in cases of vascular injury.

Summary and Perspective

Vascular smooth muscle cells represent the major type of cells in atherosclerotic plaque development. An optimal concentration and distribution of these cells represents a plaque, which can be considered stable. The migratory and proliferative capacity of VSMCs greatly depends on their interaction with their basement membrane. These cells have the potential to exist in a diverse range of phenotypes modulated by a variety of cues [182-184]; however, they are normally in a contractile state. The synthetic phenotype of VSMCs is the hallmark for restenosis and atherosclerotic plaque development and a response to vascular injury. Cells sense their surrounding by communicating with other cells and ECM, and mechanosensing is achieved by binding to their ECM via integrin receptors.

Here in this thesis, I addressed another main interaction with the ECM - via the DGC - by exploring two main proteins of the complex, dystrophin and beta-sarcoglycan, with respect to atherosclerotic plaque formation, and also the role of actin polymerization and vascular injury in the regulation of dystrophin. The deficiency of dystrophin and beta-sarcoglycan under an ApoE deficient background led to a decrease in atherosclerotic plaque and an increase in differentiated SMCs in plaques bordering the lumen in these mice. Intriguingly, this raised questions such as: Firstly, are these plaques endowed with a good fibrous cap and are they quite stable? Secondly, is targeting beta-sarcoglycan in adipose tissue, a way to reduce fat? This second question became evident from our results of reduced visceral fat and adipocyte size in WAT and PVAT. Writing this thesis resulted in a generation of a lot of questions out of curiosity, and, one such important question being, the deficiency of beta-sarcoglycan and dystrophin in ECs and their effect in lipid transportation across ECs. Is the EC function still well maintained under beta-sarcoglycan and dystrophin deficiency? As actin polymerization greatly influences actin-binding proteins, are therefore agents that target actin polymerization a possible novel therapeutic strategy in the future?

The present study addresses the basic science, rather than therapeutic potential. Considering the translational approach with the results obtained; older patients with conditions such as sarcoglycanopathies and Becker muscular dystrophy will be less susceptible to plaque development. Thus drugs that can tweak the expression of beta-sarcoglycan and dystrophin in a tissue dependent manner could have a beneficial outcome.

Popular summary

Atherosclerosis is considered as the underlying cause for cardiovascular diseases, such as stroke and myocardial infarction. Atherosclerosis is a disease in which, plaque builds up inside the artery wall and obstructs the oxygen rich blood flow. The blood vessel is made of three layers, starting from outside - the tunica adventitia, tunica media and tunica intima; each layer is separated by an elastic lamina. The media consists of vascular smooth muscle cells whereas the intima contains endothelial cells having direct contact with blood flow. A typical atherosclerotic plaque has cells, ECM and fat! One of the important functions of the VSMCs is to secrete ECM and stabilize the plaque in order to prevent them from leaking into the blood stream. This is through forming a fibrous cap, which seals the plaque component from being exposed to the blood; comparable to an intact balloon filled with water. Vascular smooth muscle cells normally reside in the media, in a contractile state. Complications such as injury to the intima, initiates them to modulate their phenotype to proliferate and migrate into the intima to repair the process. During this time, cells undergo apoptosis due to a potent inflammatory condition that exists inside the plaque, which thereby can contribute to plaque instability through forming a necrotic core consisting of cell debris and lipids. Therefore, an optimal proportion of cells are momentous to repair, but also at the same time remain alive.

To understand how VSMCs migrate, one should have a clear understanding of how cells communicate with basement membranes, a part of the ECM. One-way is through integrin signaling and a second major way through the Dystrophin glycoprotein complex (DGC). The DGC is studied extensively in muscles, since its impairment is responsible for different types of muscular dystrophies. It was previously shown that VSMCs, endothelial cells and adipocytes express the complex. Therefore, we aspired to test the important proteins of DGC in the atherosclerotic plaque development process. Two of the main components of DGC are dystrophin and beta-sarcoglycan. The VSMCs migrate by first changing from a contractile to a synthetic phenotype by down regulating their contractile filament associated molecules, such as smooth muscle actin and myosin, and up regulating the endoplasmic reticulum, and Golgi apparatus to produce ECM. To do this, these cells have to weaken or lose their connection with the basement membrane.

My thesis is focused on deciphering the role of dystrophin and beta-sarcoglycan in the atherosclerotic plaque formation. We induced atherosclerotic and restenotic types of plaques through changing the shear stress and inducing mechanical stress by using different type of collars, placed around the mouse carotid artery. In this thesis, the first paper addresses the question on the role of beta-sarcoglycan in the development of an atherosclerotic plaque formation. We found a decrease in atherosclerotic plaques in the absence of beta-sarcoglycan with plaques having a higher number of differentiated VSMCs

Concurrently, we studied the effect of beta-sarcoglycan deficiency in a mouse model of a restenotic type of plaques rich in VSMCs and ECM, which commonly develop after an angioplasty procedure in humans. In paper II we show that deficiency of beta-sarcoglycan aggravates neo-intimal hyperplasia.

To determine the role of dystrophin in atherosclerotic plaque development, we took advantage of using *mdx* mice that have a mutation in the dystrophin gene and found a reduced development of atherosclerotic plaques, with plaques having a higher number of differentiated VSMCs and a reduced systemic inflammation, notably with reduced T-cells in the plaque of these mice.

Actin polymerization and vascular injury has been shown to influence the phenotype of VSMCs, and, moreover, dystrophin interacts directly with actin. Therefore, in paper IV, we show that dystrophin is greatly regulated by actin polymerization and vascular injury.

To conclude in simple terms, imagine two people locked in a room with constant supply of fatty foods, such as hamburger, for more than a year. Among these two, one has a muscular dystrophy caused by deficiency or mutation for gene's encoding dystrophin or beta-sarcoglycan. The potential chance for developing an atherosclerotic plaque is reduced for the person having muscular dystrophy compared to the normal person (However, the person with muscular dystrophy might become incapable to eat, because the muscles required for this activity could be weak)

Our findings, although still in a basic research, might one day have a translational outcome in identifying therapeutic drugs that can tweak the expression of dystrophin and beta-sarcoglycan to modulate in such a way, to reduce the development of atherosclerotic plaque without inducing muscular dystrophy.

Populär sammanfattning på svenska

Ateroskleros anses vara den bakomliggande orsaken till hjärt-kärlsjukdomar, såsom stroke och hjärtinfarkt. Ateroskleros är en sjukdom där plack byggs upp inuti artärväggen och hindrar syrerikt blodflöde. Blodkärlet är gjord av tre lager, från utsidan - tunika adventitia, tunica media och tunica intima; Varje skikt separeras av ett elastiskt laminat. Mediet består av vaskulära glattmuskelceller medan de intima som innehåller endotelceller som har direkt kontakt med blodflödet. En typisk aterosklerotisk plack har celler och fett! En av VSMCs viktiga funktioner är att utsöndra ECM och stabilisera placken för att förhindra att de läcker in i blodflödet. Detta är genom att bilda en fiberhätta, vilken förseglar plackkomponenten från att exponeras för blodet; Jämförbar med en intakt ballong fylld med vatten. Vaskulära glattmuskelceller bor normalt i media, i kontraktillstånd. Komplikationer som skada på intima initierar dem för att modulera deras fenotyp för att proliferera och migrera till intima för att reparera processen. Under denna tid genomgår cellerna apoptos på grund av ett kraftigt inflammatoriskt tillstånd som finns inne i placken, vilket därigenom kan bidra till plackinstabilitet genom att bilda en nekrotisk kärna bestående av cellavfall och lipider. Därför är en optimal andel celler snabba att reparera, men samtidigt förblir de levande.

För att förstå hur VSMCs migrerar ska man ha en klar förståelse för hur celler kommunicerar med basalmembran, en del av ECM. Ett sätt är genom integrationssignalering och ett andra stort sätt genom Dystrophin glycoprotein-komplexet (DGC). DGC studeras omfattande i musklerna, eftersom dess nedsättning är ansvarig för olika typer av muskeldystrofi. Det visades tidigare att VSMCs, endotelceller och adipocyter uttrycker komplexet. Därför strävade vi efter att testa de viktiga proteinerna av DGC i den aterosklerotiska plackutvecklingsprocessen. Två av huvudkomponenterna i DGC är dystrofin och beta-sarkoglykan. VSMCs migrera migreras genom att man först övergår från en kontraktill till en syntetisk fenotyp genom att reglera deras sammankopplade molekyler med kontraktillfilament, såsom glattmuskelaktin och myosin, och upp reglering av endoplasmatisk retikulum och Golgi-apparat för att producera ECM. För att göra detta måste dessa celler försvaga eller förlora sin anslutning till källarmembranet.

Min avhandling är inriktad på att dechiffrera rollen av dystrofin och beta-sarkoglykan i den aterosklerotiska plackbildningen. Vi framkallade aterosklerotiska och restotiska typer av plack genom att ändra skjuvspänningen och inducera mekanisk stress genom att använda olika typer av krage placerade runt muskelkarotenären. I denna avhandling behandlas det första papperet frågan om beta-sarkoglykans roll vid utvecklingen av en aterosklerotisk plackbildning. Vi hittade en minskning av aterosklerotiska plack i frånvaro av beta-sarkoglykan med plack med högre antal differentierade VSMCs

Samtidigt studerade vi effekten av beta-sarkoglykanbrist i en musmodell av en restotisk typ av plack rik på VSMC och ECM, som vanligtvis utvecklas efter en angioplastikprocedur hos människor. På papper II visar vi att brist på beta-sarkoglykan förvärrar neo-intimal hyperplasi.

För att bestämma rollen av dystrofin vid aterosklerotisk plackutveckling utnyttjade vi användning av mdx-möss som har en mutation i dystrofinengen och fann en minskad utveckling av aterosklerotiska plack med plack med ett högre antal differentierade VSMCer och en minskad systemisk inflammation, Särskilt med reducerade T-celler i plack av dessa möss.

Actinpolymerisation och vaskulär skada har visat sig påverka fenotypen hos VSMCer, och dessutom påverkar dystrofin direkt med aktin. Därför visar vi i dokument IV att dystrofin är starkt reglerad av aktinpolymerisation och vaskulär skada.

För att avsluta med enkla ord kan du föreställa dig två personer som är låsta i ett rum med konstant matleverans, till exempel hamburgare, i mer än ett år. Bland dessa har man en muskeldystrofi orsakad av brist eller mutation för genens kodande dystrofin eller beta-sarkoglykan. Den potentiella risken för att utveckla en aterosklerotisk plack reduceras för den person som har muskeldystrofi jämfört med den normala personen (Emellertid kan personen med muskeldystrofi bli oförmögen att äta, eftersom musklerna som krävs för denna aktivitet kan vara svaga)

Våra funderingar kan, trots att de fortfarande finns i en grundforskning, en dag ha ett översättningsutfall vid identifiering av terapeutiska läkemedel som kan tweakera uttrycket av dystrofin och beta-sarkoglykan för att modulera på ett sådant sätt att minska utvecklingen av aterosklerotisk plack utan att framkalla muskeldystrofi.

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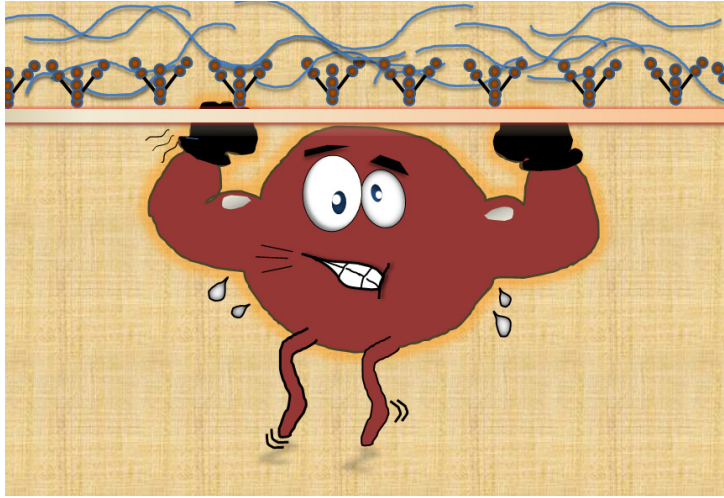


Illustration of a vascular smooth muscle cell, trying to hold on its connection with its basement membrane. Drawing by Murugesan, V.

