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NOVEL ASPECTS OF ATHEROSCLEROSIS: FOCUSING ON NEW TARGET GENES AND THE EFFECT OF CHOLESTEROL-LOWERING

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Novel aspects of atherosclerosis: focusing on new target genes and the effect of cholesterol-lowering

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To my family, my home

POPULAR SCIENTIFIC SUMMARY

Cardiovascular diseases (CVDs) are the first cause of death worldwide, accounting for over 17.7 million deaths alone per year (more than 31% of all deaths). This group of diseases often has a common underlying cause: atherosclerosis. Atherosclerosis is characterized by the accumulation of fat (lipids) in the arteries that will form plaques. These plaques will partially block the blood flow and can rupture, leading to thrombus formation and potentially death. The main risk factor for atherosclerosis is high levels of the so-called "bad" cholesterol (low-density lipoproteins, LDL), which can be obtained from a high fat diet, unhealthy habits, or genetic reasons, among others. The most common way to medically control cholesterol levels is with the use of statins (cholesterol-lowering drugs). Nevertheless, clinical events still occur, and new therapies must be developed in order to further control atherosclerosis development and its associated complications.

One of the key steps during atherosclerosis development is the movement of white blood cells (leukocytes) from the circulation to the site of inflammation where the disease takes place. This infiltration event, named transendothelial migration of leukocytes (TEML), is a key step for the immune response that the body will have while trying to control the disease. In this thesis, the role of two specific genes, Lim domain-binding 2 (*Ldb2*) in **paper I** and Poliovirus receptor-related 2 (*Pvrl2*) in **paper II**, during atherosclerosis and TEML will be discussed. *Ldb2* was found to be a general regulator of the whole migration process, while *Pvrl2* was identified to be directly involved in the process where the white blood cells precisely cross from the circulation into the site where the plaques will eventually form. The first gene, *Ldb2*, was shown to have a protective role against atherosclerosis: mice lacking this gene had more plaques that were more unstable (more prone to rupture); while *Pvrl2* was found to promote atherosclerosis: mice lacking *Pvrl2* had fewer plaques and these were more stable.

As mentioned above, the effect of cholesterol-lowering is of high importance and plays a major role in the development of CVDs. This effect was examined in **paper III** in relation to abdominal aortic aneurysm (AAA), a vascular disease involving enlargement of the diameter of the abdominal aorta often accompanied by atherosclerosis. An atherosclerotic mouse model in which we could selectively lower cholesterol was used. Moreover, the animals were treated with angiotensin II (AngII) to induce AAA. We discovered more atherosclerotic plaques and an increase of leukocytes when treating these animals with AngII. However, we did not find a high incidence of AAA in this model. This is probably because the starting cholesterol levels of our mice where not as high as in other common AAA animal models.

These three papers highlight the important role of cholesterol during CVD, especially during atherosclerosis development and AAA. The complete understanding of the newly identified gene targets could set the stage for the development of new therapeutic approaches. These would act directly on the TEML process, thus controlling the inflammatory response and reducing the associated disease complications.

RESUMEN DE DIVULGACIÓN CIENTÍFICA

Las enfermedades cardiovasculares (CVDs) son la primera causa de muerte en el mundo, siendo responsables de 17.7 millones de defunciones al año (más de un tercio de todas las muertes). Este grupo de trastornos suele tener una causa subyacente en común: la aterosclerosis. La aterosclerosis se caracteriza por la acumulación de grasa (lípidos) en las arterias formando las llamadas placas de ateroma. Estas bloquean parcialmente la circulación sanguínea y pueden romperse dando lugar a la formación de trombos, e incluso llegando a causar la muerte. El principal factor de riesgo para el desarrollo de aterosclerosis son altos niveles de "colesterol malo" (lipoproteínas de baja densidad, LDL) que proviene de una dieta alta en grasas, de hábitos poco saludables o por razones genéticas. El método más común para controlar los niveles de colesterol es el uso de estatinas. Sin embargo, a pesar de su uso siguen sucediendo eventos clínicos, por lo que deben desarrollarse nuevas terapias para controlar la evolución de la aterosclerosis y las complicaciones asociadas.

Uno de los pasos clave durante el progreso de la aterosclerosis es el movimiento de glóbulos blancos (leucocitos) desde la circulación hacia el sitio de inflamación donde se está desarrollando la enfermedad. Esta infiltración, llamada migración de leucocitos trans-endotelial (TEML), es un proceso clave para la respuesta immune del cuerpo al tratar de controlar la enfermedad. En esta tesis, se detallarán las funciones de dos genes específicos, *Ldb2* en la **publicación I,** y *Pvrl2* en la **publicación II**, durante TEML y aterosclerosis serán detallados. *Lbd2* resultó ser un regulador general de todo el proceso migratorio, mientras que *Pvrl2* resultó formar parte del proceso específico en el cuál los glóbulos blancos cruzan de la circulación a la zona donde se formarán las placas de ateroma. *Ldb2* mostró una función protectora contra la aterosclerosis: ratones sin este gen tenían más placas y estas eran más inestables (más propensas a romperse); mientras que *Pvrl2* resultó promover la aterosclerosis: ratones sin este gen tenían menos placas y estas eran más estables.

Como mencionamos anteriormente, el efecto de reducción del colesterol es de gran importancia y juega un gran papel en el desarrollo de las CVDs. Este efecto fue examinado en la **publicación III** en relación al aneurisma abdominal aórtico (AAA), una enfermedad caracterizada por el aumento del diámetro de la aorta abdominal que suele acompañarse de aterosclerosis. Para ello se utilizó un modelo de ratón al que se redujeron los niveles de colesterol selectivamente. Para inducir AAA, se trató a los animales con angiotensina II (AngII). Encontramos más placas de ateroma y un aumento de leucocitos al tratar a los animales con AngII. Sin embargo, no encontramos una alta incidencia de AAA usando este modelo, probablemente por los niveles de colesterol de nuestros ratones al inicio, que no eran tan altos como en otros modelos típicos de AAA.

Estos tres artículos destacan la importancia del colesterol en las CVDs, especialmente en el desarollo de aterosclerosis y AAA. La completa comprensión de los nuevos genes identificados podría constituir un avance para el desarrollo de nuevos enfoques terapéuticos que actuarían directamente sobre el proceso TEML controlando la respuesta inflamatoria y reduciendo las complicaciones asociadas a la enfermedad.

ABSTRACT

Atherosclerosis is most often the main underlying cause of cardiovascular diseases (CVDs), accounting for more than 31% of all deaths worldwide. It is driven by the uptake of low-density lipoproteins (LDL) by a dysfunctional arterial endothelium. It involves a complex interplay of genetic and cellular factors that result in an uncontrolled inflammatory response which can potentially be fatal. The main treatment for atherosclerosis is cholesterol-lowering drugs, statins. Despite their use, clinical events still occur. In this thesis, three papers regarding CVDs and some of the key events happening during atherosclerosis are discussed.

In **paper I** the role of Lim domain-binding 2 (*Ldb2*) as a master regulator of transendothelial migration of leukocytes (TEML) during atherosclerosis was investigated. We described its function as a modulator of the leukocyte extravasation process using *in vivo* mouse models and *in vitro* systems. By examining *Ldb2*-deficient mice we found increased atherosclerotic lesions and decreased plaque stability. Their TEML activity was increased, especially regarding monocytes and macrophages, the principal initiators of the atherosclerotic process. Additionally, the role of this gene was reinforced by a functional SNP found in coronary artery disease (CAD) cohorts associated with increased risk of myocardial infarction (MI).

In the following publication, **paper II**, we describe the function of the newly identified cholesterol-responsive gene Poliovirus receptor-related 2 (*PVRL2*) in atherosclerosis. This gene, as a member of the nectin family, plays a major role during TEML in the extravasation step. Regarding atherosclerosis development, *Pvrl2*-deficient mice showed fewer lesions and more stable plaques. An increased endothelial expression of *Pvrl2* coincided with an increase in leukocyte gene expression, strengthening its potential role during TEML. In fact, we found a significant decrease in leukocyte migration in the *Pvrl2*-deficient mice using *in vivo* assays. Moreover, we observed its endothelial expression and cholesterol-responsiveness in humans.

The effect of cholesterol-lowering on atherosclerosis is well established, and statins remain the main treatment. Since statins are prescribed to most CVD patients due to the underlying atherosclerosis, their specific effect on single diseases are not well studied. In **paper III** we aimed to identify the effect of angiotensin II (AngII)-induced AAA on atherosclerosis and the influence of cholesterol-lowering on abdominal aortic aneurysm (AAA) in an atherosclerotic mouse model. We found a low incidence of AAA formation after AngII infusion, possibly because the levels of cholesterol in our mice were not high enough. Nevertheless, AngII was found to enhance atherosclerosis and leukocyte infiltration, stressing the importance of the renin-angiotensin system on atherosclerosis and suggesting a controlling effect of cholesterol in the model.

All three papers emphasize the importance of cholesterol during CVDs. Further research in order to elucidate the underlying mechanisms and detailed role of the identified gene targets could have a major impact on the development of new drugs. These could act directly on the TEML process, thus modulating the inflammatory response and attenuating disease complications.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the papers listed below, which will be referred to in the text by their roman numerals:

I. **Lim domain binding 2: a key driver of transendothelial migration of leukocytes and atherosclerosis**
Ming-Mei Shang, Husain A. Talukdar, Jennifer J. Hofmann, Colin Niaudet, Hassan Foroughi Asl, Rajeev K. Jain, **Aranzazu Rossignoli**, Cecilia Cedergren, Angela Silveira, Bruna Gigante, Karin Leander, Ulf de Faire, Anders Hamsten, Arno Ruusalepp, Olle Melander, Torbjörn Ivert, Tom Michoel, Eric E. Schadt, Christer Betsholtz, Josefin Skogsberg, Johan L.M. Björkegren
Arteriosclerosis, Thrombosis, and Vascular Biology. 2014;34:2068-2077

II. **Poliovirus Receptor-Related 2: A Cholesterol-Responsive Gene Affecting Atherosclerosis Development by Modulating Leukocyte Migration**
Aránzazu Rossignoli, Ming-Mei Shang, Hanna Gladh, Christine Moessinger, Hassan Foroughi Asl, Husain Ahammad Talukdar, Oscar Franzén, Steffen Mueller, Johan L.M. Björkegren, Erika Folestad, Josefin Skogsberg
Arteriosclerosis, Thrombosis, and Vascular Biology. 2017;37:534-542

III. **Plasma Cholesterol Lowering in an AngII-infused Atherosclerotic Mouse Model with Moderate Hypercholesterolemia**
Aránzazu Rossignoli, Emina Vorkapic, Anders Wanhainen, Toste Länne, Josefin Skogberg, Erika Folestad, Dick Wågsäter
Submitted manuscript

Additional publication not included in this thesis:

Plasma cholesterol-induced lesion networks activated before regression of early, mature, and advanced atherosclerosis
Johan L. M. Björkegren, Sara Hägg, Husain A. Talukdar, Hassan Foroughi Asl, Rajeev K. Jain, Cecilia Cedergren, Ming-Mei Shang, **Aránzazu Rossignoli**, Rabbe Takolander, Olle Melander, Anders Hamsten, Tom Michoel, Josefin Skogsberg
PLoS Genetics. 2014 Feb; 10(2): e1004201

CONTENTS

LIST OF ABBREVIATIONS

AAA	Abdominal aortic aneurysm
AJ	Adherens junction
AngII	Angiotensin II
Apo	Apolipoprotein
CAD	Coronary artery disease
CM	Chylomicron
CVD	Cardiovascular disease
EC	Endothelial cell
ECM	Extracellular matrix
GJ	Gap junction
HDL	High-density lipoprotein
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
ICAM-1	Intercellular adhesion molecule 1
IL	Interleukin
INFγ	Interferon gamma
JAM	Junctional adhesion molecule
KLF	Kruppel-like factor
KO	*Knock out*
LDB2	Lim domain-binding 2
LDL(R)	Low-density lipoprotein (Receptor)
MI	Myocardial infarction
MMP	Matrix metalloproteinase
MTTP	Microsomal triglyceride transport protein
Mx1	Mx dynamin-like GTPase 1
NF-κB	Necrosis factor kappa B
PCL	Plasma cholesterol lowering
PCSK9	Protein convertase subtilisin/kenixint ype 9
PECAM-1	Platelet/endothelial cell adhesion molecule 1
pIpC	Polyinosinic-polycytidylic acid
PVRL2	Poliovirus receptor-related 2
ROS	Reactive oxygen species
SMC	Smooth muscle cell

SNP	Single nucleotide polymorphism
STAGE	STockholm Atherosclerosis Gene Expression cohort
TEML	Transendothelial migration of leukocytes
TG	Triglyceride
TGFβ	Transforming growth factor beta
Th1/2	T helper cell 1/2
TJ	Tight junction
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
VCAM-1	Vascular cell-adhesion molecule 1
VLDL	Very low-density lipoprotein

1 INTRODUCTION

The discovery of the central role of cholesterol in the development of atherosclerotic disease was first demonstrated by Nikolay N. Anitschkow in 1913.[1] His experiments paved the way and opened an immense field of research for cardiovascular scientists. Since then enormous progress has been made, and every year multiple papers are published highlighting and adding new findings that increase the complexity of this disease.

This introductory chapter will focus on the general characteristics of two important cardiovascular processes: atherosclerosis and aneurysm. It will also introduce the important concept of leukocyte migration during an inflammatory response. Moreover, the role of cholesterol and cholesterol-lowering therapies will be presented. Finally, the genes involved in atherosclerosis and discussed later in two of the published papers included in this thesis will be described.

1.1 CARDIOVASCULAR DISEASES

Cardiovascular diseases (CVDs) are the first cause of death worldwide, accounting for more than one third of all deaths every year. CVDs are defined as a group of diseases affecting the blood vessels and the heart, and include coronary artery disease (CAD), peripheral arterial disease, rheumatic heart disease, abdominal aortic aneurysm, thrombosis and cerebrovascular disease, among other disorders. They can lead to acute events such as myocardial infarction (MI) or stroke and eventually to death, mainly due to the development of atherosclerosis but also as a result of vessel bleeding or clot formation. Importantly, most CVDs can be prevented by addressing behavioral risk factors such as avoiding tobacco use, being physically active and having a healthy diet.[2-4]

1.2 ATHEROSCLEROSIS

Atherosclerosis is the chronic, progressive arterial disease underlying the development of CAD. It is associated with both environmental and non-environmental or genetic risk factors (Table 1). These factors have been identified in numerous epidemiological studies and include diabetes, obesity, elevated blood pressure, family history and an unhealthy lifestyle (lack of exercise, high-fat diet or smoking). The primary factor and pre-requisite for the development of the disease is elevated plasma levels of low-density lipoproteins (LDL) containing cholesterol, which will trigger an inflammatory response initiated by the migration of leukocytes from the circulation into the intima of the arterial wall. Atherosclerosis is clinically significant in half of the population, a number that increases when the risk factors accumulate, and it is responsible for 50% of all CAD deaths in Western societies.[4-7] The addition of factors occur rather easily; frequently an unhealthy lifestyle already includes several of the other risk factors associated with developing the disease. Furthermore, these are commonly associated with obesity, diabetes or hypertension and usually develop into some of the other non-environmental factors.

Table 1. Risk factors associated with atherosclerosis and CAD development.

Non-environmental factors	Environmental factors
Elevated levels of LDL and VLDL	Unhealthy diet (high-fat, salt)
Reduced levels of HDL	Smoking
Elevated levels of triglycerides	Physical inactivity
Elevated levels of lipoprotein (a)	Alcohol abuse
Elevated levels of C-reactive protein	Infectious agents
Hypertension	Air pollution particles
Metabolic syndrome	
Family history/Genetics	
Increased BMI/Obesity	
Diabetes mellitus type 2	
Male gender	
Advanced age	

The atherosclerotic process is initiated by interplay between endothelial dysfunction and retention of subendothelial lipoproteins. It is characterized by the subendothelial buildup of lipids and fibrous elements in the arterial intima layer which, over timer, lead to the formation of atherosclerotic plaques which push the intima into the lumen, narrowing the blood flow. The developed non-resolving inflammatory response triggers the arterial destruction of the intima, thrombosis, and ischemia (Figure 1).[4,8,9]
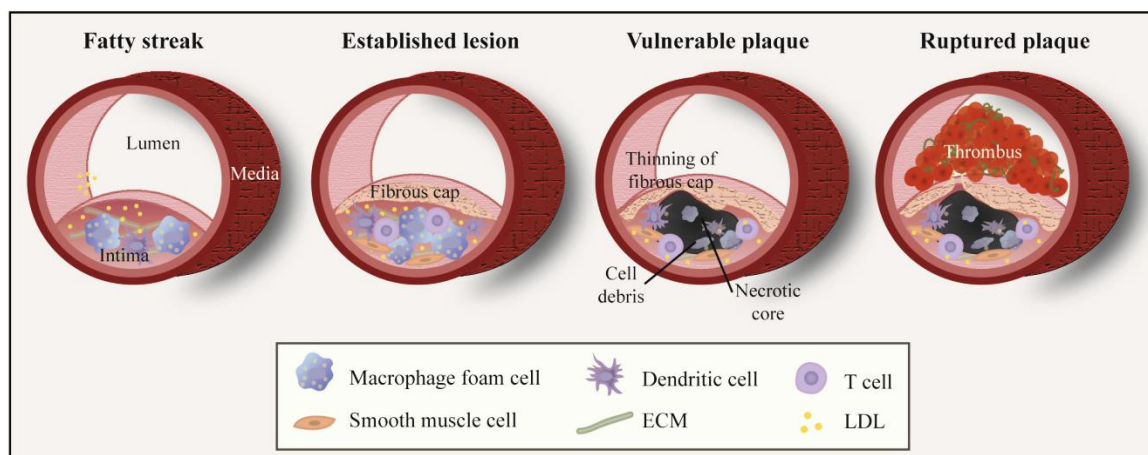

Figure 1. Progression of an atherosclerotic lesion. Evolution from an early fatty streak to rupture of the plaque, and simplified overview of the main cell types and components involved.

In brief, the disease develops when plasma levels of cholesterol rise and apolipoprotein-B (ApoB)-containing lipoproteins (mainly LDL-particles) are retained and infiltrate the arterial wall. These lipoproteins undergo various oxidative modifications that mimic pathogen/damage-associated molecular patterns that lead to the release of active phospholipids that will trigger an activation of the endothelium.[9,10] This activation can also

occur due to a disturbed blood flow and an oscillation of shear stress in the vascular wall, mainly in regions of curvature or arterial branching.[11] The activation results in the expression of adhesion molecules that will prompt a cascade of events, including recruitment of monocytes and their transmigration through the endothelium into the intima layer of the arterial wall, activation of vascular smooth muscle cells (SMCs), and accumulation of lipid, cells and extracellular material in the intimal subendothelial space. These cells include not only leukocytes such as monocyte-derived macrophages, T cells, dendritic cells, B cells and mast cells, but also SMCs that display myofibroblast characteristics.[9]

Once monocytes have migrated into the intima in response to a gradient of monocyte chemoattractant protein-1, they will differentiate into macrophages upon stimulation by macrophage-colony-stimulating factor. This results in the upregulation of pattern recognition receptors (such as scavenger receptors) that will mediate the uptake of oxidized LDL and will eventually lead to the formation of the so-called "foam cells". At the same time, in response to cytokines such as interferon gamma (INFγ), macrophages upregulate the expression of toll-like receptors (TLRs) which bind tumor necrosis factor alpha (TNFα) and interleukin 1 (IL-1), and result in the release of pro-inflammatory molecules (reactive oxygen species, ROS; pro-inflammatory cytokines; proteases and others) (Figure 2).[4,12] Foam cell apoptosis and lipid deposits, together with the exacerbated inflammation; give rise of what is known as an early atherosclerotic lesion or "fatty streak", the initial stage of atherosclerosis.
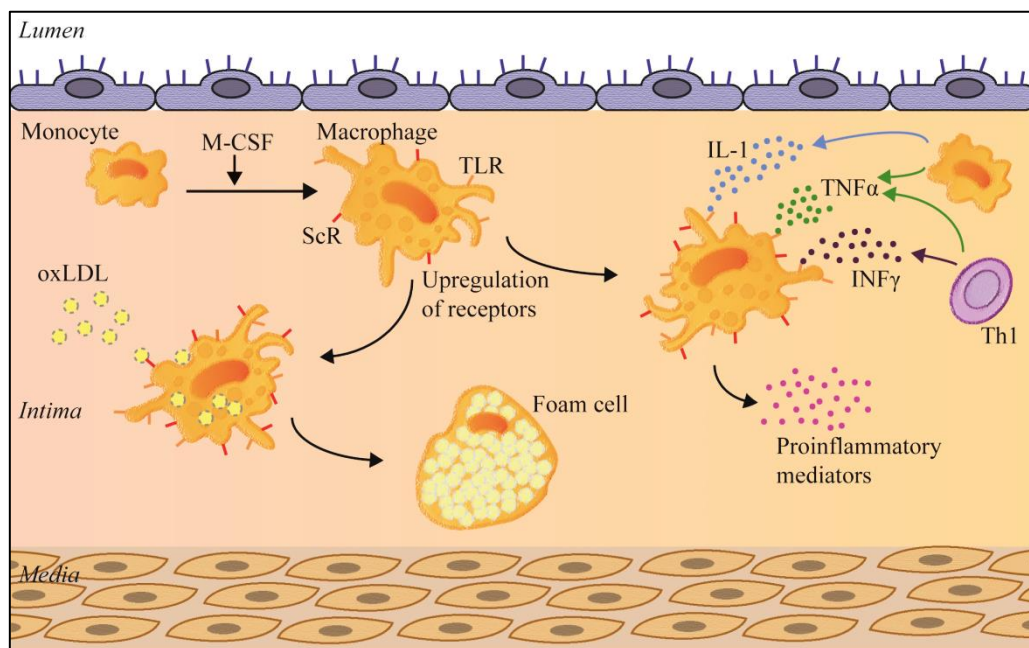

Figure 2. First steps in the progression of atherosclerosis. Differentiation of monocytes into macrophages, formation of foam cells and initiation of the inflammatory response. M-CSF: macrophage-colony-stimulating factor. ScR: scavenger receptor. oxLDL: oxidized LDL.

From this moment on, the atheroma will progress into a more complex lesion including the formation of a necrotic core and a partial resolution process depicted by the development of a covering scar.[9,13,14] The pro-inflammatory mediators released by macrophages also include growth factors that will stimulate the SMCs in the media, which will migrate and accumulate

in the lesion area and will increase the amount of extracellular matrix (ECM) and lead to the formation of the fibrous cap.[6,12] This cap offers a protective barrier between the pro-thrombotic material in the plaque and the platelets present in the circulation. Consequently, most atherosclerotic lesions do not result in acute vascular events.[15]

Nevertheless, these advanced atherosclerotic lesions usually lead to a narrowing of the vessel lumen, which can potentially result in an ischemic event. The risk of acute events escalates when there is a "vulnerable plaque" with a large necrotic core resulting from a combination of cell necrosis and defective clearance of apoptotic cells (efferocytosis).[8] The increase of apoptosis and an uncontrolled inflammatory response (activated immune innate and adaptive pathways) can destabilize the lesion (expression of proteases such as matrix metalloproteinases, MMPs; coagulation factors; free radicals and vasoactive molecules; defective collagen synthesis by SMCs), attack the collagen present in the fibrous cap, thinning and weakening it, and can eventually rupture it.[6,12,16] Plaque rupture exposes the bloodstream to the lipids and tissue factor present in the lesion, initiating a coagulation cascade that will lead to platelet adherence and thrombus formation.[6]

The lack of symptoms during the development of atherosclerosis results in substantial progression of advanced plaques that will be calcified, necrotic and close to rupture before any clinical events occur. The possibility of reversing the lesions at this point will then be of extreme difficulty.

1.2.1 Dysfunctional endothelium: athero-protective vs. athero-prone

The vascular endothelium can be regarded as dysfunctional when alterations in its phenotype occur, rendering an impaired barrier function as well as pro-inflammatory and/or pro-thrombotic characteristics. This represents a pathogenic risk factor for numerous vascular diseases, as in atherosclerosis. The situation tends to happen in unique areas within the vasculature ("lesion-prone" areas), typically around vascular curves or branch regions, often triggered by biomechanical forces (low shear stress, steep flow).[9,17] Exactly how the endothelium senses this biomechanical forces and discriminates flow patterns remain poorly described.[18] These areas are also distinguishable by their predisposition to retain LDL particles, a characteristic that exacerbates the dysfunctional phenotype of the endothelium.[9,17]

1.2.1.1 Athero-protective endothelium

When the endothelium has certain characteristics that make it resist or protect itself against an inflammatory event, it is known as "athero-protective" or "athero-resistant" endothelium. The endothelial cells (ECs) from these areas have an ellipsoidal morphology, with the nuclear and cytoplasmic components aligned in the direction of the primary blood flow and a thick glycocalyx layer.[9,19] Moreover, this phenotype involves differences in the gene expression of several transcription factors (such as Kruppel-like factors 2 and 4, KLF2 and KLF4, which are stimulated by the MEK5/Erk5 signaling pathway).[20,21] The function of these activated transcription factors is not clearly defined in the endothelium: such is the case for KLF2. On the other hand, this same factor in monocytes,[22] dendritic cells[23] and T cells[24,25] seems to

promote a general anti-inflammatory state, and in myeloid cells was shown to be athero-protective.[26] Instead, the other known upregulated transcription factor, KLF4, seems to have a much clearer athero-protective function in ECs.[27]

1.2.1.2 *Athero-prone endothelium*

Contrary to the athero-resistant endothelium, regions which seem to promote or facilitate the development of inflammation and atherogenesis are termed "athero-prone" or "athero-susceptible". ECs in these areas do not display the same pattern as the ones in the protective endothelium. In the athero-susceptible areas, the ECs present a cuboidal shape and show higher turnover rates and senescence.[9] They also seem to express markers of chronic ER stress, which cause endothelial apoptosis and therefore promote atherosclerosis.[28,29] This phenotype is also accompanied by gene expression alterations, in this case in the form of activation of the necrosis factor kappa B (NF-κB) pathway in the endothelium,[30,31] which leads to the upregulation of vascular cell-adhesion molecule 1 (VCAM-1) and TLR2,[32] as well as to an increase in pro-inflammatory chemokines, cytokines, miRNAs and ECM proteins.[33-37] Disturbed flow also modifies DNA methylation patterns. For example, this turns into the inhibition of the transcription of the previously mentioned athero-protective KLF4 factor in these vascular endothelial regions, rendering them more susceptible to the disease.[38]

1.2.2 Monocytes and macrophages in atherosclerosis

As mentioned before, endothelial activation in the early atherosclerotic lesions prompts a chemokine-induced influx of monocytes derived from the bone marrow that will initiate the inflammatory response in the intima.[39] Occasionally, the monocytes reach the lesions after proliferation and activation in the spleen, often triggered by stress-induced stimulation of the sympathetic nervous system.[40] From the different monocyte subtypes, the inflammatory Ly6^{hi} subset seems to enter the lesions faster, although both Ly6^{hi} and Ly6^{lo} groups accumulate during atherosclerosis development. While Ly6^{hi} tend to differentiate into pro-atherogenic macrophages, Ly6^{lo} monocytes have classically had a patrolling function in the circulation and their function within tissues remains unclear.[41]

Upon monocyte stimulation and differentiation into macrophages, they undergo multiple phenotypic changes.[42] These modifications contribute to segregate them into a wide spectrum of functional macrophages that will exert distinct roles (host defense and inflammation on one side of the spectrum vs. resolution and repair on the opposite flank). Inflammatory macrophages (classically activated "M1" subtype), in general, promote atherosclerosis development, while resolving macrophages (alternatively activated "M2" subtype) suppress atheroma progression and stimulate plaque regression.[8,43]

Inflammatory macrophages express and secrete cytokines, proteases such as MMPs (MMP2, MMP9), and other factors that stimulate plaque progression, rupture and thrombosis. They also promote the necrosis and thinning of the fibrous protective cap. On the other hand, resolving macrophages stabilize plaques by secreting collagen contributing to the fibrous cap, stimulating efferocytosis (clearing of dead cells) and therefore preventing necrosis, and

generating lipids (such as resolvins and lipoxins) and proteins (IL-10; transforming growth factor beta, TGFβ; and annexin A1) that promote tissue repair.[41,44]

Molecular profiling has shown extensive heterogeneity: macrophages belonging to both sides of the spectrum are found at different regions of the plaques during disease development. Therefore, consistently with the functions they exert, inflammatory macrophages tend to be concentrated in unstable plaques that are prone to rupture, while resolving macrophages gather within more stable and even regressing plaques.[41,45,46] The phenotypic modulation of lesional macrophages remains to be thoroughly studied.

Macrophages take up lipoproteins by a combination of phagocytosis, pinocytosis (native lipoproteins), and scavenger receptor-mediated internalization (modified lipoproteins).[9] This cholesterol accumulation turns them into "foam cells" due to how they look after engulfing all those particles. It has been shown that cholesterol buildup in macrophages activates TLRs and stimulates the inflammasome signaling pathways, which leads to an increased production of inflammatory chemokines and cytokines such as IL-1β.[47,48] Moreover, oxidative stress induced by modified LDL also stimulates inflammatory pathways such as the NF-κB cascade, which leads to the upregulation of monocyte chemoattractant protein-1 and a subsequent increase in monocyte recruitment to the plaques.[6,49]

1.2.3 Vascular SMCs in atherosclerosis

The layers of SMCs present in the media have the role of maintaining the muscle tone of the vessel and regulate their caliber, thus adjusting blood volume and blood pressure. During an atherosclerotic process, the SMCs are found in the intima layer of the vessel and they are phenotypically and functionally different from those found in the media under normal conditions. Moreover, the role of SMCs during the progressive stages of atherosclerosis seems to vary depending on the phenotypic state of these plastic cells.

The activation of medial SMCs occurs as a result of the accumulation of lipoproteins, endothelial activation and inflammation in the plaques. This phenotypic modulation entails a transformation from fully contractile, differentiated, mature and inactive SMCs that express both smooth muscle alpha actin (*Acta2*) and smooth muscle myosin heavy chain (*Myh11*) to a "synthetic state", a proliferating and migrating phenotype in which there has been a downregulation of the differentiation and the above mentioned genes. This synthetic phenotype is associated with an increased production of proteoglycans, ECM and other proteins related to vascular repair and fibrous cap stabilization. Conversely, the SMCs in this area may suddenly activate the expression of MMPs and inflammatory mediators and may undergo apoptosis. This occurs in response to not-well defined environmental signals, therefore changing function and promoting plaque rupture and thrombosis.[50]

Additionally, depending on the stimuli present, the SMCs seem to specialize and modify their phenotype even further. Thus, oxidized LDL leads to an intermediary phenotype with enhanced collagen expression, proliferation and migration;[51] TGFβ promotes a matrigenic phenotype associated with an increased production of ECM;[52] and inflammatory cytokines

(TNFα, IL-1) enhancing the expression of adhesion molecules and MMPs, and thus promoting an inflammatory phenotype.[53-55] Moreover, high levels of inorganic phosphate seem to boost an osteochondrogenic state which stimulates calcification.[56] Nevertheless, there is a lack of lineage-tracing studies in the field which are needed in order to show whether the SMCs truly give rise to different phenotypes *in vivo*.[50]

1.2.4 Other immune cells in atherosclerosis

1.2.4.1 Lymphocytes

Between 10% and 20% of the cells found in a plaque during atherosclerosis development are T cells.[57] They are present during all phases of the disease, whereas B cells are found in smaller numbers and more often appear in the adventitia layer. Both T cells and B cells modulate atherosclerosis and influence plaque stability.[58]

These cell subsets can be distinguished according to the expression pattern of surface and intracellular proteins, but also depending on their function (such as release of cytokines and interaction with other B cells or T cells and macrophages). In atherosclerotic plaques, the majority of T cells belong to the T helper 1 (Th1) CD4+ subset, followed by CD8+. Other subsets such as Th2, regulatory T cells, Th17 and natural killer T cells are also present.[58]

Those T cells are activated and polarized in the plaques to the Th1 subset in order to regulate the lesion growth by producing pro-inflammatory cytokines such as TNFα and INFγ, and are therefore considered pro-atherogenic.[59] Regarding Th2 function, findings are controversial, and although they are traditionally regarded as anti-atherogenic, some studies have found them to stimulate atherogenesis.[60,61] Nevertheless, Th2 cells have an important role in B cell activation, plasma cell differentiation and antibody production. Likewise, natural killer T cells and B cells seem to have a pro-atherogenic role, although an athero-protective function has also been described.[62-66] On the other hand, regulatory T cells appear to exert a more evident athero-protective function through the production of anti-inflammatory cytokines (such as TGFβ and IL-10).[67-69]

In advanced lesions B cells and T cells seem to also be activated in adventitial artery tertiary lymphoid organs, a subject that needs further investigation.[69]

1.2.4.2 Dendritic cells

Dendritic cells are antigen-presenting cells that initiate and sustain immune responses, display a selection of antigens to the T cells and have the capacity to inhibit their activation and maintain immune tolerance against self-antigens (due to their expression of co-stimulatory molecules and their cytokine production profile).[70] Most of the dendritic cells during atherosclerosis progression are located in the intima.[71] They are activated and likely participate in the early lesion development by internalizing oxidized LDL and becoming foam cells.[72,73] Additionally, they seem to promote inflammation in later stages of the disease by stimulating the production of INFγ by CD4+ T cells.[74]

1.2.4.3 Neutrophils

Neutrophils represent a significant source and an essential target for chemokines and lipid mediators.[75] These polymorphonuclear leukocytes appear in the atherosclerotic plaques as part of the blood components of an intraplaque hemorrhage or via diapedesis in the early stages of disease development.[76,77] Neutrophils are a major source of proteases that may contribute to plaque rupture and atherosclerosis progression. Moreover, the arrival of granulocytes is followed by the release of large amounts of ROS and other oxidative enzymes that exacerbate local oxidative stress and intensify plaque progression.[78,79] Furthermore, high levels of cholesterol induce neutrophilia, which is correlated to the degree of atherosclerotic lesions.[80,81]

1.2.4.4 Mast cells

Mast cells are found in their activated form in atherosclerotic plaques. They have been associated with lipid accumulation (since they facilitate the degradation of high-density lipoproteins, HDL; and impair the efflux of cholesterol) and thus with plaque progression.[82-84]

1.3 ABDOMINAL AORTIC ANEURYSM

An aneurysm is a cardiovascular event that occurs when there is a permanent and irreversible dilation of a vessel due to weakening of the wall that involves all three vascular layers (intima, media and adventitia). Abdominal aortic aneurysms (AAAs) are the most common type of aortic aneurysms. They develop in the infra-renal segment of the abdominal aorta and are defined as aneurysm when the aortic diameter exceeds the normal diameter by 50%. They are usually asymptomatic and, if left untreated, result in eventual aortic rupture and mortality in 85-90% of the cases (Figure 3). The rupture occurs when the mechanical forces on the wall (blood pressure, shear stress) exceed its strength.[85] A number of risk factors have been associated with the incidence of AAA including tobacco use, advanced age, male sex, hypertension, overweight, family history and hardening of the arteries. Oppositely, the presence of diabetes mellitus is known to be protective against the development of AAA. Aneurysms are also associated with atherosclerosis, transmural degenerative processes, neovascularization, degeneration of SMCs and chronic inflammation in the outer aortic wall; however the mechanisms that stimulate the initiation of AAA are still poorly understood.[85-89]
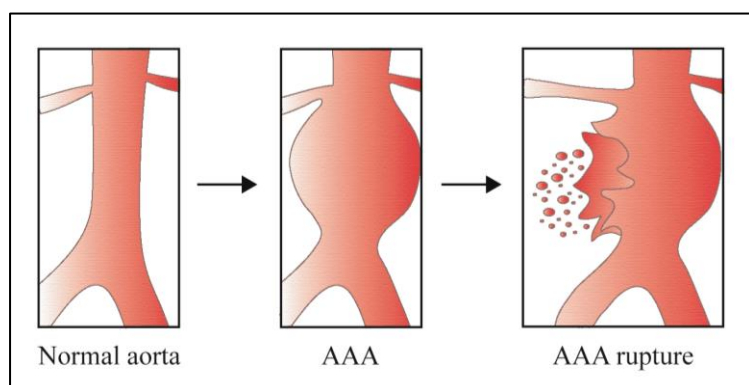

Figure 3. Schematic view of the progression of an abdominal aortic aneurysm. An AAA is developed when the normal diameter of the aorta increases 50% and can eventually lead to rupture of the wall.

The integrity of the vessel wall depends on the well-adjusted remodelling of the ECM and its components (elastin, collagen and SMCs). There are four distinct pathological events in the development of AAA: infiltration of leukocytes, destruction of collagen and elastin mediated by proteases (such as matrix metalloproteases, MMPs), loss of SMCs, and neovascularization. The initiation of an aneurysm involves the infiltration of leukocytes as a local inflammatory response. These leukocytes are associated with an increased production of ROS, inflammatory cytokines and chemokines which, in combination with leukotrienes and immunoglobulins, lead to loss of elastin and collagen, an upregulation of local adhesion molecules, SMC apoptosis and neovascularization.[85,87,88,90,91] Adventitial collagen is responsible for the resistance of the aorta in the absence of elastin in the media: when the collagen turnover is not enough to compensate for its loss (meaning there is an imbalance between synthesis and degradation), it may result in further dilation of the vascular wall and the risk of rupture increases exponentially (Figure 4).[86,90,91]
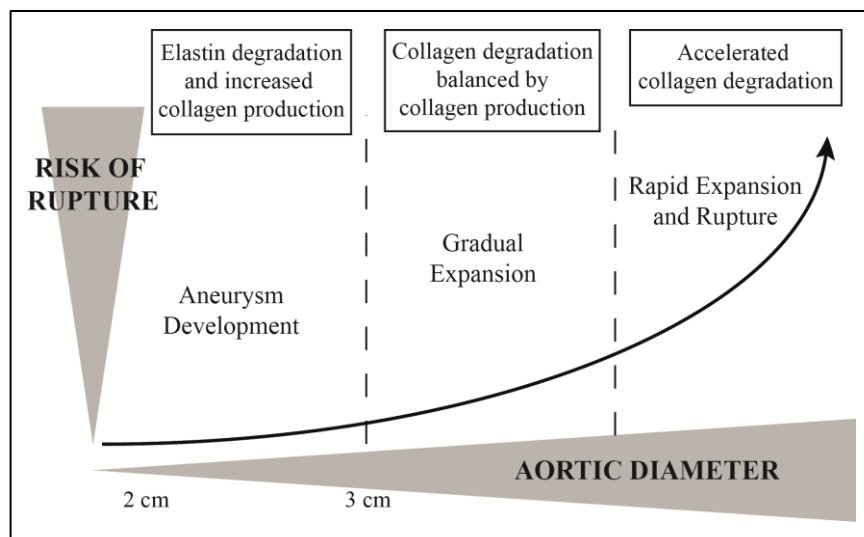

Figure 4. Biochemical and proteolytic events in AAA.

The development of AAA is also associated with mural thrombus formation. Aortic blood flow is maintained, in contrast with other occlusive cardiovascular diseases like atherosclerosis, which results in a continuous remodelling of the components of the thrombus.[86] This thrombus also acts as a source of proteases, since the neutrophils present therein store MMPs; and it also contains plasminogen which can result in local generation of the MMP activator plasmin.[92] The increasing thickness of the wall induces local hypoxia in the media, causing increased medial neovascularization and resulting in further inflammation.[93]

1.4 TRANSENDOTHELIAL MIGRATION OF LEUKOCYTES

The EC layer is the first obstacle leukocytes have to overcome during their recruitment to inflammation sites. The adhesion of leukocytes from the circulation and their consequent transmigration through the endothelium into the wall of blood vessels is a crucial event in the pathogenesis and inflammation processes of atherosclerosis and AAA. The extravasation cascade (commonly referred to as transendothelial migration of leukocytes, TEML), is a

complex multistep route that entails the activation of numerous adhesion molecules and signaling pathways.[94]

In brief, when the endothelium is activated due to an inflammatory stimulus, pro-inflammatory cytokines induce the expression of several adhesion molecules that will attract and recruit leukocytes.[95] These leukocytes, assisted by their endothelial ligands, will go through the different steps of the TEML cascade until their complete extravasation (Figure 5). The key steps of the cascade start with the rolling of leukocytes which brings them close enough for their chemokine receptors to be activated by E-selectin and P-selectin on the endothelial surface.[96] It continues with the leukocyte adhesion to the endothelial surface, a step facilitated by VCAM-1 and intercellular adhesion molecule 1 (ICAM-1).[97,98] Leukocytes can keep crawling over the endothelium until they reach the junction where they will migrate through. The last step includes the diapedesis of the leukocytes (the actual transmigration phase) and it is the only process that is irreversible. Diapedesis occurs when the leukocytes move across the ECs via a transcellular route (across the EC body), or most commonly in a paracellular way by passing in between two ECs. This later route implies breaching the array of endothelial connections, namely tight junctions (TJs), adherens junctions (AJs) and gap junctions (GJs) that control vascular permeability and maintain the integrity of the endothelium.[99,100] This phase is mediated by multiple proteins present in the different junctions including platelet/endothelial cell adhesion molecule 1 (PECAM-1), poliovirus receptor (PVR/CD155), junctional adhesion molecules (JAMs), CD99, nectins such as poliovirus receptor-related 2 (PVRL2), and others.[101] Some of these molecules such as CD99 and PECAM-1 are expressed both on the endothelium and on the leukocytes, suggesting that homophilic interactions occur during this migration step.
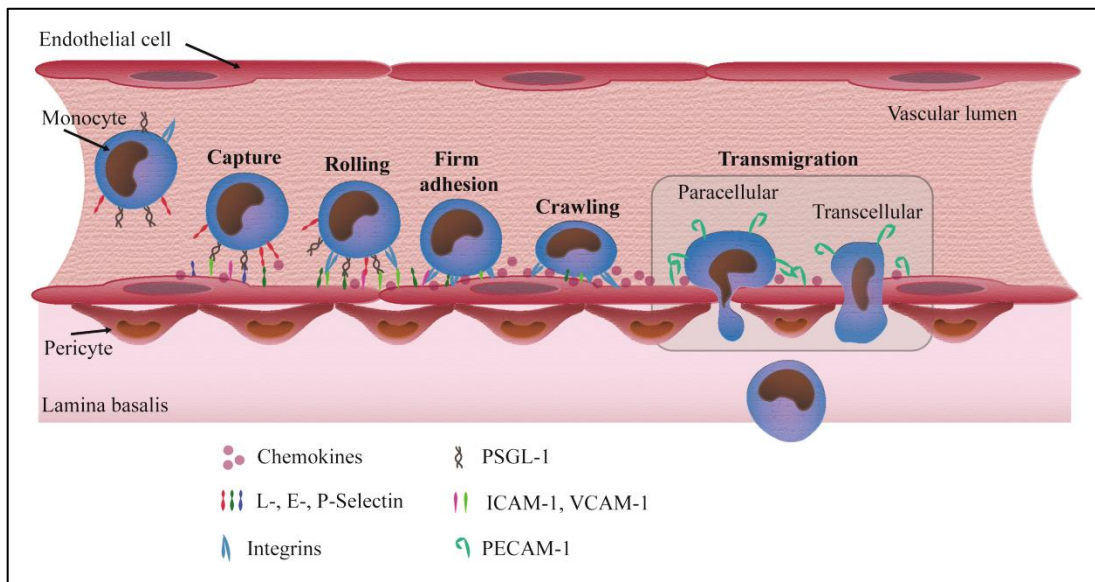

Figure 5. Schematic view of the TEML adhesion cascade and some of the main players involved.

The three dynamic endothelial junctions mentioned above consist of transmembrane and cytoplasmic proteins that are associated with the actin cytoskeleton (Figure 6). The TJs are localized in the most apical border (close to the lumen of the vessel), control the diffusion of

ions and prevent large macromolecules from penetrating the ECs. They include transmembrane proteins from the JAMs family (JAM-A, JAM-B, JAM-C and CAR), occludins, and claudins associated with integrins, zona ocludens 1 (ZO-1) or afadin as adaptors to the cytoskeleton. AJs provide mechanical strength between ECs and contain transmembrane nectins and VE-cadherin associated with cytosolic catenins that serve as anchor to the intracellular actin network. Lastly, GJs participate in endothelial cell-cell signaling and communication, although these junctions do not seem to contribute to TEML. PECAM-1 and CD99 are excluded from the TJs and GJs, and are sometimes included as non-classical proteins belonging to the AJ system. [99,102-104]
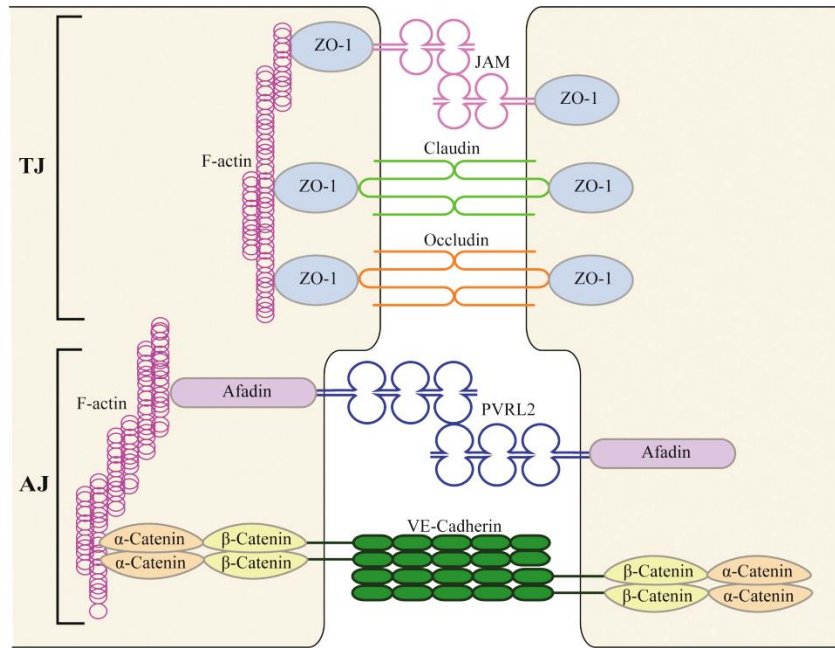

Figure 6. Schematic view of the classical endothelial proteins participating in TJs and AJs.

The order of events in which these proteins appear to function on the EC basolateral membrane seem to be quite distinct. For some of the main molecules involved, this process has been studied using sequential blocking experiments. In this fashion, PECAM-1 was shown to act first, followed by PVR/DNAM-1 (endothelium/leukocyte) and afterwards by CD99.[105] These are just three of all the players involved in the complex migration process, which involves complicated signaling and communication mechanisms that are acting during all the stages of the TEML process.

Moreover, a novel and still not completely understood structure involved in the diapedesis phase of TEML has been defined: the lateral border recycling compartment. This structure seems to form vesicles that cluster PECAM-1 and also contains JAM-A and CD99. It is localized close to the endothelial basolateral membrane and moves towards the sites of migrations during TEML.[106-108]

Once leukocytes have passed the EC layer they have to cross through the basement membrane and pericytes (in venules and microvasculature) to complete extravasation. This

takes place via gaps in between pericytes that concur with areas of the basement membrane containing less ECM.[109]

1.5 CHOLESTEROL AND LIPOPROTEIN METABOLISM

Cholesterol is a type of dietary lipid (exogenous uptake), but also synthesized *de novo* in liver and intestine. The body requires cholesterol for vital biochemical functions (formation of fluid cell membranes, steroid hormone production, metabolism of vitamin D), but abnormalities in its metabolism, or excessive plasma levels, can lead to health complications.[110,111]

The chemical formulation of cholesterol is 3-hydroxy-5,6-cholestene, and it is synthesized from acetyl-coA in the cytosol in three stages. In short, the first set of reactions includes the formation of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA), which is reduced to mevalonate by HMG-CoA reductase, and its further conversion into isopentenyl pyrophosphate. The second stage concludes with the synthesis of squalene (passing through geranyl pyrophosphate subsequently converted to farnesyl pyrophosphate). The last stage comprises the cyclization of squalene to squalene epoxide, lanosterol, and finally cholesterol.[112]

Cholesterol is released into the bloodstream inside the hydrophobic core of lipoproteins. These hydrophilic particles are secreted by the liver and small intestine in order to deliver cholesteryl esters, triglycerides (TGs) and phospholipids to the peripheral tissues and to recycle them back to the liver for clearance.[113] The lipoproteins also contain specific proteins (apolipoproteins) in their membrane.

There are five major lipoproteins, classified according to their size and density: chylomicrons (CMs), very low-density lipoprotein (VLDL), intermediate-density lipoprotein, low-density lipoprotein (LDL), and high-density lipoprotein (HDL). CMs contain ApoB-48 and are produced in the gastrointestinal tract using dietary lipids. They are transported through the lymphatic channels into the blood circulation, where they become remnant CMs after the removal of the TGs by the enzyme lipoprotein lipase.[114] While the enterocytes synthesize ApoB-48, hepatocytes in the liver synthesize ApoB-100 and assemble VLDL with the aid of microsomal triglyceride transfer protein (MTTP).[115] Once secreted, the ApoB-containing lipoproteins obtain further proteins, namely ApoC-II, ApoC-III, and ApoE. The enclosed TGs are hydrolyzed by lipoprotein lipase, releasing free fatty acids for cellular uptake by peripheral tissues, and leading to the formation of smaller and denser lipoprotein remnants.[113] HDL particles contain ApoA in their surface. VLDL and LDL transport hepatic lipids to the peripheral tissues, while HDL returns cholesterol to the liver where it can be excreted via bile acid metabolism.[111] In order to do so, HDL interacts with cholesteryl ester transfer protein, which facilitates the exchange of cholesterol esters and TGs with LDL particles. The cholesterol acquired by HDL is excreted through the bile, and the HDL particle is either returned to the bloodstream or hydrolyzed.[113,116,117] Therefore, LDL is traditionally considered as "bad cholesterol"

whereas HDL is considered "good cholesterol". Furthermore, a high HDL/LDL ratio is linked with a decreased risk of developing heart disease.

It is generally accepted that LDL-cholesterol particles are being deposited in the dysfunctional arterial wall and will initiate the atherosclerotic disease development. These particles are transporting the largest amount of cholesterol of all lipoproteins. The LDL particles bind to LDL-receptors (LDLRs) mainly in liver and intestines. The LDL, once bound to the LDLR, forms a complex that will be endocytosed, free cholesterol will be released and the receptor recycled to the cell surface. At the same time, proprotein convertase subtilisin/kexin type 9 (PCSK9) stimulates LDLR lysosomal degradation, therefore indirectly decreasing LDL clearance.[113]

1.5.1 Cholesterol-lowering effects and CVDs

Hyperlipidemia, and more specifically, high levels of LDL-cholesterol are, as stated earlier, the most important risk factor for CAD and other CVDs. The pharmacological way to control elevated lipid levels in patients with this disease is therefore lipid-lowering therapy.

The main group of lipid-lowering drugs are statins (HMG-CoA inhibitors), even though other options are still being used. In addition, newly developed drugs such as PCSK9 inhibitors that reduce the LDLR degradation have been recently approved for the treatment of homozygous familial hypercholesterolemia.[118-121] However, nowadays statins are the keystone of medical treatment for dyslipidemia and do not seem to have competition in the market. Statins block hepatic cholesterol synthesis by inhibiting HMG-CoA reductase, which results in less cholesterol being released into the circulation and a decrease in the inflammatory response (Figure 7). They are structural analogues to HMG-CoA reductase and are competitive inhibitors with 1000-10000 times greater affinity for its substrate. It is commonly accepted that the decrease of LDL-cholesterol with statin treatment reduces the risk of major coronary events, ischemic stroke and revascularization, and stabilizes the fibrous cap.[118] However, residual cardiovascular risks, even with high doses of statins, remain high and additional LDL-cholesterol lowering therapies are needed.
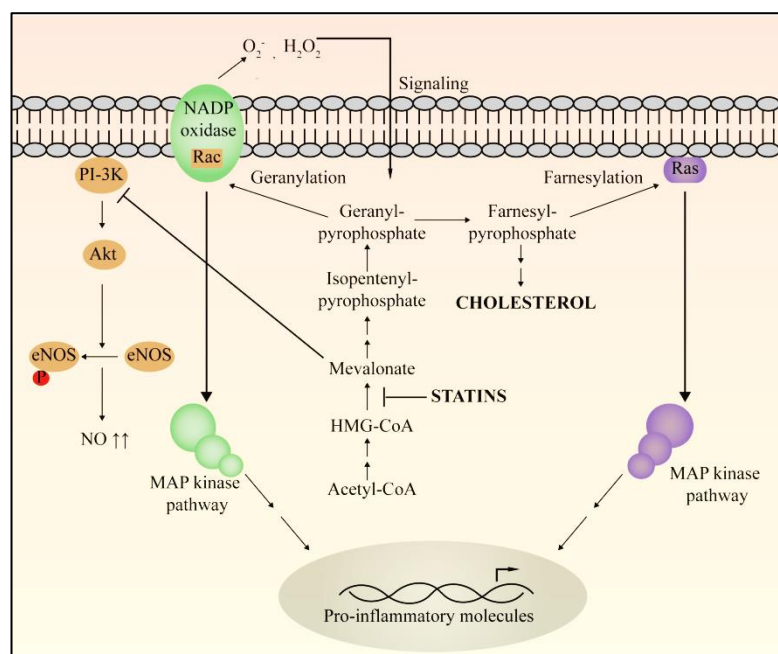
Figure 7. Schematic diagram showing the various functions of statins. PI-3K: phosphoinositide-3-kinase; Akt: protein kinase B; eNOS: endothelial nitric oxide synthase; NADP oxidase: nicotinamide adenine dinucleotide phosphate oxidase; MAP kinase: mitogen-activated protein kinase.

1.5.1.1 *Atherosclerosis and cholesterol-lowering therapy*

The accumulation of modified LDL-cholesterol in the arterial wall leads to the formation of foam cells contributing to the pathogenesis and development of atherosclerotic plaques. A reduction in plasma LDL-cholesterol levels has been shown to slow its progression. Statins have proven to decrease the cardiovascular events in patients with atherosclerosis. Several studies have shown that high-dose statin treatment not only decreases progression, but also induces regression of plaques.[122] Statins seem to modify the biology of atherosclerosis through multiple pleiotropic effects: by indirectly lowering plasma cholesterol through upregulation of the LDLR; by decreasing vascular inflammation via reduction of C-reactive protein levels in serum; by improving the endothelial function and increasing endothelial nitric oxide synthase when interfering with the PI-3 kinase/Akt pathway (Figure 7); by increasing EC repair; by reducing the expression of pro-inflammatory molecules and interfering with the MAP kinase and Ras pathways; by inhibiting platelet aggregation; and by stabilizing atherosclerotic plaques through an increase in collagen and SMCs and reducing the inflammatory cell recruitment and matrix-degrading enzymes.[123,124] Statins also act on macrophages, reducing their accumulation inside the plaque and downregulating CD36 expression, which decreases the uptake of oxidized LDL.[125,126]

Even though statins have shown to significantly decrease the mortality and morbidity of CAD patients, the disease remains as the number one cause of death worldwide. Almost 1 in 10 high-risk patients treated with an intensive statin therapy, who respond to the treatment with low levels of LDL-cholesterol, have still suffered from subsequent events in a two-year period.[127] In addition, patients on standard statin therapy due to diagnosed atherosclerosis still develop a major cardiovascular event in more than 20% of the cases

within five years of treatment.[128] This may be caused by the fact that there is no reasonable and inexpensive non-invasive imaging method that would allow the detection and visualization of early atherosclerotic plaque progression, inflammation and remodelling; meaning that most patients will not be prescribed statins until they have a cardiovascular event later in life. Since atherosclerosis starts to develop in the first decade (Figure 8), those patients will already have a substantial burden of pre-existing advanced plaques at the onset of cholesterol-lowering therapy. Nowadays, hybrid morphology-functional techniques such as PET/CT and PET/MRI are promising methods that allow the detection of high-risk plaques in a non-invasive way to help select patients at increased risk.[129] Additionally, biomarkers of early atherosclerosis development are needed to improve the identification of patients who would benefit from early statin therapy.
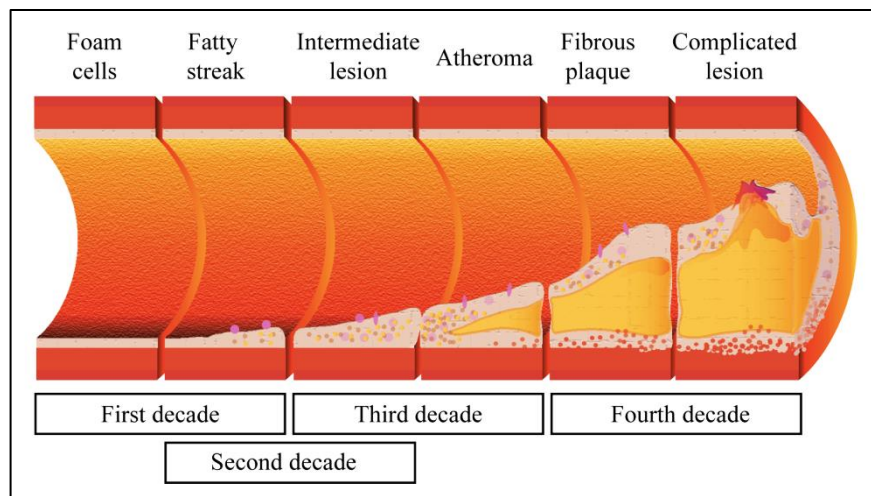

Figure 8. Timeline for the development of atherosclerosis.

1.5.1.2 *Abdominal aortic aneurysm and cholesterol-lowering therapy*

Although atherosclerosis and AAA are both chronic inflammatory diseases of the vascular wall and patients with AAA frequently have atherosclerosis,[130] the diseases have different pathogenic mechanisms and therefore the medical approaches differ. Even though the current guidelines recommend statin therapy to patients diagnosed with AAA due to their increased risk of cardiovascular disease caused by atherosclerosis, the effects of statins have not been fully defined yet, and multiple studies show controversial results on the matter. Besides the cholesterol-lowering effect, statins also reduce the expression of inflammatory molecules as stated before (Figure 7), and this includes downregulation of MMPs.[131] Treatment with atorvastatin in an elastase-induced AAA model suppressed AAA development through inhibition of macrophage migration in rats.[132] Combination treatments with atorvastatin and amlodipine, a calcium channel blocker, supressed AAA formation in an angiotensin II (AngII)-induced mouse model via inhibition of the Rho-kinase pathway.[133] A lower mortality after AAA rupture and reduced risk of rupture in AAA was associated with statin treatments in a nationwide population-based case-control study.[134,135] A meta-analysis of clinical trials from statin-treated AAA patients showed a decreased mortality of 43% and a delayed progression of AAA over a five-year period. On the other hand, the same authors failed to

show significant reduction in AAA growth rates in patients on statin treatment.[136] Another meta-analysis showed a beneficial effect of statins on preventing the growth of small AAAs, which was enhanced as the baseline diameter increased.[137] Others have shown the lack of beneficial effect for both atorvastatin and rosuvastatin in the AngII-infused mouse model of AAA.[138] The evidence that statins should attenuate AAA growth is still unclear, and randomised controlled-trials to assess the true impact of statins therapy on the disease are unlikely to happen given the high cardiovascular morbidity among patients.[139] Therefore, animal models of AAA are so far the only way to further investigate the effects of plasma cholesterol-lowering therapies for the disease development.

1.6   GENES OF INTEREST

Atherosclerosis is a complex condition that involves a large number of differentially expressed genes which will affect the disease progression and development. Genetic alterations could have a major impact in the plaque region as well as in other related processes. From the initial steps of the dietary absorption of cholesterol, to its packing into lipoproteins in the liver, the modification of the LDL particles within the intima, the immune response cascade or the activation of the SMCs from the media, among others, every single step is of vital importance.

In this thesis, two genes previously found to be affected while studying different aspects of atherosclerosis and response to cholesterol were taken into examination to elucidate their function and real effect within the disease: Lim domain-binding 2 (*LDB2*) and Poliovirus receptor-related 2 (*PVRL2*). In the next section these two genes will be introduced and generally described. They were both found to influence atherosclerosis development by altering the migration of leukocytes across the endothelium, although in opposite manners. Thus, papers I and II focus on the study of these two genes and their involvement in atherosclerosis and TEML using mouse models.

1.6.1   Lim domain-binding 2

LDB2, also known as *CLIM1*, is a gene that belongs to the LIM domain-binding family. It is located on human chromosome 4p15.32. All members of this family have two characteristic domains: a carboxy-terminal LIM interaction domain and an amino-terminal homodimerization domain, and have a conserved sequence for nuclear localization. The LIM acronym comes from three homeodomain proteins: lin-11, isl-1 and mec-3. Most LIM proteins have more than one LIM domain, and are widely spread in nature.[140] These proteins bind to transcription factors and function as adapter molecules to allow assembly of transcriptional regulatory complexes or to block their formation,[141] and are essential regulators of embryonic development.[142-146] The family of LIM domain-binding proteins contains two members: Ldb2/Clim1[144] and Ldb1/Clim2,[144,147] both isolated in mice although homologs have been isolated in other species (fly, frog, chicken). The human genes have also been isolated and cloned and show a high degree of identity with the other species homologs.[148,149]

LDB2 is expressed across multiple organs and tissues in the human body.[150] Genome wide association studies have found it to be involved in cytoskeletal organization and cell migration during a retinal disease development linked to vision loss.[151] Besides, it has recently been indirectly associated with transcriptional regulation of the endothelium via vascular endothelial growth factor (VEGF)-modulation of angiogenesis and vascular remodeling.[152] Gene ontology annotations for biological functions associated with the *LDB2* gene include, besides the already mentioned regulation of transcription, regulation of cell migration, kinase activity, cellular component biogenesis, hair follicle development, and epithelial structure maintenance. It is also annotated as being part of the nucleus, transcription factor complexes and plasma membrane.

In a previous publication from our group, *LDB2* was found to be the most connected gene in a transcription factor regulatory network inferred from TEML and atherosclerosis genes in CAD macrophages (blood monocytes from CAD patients differentiated to macrophages *in vitro*). In this study, it was found to be expressed in the endothelium, macrophages and SMCs in atherosclerotic mouse samples.[153] In order to elucidate the role of *Ldb2 in vivo* in the disease development, we used a mouse model of atherosclerosis were we have analysed *knocked out* (KO) mice. The results and conclusions from these experiments will be discussed in Paper I.

1.6.2   Poliovirus receptor-related 2

PVRL2, also known as *NECTIN2*, *CD112*, *PRR2* or *HVEB,* is a member of the nectin immunoglobulin family and encodes for a single-pass type I membrane glycoprotein. It is located on human chromosome 19q13.2 close to the cluster of apolipoprotein genes *APOE*, *APOC1*, *APOC2* and *APOC4*, a locus which has previously been associated with CAD and carotid intima-media thickness.[154,155] It is expressed ubiquitously in neuronal, epithelial, endothelial, and fibroblastic cells, as well as in many tumors.[156] Its most recognized roles include being an entry receptor for viruses,[157] to coordinate the Sertoli junctions in the testis,[158] being involved in Alzheimer's disease[159,160] and in breast and ovarian cancers,[161] in addition to participate during neuron synapse formation.[162] It plays a central role in the endothelium as a plasma component of the AJs, where it serves as a cell adhesion molecule.[163] At the endothelium, Pvrl2 binds to afadin, an actin-filament binding scaffold protein, through its intracellular region in order to recruit, regulate, and interact with other proteins in the AJs (VE-cadherin) or in TJs (JAM-A).[164]

Up to now, the function of Pvrl2 in CVD has not been extensively studied, although some recent publications suggest that it might participate in TEML during disease development by promoting leukocyte diapedesis.[165,166] Lately it has also been shown how its expression was induced by oxidized LDL stimulus in the murine aortic endothelium.[167] Moreover, we previously found *Pvrl2* to be downregulated when lowering cholesterol in atherosclerosis-prone mice.[168,169] This, together with previous knowledge of PVRL2 being part of the AJs in the endothelium and appearing to affect TEML, were the main reasons for our interest in its further study within the atherosclerosis environment. In order to do so, we generated a *Pvrl2*

KO atherosclerotic mouse model. The results of the experiments that followed will be discussed in Paper II.

2 AIMS OF THE THESIS

The main goal of my thesis is to study the cholesterol-lowering effect and assess new potential therapy targets for atherosclerosis and CVD. In order to do so, different particular mouse models (explained in detail in the following section) have been used.

The sub-aims to achieve these objectives include:

- To investigate the role of *Ldb2* on atherosclerosis and TEML using the atherosclerosis-prone $Ldlr^{-/-}Apob^{100/100}$ mouse model (Paper I).

- To investigate the role of the cholesterol-responsive gene *PVRL2* on atherosclerosis development and its potential role as a novel target for atherosclerosis intervention (Paper II).

- To investigate the effect of plasma cholesterol lowering (PCL) and atherosclerosis development on AAA formation using the unique mouse model $Ldlr^{-/-}Apob^{100/100}Mttp^{flox/flox}$Mx1-*Cre* combined with AngII-induction of AAA (Paper III).

3 MOUSE MODELS OF ATHEROSCLEROSIS AND AAA

The use of experimental animal models in research is of great importance in order to study the mechanisms underlying human diseases and identify potential therapeutic treatments.[170] Several animals, including rats, mice, rabbits, hamsters, dogs and pigs are being used in cardiovascular research. However murine models are still the first choice due to the small size of mice, high fertility, cost-effective maintenance, short gestational time, possibility of inducing genetic modifications and easy access.[170,171] Moreover, mice are fairly homogeneous in genetic background, food intake, circadian rhythms and living conditions, which minimize confounding factors. Furthermore, a great advantage is also the possibility to follow disease progression and stages over a relatively short period of time.

Two unique mouse models were used to test the hypotheses in the experimental section of the publications included in this thesis and they will be described below.

3.1.1 Atherosclerosis mouse models

Atherosclerosis is a challenging condition to study in humans due to the length of the disease progression. However, in small animals like mice atherogenesis can be easily induced by dietary or genetic manipulations that allow studying the whole disease progression in a relatively short period of time.[171] The most common murine models used in this field are those with a C57BL/6 background (the inbred strain most prone to develop atherosclerosis) in which genetic modifications have been made in the *Apoe*, the *Ldlr* or the *Apob* genes to rapidly induce atherosclerosis.[170,172,173] The differences between these three models are discussed in Table 2. Briefly, the *Apoe* KO, or *Apoe*-deficient, mice have elevated levels of plasma cholesterol on a chow-diet at already 10 weeks of age due to the accumulation of VLDL-cholesterol particles.[174,175] When fed a high-fat diet, their plasma cholesterol levels rise up to 2000 mg/dl, which result in the appearance of large atherosclerotic lesions at an early age. These lesions develop throughout the aortic tree with fibrous caps, necrotic cores and calcification, but without rupture of the plaques or thrombus formation.[176,177] The *Ldlr*-deficient mice fail to develop lesions when fed a chow diet and require a high-fat diet to increase their plasma cholesterol levels to around 400 mg/dl. This elevation is due to a rise of LDL particles, which makes the model more human-like in the context of hyperlipidemia, and the mice develop large atherosclerotic lesions in the aortic root area.[178,179]

Regarding the total expression of apolipoprotein B, mice express 70% of Apob-48 and 30% of Apob-100 in the liver, in contrast to human that express only APOB-100. Apob-100 is the main protein in the LDL particles and is needed for them to bind to the LDLR, while Apob-48 is primarily required for the assembly of CMs in the intestine.[170,173,180] Modifications in the APOB region have traditionally been introduced in mice in two different ways: as a *knock in* from the mutant human *APOB* gene, or as a targeted mutagenesis of the mouse *Apob* gene, to generate mice that exclusively synthesize APOB-100 or Apob-100, respectively. These mice have been crossed with *Ldlr*-deficient animals to generate the transgenic *Ldlr*^{-/-}

Apob[100/100] mice that exhibit plasma levels of cholesterol of 300-400 mg/dl on a regular chow diet, and develop atherosclerosis along the whole aortic tree.[168,173]

Table 2. Characteristics of the three principal mouse models of atherosclerosis.

Genetic modification	Cholesterol levels	Characteristics
Apoe^{-/-}	CD: 400-800 mg/dl HCD: 2000 mg/dl	Extensive lesions. Cholesterol in VLDL particles.
Ldlr^{-/-}	HCD: 400 mg/dl	Requires HCD. Large lesions. Cholesterol in LDL particles.
Ldlr^{-/-}*Apob*[100/100]	CD: 300-400 mg/dl	Pronounced atherosclerosis. Cholesterol in LDL particles.

CD: chow diet. HCD: high cholesterol diet.

One of the drawbacks of using mouse models to study atherosclerosis development is that none of these models have so far demonstrated plaque rupture as it occurs in humans. Another disadvantage is that the plasma lipid profile of mice is different compared to humans. However, the plasma lipid profile of the *Ldlr*^{-/-}*Apob*[100/100] model mimics the profile of patients with familial hypercholesterolemia, and these mice develop advanced atherosclerotic lesions on a normal diet.

This model has also been further modified to include a "genetic switch" which, when activated, turns off hepatic lipoprotein secretion: the *Ldlr*^{-/-}*Apob*[100/100]*Mttp*[flox/flox]Mx1-*Cre* mouse model. This model allowed us to abruptly lower plasma cholesterol levels by Cre-induced recombination of the floxed *Mttp* gene. The induction of *Cre* expression is regulated by the Mx dynamin-like GTPase 1 (Mx1) promoter, which is activated by polyinosinic-polycytidylic acid (pIpC) injections in an interferon-dependent manner.[181]

The two atherosclerosis models that have been used for the publications included in this thesis are the *Ldlr*^{-/-}*Apob*[100/100] and the *Ldlr*^{-/-}*Apob*[100/100]*Mttp*[flox/flox]Mx1-*Cre*. The first model (*Ldlr*^{-/-}*Apob*[100/100]) has been further developed to include modifications in the genes of interest (*Lbd2* and *Pvrl2*) for the first two publications (Paper I and II), therefore obtaining an even more complex genetic picture.

3.1.2 Abdominal aortic aneurysm mouse models

In order to identify potential new treatments and the mechanisms involved in the development of AAA in more detail, rodents have been widely used.[182] Chemical or genetic induced AAA in rodents is similar to the human disease, including inflammation, thrombus formation, medial degeneration and rupture.[183] The most commonly used murine models of AAA are the angiotensin II infusion in hyperlipidemic mice (*Apoe*^{-/-} or *Ldlr*^{-/-}) and the calcium chloride (CaCl$_2$) chemical treatment. Other models used include elastase-perfusion, genetically modified *Mmp*^{-/-} mice or surgical induction of AAA using vein patches or xenografts.[182,183]

In the AngII model, angiotensin II infused into *Apoe*^{-/-} mice at 500-1000 ng/kg/min via subcutaneously implanted mini-osmotic pumps has been shown to promote aortic aneurysm formation within a month period,[184] although not all mice develop AAA.[185] In this model the aneurysm forms in the suprarenal aorta with a number of rupture and dissection cases during the first week of AngII exposure.[186] The AAAs developed in these mice are associated with some of the main pathological hallmarks of the disease in humans, including the presence of macrophages in the media within days of the infusion, dilation of the lumen, degradation of the ECM and thrombus formation within a week; plus subsequent remodelling of the vessel and neovascularization.[183] Also, as in the human setting, the males are more prone to develop AAA when using this model.[187] Importantly, it has been reported that potential increases in blood pressure using the AngII model do not influence AAA development.[188]

In the $CaCl_2$ model, the animals receive a direct periaortic application of $CaCl_2$ between the iliac bifurcation and the renal branches which increases the aortic diameter 50-110% within 2-4 weeks. The formed AAA in this model includes calcium deposition throughout the media, disruption of SMCs and elastin, and influx of inflammatory cells. However, in contrast with the AngII-infused model, no thrombi are formed.[182,183,189]

Consequently, in this thesis the AngII-infused model was chosen for the animal experiments related to AAA formation. In order to test the hypothesis for paper III, this AngII-induction method was performed for the first time (to our knowledge) in the previously mentioned *Ldlr*^{-/-}*Apob*[100/100]*Mttp*[flox/flox]Mx1-*Cre* mouse model, and was further combined with the cholesterol-lowering treatment.

4 PAPERS AND DISCUSSION

This thesis includes the three publications mentioned at the beginning, named Paper I, II and III. The main findings and conclusions drawn from all of them will be briefly presented below. The actual papers containing all detailed methods and results can be found attached at the end of this thesis.

4.1 PAPER I - LIM DOMAIN BINDING 2: A KEY DRIVER OF TRANSENDOTHELIAL MIGRATION OF LEUKOCYTES AND ATHEROSCLEROSIS

As mentioned before, *LDB2* had been found to be the most connected gene in a regulatory network of transcription factors inferred from TEML and atherosclerosis genes in CAD macrophages in a previous publication from our group.[153] This means that LDB2 was found to be a master regulator of a TEML gene network in relation to CAD, but it does not indicate that it was necessarily the main driver of the disease *in vivo*. *LDB2* had not been studied in relation to CAD before, so nothing more was known in this regard. All this information pointed us towards the direction of validating its potential effect in TEML during an atherosclerotic process, which was examined in detail in this publication. In this regard, we performed both animal experiments *in vivo* using our atherosclerosis-prone mice ($Ldlr^{-/-}Apob^{100/100}$) and cell experiments *in vitro*. Moreover, a genetic validation in well-characterized human CAD cohorts was included.

Regarding the migration experiments carried out with primary leukocytes from mice (either from spleen or blood), lack of *Ldb2* led to an increased migration, a result opposite to that observed in the monocytic THP-1 cell line. Overexpression of *LDB2* in THP-1 cells resulted in an increase in leukocyte migration, whereas nearly complete depletion by siRNA-targeting caused a decrease migration of leukocytes. These conflicting results can be explained as a result of the key driver role of the gene. When a main regulator of a gene network is completely depleted (KO mice do not have the gene from the beginning of their life), downstream genes often lose control and result in increased activity, as it happened with the primary leukocytes in our study. When this situation occurs in a more physiological condition, as in the THP-1 cells, the effects can be reversed and stimulation leads to increased network activity, whereas depletion leads to a loss of network activity. This intricate modulation denotes the complex nature of biological processes.

In addition, in order to study the effect of *Ldb2* deficiency in atherosclerosis development and to try to pin-point its specific function within the disease we used our athero-prone mice crossed with *Ldb2* heterozygotes. Loss of this gene in $Ldlr^{-/-}Apob^{100/100}$ mice increased atherosclerotic lesion size and decreased plaque stability, suggesting an athero-protective role for *Ldb2* in mice. This was found to be due to increased TEML activity, corresponding with our previous hypothesis as a genetic modulator. To confirm that, we performed multiple migration assays: air pouch and retinal vasculature models *in vivo*, leukocyte migration *in vitro*, and *ex vivo in situ* perfusion of primary leukocytes into our mouse model. As stated above, *in vitro* experiments showed increased migration of leukocytes in

the *Ldb2*-depleted animals. The air-pouch model showed similar results, confirming an overall increase of leukocytes for the KO mice, as well as specifically an increase in monocytes and macrophages. This was further established with the retina model. Moreover, the *in situ* experiments allowed us to define that the lack of *Ldb2* affects the leukocytes and not the arterial wall. Therefore, *Ldb2*-deficiency leads to increased TEML due to its effect on leukocytes. Nevertheless, an increase in *Vcam-1* expression in leukocyte and the aortic wall was found in the KO mice, suggesting an indirect effect that further increases TEML by increasing monocyte adhesion to the vessel wall.

Moreover, a functional SNP of *LDB2* (rs10939673) was associated with the extent and risk of MI in multiple CAD cohorts, which strengthens the fact that it is a player with an important role on atherosclerosis and a potential target that should be investigated further.

4.2 PAPER II - POLIOVIRUS RECEPTOR-RELATED 2: A CHOLESTEROL-RESPONSIVE GENE AFFECTING ATHEROSCLEROSIS DEVELOPMENT BY MODULATING LEUKOCYTE MIGRATION

In this study, the atherogenic role of *PVRL2*, a newly identified cholesterol-responsive gene, was investigated. As mentioned previously, *PVRL2* had not been studied in the context of CVD before, although recent studies had suggested it might play a role in TEML during atherosclerotic disease development.[190]

Pvrl2 was earlier identified in our group as part of a set of genes involved in the prevention of atherosclerosis lesion development in response to PCL using the *Ldlr*^{-/-}*Apob*[100/100] *Mttp*[flox/flox]Mx1-*Cre* mouse model.[168,169] In the mentioned studies, *Pvrl2* was one of the genes that responded to PCL in mice just before atherosclerosis regressed both in early (30 weeks of age) and mature (40 weeks) atherosclerotic lesions,[169] and thus was one of the genes potentially driving atherosclerosis regression.

In the present publication, gene expression levels of *Pvrl2* in the aortic arch were examined during the disease development. *Pvrl2* expression was significantly upregulated in mice at 30 weeks of age, the same time-point as when the atherosclerotic lesions start to expand rapidly.[168,169] Subsequently, we bred *Pvrl2*^{+/-} mice into our atherosclerosis-prone *Ldl*^{-/-} *Apob*[100/100] mice and we found out that the *Pvrl2*-deficient mice had less atherosclerotic lesions and more stable plaques. When examining the plaque composition, macrophages were also significantly decreased, whereas no differences were found in SMCs, T cells, neutrophils, necrotic core area or proliferating cells.

As indicated above, PVRL2 may play a role in TEML which could be the cause of the decrease in atherosclerosis lesion size in the *Pvrl2*^{-/-} mice. We investigated the role of TEML by different migration assays, using both our mouse model as well as human umbilical vein endothelial cells (HUVEC). As expected, we found out that leukocytes migration was decreased in the *Pvrl2*-deficient mice, especially in the monocyte and macrophage fractions. Moreover, monocytes seemed to continue to adhere to the endothelium as usual even when

Pvrl2 was lacking, which confirmed the hypothesis of the importance of endothelial Pvrl2 during the transmigration or diapedesis process.

In order to validate the atherogenic role of PVRL2 in humans, the Stockholm Atherosclerosis Gene Expression (STAGE) study was used. In this cohort, *PVRL2* was highly expressed in the atherosclerotic arterial wall and its expression in this tissue correlated with plasma cholesterol levels (total cholesterol, LDL, and VLDL). In contrast, the expression of *PVRL2* in blood was low and no correlations with any CAD phenotypes were found in this tissue. Thus, indicating that the expression of *PVRL2* in leukocytes *per se* is not important for the atherogenic role of the gene, but rather its endothelial expression.

Taken together, the results from this publication point towards a pro-atherogenic role of *PVRL2* through an increase in TEML and thus an increased inflammatory response. In order to corroborate this, further experiments would be needed. Nevertheless, these findings highlight the importance of TEML as a crucial event during atherosclerosis. TEML is such a critical process that overall targeting means it would completely modify the outcome of any inflammatory disease. Clearly, inflammation is a much needed process to fight pathogens and disease, so completely haltering it is not feasible. Hence, an increased emphasis in TEML within disease development is required in order to potentially improve the outcome of inflammatory diseases.

4.3 PAPER III - PLASMA CHOLESTEROL LOWERING IN AN ANGII-INFUSED ATHEROSCLEROTIC MOUSE MODEL WITH MODERATE HYPERCHOLESTEROLEMIA

AAA is the tenth most common cause of death in industrialized countries, and prevention strategies aiming at reducing the disease progression and risk of aneurysm rupture are needed.[86] The aim of this project was to investigate the relation between the effect of PCL on AngII-induced AAA and atherosclerosis. In order to do so, we used our atherosclerosis-prone mice with the genetic switch that enables us to lower plasma cholesterol levels by 80% or more at any desired time-point, as described earlier ($Ldlr^{-/-}Apob^{100/100}Mttp^{flox/flox}$Mx1-*Cre*). To our knowledge, this model had never been used before to study AAA development. In order to induce AAA formation we used the AngII infusion method explained before in 20 week-old mice. PCL was performed one week before the AngII infusion, and mice were observed during the following 8 weeks prior to sacrifice and organ collection. The mice are divided into three groups: a control group were no PCL was induced and with NaCl in the pumps; a second group ("AngII" group) were no PCL was induced either but which had AngII to promote AAA formation; and a third group ("PCL+AngII" group) were PCL was induced just before AngII-containing pumps were implanted to promote AAA formation.

The sole infusion of AngII into our mice caused a clear increase of atherosclerotic lesions, as well as an elevation of inflammatory leukocytes. This was not seen in those mice were PCL was induced, which strengthens the importance of high levels of plasma cholesterol for the disease development. Moreover, an increase in systemic blood pressure by AngII was

detected, and this was prevented by PCL. Still, the effect of AngII on blood pressure is debatable, as both increases and decreases have been previously reported.

Yet, infusion of AngII in our mouse model did not allow for the development of classical AAA but rather a modest aneurysmal phenotype with a slight remodelling of the arterial wall. Additionally, PCL did not have any detectable effect on AAA formation. The incidence of AAA in our model is reported to be between 20-30% after AngII infusion, a much lower frequency that the one described using the classical $Apoe^{-/-}$ or $Ldlr^{-/-}$ mouse models (usually around 70-80% prevalence), presumably caused by the differences in plasma cholesterol of the models..

Furthermore, collagen content, a characteristic hallmark of AAA progression, was not altered. Elastin was found slightly increased after AngII infusion, although this difference was not detected when lowering plasma cholesterol. Additionally, the gene expression of selected targets usually affected by AAA development was not changed, except for an increase in the macrophage marker $Cd68$ after AngII infusion. This coincides with the previously mentioned increase of this cells detected by immunohistochemistry, although this effect is reverted when levels of plasma cholesterol are lowered.

The main reason why AAA could not be achieved is probably, as mentioned above, the modest hypercholesterolemia of our mice. As stated before, the classic models to study AAA using AngII infusion ($Apoe$ and $Ldlr$ KOs) have much higher plasma cholesterol levels, thus reinforcing the statement that high cholesterol levels are needed in order to develop AAA in mice.

5 FUTURE PERSPECTIVES

In the past decades, clinical, epidemiological, genetic and experimental studies in humans and mice have helped to identify a number of risk factors for CVD. Among the most important is cholesterol contained in the LDL particles. Despite the recognition of LDL-cholesterol as a major risk factor for CVDs and atherosclerosis, and the development of powerful LDL-cholesterol-lowering drugs (statins), complications due to atherosclerosis remain the first cause of death in the western society. A reason for this is that the atherosclerotic plaques are already advanced at onset of therapy as only a minor percentage of individuals at CAD/MI risk are qualified to primary statin treatment. Thus, we need to better understand markers and signs of early atherosclerosis development and the molecular mechanisms that are involved to define those who could benefit from early therapies, and we need to seek new therapies to reduce plaque burden besides statin treatment. As briefly mentioned before, the newly developed therapies against PCSK9, especially when combined with a statin treatment, reveal outstanding results and have shown to decrease up to 70% the levels of LDL-cholesterol in patients.[191] Some ambitious randomized controlled clinical trials aiming to validate the overall cardiovascular benefit of targeting PCSK9 are still ongoing (such as the "Evaluation of cardiovascular outcomes after an acute coronary syndrome during treatment with Alirocumab", the ODYSSEY Outcomes trial)[192] and their results are eagerly awaited, while others have just recently been completed (such as the "Further cardiovascular outcomes research with PCSK9 inhibition in subjects with elevated risk", the FOURIER trial)[193] showing a highly significant reduction in major acute cardiovascular events of up to 20%. The promising results of these new lipid-lowering drugs shed some light into the dark and complex scenario of CVD and its associated complications. However, the high cost of these therapies is still a major drawback, which encourages the need for further investigations regarding cholesterol-lowering therapies and their positive effects.

In the cardiovascular field, as in many others, every seemingly minor discovery may boost research in unexpected ways. In particular, leukocyte transendothelial migration has not obtained the attention it needs. Such an important step in any inflammatory event is crucial for the development of disease. Understanding all the actors (and not only the major ones) that participate in the complex TEML process will open new doors to potential future targeted therapies. As previously mentioned, a complete inhibition of this process will be counterproductive. The inflammatory response is necessary in order to fight pathogens and disease, but this response could potentially be modulated in those sites where is no longer providing a beneficial effect. Moreover, anti-TEML therapy could ideally be spatially directed towards an organ or tissue, therefore specifically targeting the process at the desired place. Some marketed drugs already target key processes in TEML,[194] such as natalizumab, which is directed against a cell adhesion integrin inhibiting leukocyte adherence and is prescribed for the treatment of multiple sclerosis.[195,196] Another drug that also targets adhesion integrins involved in TEML is vedolizumab, used for the treatment of ulcerative colitis and Crohn's disease.[197] However, as pointed out, this type of treatments

entail potentially high associated side effects, as it happens with natalizumab (opportunistic viral brain infection that leads to progressive leukoencephalopathy).[196] Indeed, lack of leukocyte migration is consequence of a genetic disease termed "leukocyte adhesion deficiency" which leads to impaired wound healing and recurrent bacterial infections.[198] Therefore, one should be extremely careful when interfering with the TEML process.

In this thesis, two new possible targets, *LDB2* and *PVRL2* have been examined in the context of TEML in atherosclerosis disease. Both seem to play part in the extravasation process, although in different ways: *LDB2* appears to be a master regulator of the whole TEML process, while *PVRL2* plays a smaller and more specific role within the diapedesis step. In both cases, a complete inhibition would most likely correlate with an increase of opportunistic infections. Moreover, specific malfunctions of the tissues and organs where they exert their main functions, as both are broadly expressed, would also increase the incidence of adverse effects. These facts highlight the importance of developing drugs against organ or tissue specific targets. Controlling the migration process in a more specific and targeted way could turn these genes into very interesting potential targets. Nevertheless, each specific situation should be carefully assessed to minimize off-target side effects. Improved experimental models mimicking human disease, such as synthetic microfluidic systems or improved animal models, should be further developed in order to broaden our knowledge on the molecular and mechanistic cell interactions involved, to provide better medical solutions in the future.

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