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Assessment and Modulation of the Lymphatic Function Throughout the Onset and Progression of Atherosclerosis

par
Andreea Milasan

Sciences Biomédicales, Université de Montréal
Médecine

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Cette thèse intitulée:

Assessment and modulation of the lymphatic function throughout the onset and progression of atherosclerosis

Présentée par:
Andreea Milasan

A été évaluée par un jury composé des personnes suivantes:

Éric Thorin, PhD
Président-rapporteur

Catherine Martel, PhD
Directrice de recherche

Matthieu Ruiz, PhD
Membre du jury

Pierre-Yves von der Weid, PhD
Examinateur externe

Guy Rousseau, PhD
Représentant du doyen de la FESP

Résumé

L'athérosclérose est la principale cause de maladies coronariennes, affectant les artères de grand et moyen calibre. C'est une maladie inflammatoire chronique caractérisée par des plaques situées dans la couche de l'intima, composées de cellules inflammatoires, de cellules musculaires lisses, de composants fibreux et de lipides. Qu'il provienne de source alimentaire ou hépatique, le cholestérol qui s'accumule dans les macrophages des tissus périphériques, comme la paroi artérielle, engendre une réaction inflammatoire et doit être conséquemment mobilisé à l'aide d'accepteurs de cholestérol comme les lipoprotéines de haute densité (HDL). Ce processus spécifique est appelé transport inverse du cholestérol (mRCT). Des études ont démontré que l'apolipoprotéine A-I (apoA-I) pourrait être un acteur clé dans la régulation du mRCT, exerçant des effets différents de ceux du HDL. Plus important encore, le système lymphatique a récemment été identifié comme un nouvel acteur essentiel dans l'élimination du cholestérol de la lésion athérosclérotique (Martel et al., JCI 2013). Il a été démontré que sans vaisseaux lymphatiques fonctionnels, la mobilisation du cholestérol hors de la plaque ne peut pas être réalisée correctement et aggrave la maladie.

Le réseau lymphatique est parallèle au système sanguin et il est présent dans presque tous les tissus du corps. C'est un acteur essentiel dans le maintien de l'homéostase des fluides, dans le transport des cellules immunitaires de la périphérie vers les ganglions lymphatiques correspondants, ainsi que dans l'absorption des lipides alimentaires de l'intestin vers la circulation sanguine. Le système lymphatique comprend les vaisseaux lymphatiques (LVs) initiaux et collecteurs, ainsi que les ganglions lymphatiques, qui ont une anatomie spécifique et des rôles distincts. La lymphe, le liquide clair qui circule dans les LVs, se jette dans la circulation sanguine au niveau de la veine sous-clavière. Les plaquettes sont responsables de la régulation de cette séparation des vaisseaux sanguins et lymphatiques via la formation d'un thrombus formé lors de l'interaction de leur récepteur CLEC-2 avec la podoplanine présente sur les cellules endothéliales lymphatiques. Il a également été démontré que l'activité plaquettaire était nécessaire tout au long de la vie pour maintenir l'intégrité des jonctions des LVs.

L'athérosclérose est également caractérisée par une activation cellulaire et une apoptose accrue. Par conséquent, ces activités cellulaires peuvent entraîner la formation de particules submicroniques appelées vésicules extracellulaires qui ont des effets variables, mais

souvent néfastes, sur l'endothélium sanguin et l'évolution de la plaque. La maladie cardiovasculaire a été associée à une augmentation du nombre des vésicules extracellulaires (EVs) en circulation, et nous croyons que ces véhicules pourraient être impliqués dans le dysfonctionnement lymphatique lié à l'athérosclérose.

D'après des données récentes publiées au cours de ma maîtrise, l'amélioration du transport lymphatique pourrait limiter la progression de l'athérosclérose et favoriser la régression de la plaque. Nous avons montré que le transport lymphatique est altéré chez les jeunes souris prédisposés à développer l'athérosclérose, même avant l'apparition de la plaque. Nous avons prouvé que cet effet est d'abord associé à un défaut au niveau des vaisseaux collecteurs et nous suggérons que l'amélioration de la liaison du VEGF-C/ VEGFR3 puisse supprimer ce défaut spécifique.

L'objectif global de cette thèse était de poursuivre dans cette voie et de mieux définir le rôle de l'important facteur de croissance lymphatique, VEGF-C, et de la lipoprotéine apoA-I dans la maintenance de l'intégrité et la fonction des vaisseaux lymphatiques. En outre, une meilleure description des composants de la lymphe, en particulier des agents libérés par les cellules, a été jugée nécessaire.

La première publication nous a permis de montrer que, lorsqu'elles étaient injectées avec un mutant du facteur de croissance VEGF-C ciblant spécifiquement le récepteur VEGFR-3 (VEGF-C 152s), avant l'administration d'une diète pro-athérogène, les souris *Ldlr*$^{-/-}$ étaient protégées contre l'accumulation excessive dans la plaque et celle-ci était plus stable à long terme. La capacité de contraction soutenue des vaisseaux lymphatiques collecteurs et l'expression accrue de VEGFR-3 et de FOXC2 observée chez ces souris traitées avec VEGF-C-152s ont contribué à la clairance des composants nocifs contenus dans les tissus périphériques tels que les macrophages et le cholestérol.

La deuxième publication a montré que des souris *Ldlr*$^{-/-}$ athérosclérotiques traitées à faible dose avec de l'apoA-I, présentaient un transport lymphatique accru et une hyperperméabilité des vaisseaux lymphatiques collecteurs abrogée, possiblement par une modulation de l'activité plaquettaire.

La troisième publication est la première à démontrer la présence de vésicules extracellulaires d'origines hétérogènes dans la lymphe des souris et que le nombre de différents sous-types augmente chez les souris athérosclérotiques.

Collectivement, ces études confirment la présence d'un dysfonctionnement lymphatique chez la souris avant même l'apparition de la plaque, et il est intéressant de noter que ce dysfonctionnement est principalement associé à un défaut des vaisseaux lymphatiques collecteurs, limitant ainsi le transport de la lymphe des tissus périphériques vers le sang. Différents traitements avec des facteurs de croissance et des lipoprotéines peuvent potentiellement moduler l'apparition et la progression de la lésion en améliorant la fonction lymphatique à différents stades de la maladie athérosclérotique. Nos découvertes concernant la présence de EVs dans la lymphe représentent leur potentiel en tant que biomarqueurs, mais également une nouvelle cible pour mieux comprendre la dysfonction lymphatique.

Mots-clés: athérosclérose, vaisseaux lymphatiques, cellules endothéliales lymphatiques, cellules musculaires lisses lymphatiques, lipoprotéines, transport cellulaire, inflammation, vésicules extracellulaires, plaquettes.

Abstract

Atherosclerosis is the principal cause of coronary artery disease (CAD), affecting large- and medium-sized arteries. It is a chronic inflammatory disease characterized by intimal plaques composed of inflammatory cells, smooth muscle cells, fibrous components and lipids. Cholesterol that accumulates within macrophages in peripheral tissues, like the arterial wall, whether from dietary or synthetic sources, promotes inflammatory responses and needs to be excreted with the help of the cholesterol acceptor high density lipoprotein (HDL). This specific process is termed macrophage reverse cholesterol transport (mRCT) and studies have demonstrated that lipid free apolipoprotein A-I (apoA-I) could be a key player in mRCT regulation, exuding different effects than HDL. More importantly, recently, the lymphatic system has been identified as a novel prerequisite player in the removal of cholesterol out of the atherosclerotic lesion (Martel *et al.*, JCI 2013). It has been demonstrated that without functioning lymphatic vessels cholesterol mobilization from the plaque cannot be properly achieved and aggravates the disease.

The lymphatic network runs in parallel to the blood vasculature and is present in almost all the tissues of the body. It is a crucial player in maintaining fluid homeostasis, trafficking immune cells from the periphery to corresponding lymph nodes, as well as transporting lipids from the intestine to the circulation. The lymphatic system comprises the initial and collecting lymphatic vessels (LVs), as well as lymph nodes, all with a specific anatomy and distinctive roles. Lymph, the clear fluid that circulates within LVs drains towards the bloodstream at the level of the subclavian vein. Platelets are responsible to regulate this blood/lymphatic vessel separation by forming a clog, upon the interaction of their C-type lectin-like receptor 2 (CLEC-2) with podoplanin, present on lymphatic endothelial cells. Platelet activity has also been shown to be required throughout life in order to maintain LV junction integrity.

Atherosclerosis is also characterized by increased cellular activation and apoptosis. Consequently, these cellular activities may result in the formation of submicron particles called extracellular vesicles (EVs) that have variable effects on the blood endothelium and subsequent plaque evolution. CAD has been associated with increased circulating EVs, and we suspect that these EVs might be involved in atherosclerosis-related lymphatic dysfunction.

Based on recent data collected during my master's degree, there is evidence that enhancing lymphatic transport could limit atherosclerosis progression and favour plaque regression. We showed that lymphatic transport is impaired in young, atherosclerosis-prone mice, even before atherosclerosis onset. We believe it to be potentially associated with a defect in the lymphatic pumping capacity, and we suggest that enhancing VEGF-C/VEGFR-3 binding can abolish this specific defect.

The global objective of this thesis was to pursue along this path and better delineate the role of the important lymphatic-specific growth factor, VEGF-C and the lipoprotein apoA-I, on collecting LVs function. Furthermore, a better understanding of lymph components, especially cellular releasants was deemed necessary.

The first publication allowed us to show that when injected with VEGF-C 152s, before the administration of a pro-atherogenic regimen, *Ldlr*$^{-/-}$ mice were protected from excessive plaque formation and long-term, had a more stable plaque. The sustained contraction capacity of the collecting lymphatic vessels and the enhanced expression of VEGFR-3 and FOXC2 observed in these VEGF-C-152s treated mice contributed to the clearance of harmful components contained in peripheral tissues such as the macrophages and cholesterol.

The second publication showed that atherosclerotic *Ldlr*$^{-/-}$ mice treated with low-dose lipid-free apoA-I had enhanced lymphatic transport and abrogated collecting LV permeability possibly through modulation of platelet activity.

The third publication is the first ever to demonstrate the presence of extracellular vesicles of heterogeneous origins in the lymph of mice, and that their levels differ in atherosclerosis.

Collectively, these studies confirm that lymphatic dysfunction is present before the onset of atherosclerosis, and particularly of interest, that this dysfunction is primarily associated with a defect in the collecting vessels, thereby limiting the lymph transport from peripheral tissues to the blood. Different treatments with growth factors and lipoproteins have the potential to modulate the lesion onset and progression through the enhancement of lymphatic function, while our findings regarding the presence of EVs in lymph represents their potential as biomarkers, but also a new venue to better understand lymphatic dysfunction.

Keywords: atherosclerosis, cholesterol, lymphatic vessels, lymphatic endothelial cells, smooth muscle cells, lipoproteins, cellular transport, inflammation, extracellular vesicles, platelets.

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List of acronyms

Akt	Protein kinase B
AngII	Angiotensin II
ApoA-I	Apolipoprotein A-1
ApoB	Apolipoprotein B
ApoE	Apolipoprotein E
$ApoE^{-/-}$	Mouse strain deficient in apoE
Ca^{2+}	Calcium ion
CAD	Coronary artery disease
CD62P	P-selectin
CETP	Cholesterylester transfer protein
CFDA	Carboxyfluorescein diacetate
CFSE	Carboxyfluorescein succinimidyl ester
CLEC-2	C-type lectin-like receptor-2
CM	Chylomicron
CO_2	Carbon dioxide
CVD	Cardiovascular disease
Connexin-37	Cx-37
EC	Endothelial cell
ECM	Extracellular matrix
eNOS	Endothelial nitric oxide synthase
Erk1/2	Extracellular-signal-regulated kinase 1/2
ET-1	Endothelin 1
EVs	Extracellular vesicles
FITC	Fluorescein isothiocyanate
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPIa/IIa	Glycoprotein Ia/IIa
GPIIb/IIIa	Glycoprotein IIb/IIIa
hApoB-100	Human apolipoprotein B-100
HDL	High density lipoprotein
HMG-CoA	3-hydroxy-3-methylglutaryl CoA
HMG-CoA reductase	3-hydroxy-3-methyl-glutaryl-coenzyme A reductase
HMVEC-dLyAd	Human dermal microvascular lymphatic endothelial cells
HSP	Heat shock protein
IBD	Inflammatory bowel disease
ICAM-1	Intercellular Adhesion Molecule 1
IDL	Intermediate-density lipoprotein
IL-(1,8,…)	Interleukin-(1,8,…)
iNOS	Inducible nitric oxide
IVUS	Intravascular ultrasound
LCAT	Lecithin–cholesterol acyltransferase
LDL	Low density lipoprotein
LDLR	Low density lipoprotein receptor

Ldlr$^{-/-}$	Mouse strain deficient in LDLR
LEC	Lymphatic endothelial cell
LMC	Lymphatic muscle cell
LN	Lymph node
LOX-1	Lectin-like oxidized low-density lipoprotein receptor-1
Lp(a)	Lipoprotein(a)
LPL	Lipoprotein lipase
LPS	Lipopolysaccharides
LV	Lymphatic vessel
LYVE-1	Lymphatic vessel hyaluronan receptor 1
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein 1
MHC	Major histocompatibility complex
MI	Myocardial infarction
MMP	Metalloproteinase
MLC_{20}	Myosin light chain of 20 kDa
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
mRCT	Macrophage reverse cholesterol transport
mRNA	Messenger ribonucleic acid
miRNA	Micro ribonucleic acid
MV	Microvesicle
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIRF	Near infrared fluorescence
NO	Nitric oxide
NOS	Nitric oxide synthase
nNOS	Neuronal nitric oxide synthase
OVA488	Ovalbumin 488
oxLDL	Oxidized LDL
PAF	Platelet activating factor
PAMP	Pathogen-associated molecular complexes
PCSK9	Proprotein convertase subtilisin/kexin type 9
Pcsk9$^{-/-}$	Mouse strain deficient in PCSK9
pdEV	Platelet-derived extracellular vesicle
PDGF	Platelet-derived growth factor
PDPN	Podoplanin
PE	Phenylephrine
PI3K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
PROX-1	Prospero homeobox 1
PUFA	Polyunsaturated fatty acids
qPCR	Quantitative polymerase chain reaction
RBC	Red blood cell
RNAi	RNA interference
ROS	Reactive oxygen species
S1PR1	Sphingosine-1-phosphate receptor 1

siRNA	Single interfering ribonucleic acid
SMC	Smooth muscle cell
SREBP	Sterol regulatory element-binding protein
SR-B1	Scavenger receptor class B type 1
TF	Tissue factor
TG	Triglyceride
TGFβ	Transforming growth factor beta
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
Treg	Regulatory T cell
TXA_2	Thromboxane A_2
VCAM-1	Vascular cell adhesion protein 1
VEGF-C	Vascular endothelial growth factor-C
VEGFR-3	Vascular endothelial growth factor receptor-3
VLDL	Very low-density lipoprotein
vWF	von Willebrand factor
WT	Wild type

You can do anything as long as you have the drive, the passion,
the focus, and especially the ***support****.*

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INTRODUCTION

1 CARDIOVASCULAR DISEASE

Cardiovascular disease (CVD), characterized by disorders of the heart and blood vessels, is the number one cause of death globally[1]. Its prevalence is increasing with the age of the population, and the incidence is five times more common in men than in women. This difference, between the sexes, decreases with age[2]. Most deaths are from heart attacks caused by sudden blood clots in the heart's arteries[3]. CVDs include coronary artery disease (CAD), which affects the coronary vessels supplying the heart muscle; cerebrovascular disease, which affects the vessels supplying the brain; peripheral arterial disease, that affects arteries of the arms and legs; rheumatic heart disease, triggered by rheumatic fever that is caused by streptococcus; cardiac birth defects; and venous thrombosis which is a blood clot that forms in a vein[4].

1.1 The circulatory system

To keep the body alive, each of its cells must be able to benefit from a continuous supply of nutrients and oxygen. In addition, carbon dioxide (CO_2) and other metabolic waste produced by the cells must be collected and disposed of. This task belongs to the circulatory system, a network of vessels that allows the heart to circulate blood throughout the body[5]. Deoxygenated blood from the periphery is transported back to the heart by the capillaries, to the venules, to the veins, all the way to the right side of the heart, to ultimately make its way to the lungs. Oxygenated blood from the lungs is sent back to the left side of the heart from where it is ejected into the aorta, and makes its way to the arteries, arterioles, and finally reaches the capillaries where the exchange of nutrients occurs[6].

1.1.1 The heart

The heart is at the core of the circulatory system and acts as a pump responsible of circulating blood throughout the body to supply the necessary physiological needs. Blood carries the essential elements required by the cells, namely nutrients, oxygen and hormones. In addition, blood contributes to the elimination of cellular waste, the protection of the body via the immune system, and regulates internal homeostasis[7].

The wall of the heart consists of three tunics, the epicardium, located on the outside, the myocardium, located in the central part of the wall, and the endocardium lining the cavities of the heart. The latter consists of endothelium and a thin layer of connective tissue. Thus, this layer allows the cavities of the heart and the valves to be smooth in order to reduce friction of the blood during each propulsion[8]. The myocardium is mainly composed of cardiomyocytes which contribute to cardiac contraction, and therefore, to the pumping action of the heart[9]. The epicardium is made up of mesothelial cells and connective tissue, which makes the surface of the heart smooth and slippery, thus avoiding friction[10]. Moreover, the epicardium is a constituent of the pericardium, which is a double-walled sac, representing the inner layer called the visceral pericardium. As a whole, the pericardium's main function is to protect the heart and avoid sudden dilation, especially caused by the right chamber, while it also helps keep the heart in place within the mediastinum which is a division of the thoracic cavity[11]. Since it contains pericardial fluid, it also provides the heart with the freedom of movement necessary for contractions that occur quickly and vigorously[12].

The heart is composed of four different cavities, the right and left atria, as well as the right and left ventricles. The right atrium serves to receive deoxygenated blood from the systemic circulation via the superior and inferior vena cava, and the coronary sinus that carries blood from the heart. The blood then passes to the right ventricle when opening the right atrioventricular valve, also called the tricuspid valve[11]. In both the right ventricle and the left ventricle, atrioventricular valves are connected to papillary muscles via tendinous cords. Thus, the opening of these valves occurs when the blood pressure of the atria is greater than that of the ventricles, resulting in the relaxation of the papillary muscles and tendinous cords[13]. Subsequently, the blood is propelled from the right ventricle to the pulmonary trunk tendinous ropes. This allows the blood to be delivered to the lungs via the right and left pulmonary arteries. Once cleared of CO_2 and reoxygenated, the blood returns to the heart via the pulmonary veins that carry the blood to the left atrium. The blood then passes from the left atrium to the left ventricle following the opening of the left atrioventricular valve, also called bicuspid or mitral valve. Finally, the oxygenated blood is propelled to the systemic circulation through the opening of the aortic valve, which carries the blood into the aortic arch, and then the ascending and descending aortas[14].

Both ventricles contract simultaneously to propel similar volumes of blood. However, their morphology is distinct. The left ventricle is much thicker, since it must propel blood towards the systemic circulation, where it is opposed by a greater resistance and has more distance to cover. The right ventricle sends blood to the nearby lungs, which offer lower resistance, and this could explain why the right ventricular myocardium is less bulky[15]. Finally, the septum separates the right and left ventricles and includes elements of the cardiac conduction system[16].

1.1.2 The blood vessels

The circulatory system is composed of three main types of blood vessels: arteries, veins and capillaries. The aorta is the primary and largest artery in the human body and is emerging straight out of the heart. It represents an important conduit and an elastic reservoir that accommodates blood flow during contractions[17]. From the rising aorta, at the level of the aortic arch there is a bifurcation of three main branches, namely the brachiocephalic trunk, left common carotid artery, and left subclavian artery. From the ascending aorta, two other coronary arteries originate, the right coronary artery and the left main coronary artery, that supply the heart muscle with blood[18]. Blockade of these arteries or their branches, by pathological conditions such as atherosclerosis can lead to angina. The latter is due to insufficient blood supply and causes chest pain, and even a heart attack[19].

Blood vessels are composed of three different layers, except for the capillaries, and within each one of these layers there are differences in muscle and collagen content that varies with size and the location of the vessel[20]. From the inside outwards, are the intima, the media and the adventitia (Figure 1). The intima is the finest and innermost tunic, and it is at this level that atherosclerosis develops. It is composed of a single layer of endothelial cells (ECs) portraying different properties like metabolic activities, thromboresistance, immune functions and elasticity. It also contains an elastic membrane made of extracellular matrix (ECM) on which ECs reside[21]. Also referred to as a basement membrane, it is dotted with fenestrations whose number and appearance vary depending on the location of the vessel in the arterial tree or vascular bed[22]. Following, is the tunica media, which is the thickest. It is the main constituent of the artery and consists of smooth muscle cells (SMCs) surrounded by an ECM composed of elastic and fibrous proteins, such as collagen and elastin[21]. In majority, the media

is avascular, except in its external part that receives irrigation by the *vasa vasorum* of the adventitia (tunica externa)[23]. The latter is the external coat of the blood vessel that consists of the external elastic lamina with loosely organized connective tissue rich in collagen and elastic fibers, as well as fibroblasts and adipocytes[24]. The adventitia ensures the anchorage of the arteries to the surrounding structures and is sometimes also traversed by longitudinal smooth muscle fibers[23].

Figure 1. Structure of the artery wall.

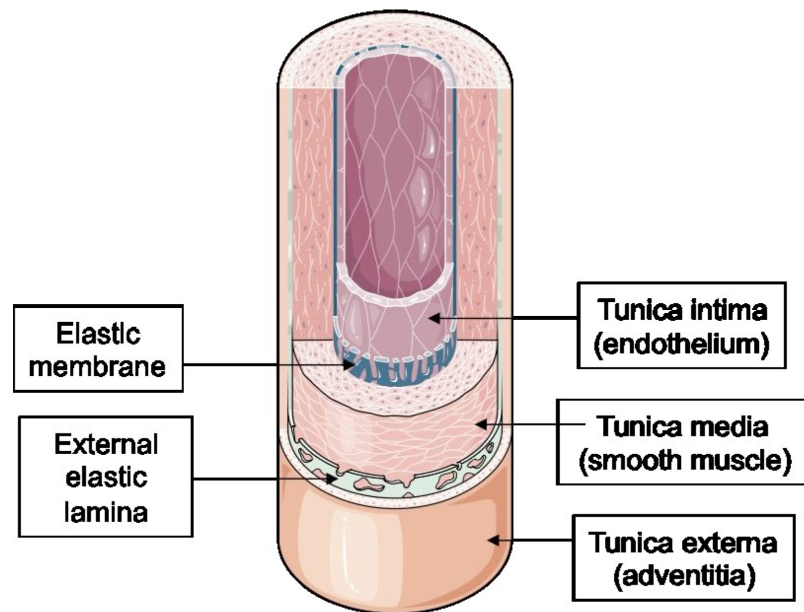


Illustration showing the different layers of a blood vessel wall. Adapted from Servier Medical Art http://www.servier.com/slidekit/?item=16

In the arteries, blood circulates under very high pressure. As such, the structure of an artery must allow it to withstand these variations in pressure generated by the rhythmic pumping of the heart. This is why the arteries are covered with thick walls, are wrapped in elastic tissue, and contain less muscle, which allows them to expand. To withstand hemodynamic loads, elastin is present in these vessels to allow them to increase in size and modulate their diameter as needed. The more muscular arteries, like the brachial artery, the radial artery, and the femoral artery are characterized by a higher number of SMCs in the media than do the elastic arteries.

The latter, which include the aorta and pulmonary arteries, since they are in close proximity to the heart, they are mainly composed of elastic tissue in the media. This allows them to maintain the necessary pressure gradients while supporting the continuous pumping of the heart[6].

Arterioles are blood vessels in the microcirculation that extend and branch out from arteries and are consequently smaller in diameter. They are composed of one to two layers of SMCs and due to lack of elastic tissue they represent the main site of vascular resistance[6].

Blood capillaries are the smallest subunit of the microcirculation and are composed of a single layer of ECs. Although they lack SMCs, capillaries can be surrounded by cells with a contractile function called pericytes[25] that control blood flow by varying the diameter of the capillaries[26]. Since the walls of the arteries and veins are too thick to allow diffusion of molecules, the thin walls of the capillaries allow for this exchange of nutrients and metabolites, to and from the bloodstream[6].

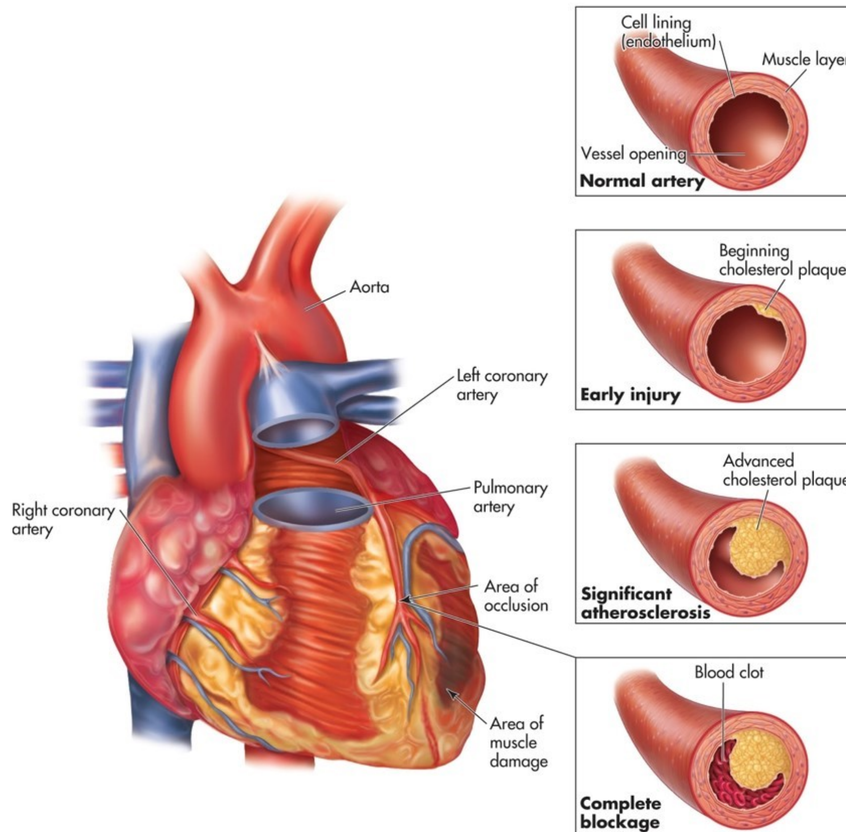
Venules emerge from veins and receive blood from capillaries. They also participate in the exchange of oxygen and nutrients for water products. Venules are fragile as they are very thin-walled, making them likely to break easily when the volume exceeds their normal capacity[6].

Blood will flow from venules into larger veins, similar to the arterial system. Contrarily, venous pressure is low and as such, veins are thin walled and less elastic. This permits veins to hold the majority of the blood in circulation, due to their high capacitance in accommodating a large volume of blood at relatively low pressure. Blood flow in the leg veins is helped by muscle contractions and the one from lower extremities is promoted by continuous respiratory changes that affect pressure gradients in the abdomen and chest cavity. Interestingly, one-way valves are present inside the veins to ensure forward flow of blood towards the heart[6].

1.2 Atherosclerosis

Atherosclerosis is derived from the Greek words ‘sclerosis’ meaning hardening and ‘athera’ that relates to gruel, which is the accumulation of lipids (Figure 2)[27].

Figure 2. Brief overview of atherosclerosis development.



Atherosclerosis is due to fatty deposition, which clogs the artery and causes the vessel to lose elasticity. With this plaque accumulation, lumen space is decreased and thus leads to reduced blood flow, or even worse, a total blockade of the vessel. This leads to a multitude of adverse reactions implicating different cells and altering their functions[28]. Illustration used from *http://medwords.blogspot.com/2013/09/atherosclerosis-disease-symptoms.html (Copyright* © 2011 Medicinal Words | Powered by Blogger

Atherosclerosis is initially asymptomatic[29]. While clinically it often manifests later on in life, the onset of atheroma can begin during childhood, and the rate of progression is a function of risk factors that can be modulated, as well as unmodifiable factors such as age, sex and family history. Risk factors influence the development of atheroma, the frequency of occurrence of cardiovascular complications and their recurrence[30]. With time, atherosclerotic plaques can evolve to become particularly complex. While more advanced lesions can grow and

end up completely blocking blood flow, the most significant clinical complication is detachment of the thrombus that leads to acute vessel occlusion, thus resulting in myocardial infarction (MI), pulmonary embolism or stroke[30]. Additionally, the atherosclerotic plaque is under dynamic changes that result in increased cellular activation and apoptosis, thus resulting in the release of extracellular vesicles (EVs) of various cellular origins[31]. Despite being considered simple cellular debris for decades, EVs are now well documented to interact with neighboring cells and are suspected to be key players in many physiopathological processes such as thrombosis, autoimmune diseases and inflammation. EVs have been associated with several pathologies, including rheumatoid arthritis[32], tumor progression[33], angiogenesis[34], metastasis[35], diabetes[36], hypertension[37], metabolic syndrome[38], hypercholesterolemia and CVDs including atherosclerosis[39]. As EVs are increasingly associated with the key steps of atherosclerosis, and participate in vascular remodelling, they represent important new potential biomarkers and pharmacological targets [40]. A more thorough review of their characterization and specific functions will be described in the following chapters.

The effects of atherosclerosis can be fatal, so a more thorough understanding of the causes and diverse mechanisms of CAD pathogenesis is of upmost importance. As knowledge about all the players involved in atherosclerosis onset and progression improves, it will lead to superior optimization of disease management.

1.2.1 A historical perspective

Atherosclerosis is often thought to be characteristic of the modern world and largely due to contemporary lifestyles. The earliest known case of atherosclerosis was diagnosed by tomography in the aorta of the mummy of an Egyptian princess who died in her early 40s, more than 3500 years ago[41]. Calcification of an atherosclerotic lesion was also identified by computerized tomography scan in a mummified iceman who lived around 3000 years ago[42]. More evidence was supplied recently, when 44 out of 52 mummies had identifiable cardiovascular structures, with 20 of them categorized with either definite atherosclerosis, due to calcification within the wall of a specific artery, or probable atherosclerosis, with calcifications along the longitude of an artery[43]. Since Egyptian culture might have had unique attributes, analysis of other ancient societies was also performed. In fact, probable or definite atherosclerosis was found in 47 of the 137 mummies originating from four different

preindustrial populations such as, 29 ancient Egyptians, 13 ancient Peruvians, 2 Ancestral Puebloans, and 3 Unangan hunter gatherers[43]. While their diets were different, as well as their climates, what they all had in common is smoke inhalation from the fire used for warmth and cooking. Furthermore, a high level of chronic infections and inflammation present in premodern conditions could have promoted the inflammatory aspects of atherosclerosis[43].

The presence of atherosclerosis in pre-modern individuals suggests that the disease is part of human ageing more than it is characteristic of any specific diet or lifestyle[44]. While there may be a genetic predisposition to the disease, the longer you live and survive a multitude of threats, the more susceptible you are to a multitude of risk factors that cause atherosclerosis development.

1.2.2 Risk factors

While scientists thought that cardiovascular disease was primarily a consequence of age, the Framingham study, which began in 1948, showed the importance of other risk factors like tobacco, lack of exercise, stress, diabetes, high blood pressure, and a diet high in cholesterol, just to name a few[4]. In 1976, based on this study, a risk score, which was improved in 1998, was developed to detect the individuals most likely to trigger a cardiovascular event according to these different factors[45]. It was a longitudinal study that aimed to assess similarities in characteristics and factors between subjects, that may cause CAD. The studies included numerous participants who did not yet have any symptoms or any previous incidents of MI or stroke[4, 46]. Up to 90% of CVD may be preventable and improving some of the risk factors such as healthy eating, exercise, no smoking and limiting alcohol intake can make a significant difference[47]. Furthermore, treatment against predisposing factors such as high blood pressure, high content in blood lipids and lipoproteins, as well as diabetes is also beneficial[28]. Nonetheless, as clarified by the mummies studies, by living longer we are still prone to a multitude of advert situations that increase the risk to develop atherosclerosis. At the very least, by narrowing down some of the main factors that increase susceptibility, some prevention becomes possible.

1.3 Cholesterol

Cholesterol is an organic molecule and a hydrophilic lipid. Cholesterol plays an important role in the structure of the cell membrane, which is why it is the principal sterol synthesized by all the cells of an animal. Furthermore, several compounds are synthesized from cholesterol, such as sex hormones, bile acids and vitamin D[28]. Cholesterol is obtained from both synthesis and dietary sources, and in vertebrates, the hepatic cells produce the highest amount. It is absent in prokaryotes, with the exception of *Mycoplasma* which requires it for growth[48].

François Poulletier de la Salle is responsible for finding cholesterol in gallstones, back in 1769. More than a century later, the first link between cholesterol and atherosclerosis was brought to light by Adolf Windaus, where patients' aortic plaques were shown to contain 25 times more cholesterol than normal aortas[49].

1.3.1 Function

Cholesterol is a vital component of the body, which is why each cell is able to synthetize it in a complex process[50]. As organisms evolved, cells required membranes that would allow for a wide variety of integral membrane proteins, such as channels, transporters, and enzymes. As such, cholesterol maintains proper fluidity, contributes to intracellular transport and can modulate important properties of the vertebrate plasma membrane, as it composes it at around 30%[51]. Part of its structural role also includes modulating the permeability of the plasma membrane, to allow for proper regulation of ion leaks[52]. Cholesterol is also required for the structure and function of lipid rafts which are required for endocytosis of various substances inside cells, as well as facilitating certain signalling pathways[53, 54].

1.3.2 Biosynthesis

Endogenous synthesis of cholesterol takes place in the cytoplasm of the liver and intestinal cells, where the condensation of three acetyl-CoA forms 3-hydroxy-3-methylglutaryl CoA (HMG-CoA), a reaction catalyzed by HMG-CoA synthase. Following, HMG-CoA becomes mevalonate with help from the enzyme HMG-CoA reductase, a reaction that represents the rate-limiting step in cholesterol biosynthesis. Finally, mevalonate is converted to isopentenyl

pyrophosphate and further down leads to dimethylallyl pyrophosphate, both which are used to make isoprenoids, a diverse class of biomolecules such as cholesterol, heme and vitamin K[55].

All animal cells can produce cholesterol for diverse uses and this process differs between cell types depending on their needs, as well as the organ function. Primarily the liver newly synthesizes close to 70% of cholesterol, endogenously, while close to 30% comes from dietary intake, exogenously. In total, the human body contains approximatively 70 g of cholesterol[56].

1.3.3 Dietary sources

Since all animal cells produce cholesterol, animal-based foods such as red meat, eggs, fish oil, butter and even breast milk contain cholesterol in varying amounts[57]. It is at the level of the small intestine that cholesterol absorption takes places daily. Specialized lymphatic vessels called lacteals are located within the villi of the intestine and are responsible for the uptake of dietary fats, to then deliver them to the blood via the mesenteric LVs that drain into the thoracic duct[58]. Interestingly, following increased cholesterol ingestion, the body is able to compensate by reducing its own cholesterol synthesis. The intestine tightly regulates how much cholesterol of dietary origins enters the body and this ingested cholesterol is mainly esterified after reaction with fatty acids. The conversion of free cholesterol to cholesteryl ester is catalyzed by lecithin:cholesterol acyltransferase (LCAT) in peripheral tissues, while dietary cholesterol absorbed by enterocytes, the intestinal cells, is esterified by acyl-coenzyme A: cholesterol acyltransferase 2. This conversion allows for more cholesterol to be packaged into the interior of lipoproteins, which allow for more efficient cholesterol transport through the blood stream[59].

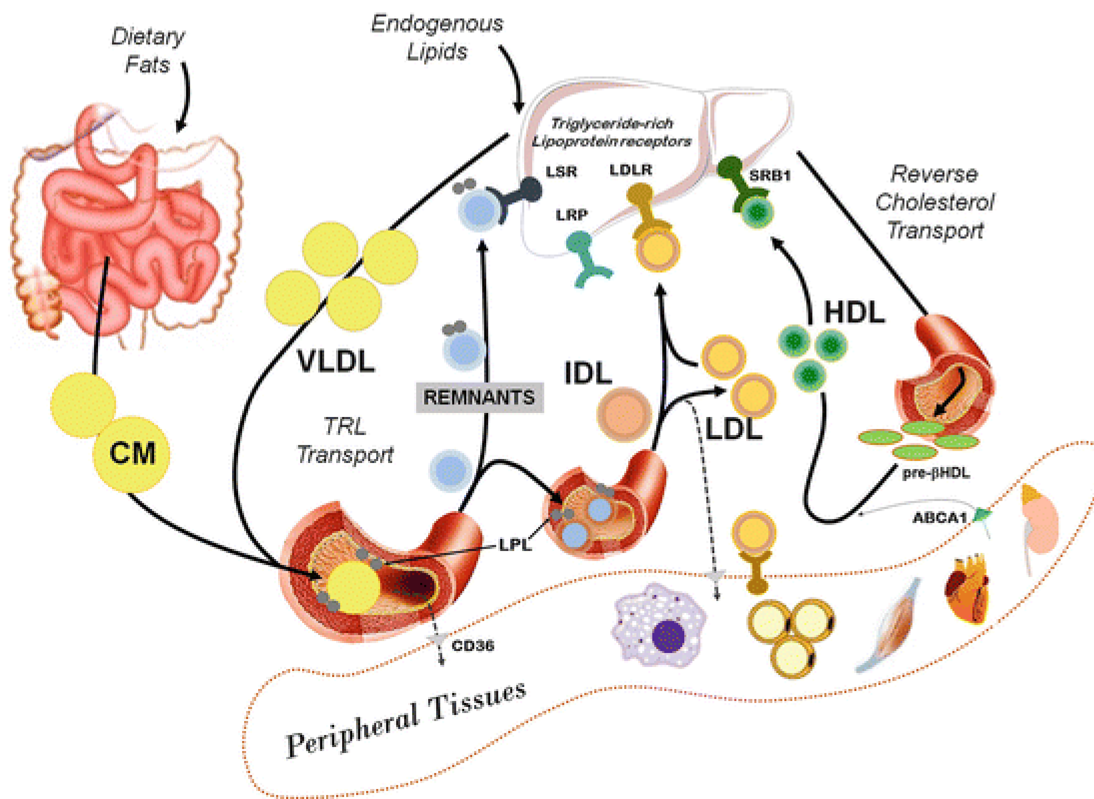
1.3.4 Lipid metabolism and lipoprotein transport

Lipids, like cholesterol and triglycerides (TGs), are insoluble in plasma[59]. In the circulation, they must be transported in association with proteins, to undertake a broad array of functions such as to serve as energy for cells, deposition of lipids and to form bile acids. Their classification, along with their function and pathways of metabolism, play an important role in different disorders and how they can promote the development of atherosclerosis.

1.3.4.1 Classification

There are five major classes of particles that transport lipids between organs, tissues, and cells, listed from larger and less dense to smaller and denser: chylomicron (CM), very low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL). Lipoproteins are larger and less dense when their lipid to protein ratio is high[60]. A thorough comprehension of the main functions of all the lipoproteins is necessary, as they each play a critical role in proper metabolism and lipid handling, among other various responsibilities (Figure 3).

Figure 3. Lipoprotein transport pathways.



A brief overview of the different pathways involved in lipoprotein transport is illustrated by de Guia *et al.*[61] with permission and will be discussed with more depth in the sections below. Triglyceride-rich lipoprotein (TRL). Permission obtained from © 2017 Elsevier Ltd.

Chylomicrons are the largest lipoproteins that form during digestion and are composed of several apolipoproteins such as A-I, B-48, C-II, C-III, and E[59]. Their main role is to transport exogenous lipids like cholesterol and fatty acids from the small intestine to peripheral adipose tissues where they are reprocessed. Within enterocytes, free fatty acids combine with glycerol and form TGs, while cholesterol is esterified[62]. Following, in order to form CMs, TGs and cholesterol are assembled, and the main apolipoproteins contained are B-48, along with C-II and E. Importantly, apoB-48 does not bind to LDL receptor (LDLR) who has an apoB-100 and not an apoB-48 binding site, and whose main ligand is LDL[59]. Along the way, CMs exchange apoproteins with HDL, as they acquire apoE and apoC-II from HDL to convert into a mature form[63]. Lipoprotein lipase (LPL) reduces CM size by hydrolyzing core TGs and releasing free fatty acids that can then be directly used as energy, in combination to form more TGs or deposited in adipose tissue[59, 64]. At the end, CMs are broken down to CM remnants that have a smaller core of lipids and are cleared away by liver CM remnant receptors that bind to apoE[65].

Conversely, the endogenous pathway of lipid metabolism commences with the synthesis of VLDL by the liver, which contains a core composed in major part of TGs and also some cholesterol esters (CEs). The surface apolipoproteins for VLDL include apo C-II, which activates LPL; apoC-III, which inhibits LPL; and apoB-100 and E, which bind to the LDL receptor[59]. Similar to CMs, the TG core of nascent VLDL is hydrolyzed by LPL, thus generating IDL. Circulating cholesteryl ester transfer protein (CETP) mediates the transfer of cholesteryl esters from HDL particles to VLDL, while TGs are transferred in the opposite direction, which promotes cholesterol removal from peripheral cells and uptake by the liver. IDL remnants can then be cleared from the circulation by remnant receptors on the liver, or remodeled by hepatic lipase to form LDL particles[66].

Two of the main cholesterol carriers in the body are LDL, clinically often referred to as the “bad” type of cholesterol, and HDL, considered as the “good” cholesterol. A high ratio of HDL: LDL in the body is associated with a lower risk of CAD[67], and both entities play a pivotal, yet opposite role, in the modulation of atherosclerosis. The main function of LDL is to transport cholesterol and deliver it to cells, where it is used for a variety of functions. LDL contains a core of CEs, lesser amounts of TGs, and its most important and atherogenic component is apoB-100 which is synthetized by the liver[68]. If not enough LDL receptors are

present, cholesterol uptake by the cells is reduced, leading to increased cholesterol circulating in the blood vessels[69]. Knocking out LDLR in transgenic mice leads to a significant increase in total cholesterol levels which can be reversed by restoring the *Ldlr* gene[70].

LDL internalization is thoroughly regulated by the body through a negative feedback control mechanism depending on cellular cholesterol requirements. Decreased HMG CoA reductase activity, which leads to a fall in *de novo* cholesterol synthesis by the cell, upregulates LDLR expression and cholesterol uptake from the circulation. The main protein responsible for the regulation of cholesterol in the endoplasmic reticulum is the sterol regulatory element-binding protein (SREBP)[71].

LDLR is also modulated by the proprotein convertase subtilisin/kexin type-9 (PCSK9), which binds to it and targets it for lysosomal degradation in cells, leading to decreased hepatic clearance of plasma LDL[72]. In addition to its influence on cholesterol transport, overexpression of PCSK9 also affects production of apoB-containing lipoproteins in the intestine by increasing apoB mRNA and enhancing apoB protein stability through activation of the microsomal triglyceride transfer protein (MTP), which is required for the assembly and secretion of VLDL in the liver and also participates in the association of TGs with apoB-48 in CMs[73]. Since PCSK9 significantly impairs the clearance of LDL from the blood, its loss-of-function mutations led to nearly 85% lower plasma LDL levels[74] and as such, offer an important mechanism for protection from CVDs like atherosclerosis[75]. PCSK9 is now considered a potential target for cholesterol-lowering therapies that are especially beneficial for patients who do not tolerate other medications such as statins which have pleiotropic effects[72, 76].

HDL is the smallest and most dense lipoprotein, as it contains the highest proportion of proteins to lipids. Its most abundant apolipoprotein is apoA-I, followed by apoE[77]. However, more than ninety-five proteins are believed to be associated with HDL, ranging up to two hundred twenty-five, as compiled by Dr. Sean Davidson and his team to date. Its main lipoprotein, apoA-I, is synthesized in the liver and the intestine. ApoA-I interacts with receptors in various cell types, including hepatocytes, enterocytes, and macrophages[78]. While apoA-I is lipid-free or at most lipid-poor, which is the preferred substrate of the ABCA1 receptor, in plasma, the preferred state of apoA-I is in association with HDL[79]. ApoA-I in circulation interacts with phospholipids to form nascent discoidal HDL which, once generated, promotes

cholesterol efflux in macrophages present within the lesion[80]. Externalized cholesterol is absorbed by nascent discoidal HDL, and then esterified by LCAT, which sequesters it and eventually makes the newly synthesized HDL spherical. This esterification of cholesterol is particularly important for HDL uptake by the liver and any impairment leads to detrimental HDL dysfunction[81]. Further discussions regarding HDL and apoA-I are detailed in chapter 3 and study #2.

1.3.5 Recycling and excretion

Bile acid synthesis occurs in the liver following cholesterol oxidation. This process is responsible for a daily turnover of a majority of the cholesterol present in humans. Following, bile acids get secreted into the bile and can be stored temporarily in the gallbladder[82]. Bile salts solubilize fats in the digestive tract and facilitate digestion and absorption of fat molecules in the small intestine. In humans, around 50% of the cholesterol is reabsorbed by the small intestine and brought back into the bloodstream, while the rest pursues the path of fecal excretion [83]. Around 95% of bile acids secreted by the hepatocytes are reabsorbed from the intestines, via the portal circulation, while the remainder are excreted in the feces[84]. On a daily basis, close to 1g of cholesterol enters the colon, originating from the diet, bile, or intestinal cells and is metabolized by bacteria present in the colon[85]. Under conditions where cholesterol is more concentrated, as in the gallbladder, it crystallizes and forms gallstones[86].

1.3.6 Dyslipidemia

Dyslipidemia is characterized by an abnormally high amount of lipids, such as total cholesterol, LDL or TGs and low HDL or apoA-I in the blood. Prevalence of dyslipidemia is highest in patients with premature CAD and the disturbance is most often familial[87].

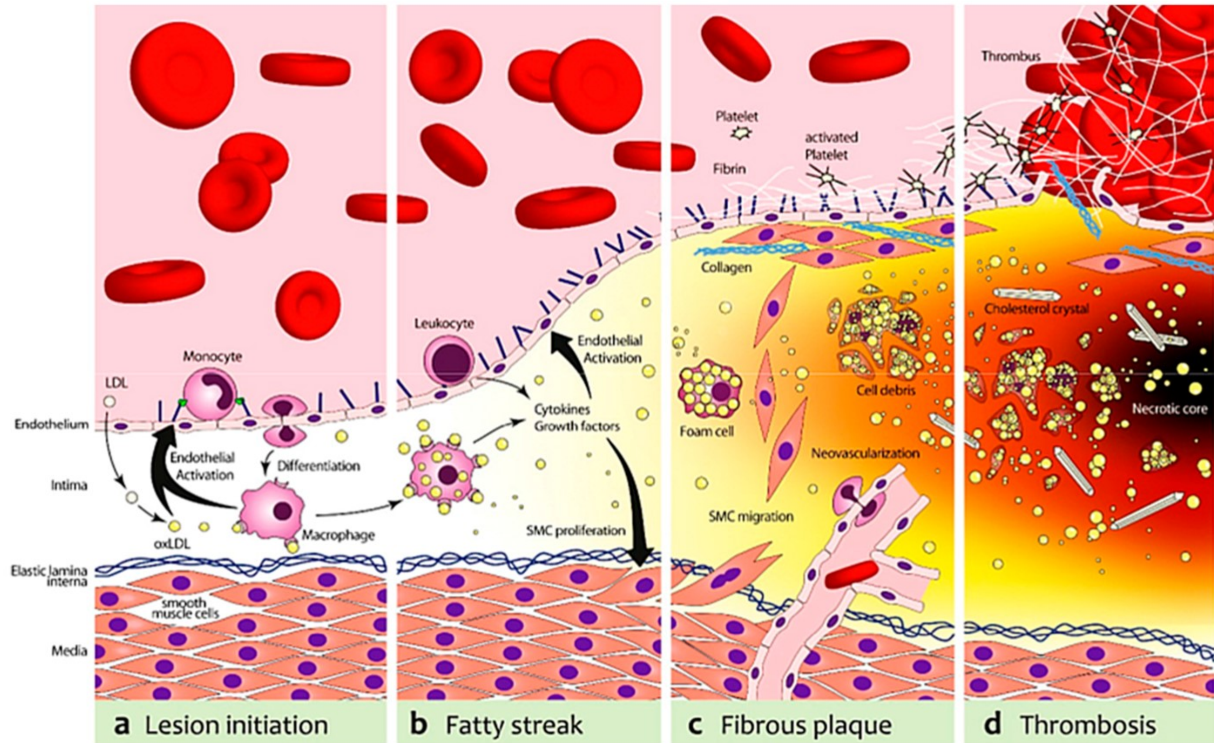
Although polygenic, dyslipidemia is strongly influenced by obesity, particularly visceral adiposity, as well as the amount of saturated fat and cholesterol from the diet. A commonly known genetic disorder, familial hypercholesterolemia (FH), is the leading clinical phenotype resulting from dominantly inherited defects in LDL catabolism. Genetic variations in *Ldlr*, *apoB* or *Pcsk9* genes are the main ones associated with FH. Genetic variations in the *Ldlr* gene are mostly due to loss-of-function mutations, thus leading to increased plasma LDL levels. Defects in the LDLR binding region of *apoB* and rare gain-of-function of the *Pcsk9* gene mutations give

rise to the same lipid homeostasis functional defects. In addition, other genes are also associated with lipid control and regulatory regions such as *Upstream Transcription Factor 1*, *apoE*, *LPL*, *Fibrinogen Beta Chain*, and *Hepatic Lipase*, which all lead to hypercholesterolemia and have been shown to predispose to premature cardiovascular diseases[88]. The *apoE*$^{-/-}$ mouse is particularly representative of familial combined hyperlipidemia, in that it leads to both elevated LDL and TG plasma levels[89]. Similarly, in a Japanese family, heterozygous LPL deficiency led to increased LDL levels in plasma despite normal LDLR activity[90].

1.4 Evolution of an atheroma

Atherosclerotic plaque formation is a continuous process and can extend longitudinally along the entire circumference of the vessel (Figure 5). A thorough description of each underlying step of atherosclerosis onset and progression follows.

Figure 5. The evolution of an atherosclerotic lesion.



Gargiulo *et al.* provide us with a general summary of the main steps that characterize the atherosclerotic plaque initiation and progression. (a) In the first stage, endothelial cell injury allows for LDL to gain entry and then be modified by oxidation thus becoming oxidized LDL. The latter then promotes leukocyte recruitment and differentiation into macrophages, by upregulating adhesion molecules and various chemokines. (b) The modified LDL is ingested by macrophages to create foam cells that form a fatty streak in the arterial wall and secrete pro-inflammatory cytokines.(c) Smooth muscle cells then migrate to the surface of the plaque and create a fibrous cap further reinforced by collagen, fibrin and activated platelets. (d) When the cap is thick, plaque is considered stable. However, proteases secreted from foam cells can destabilize plaque, making it prone to rupture and thrombus formation. As well, foam cells undergo apoptosis where they release various debris and lipids, resulting in the formation of a necrotic core[91]. Open Access article distributed under the terms of the Creative Commons Attribution-Noncommercial 3.0 Unported License

1.4.1 Endothelial dysfunction

The vascular endothelium has long been perceived as a mere physical barrier between blood and the components of the vessel wall[92]. However, it does not only physically limit the access of blood components to cells, tissues and organs, it is also significantly implicated in atherosclerosis development[93].

A broad array of regulatory functions of the vascular endothelium have been highlighted by Robert F. Furchgott, among others. In 1980, he demonstrated the existence of an endothelium-derived gas, later identified as nitric oxide (NO)[93], that led to vasorelaxation following achetylcholine addition to the endothelium[94]. Acetylcholine is recognized to date as a potent vasodilator *in vivo*. Conversely, phenylephrine (PE) binds to α_1-receptors and leads to vasoconstriction which increases blood pressure[95], while exerting a minimal relaxing effect at concentrations higher than 1 μM in the mouse aorta[96].

NO has both protective and toxic properties depending on its concentration and is an important regulator of vascular tone. It is also involved in inhibiting platelet adhesion and aggregation, leukocyte adherence, SMC proliferation and LDL uptake[27, 97, 98]. Reduced bioavailability of NO leads to impairment of endothelial vasodilation that in turn leads to detrimental effects. The amino acid L-arginine is the biological precursor of NO, which is provided by NO synthases (NOSs), either the neuronal (nNOS), endothelial (eNOS) or inducible (iNOS) isoforms[99]. Both nNOS and eNOS constitutively produce low levels of NO when intracellular calcium concentrations increase[100]. More complex, iNOS which is present in macrophages and SMCs among other types, starts releasing NO under inflammatory conditions as induced by certain cytokines[27, 101, 102]. Being a free radical, NO produces endothelial damage, as it can act as both an oxidant and an antioxidant[102]. In the presence of reactive oxygen species (ROS), which cause cellular damage and alter DNA, NO combines with them to form peroxynitrite which is believed to be implicated in lipoprotein oxidation, a key early stage in the development of atherosclerosis[103]. ROS are produced by a variety of the risk factors associated with atherosclerosis such as smoking, stress and radiation[104]. Therefore, during atherosclerosis, the damage sustained to the endothelium impairs eNOS function and NO release is altered. Administration of L-Arginine demonstrated favorable effects in atherosclerosis and disturbed shear stress by increasing eNOS expression in cells and *in*

vivo[105, 106]. Other potent vasodilators such as prostacyclin and tissue-type plasminogen activator are also produced by the endothelium[107]. While NO in itself cannot be simply categorized as good or bad, it is important to delineate the underlying problem of the endothelial dysfunction that attenuates its protective role. NO insufficiency due to inefficient eNOS activity may be at play in some disease states, however, insensitivity of the SMCs or altered responsiveness despite sufficient NO release are also likely[108].

Atherosclerotic lesions occur mainly at sites that are exposed to disturbed blood flow, which cause low or oscillatory shear stress on the vessel wall. Such areas occur at bends, branches and bifurcations of the arterial tree[109]. Disturbed flow alters EC function[110] and impairs its atheroprotective role[111]. These changes can be mediated by stimulation of the release of NO from the ECs[112]. In addition, low shear stress was shown to increase the intima-media thickness of the common carotid artery in healthy men[113].

When the endothelium is altered due to damage and increased activation, ECs release vasoconstrictor factors such as endothelin-1 (ET-1) and angiotensin II (AngII)[27]. ET-1 can significantly contribute to the pathogenesis of atherosclerosis and can also stimulate SMC migration and growth[114]. ET-1 is released from intracellular and extracellular compartments of human coronary atherosclerotic tissue in response to mechanical stress[115]. Furthermore, increased plasma concentrations of AngII also promote the development and severity of atherosclerosis, especially in cases of hyperlipidemia[27]. AngII modulates vascular SMC proliferation and the production of ECM[116]. Both these effectors can promote leukocytes and platelets recruitment and increase several adhesion molecules at the surface of ECs[117, 118]. Intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) are cell surface glycoproteins induced at endothelial sites of inflammation able to mediate the adherence of leukocytes to the endothelium. While a low level of ICAM-1 is expressed on normal endothelial cells, VCAM-1 expression occurs during inflammation and is present in the microvessels of human atherosclerotic lesions[119]. As such, although both adhesion molecules increase in atherosclerotic lesions, VCAM-1 seems more important in the initiation of atherosclerosis[120]. P-selectin (CD62P) is an activated platelet and blood EC (BEC) receptor that mediates adhesion between vascular cells and was also shown to promote migration of inflammatory cells into early and advanced atherosclerotic lesions[121].

At sites of injury and inflammation, a variety of proinflammatory cytokines such as interleukin-1 (IL-1) and tumor necrosis factor alpha (TNF-α) promote leukocyte adhesion and activation, as well as neutrophil activators, such as granulocyte macrophage colony stimulating factor (GM-CSF), plasminogen-activating factor (PAF) and interleukin-8 (IL-8)[122]. These markedly potentiate neutrophil activation by increasing their adhesiveness, which then contributes to further endothelial damage and injury by producing ROS[123]. As such, antibodies that block adhesion molecules could improve the inflammatory response in atherosclerotic plaques[124].

1.4.2 Lipoprotein modification

Abnormal lipoprotein metabolism is a major predisposing factor to atherosclerosis. Dyslipidemia is estimated to be present in over 70% of patients with premature CAD[87]. Different players have an instigative role in atherosclerotic lesion development and modulation.

LDL particles contain cholesterol, TGs, phospholipids, apoB-100, apoE and apoC, among the twenty-two associated proteins identified to date, a number that may range up to sixty as reflected when studies were compiled by Dr. Sean Davidson and his team. Since all LDL particles contain one copy of apoB-100, and only 10 to 20 percent contain apoC-III, there is a direct relationship between apoB-100 and LDL particle number[59]. Interestingly, elevated plasma concentrations of apoB-100 containing lipoproteins can induce the development of atherosclerosis even in the absence of other risk factors. This is due to LDLs getting trapped in the subendothelium of the vessel via a charge-mediated interaction with proteoglycans in the ECM[125] and are more prone to ROS modification of surface phospholipids and unesterified cholesterol[126]. Compared to mice with regular LDL particles, mice expressing LDL with defective proteoglycan binding developed significantly less atherosclerosis[127].

Oxidized LDL (OxLDL), produced when LDL cholesterol is damaged by chemical interactions with free radicals, is responsible for a panoply of aggravating effects and elevated plasma concentrations are associated with CAD[128]. OxLDL promotes recruitment of proinflammatory factors[27] and increases monocyte binding through activation of monocyte ß1 integrin[129]. Macrophage mobility is thus reduced, trapping them within the vessel wall. In a study performed on patients with FH, hyperlipidemia displayed elevated levels of ICAM-1 and VCAM-1, which upregulate endothelial adhesiveness, but these levels were reduced

following LDL apheresis[130]. Additionally, OxLDL disrupts EC surface and as a result impairs eNOS function, thus reducing NO release[131]. High levels of cholesterol are also known to increase endothelial production of oxygen free radicals, which in turn bind to and inactivate NO[132]. Furthermore, oxLDL inhibits NOS expression in platelets, leading to a concentration-dependent increase in thrombin-induced platelet activation and aggregation, thus contributing to vasoconstriction and intravascular thrombus formation [133]. When supplemented with dietary L-arginine, platelet aggregation was normalized in hypercholesterolemic patients by restoring endogenous levels of NO and helped attenuate platelet reactivity and aggregation[134]. Moreover, oxLDL promotes transcription factors like nuclear factor kappa B (NF-κB) that increases the expression of multiple proinflammatory cytokines[135]. OxLDL is also involved in cellular death of SMCs and ECs, further supporting the response to injury hypothesis of atherosclerosis[136]. In addition, OxLDL also upregulates the angiotensin II type I receptor, thereby increasing the functional response of vascular SMCs to angII stimulation[137, 138]. More importantly, this receptor regulates the induction of the lecithin-like oxidized LDL receptor-1 (LOX-1), important in the process of oxidized LDL-mediated monocyte adhesion to coronary artery endothelial cells[139]. The prevention of oxidative modification of lipoproteins, as with paraoxonase[140], or lowering of cholesterol with a statin drug is associated with less severe CVD[141].

Although LDL is the main culprit in the progression of atherosclerosis, some studies showed that IDL levels can also be predictive of an increased incidence of CVD, independent of any other factors[142, 143]. Furthermore, IDL and VLDL also contain TGs and apoC-III, each of which is associated with atherosclerosis. High concentrations of CM remnants or VLDL particles were shown to lead to lower levels of HDL, while studies in the plasma of CAD patients pinpointed that apoC-III-enriched HDL particles are significantly higher when compared to control and may lead to reduced macrophage cholesterol efflux capacity[144].

Lipoprotein(a) (Lp(a)) also carries cholesterol and is assembled intrahepatically from apoA and LDL[128]. ApoA links to apoB-100 on the surface of LDL with the help of two covalent disulfide bridges. The formation of apoA:apoB and its structural integrity is modulated by LCAT[145]. As it is similar to plasminogen, Lp(a) becomes a competitive inhibitor of fibrinogen and fibrin, thereby interfering with fibrinolysis and favoring thrombus accumulation[146].

1.4.3 Immune cell infiltration

Oxidatively modified lipoproteins promote immune cell infiltration, release of inflammatory mediators and upregulation of adhesive markers, with the help of the endothelium which provides the necessary support for leukocyte migration[97].

1.4.3.1 Monocytes

Circulating monocytes display heterogeneity in mice and humans and play a significant role in lipid accumulation and atherosclerosis progression[147]. Reduction of lesion formation in monocyte-deficient *apoE*$^{-/-}$ and *Ldlr*$^{-/-}$ mice was observed[148, 149]. Similarly, reducing the activity in circulation of monocyte chemoattractant protein 1 (MCP-1), significantly decreased atherosclerosis in these two mouse models[150]. Human monocytes are bone marrow-derived leukocytes that after migrating from the circulation to the intima of the blood vessel, can differentiate into monocyte-derived macrophages and monocyte-derived dendritic cells (DCs) that govern immune responses. Additionally, they can internalize modified lipoproteins, like oxLDL, and transform into foam cells[151].

Humans possess three main subsets of monocytes, phenotypically and functionally different. There is a classical (CD14^{++}, CD16^{-}), intermediate (CD14^{+}, CD16^{+}) and non-classical (CD14^{+}, CD16^{++}) subset, and each is characterized by the secretion of different kinds on interleukins and cytokines[152]. In the mouse, monocyte subsets are based on expression levels of Ly6C on the cell surface, from which two subsets arise, the inflammatory Ly6C^{high} and Ly6C^{middle} with chemokine receptors CCR2^{high}CX3CR1^{low}, or patrolling Ly6C^{low} with chemokine receptors CCR2^{low}CX3CR1^{high}. The CCR2^{high} subset are highly migratory and have a superior infiltrating capacity compared to the CCR2^{low} subset, while the CX3CR1 receptor mediates resident monocyte accumulation[153]. The Ly6C^{high} monocyte subset doubled each month in hypercholesterolemic *apoE*$^{-/-}$ mice on a HFD, and further activated the endothelium, infiltrated the injury site, and became lesional macrophages, thus confirming their role as important modulators of the inflammatory response in experimental atherosclerosis[154]. Following chemokinesis, monocytes adhere to and roll on ECs by interacting with E- and P-selectins[155]. Alternatively, monocytes can also reside in the spleen and in response to ischemic MI they were shown to participate in wound healing of the injured cardiac tissue[156]. Conversely, in stroke-induced injury, splenectomised mice had less Ly6C^{high} and Ly6C^{low}

monocytes in the brain, but no effects were observed in swelling of the tissue and infarct size[157]. Nonetheless, since they are the most abundant cell type in atherosclerotic plaques, macrophages are now known to play a central role in cholesterol homeostasis within the lesion, to mediate several inflammatory pathways, affect the necrotic core, and modulate ECM degradation[158].

1.4.3.2 Dendritic cells

Dendritic cells, the most efficient antigen presenting cells[159], modulate the immune response and are able to activate or inhibit T cells, with the help of different cytokines and cell surface co-stimulatory molecules. As such, they are important regulators of the innate immune response. DCs originate from monocytes or DC precursors present in the bone marrow and constitute a diverse population principally known as: conventional DCs, plasmacytoid DCs, monocyte-derived DCs, and Langerhans cells[151]. When inflammation occurs, monocytes are converted to monocyte-derived DCs following release of GM-CSF or Toll-like receptors (TLRs) ligands. TLRs are pattern recognition receptors present on a variety of cells such as DCs, leukocytes and T and B lymphocytes. They are needed for downstream cell signalling and to recognize PAMPs[160]. Proinflammatory cytokines, such as TNF-α, IL-6, and IL-12, can be generated by TLR binding, but they can also generate IL-10, an atheroprotective cytokine[151]. DCs express CD11c and are predominantly found in the adventitia of the blood vessel under physiological conditions, instead of the intima of atherosclerotic aortas. Of notice, in mice, CD11c messenger RNA (mRNA) is higher in the sites of the aortic arch susceptible to atherosclerosis, compared to those more resistant[161]. In carotid cross-sections of patients, a much lower number of DCs was detected in initial lesions or stable plaques kept under control by statins, in comparison to more advanced lesions[162]. Furthermore, interactions with T cells are mainly observed at vulnerable sites of the plaque predisposed to rupture. As well, during inflammation, recruitment of monocytes, T cells, and DCs to the affected region is mediated by MCP-1, which is secreted by DCs[59]. The interaction with T cells is dependent on the toll receptor MYD88 and when inhibited, both T cell effectors and regulatory T cells (Tregs) were downregulated. Lesion size however was surprisingly increased, and the authors attribute this effect to alternative recruitment of myeloid-derived inflammatory cells[163]. DCs deposition in the intima was shown to increase with age and be dependent on the receptor CX3CR1. It is

suspected that they originate from Ly-6C^{lo} monocytes[151, 164]. DCs also express scavenger receptors LOX-1, SR-A and CD36 to facilitate oxLDL uptake and become proinflammatory[165]. Activated DCs will move to the draining lymph nodes (LNs) and promote T cell proliferation against oxLDL[166]. In such inflammatory conditions, DC migration relies on VCAM-1 and ICAM-1[167]. In fact, expression of these two molecules is induced on ECs by mediators such as ROS and are used as scaffold for leukocytes, but also to open an intercellular passageway for the latter to migrate through[168]. The role of DCs in atherosclerosis is multifunctional as they modulate a panoply of proinflammatory factors and cells, and can uptake lipids thus further promoting inflammation[151].

1.4.3.3 T cells

The role of T cells in atherosclerosis was confirmed by oligoclonal expansions of T cells and antigen-driven proliferation in lesions[169]. Interferon gamma (IFN-γ) is produced in high levels by T cells and this cytokine activates macrophages and worsens disease development. Furthermore, it also recruits SMCs that inhibit the synthesis of collagen, leading to a more unstable lesion as characterized by a thinner fibrous cap. These SMCs can further become macrophage-like and accumulate as foam cells[170]. It has been demonstrated that the removal of IFNγ or associated receptors reduces atherosclerosis, while injections of recombinant IFNγ promote plaque growth[151].

Distinction between the different T cell subtypes is non-negligible. Helper T (Th) cells promote atherosclerosis with the help of two different subtypes that differ, but both end up contributing to atherosclerosis progression. Unlike Th1, Th2 was shown to decrease IFN-γ production and is believed to exert minimal atheroprotection[151]. Th2 cells are particularly important in activating B cells and atherosclerotic progression in mice was inhibited following B cells blockage with an antibody against CD20[151, 171]. Alternatively, Tregs are protective as they inhibit and supress inflammation. Furthermore, they induce IL-10, an anti-inflammatory cytokine[172]. In mice that had IL-10-deficiency and fed a HFD, cholesterol accumulation within the atherosclerotic lesion was three times more significant than control[172].

1.4.4 Foam cell formation

Onset and progression of atherosclerosis is tightly associated with foam cell formation. The transformation into macrophages is a crucial step, as M-CSF-knockout *apoE*$^{-/-}$ mice had reduced lesions in the proximal aorta[173]. Once monocytes reach the intima, they undergo differentiation to macrophages and exert enhanced expression of scavenger receptors including CD36, CD68 and LOX-1 to be able to uptake lipoproteins[27]. Their main role is to clear the lipids from plaque, and in doing so they transform into foam cells[174]. Once internalized, lipids are transported to the lysosome to be further broken down into free cholesterol by the action of lysosomal acid lipase[175].

Measures of macrophage cholesterol efflux are correlated with the incidence of cardiovascular events. Similar to scavenger receptors used to engulf lipids, foam cells also have several cholesterol transporters that aid in cholesterol efflux, including ATP-binding cassette transporters ABCA1 and ABCG1, as well as scavenger receptor B type 1 (SR-B1) which mediate bidirectional lipid transport in the macrophage[175, 176]. Under pathological conditions such as atherosclerosis, proinflammatory stimuli upregulate expression of scavenger receptors, especially LOX-1, and in turn downregulate expression of cholesterol transporters within the cell. The resultant is increased foam cell formation, leading to plaque augmentation[175]. Importantly, both ECs and vascular SMCs can become foam cells. Hyperlipidemia was shown to promote EC activation and altering their characteristics thus rendering them more permeable to lipoprotein uptake. Vascular SMCs are thought of as more plastic and thus adaptable to environmental cues. It is believed that they are able to migrate towards the intima, from the media, and uptake lipoproteins as needed[177].

Modified LDL can also be uptaken by the macrophages through those scavenger receptors whose expression is low under physiological conditions, but can be upregulated via JNK, Wnt and NF-κB signaling[178]. Worrisome is that these foam cells can rupture and release oxLDL as well as intracellular enzymes and oxygen free radicals that can further cause damage to the vessel wall. *In vitro*, LDL isolated from the blood of atherosclerotic patients induced cellular lipidosis in cultured macrophages and was characterized as atherogenic, while LDL from healthy subjects had no effect. Furthermore, the atherogenic LDL promoted the release of TNF-*α*, but also of the anti-inflammatory C-C Motif Chemokine Ligand 18 (CCL18) at the

transcriptional level[147]. A proposed treatment to prevent the development of atherosclerosis is recombinant adenovirus-mediated gene transfer of decoy macrophages to compete for the uptake mediated via other related scavenger receptors and block foam cell formation[179].

In recent years, due to the pleiotropic functions of macrophage phenotypes, a more thorough classification has emerged. As macrophages have a high level of adaptability, this allows them to refine their mechanisms of action depending on the environment at play[180]. Several lipids, growth factors, and cytokines modulate macrophage polarity and preferred phenotype. As such, two main phenotypes were initially identified, classically activated M1 macrophages which are proinflammatory, and M2 macrophages that have a protective role in plaque stabilization[178]. M1 macrophages are believed to differentiate in response to TLRs and IFN-γ signalling and they can also be activated by pathogen-associated molecular complexes (PAMPs) and lipopolysaccharides (LPS)[147, 181]. They secrete proinflammatory factors, such as TNF-α, IL-1β, IL-12, and IL-23, as well as chemokines CXCL9, CXCL10, and CXCL11. Furthermore, they produce high levels of ROS and NO, as they activate iNOS and further aggravate the inflammatory response[182]. Contrarily, M2 macrophages respond to certain cytokines released by Th2 cells, IL-4 and IL-13, and in turn release IL-10 and other protective cytokines that aid in wound healing[183]. While M1 macrophages were increased in growing plaques, protective M2 macrophages were mainly observed in lesions undergoing regression[147].

Recently, the M2 type was further divided into different subgroups based on the associated activation stimuli and protein expression pattern[147]. Briefly, M2a macrophages are activated by IL-4 and IL-13 and express high levels of CD206; M2b macrophages are activated by TLR signalling, as well as immune complexes and ligands, and produce both anti-inflammatory IL-10, as well as proinflammatory IL-6 and TNF-α cytokines; M2c macrophages can be induced by IL-10 and transforming growth factor-β (TGF-β), and are known for their anti-inflammatory properties as well as clearance of apoptotic cells; M2d macrophages respond to TLR signalling and exert angiogenic properties that can promote atherosclerotic progression[147].

As macrophage function and variety in atherosclerosis is important, they represent an attractive therapeutic target. Further investigation of the roles of each different type in atherosclerosis progression and development needs more thorough investigation.

1.4.5 Vascular smooth muscle cell proliferation and modulation

The engulfment of modified lipids by macrophages produces a variety of cytokines and growth factors which not only promote monocyte, but also vascular SMC recruitment. Release of IL-1 and TNF-α stimulates the production of platelet-derived growth factor (PDGF) and fibroblast growth factor following EC and platelet activation which leads to migration of SMCs from the media to the intima[27]. Usually, SMCs in the media express a variety of specific markers including myosin heavy chain, smooth muscle 22α langelin, smooth muscle actin, smoothelin and others. However, in atherosclerosis, their expression is reduced and they are more prone to proliferation, migration and production of diverse ECM proteins, growth factors and cytokines[184].

One of the more important steps in the migration and proliferation of SMCs is the secretion of metalloproteinases (MMPs), responsible of ECM degradation. MMP9 is responsible for enhanced SMC migration and causes disruption of the elastic lamina in cerebral arteries and the aorta, thereby weakening the artery[27]. Both MMP-9 and MMP-2 are believed to be the most significant proteases associated with the progression of the atherosclerotic lesions, as assessed in *Ldlr*$^{-/-}$*;apoB*$^{100/100}$ mice[185]. LPL, secreted by the endothelium, promotes SMC proliferation through a process that leads to switching off contractile proteins genes and conversely, switching on genes associated with synthetic activity[27]. Following, SMCs produce collagen in the hopes to stabilize plaque progression. TGF-β is also implicated in the production of collagen and is associated with the development of intimal hyperplasia[186]. Inflammatory cytokines such as IFN-γ, produced by T cells, inhibit the synthesis of collagen by SMCs along with IL-1β and TNF-α[187]. Collagen represents close to 60% of the total plaque and promotes plaque growth which leads to arterial lumen narrowing[188]. It also contributes to plaque structural integrity and stimulates further lesion progression as it acts to allow a depot of proatherogenic molecules like modified lipoproteins, growth factors and modulates thrombus formation[189]. Importantly, a good balance of collagen is necessary since too much collagen causes unwanted fibrosis that leads to arterial stenosis, while unrestrained collagen breakdown or aberrant synthesis results in a weaker and vulnerable plaque that is increasingly prone to rupture[188].

Vascular SMCs can also undergo phenotypic switching following cholesterol loading and acquire increased mRNA expression of macrophage-related genes CD68, Mac-2, and ABCA1, as well as their foam cell properties[190]. As such, particularly important for targeted therapies, is that not all foam cells originate from macrophages. Nearly half the foam cells in an atherosclerotic lesion are derived from SMCs and those present in the coronary intima have reduced expression of ABCA1 in comparison to macrophage-derived foam cells, thus rendering them less efficient in cholesterol efflux, as well as more inflammatory[191].

1.4.6 Platelet adhesion and aggregation

Atherosclerosis involves the interactive processes of the atherosclerotic lesion and later on, the formation of thrombi. Under normal conditions, resting platelets do not adhere to the endothelium. However, when they are activated by inflammatory processes or by arterial hypertension, platelets interact with the endothelium and inflammatory cells from the premature stages of atherosclerosis[192].

Platelets circulate in an isolated fashion within blood in the arterial wall, but in response to vascular damage, they stick to the subendothelial matrix. This is aided by adhesive proteins that are now at the surface of the endothelium, which physiologically would not be expressed, such as collagen and Von Willebrand factor (vWF). Glycoprotein Ia/IIa (GPIa/IIa) is a major platelet receptor for collagen and particularly important under low flow conditions[193], whereas the GPIb-IX receptor enables platelets to remain tethered to a vWF matrix under conditions of high shear stress[194]. GPVI along with GPIa/IIa is also a collagen receptor and on the surface of the platelet forms a complex with the receptor Fcγ, which leads to phosphorylation of the receptor and platelet activation. When platelets are activated, they secrete α and dense granules. The α granules store large amounts of proteins such as fibrinogen, vWF, platelet factor 4, GPIIb/IIIa and PDGF, among others, which participate in platelet aggregation. Dense granules release calcium, phosphorus, ADP and ATP. Thromboxane A_2 (TXA_2) is also released by activated platelets and promotes the activation of new platelets, and platelet aggregation[195]. Once the released factors bind to platelets, receptor activation leads to an increase in intracellular calcium ions (Ca^{2+}) as it gets released from platelet endoplasmic reticulum Ca^{2+} stores. This triggers a calcium-dependent association of GPIIb and GPIIIa to form the complex GPIIb/IIIa, which binds fibrinogen and promotes inter-platelet binding,

ultimately resulting in a clot. Interaction between GPIIb/IIIa on activated platelets and ICAM-1 in ECs causes platelets to adhere to the injured endothelium. Similar to how CD62P is expressed on all platelets following activation and can also bind the endothelium, P-selectin glycoprotein ligand-1 is present on most leukocytes and plays an important role in their recruitment to the site of injury during inflammation[196].

Thrombin is one of the most potent platelet activators and in inactivated platelets CD62P is stored in α granules. The A disintegrin and metalloproteinase 15 (ADAM15) is also expressed by ECs and binds to platelets via GPIIb/IIIa, further inducing their activation[197]. Furthermore, once platelets are activated they can also release CD40 ligand (CD40L) which they store and express on their surface, that once solubilized, gets deposited onto the endothelium and induces expression of molecule adhesion cells E-selectin, ICAM-1 and VCAM-1[198]. A direct positive correlation between the levels of soluble CD40L and thrombus formation were observed *in vivo*[199].

Platelets greatly contribute to the aggravation of the atherosclerotic lesions as they modulate MCP-1 release and ICAM-1 presence on ECs through an NF-κB mechanism[200]. Platelets also stimulate the release of interleukin 1 beta (IL-1ß), the main EC activator, and further promote the release of IL-6 and IL-8, responsible to attract leukocytes[201]. PF4 is released in majority by activated platelets and causes chemotaxis of monocytes and other leukocytes. It inhibits binding and degradation of LDL by its receptor and led to a nearly 10-fold increase in ox-LDL within the artery wall[202]. PDGF is released during platelet activation and stimulates the proliferation of muscular cells, thereby causing hyperplasia of the inner section of the vascular wall[192].

Particularly of interest in recent years is the C-type lectin-like receptor 2 (CLEC-2), constitutively expressed at the surface of platelets. CLEC-2 is activated by podoplanin (PDPN), a small membrane glycoprotein present on lymphatic vessels, in the lungs and in the kidney[203]. CLEC-2 mediates platelet leakage from hyperpermeable vessels in order to maintain vascular integrity during inflammation and the PDPN/CLEC-2 interaction is particularly of notice in deep vein thrombosis when PDPN is upregulated to facilitate thrombus formation[204].

Platelets, therefore, affect atherosclerosis development and progression in a multifactorial manner, led mainly by their interaction with ECs.

1.4.7 Plaque vulnerability

Atherosclerosis *per se* is not deadly, what poses an actual risk is the vulnerability of the arteriosclerotic plaque to breakage. A plaque is prone to rupture when composed of a significant lipid core, as well as an excessive amount of necrosis and debris. This agglomerate is separated from the lumen by a fibrous cap, which is primarily composed of collagen, proteoglycans, and SMCs, but becomes unstable when disbalance between lytic processes and lack of repair occur[205]. A multitude of inflammatory cells erode the fibrous cap by various mechanisms, but the activation of MMPs seems to be one of the most significant factors. The resultant is a plaque fissure which attracts platelets to the lipid core, along with blood coagulation factors. As such, thinner the fibrous cap, higher the risk of rupture[205]. Recently, it has been proposed that plaque rupture occurs at multiple sites and not just an isolated one, as demonstrated by the widely spread activation of neutrophils across the coronary vasculature in patients suffering of unstable angina[206].

As plaque complexifies, it shows evidence of multiple ruptures and repairs, and over time this accumulation leads to luminal narrowing[184]. Plaque repair requires SMCs to synthetize collagen, which is altered by cellular death and thereby further promotes cap thinning[205]. Apoptosis is predominantly observed in macrophages and vascular SMCs, although all cell types within the vessel wall can undergo apoptosis[184]. SMCs are suspected to undergo apoptosis through death ligand/death receptor interactions with macrophages and symptomatic plaques were shown to exert a higher level of SMC apoptosis compared to stable lesions[207]. This is due to the fact that the consequence of SMC apoptosis is thinning of the fibrous cap, enlarged necrotic core, macrophage infiltration into the cap and calcification. In fact, M2 macrophages induce macroscopic calcium deposition, often referred to as macrocalcification, by inducing osteoblast differentiation in bone with the help of Oncostatin M. However, clinically, macrocalcification leads to plaque stability, while microcalcification seems associated with plaque rupture[208]. While some studies debate whether or not calcification is helpful to identify a vulnerable plaque, Hermann *et al.*, among others,

demonstrated that patients that had suffered a stroke have significantly increased coronary artery calcification values at baseline when compared to patients without any episodes[209].

SMC death further impedes on the atherosclerotic plaque through cytokine release, as IL-1β is released following apoptosis and necrosis releases IL-1α. Although SMCs clearance is usually done quickly, especially by the phagocytic properties of the SMCs themselves, in hyperlipidemia this process is greatly impaired potentially due to their phenotype switching to macrophage-like cells[184, 210].

The vulnerable plaque is complex, and many mechanisms are yet to be further elucidated upon to better understand treatment and prevention of thrombus formation. With the help of pathologists, a thorough classification of the lesions has been obtained (Table 1).

Table 1. Classification of the atherosclerotic lesion.

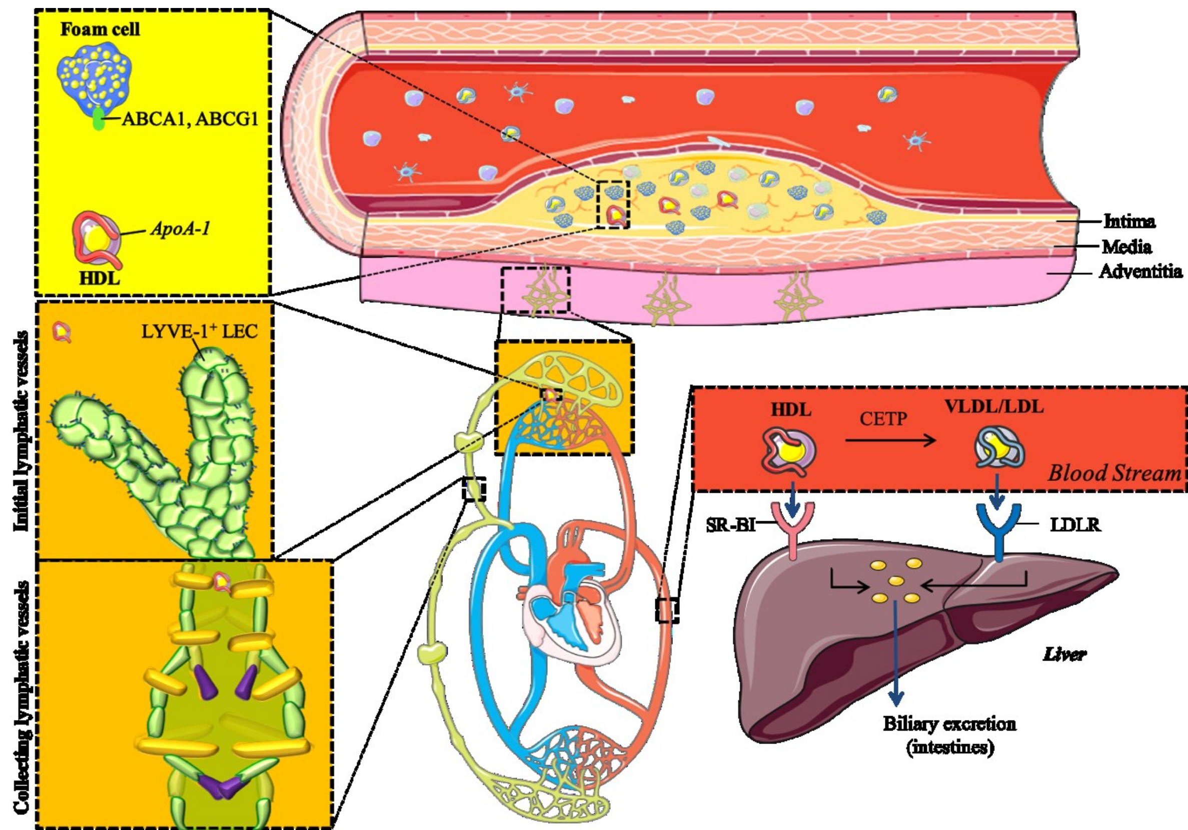
Type	Histology
Type I (initial)	Atherogenic lipoproteins and infiltrates mononuclear leukocytes, with minimal intimal thickening
Type II (fatty streaks)	Layers of macrophages or foam cells with SMC infiltration from the media into the intima
Type III (intermediate)	Scattered coarse lipid granules or particles, and disruption of the integrity of SMCs
Type IV (atheroma)	Large extracellular lipid core covered mainly by the intima
Type Vb and Vc (fibroatheroma)	Large extracellular lipid core and developing fibrous caps, increase in collagen and SMC content Vb mainly calcific Vc mainly fibrous
Type VI (complicated)	Ruptured plaque, fissure formation or hematomas, hemorrhage and thrombus formation

This table summarizes a brief description of the atherosclerotic lesion during different stages of atherosclerosis progression. The information has been adapted from multiple sources[27, 211, 212].

1.4.8 Reverse cholesterol transport

The physiological mechanism through which cholesterol homeostasis is obtained in the artery wall, characterized by its mobilization from foam cells and its subsequent transfer from the peripheral tissues to the liver and the intestines for further excretion, is called macrophage reverse cholesterol transport (mRCT)[213]. In recent years, mRCT has been redefined and the transport of cholesterol from peripheral tissues, such as the atherosclerotic lesion, to the bloodstream, has been clarified[214]. The lymphatic vessels were shown to be the critical route that facilitates transport of cholesterol, by cholesterol acceptors such as HDL, from the aortic wall to the bloodstream where it can be eliminated (Figure 4)[215].

Figure 4. Overview of the updated reverse cholesterol transport pathway.



Within the atherosclerotic lesion, cholesterol is effluxed from macrophages and uploaded onto cholesterol acceptors like HDL, which will then cross the medial layer of the artery to reach the adventitia. From there, it will enter the adventitial lymphatic capillaries and get propelled through the afferent collecting lymphatics to attain the draining lymph node. It sequentially

passes through the efferent collecting lymphatic to then join the bloodstream at the level of the subclavian vein[215]. The next steps imply an internalization of the solubilized cholesterol in circulation, by the liver *via* the SR-B1 receptor[216]. In parallel, cholesterol from HDL can also be transferred to VLDL and LDL *via* CETP[217], a pathway that is not present in mice, and be internalized by the liver *via* LDLR, to also be secreted into the bile[217, 218]. Figure was adapted from Servier Medical Art http://www.servier.com/slidekit/?item=16

Considering that atherosclerosis is a chronic inflammatory disease and that stimulation of lymphatic function has been associated with the amelioration of inflammatory conditions in several other disease settings[219-223], it then becomes possible that modulating the lymphatic function could in turn also modulate atherosclerosis. In the heart, evidence shows that blocking lymph flow may contribute to cardiac lymphedema and poor heart performance in animal and human heart studies[224]. Lymphedema is characterized by overaccumulation of interstitial fluid in cases of impaired lymphatic drainage. There are two types of lymphedema, one is primary which is inherited and generally affects the lower extremities. The secondary type is caused by cancer-related surgeries, therapies or infections[225].

It was back in 1981 that Lemole observed intimal vessel thickness following lymphatic vessel blockage. He proposed that the accumulation of interstitial fluid in the artery wall may contribute to the development of atherosclerosis due to factors present in the intimal edema[226]. Albeit only descriptive, studies analyzing the morphology of LVs in the artery wall allowed for insights between their associations with atherosclerosis. In animal models, LVs have been observed in the adventitia of the artery wall[227], an effect believed to be an important requirement for the draining of local inflammatory cells and cytokines from peripheral tissues[228]. Any alterations in lymphatic drainage aggravate plaque development in part by T cell accumulation[229]. In a model of hypercholesterolemia, lymphatic function was significantly altered and led to impaired dendritic cell migration[230], showcasing the important role of mRCT in removing excess cholesterol from the periphery. In atherosclerotic patients, the presence of lymphatics in the adventitia of the internal carotid demonstrated that their number increases with the severity of atherosclerosis[231]. Furthermore, they showed that a dense LV network can offer protection against atherosclerosis development[232]. A thorough overview of the lymphatic vasculature will be extensively discussed as of chapter 3.

1.5 Treatment

When the body is unable to achieve and maintain healthy cholesterol levels, treatments for atherosclerosis come in many forms and include heart-healthy lifestyle changes, medication, surgery, as well as ongoing innovative clinical trials. The ultimate goal of all these treatments is to prevent atherosclerosis and relieve its symptoms, by reducing specific risk factors in an effort to slow or stop the buildup of plaque.

Generally, medication is the first line of treatment even in the later stages of atherosclerosis. However, in some cases, more aggressive care is needed when narrowing of the artery becomes significantly at risk for complete occlusion. Surgical opening of the affected arteries is performed with placement of a stent or bypass surgery, as follows[233].

Coronary angioplasty improves flow to the heart and relieves chest pain through placement of a small mesh tube, called a stent, that is inserted to mechanically keep the artery open following the procedure. Coronary bypass surgery involves harvesting of a small segment of vein from other areas of the body, such as the leg, arm, or chest, and is then used to bypass a narrowed artery. This redirects the blood and improves blood flow to the heart, relieves chest pain and possibly prevents a heart attack. A carotid endarterectomy can also be performed to remove plaque buildup from the carotid arteries in the neck, thus restoring blood flow to the brain and helps prevent a stroke[234]. Since these methods are invasive and at high risk, a multitude of options are currently available to prevent such drastic measures against CAD, unless otherwise deemed necessary.

1.5.1 Diet and exercise

A healthy diet is part of an overall better physiological state with respect to any disease. Particularly, consumption of fruits and vegetables has been reported to reduce the risk of atherosclerosis. Antioxidant vitamins C and E, along with green and yellow vegetables contain carotenoids and polyphenols, which have been shown to be inversely associated with CAD. Fish is another aliment that is associated with lower risk of CAD and stroke, due to its richness in n-3 polyunsaturated fatty acids (PUFAs). The latter are responsible for reduced platelet aggregation, TG lowering, anti-inflammatory effects and plaque stabilization, all crucial factors in the prevention of atherosclerosis[235]. Reduced PUFA levels were associated with increased

risk of CVD[236]. Even soy beans have recently been reported to be associated with a reduced risk of CAD and stroke in women, as they contain isoflavone which has a similar structure to that of estrogen and bonds with estrogen receptors, thus limiting atherogenesis[235, 237].

Any diet is well accompanied by physical activity to further help improve the blood circulation and lower high blood pressure, a key risk factor for atherosclerosis. A good rule of thumb is to get 30 minutes of moderate aerobic exercise most days of the week. Regardless of the pace, distance or amount, exercise reduces risk of premature death by nearly 40%. Running consists as the main benefactor in life expectancy, adding gains of up to 3 additional years following an input of around 4 hrs/week[238]. However, excessive exercising, as observed with marathoners, has given rise to the "Extreme Exercise Hypothesis" and suggests that adverse cardiovascular manifestations may occur following intense exercise training, which counterbalances the health benefits of a physically active lifestyle. Marathoners had a lower heart rate, body mass index (BMI) and TG count, as well as higher HDL, but coronary atherosclerosis was actually increased, and a higher risk of sudden cardiac death was reported. This was suggested to be due to persistent and prolonged tachycardia and increased blood pressure. Both metabolic and mechanical stress of coronary arteries was generated by excessive functioning[239]. Oxidative stress also was increased, leading to vascular endothelial dysfunction and deterioration of vascular elastic properties, but improvements occurred following antioxidant supplementation[240].

Nonetheless, regular exercise helps prevent atherosclerosis in varying ways. It keeps the arteries healthy by lowering cholesterol and boosting the production of NO by the cells lining the arteries, thus helping with blood circulation. Furthermore, moderate exercise reduces atherosclerotic risk factors such as blood clots, diabetes, obesity and stress[241].

Despite these benefits, lifestyle changes alone are sometimes insufficient to control cholesterol levels or other risk factors and to decrease the chance of having a heart attack or stroke.

1.5.2 Low Density Lipoprotein management

Without lipid-lowering medications, 5-year intensive lifestyle changes reduced LDL by 37% and in turn reversed the progression of coronary atherosclerosis. Despite this impressive

achievement, some patient's cholesterol levels still remain elevated and established lipid-lowering agents need to be prescribed[242].

1.5.2.1 HMG-CoA inhibitors

Statins are first-line drugs for the treatment of dyslipidemias and CAD prevention through their effects of lowering LDL levels by up to 60%[243]. As analogues of the HMG-CoA reductase substrate, they bind to it and inhibit the conversion of HMG-CoA to mevalonate, which is the rate-limiting step in *de novo* cholesterol synthesis[244]. As such, this inhibition decreases cellular cholesterol concentrations by increasing the expression of LDLR[245]. A variety of statins are currently available including atorvastatin, fluvastatin, lovastatin and pravastatin[246].

Lowering LDL is considered the main contributor to reduced risk of CAD. Statins inhibit immune cell proliferation and activation, improve endothelial function by increasing NO production which reduces ROS production and limits LDL oxidation, increase plaque stability, and inhibit platelet adhesion and aggregation[242].

This lipid treatment focuses mainly on endogenous LDL, and results neglect other important aspects of lipoprotein metabolism[247]. Furthermore, the most important statins side effect is severe muscular pain[248]. Among others, statin therapy has been associated with new-onset diabetes mellitus, very rarely increased liver injury, and a small increase in risk of stroke in patients with prior stroke. However, long-term statins treatment is considered safe with a low risk of pertinent adverse effects. Nonetheless, the established cardiovascular benefits of statin therapy are considered to heavily outweigh the risk of adverse effects[249].

1.5.2.2 Inhibitors of intestinal sterol absorption

Ezetimibe inhibits the intestinal cholesterol uptake at the level of the brush border of the enterocyte by inhibiting the absorption protein Niemann-Pick C1-Like 1 (NPC1L1) present on jejunal intestinal cells. This effectively inhibits intestinal cholesterol absorption from dietary sources as well as from bile and can reduce hepatic cholesterol concentration and circulating LDL[250]. Therapy that combines ezetimibe with a statin decreased the risk of cardiovascular events when compared to statin administration on its own[251].

1.5.2.3 Bile acid-sequestering agents

Resins, such as cholestyramine, colestipol and colesevelam were used as primary cholesterol lowering agents prior to statins and can reduce LDL up to 30%[252]. Following oral administration, these molecules are positively charged and non-digestible, so they stay in the GI tract and bind to bile acids to form insoluble and non-absorbable complexes that end up being excreted into the feces. Under normal conditions, close to 95% of bile acids are reabsorbed, but reducing their absorption leads to an increase in endogenous synthesis. Furthermore, similarly to statins and ezetimibe, resins upregulate LDLR which increases LDL uptake and removal of VLDL remnants[242].

1.5.2.4 Proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors

PCSK9, responsible for the degradation of LDLR and reduced metabolism of LDL, was discovered more than a decade ago in patients with FH and led to subsequent experimental studies of possible PCSK9 inhibitor approaches. In 2015, two PCSK9 antibodies, alirocumab and evolocumab were approved by the Food and Drug Administration (FDA) as additional therapy for patients with hypercholesterolemia who need further LDL lowering despite a healthy diet and a maximal dose of statins. These human monoclonal antibodies bind to PCSK9 and prevent its binding to LDLR, which reduces its degradation and increase hepatic uptake and metabolism of LDL. Clinical trials showed significantly reduced LDL and major cardiovascular events at 24 and 78 weeks over other therapeutic options[253, 254]. Large clinical trials support the use of PCSK9 monoclonal antibodies in addition to statins, in patients at high risk of developing CAD[255]. Therefore, the goal is to develop PCSK9 inhibitors that work together with statins to provide maximal efficiency in LDL lowering. Since SREBP also controls the expression of PCSK9, thus increasing PCSK9 serum levels following a moderate statin dose, this could explain why doubling the dose of statins only reduces LDL by another 6%[256, 257]. Despite these promising results, long term effects need further investigation.

In addition, trials are also testing other classes of agents that target PCSK9 such as RNA interference (RNAi), vaccines and antisense targeting. PCSK9-directed RNAi binds to RNA-induced silencing complex (RISK), leading to degradation of PCSK9 mRNA, reducing its translation. Inclisiran, a PCSK9 RNAi, reduced LDL by 59% at the end of two months following one injection and 57% at the end of 4 months following two injections, indicating that RNAi

has a long-lasting effect and requires fewer injections[258]. Anti-PCSK9 vaccines show promising effects on animal models that can last for up to 10 months. The PCSK9Qβ-003 vaccine decreased plasma PCSK9 levels and in turn increased LDLR expression in liver[259]. Several PCSK9 orally effective antibodies are also in development. Effective oral agents, such as Adnectin (BMS-962476), could provide an alternative to administration by injection[260]. Similarly, using antisense oligonucleotides to decrease intracellular expression of PCSK9 led to promising results in preclinical and phase I trials, where a reduction in LDL of 38 to 50% and doubled LDLR expression in the liver were observed. However, further investigations were discontinued for now, especially in human subjects[261]. PCSK9 inhibitors have nonetheless been incorporated since 2018 in the Cholesterol Guideline algorithm to be used as secondary prevention in patients whose LDL levels are still higher than 70 mg/dL, despite maximally tolerated statin plus ezetimibe dual therapy[262].

1.5.2.5 Orphan drugs to treat homozygous familial hypercholesterolemia

FH is inherited hypercholesterolemia that is mainly caused by the deficiency of LDLR that leads to reduced LDL metabolism. Patients that have homozygous FH (HoFH) have two abnormal copies of the LDL receptor gene and LDL receptor activity less than 2% of normal individuals[242]. The focus in these patients is to pharmacologically target the production of LDL rather than its uptake, and so, statins, bile acid sequestrants and PCSK9 inhibitors are not sufficient to lower LDL levels, due to minimal LDLR activity. Currently two drugs are approved for HoFH, mipomersen, an antisense of apoB-100, and lomitapede a microsomal triglyceride transfer protein (MTP) inhibitor. Since HoFH is rare, with less than 200 000 people suffering from this disorder, these drugs are considered orphan drugs.

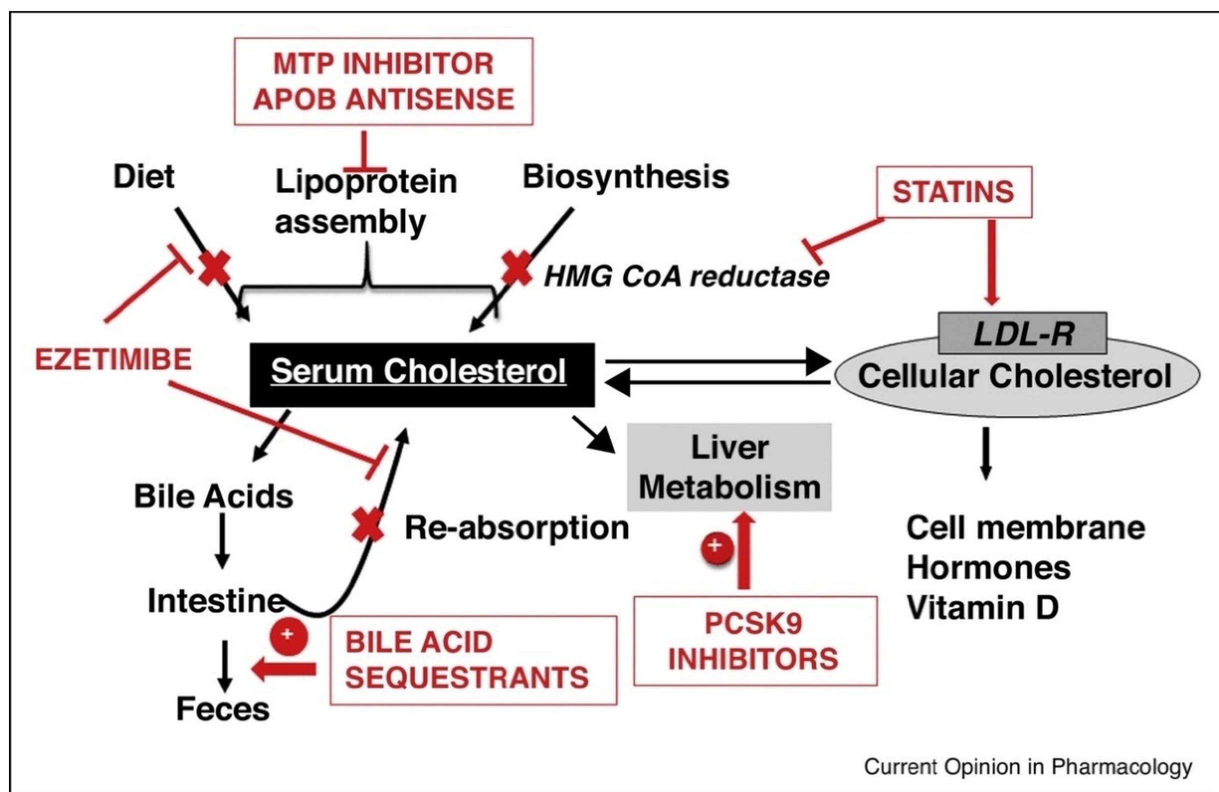
Mipomersen is an apoB-100 antisense, that enters hepatocytes and interferes with translation to reduce synthesis of apoB-100, the main lipoprotein of LDL and VLDL. Clinical studies revealed that weekly injections with mipomersen for 26 weeks reduce LDL by up to 36%, with no changes observed in HDL[263].

Lomitapide binds to MTP and inhibits its function, thus reducing biosynthesis and plasma levels of both CMs and VLDL, and consequently decreases plasma levels of LDL, TGs, and Lp(a)[242]. Inhibition of MTP with different doses of lomitapide resulted in the reduction of LDL cholesterol levels by up to 51%. Since preventing VLDL formation is highly cytotoxic

for the liver, the FDA has mandated that patients should be monitored for this potential side effect[264].

Despite the reduction of LDL by using cholesterol-lowering medication (Figure 6), the risk of cardiovascular events remains especially increased in patients with low HDL levels and high levels of TGs[242].

Figure 6. Summary of the mechanisms of action of cholesterol lowering drugs.



An overview by Chang *et al.* of the different levels at which a broad array of cholesterol lowering medications act to keep under control circulating cholesterol levels, particularly LDL[242]. Permission obtained from © 2017 Elsevier Ltd.

1.5.3 High Density Lipoprotein pharmacotherapeutic strategies

The inverse relationship between HDL levels and CAD incited a lot of interest in pharmacological agents that aim to elevate plasma levels of HDL. Niacin and Vitamin B3 are effective HDL raising agents currently on the market that first emerged as dyslipidemia treatment[265]. However, two large randomized trials of niacin, AIM-HIGH and HPS2-THRIVE, disappointingly came to the conclusion that despite acting to raise HDL levels, this did not translate to a decrease in the incidence of cardiovascular events and may even have caused more harm by placing patients at risk of significant side effects[266].

As statins offer little to no effect on increasing HDL levels, efforts were rather focused on alternative ways to increase these levels, such as directly through the inhibition of CETP, which transfers cholesteryl esters from HDL, to VLDL or LDL in exchange of TGs[267]. Several CETP inhibitors have been tested, like anacetrapib, evacetrapib, and dalcetrapib, and have shown that in patients already receiving statin therapy, the three CETP inhibitors increased HDL levels by approximately 140%, 80% and 30%, but similar to the niacin trials, these failed to reduce cardiovascular events[242]. Disappointingly, torcetrapib actually ended up showing an increase in cardiovascular risk[268]. Interestingly, inhibiting CETP solely changes the pathway of cholesterol excretion, thus occurring through HDL instead of VLDL/LDL, but mRCT efficacy remains unchanged. This means that although cholesterol efflux may be augmented, since there are more acceptors in circulation, their proper transport lacks. Additionally, in humans it is believed that the main uptake of CE by the liver is through particles that contain apoB, more so than HDL itself[269]. HDL may also transfer apoE to LDL which could also bind and be uptaken by the liver through alternative receptors, in parallel to LDLR-mediated cholesterol uptake. Alternatively, recombinant LCAT restored HDL-mediated NO production *in vitro*, supporting LCAT as an interesting new target that may aid in restauration of HDL functionality in different setting such as acute coronary syndrome[270].

Currently, more and more experts agree that it is not the HDL cholesterol amount that matters most, but rather, the functionality of HDL particles, that has a greater potential in some diseases such as CAD. HDL particles also have antiatherogenic properties that extend to antioxidation, protection against thrombosis, maintenance of endothelial function, and

maintenance of low blood viscosity through an action on red cell deformability[271]. The antioxidant properties of HDL are suspected to be due to the association of HDL with paraoxonase. Small and dense HDL particles exhibit more antioxidant activity than large particles, due to stronger attachment of paraoxonase to these particles[272]. Further contributing to plaque impediment, HDL improves endothelial function by increasing eNOS production[273] and inhibiting cellular adhesion molecule expression[274].

In recent years, discrepancies in clinical responses have been shown to depend on patients' genetic profiles. In a pharmacogenomic genome-wide association study (GWAS), beneficial dalcetrapib effects on cardiovascular outcomes benefited patients with polymorphisms in the adenylate cyclase isoform 9 (*ADCY9*) gene[275]. To date, patients treated with dalcetrapib that had the AA genotype of the *ADCY9* gene had a 39% reduction in cardiovascular events, whereas those with the GG genotype had a 27% increase in events. Of notice, is that only about 20% of patients have the AA genotype and only to these specific patients dalcetrapib may be beneficial[276]. Recently, a phase 3 trial using anacetrapib, specifically targeting patients with the AA mutation announced no significant differences between groups. Nonetheless, the dal-GenE study continues and is currently underway in which 6 000 patients with the AA genotype will be randomized to dalcetrapib or placebo, since the two drugs inhibit CETP in different ways.

GWAS has emerged as an essential tool to identify gene variants associated with altered lipoprotein metabolism and the risk of CAD. This has already allowed to develop specific targets for drug therapy and has resulted in novel study designs such as Mendelian randomization that aims to demonstrate causality between the trait and CAD[277]. A lot of evidence based on this concept confirmed LDL, using PCSK9 nonsense mutations, and Lp(a) as causal risk factors for CVD but negated the role of CRP, HDL and homocysteine as causal factors. As such, Mendelian randomization studies emphasize the importance of some inflammatory markers such as IL-6, as causal factors for CAD[278]. This will further help identify markers of increased cardiovascular risk and promote development of new drug therapies to relieve atherosclerosis burden.

The intravenous recombinant form of apoA-I Milano is a naturally occurring variant of apoA-I associated with protection from vascular disease despite very low levels of HDL cholesterol. The ETC-216, showed promise when a significant reduction in coronary atheroma

volume using intravascular ultrasound (IVUS) was observed[279]. Unfortunately, recently, the MILANO-PILOT trial where infusion of a new mimetic preparation MDCO-216 of apoA-I Milano was administered to patients with an acute coronary syndrome did not lead to significant plaque regression[280]. Similarly, another trial infusing apoA-I, more specifically therapy with CER-001 which is an engineered lipoprotein particle mimicking pre-βHDL, to promote cellular cholesterol efflux and mRCT have provided negative results to date[281]. However, Rader *et al.* are not discouraged, rather, they believe short-term plaque regression as assessed by IVUS may not be an appropriate measure for the reduction in cardiovascular events. Furthermore, they suspect that apoA-I has stabilizing effects that do not decrease the size of plaque. A third apoA-I product, CSL112, is still in clinical development and has recently entered phase III clinical trials[282]. Importantly, the functionality of each molecule can differ in composition, dosing, pharmacokinetics and pharmacodynamics and as such, results may differ and may need to be studied differently. Even the way they are reconstituted can affect their functionality. Although the MILANO-PILOT trial showed that administering MDCO-216 in patients with CAD increased cholesterol efflux, this did not result in any favorable effects on lesion size and LDL levels[283]. This is why alternative venues urge to be studied.

1.5.4 Triglyceride management

Hypertriglyceridemia is generally associated with acute pancreatitis, and while some studies have shown its causative relationship to CAD, others showed failure to provide cardio-protective effects[242]. Nonetheless, several classes of drugs can be used to lower TGs, such as fibric acid derivatives like gemfibrozil, bezafibrate and fenofibrate, niacin, omega-3 fatty acids, and even statins[284].

Fibrates can reduce plasma TG levels by up to 50% and raise plasma HDL concentrations as much as 20%[285]. Fibrates bind to peroxisome proliferator-activated receptor alpha in muscle, liver, and other tissues, and induce the expression of LPL, inhibit expression of apoC-III, and reduce production of apoB-100 and VLDL[286].

Niacin can lower plasma TG levels by up to 45%, raise HDL by up to 25%, and reduce LDL by up to 20%[287]. The best known side effect of niacin is flushing[288] and since it is a drug that has been discovered many years ago, it is commonly looked at as ineffective.

Nonetheless, niacin was shown to reduce DC accumulation into draining LNs highlighting some hopeful anti-inflammatory effects[289].

PUFAs, although not very well understood, reduce hepatic production of VLDL and promote the clearance of TGs. Omega-3 PUFAs can reduce TG levels by 20 to 50% and are currently approved for patients with severe hypertriglyceridemia[242].

1.5.5 Antiplatelet and atherothrombosis drugs

Platelets participate not only in arterial thrombosis, but also in the initial development of atherosclerosis, which motivated the use of anti-aggregation drugs in the hopes of limiting the disease. The first line of protection was the use of aspirin, recommended for the primary prevention of CVD for patients with moderate-to-high risk. Aspirin irreversibly inhibits cyclooxygenase-1, an enzyme responsible for the formation of prostaglandin H2, the precursor of TXA_2[290]. However, data regarding aspirin efficacy has debatably overestimated its beneficial effects, since the incidence of patient cardiovascular events during aspirin treatment remains high[291].

Clopidogrel belongs to the thienopyridine family and is one of the most used oral platelet antiaggregant that selectively and irreversibly prevents the linkage of ADP to its platelet receptors P2Y1 and P2Y12. This hinders the activation of the complex GPIIb/IIIa and therefore, the linkage to fibrinogen, thus inhibiting platelet aggregation[192]. Clopidogrel has often been used to prevent atherosclerotic events in patients with a history of acute MI and in patients with CVD that presented exaggerated formation of leukocyte-platelet aggregation. Following clopidogrel treatment, the latter had decreased expression of such formations, and a decreased expression of P-selectin and CD40L[292, 293]. Results from the CAPRIE trial show that in patients with a history of ischemic disease, clopidrogel was more effective than aspirin in reducing the production of new ischemic events or vascular death[294]. Furthermore, the use of clopidogrel for five weeks in patients with coronary artery disease resulted in a decrease in the markers of inflammation such as CRP and CD40L, as well as in an improvement in the bioavailability of NO at the endothelial level[295].

Thrombin is one of the most potent platelet activators and its receptors, called protease-activated receptors (PAR), have a very high affinity, with the most important one of these in humans being the PAR-1 receptor[296]. In preclinical and small clinical trials PAR-1 inhibition

has been shown to inhibit thrombin mediated platelet activation without an increase in bleeding, which is important as it protects haemostasis unlike the ADP and TXA_2 pathway inhibitors[297]. Two oral antagonists of PAR-1 are in clinical development, vorapaxar and atopaxar. Phase 3 clinical evidence for vorapaxar shows promise regarding its use for the reduction of thrombotic cardiovascular events in higher-risk patients with a history of MI or peripheral artery disease. Nonetheless, careful patient selection is needed for its future development. Phase 2 clinical evidence for atopaxar reported an increased bleeding risk, as well as liver and cardiac toxicity, which halted progression of trials for the moment[298].

Activation of the GPIIb/IIIa receptor is another pathway of platelet aggregation and is inhibited by abciximab, eptifibatide and tirofiban. These potent inhibitors are intravenous agents and have been well studied clinically in acute coronary syndromes[297].

Dual antiplatelet therapy has more recently emerged as a standard of care in acute coronary syndromes, and aspirin is usually used in combination with clopidogrel or one of the newer more potent antiplatelet agents[299].

An emerging therapeutic venue is plasma apheresis, particularly important in patients with FH and thrombotic thrombocytopenic purpura. While LDL apheresis has proven efficient, and the plasma exchange has also reduced blood viscosity, negative effects were observed due to oxidative injury to red blood cells (RBCs)[300].

Numerous notable research and advances in atherosclerosis have been made to date. The ongoing interest in reducing residual cardiovascular risk in patients who currently are believed to undergo optimal treatment for CVD is forefronting the search for new targets and drugs to modulate the atherosclerotic process. Atherosclerosis represents a complex multifactorial system and more thorough work is needed to bring forward novel biomarkers, imaging techniques and therapeutic targets for atherosclerosis. As described in the sections to come, very promising venues lie ahead.

2 EXTRACELLULAR VESICLES

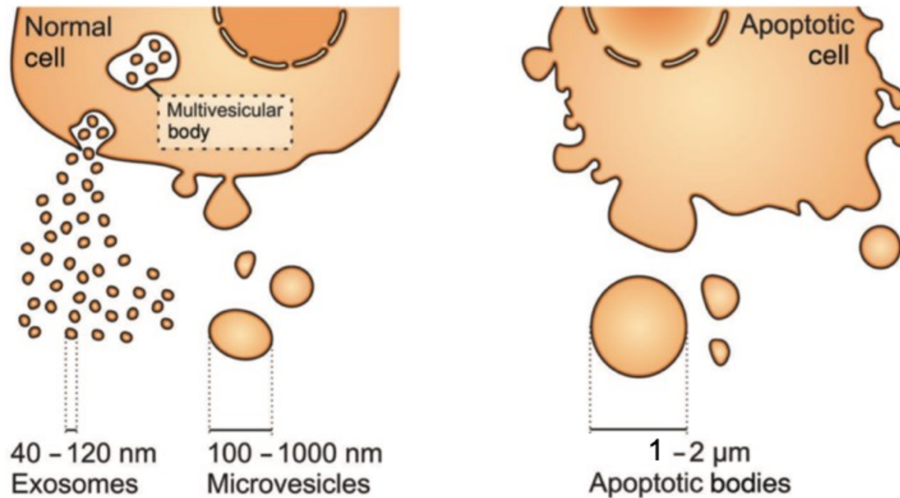
The release of EVs is a process conserved through evolution, which indicates how essential their role is physiologically. Hence, prokaryotic, plant, and eukaryotic cells all produce EVs[301]. Recently, increased levels of EVs, especially from specific cell types such as platelets, RBCs and ECs have emerged as potent atherosclerotic modulators as some may cause vascular damage leading to permeability, promote thrombogenesis and release inflammatory stimuli, alongside other diverse functions[40].

EVs constitute a heterogeneous population of submicron vesicles harboring biological material and surrounded by a lipid bilayer similar to that of a cell plasma membrane, in contrast to the single-layered HDL and LDL found in all body fluids[302, 303]. Cellular EVs in body fluids differ in morphology, cellular origin, size, number, antigenic composition and functional properties[304]. Their composition varies as they may contain proteins, lipids, nuclear material, DNA, microRNAs[305], non-coding RNAs, and/or surface receptors and antigens that originate from different cells, and that depend on physiological conditions[306]. EVs participate in intercellular communication[306], and due to their diversity and prominent presence in almost all tissues, they are considered important potential biomarkers[307]. Once uptaken by different cells, EVs are able to transfer their functional cargo and alter their status[308]. Therefore, it is critical to assess EVs in various biological fluids to delineate their functions.

2.1 Classification

The classification and characterization of vesicles remains a subject of debate, but some consensus has been reached (Figure 7). Classified according to their size and mechanism of formation, it is now possible to distinguish these distinct released populations as follows: exosomes, exocytosed from multivesicular bodies; microvesicles (MVs) that bud directly from the plasma membrane; and apoptotic bodies which result from apoptotic blebbing following cell death[309].

Figure 7. Cells produce different types of extracellular vesicles that vary in size.



This illustration was adapted from Zoborowski *et al.* to reflect most recent consensus regarding EVs size. Furthermore, it demonstrates how exosomes are formed and released from intracellular compartments, alongside MVs that emerge from the cell surface, in either healthy or diseased cells. Alternatively, apoptotic bodies are created when apoptotic cells disassemble[308]. Permission granted from Copyright © 2015, Oxford University Press.

2.1.1 Exosomes

Exosomes constitute the smallest subpopulation of EVs with a size ranging between 40 to 120 nanometers (nm)[308]. Confusion on the origin and nomenclature of EVs has been highly debated numerous times due to the fact that vesicles with the size of exosomes that bud at the plasma membrane have also been called exosomes[310]. Secreted by various types of cells, exosomes are formed by the fusion between multivesicular bodies and the plasma membrane, which is an active process[311]. The multivesicular bodies are formed by budding from an endosome[312], itself the result of an internal budding of the plasma membrane, and then contain organelles of the endosomes[313]. They are involved in several functions related to endocytosis and protein trafficking such as sorting, recycling, transport, storage and release[314]. These multivesicular bodies can thereafter fuse with the plasma membrane and be released into the extracellular medium to form exosomes. They then contain endosome-

associated proteins, such as GTPase Rab, SNAREs, Annexins and flotillin[309]. They also harbor a set of evolutionally conserved proteins, such as Tsg101, Alix and are rich in lipids, such as cholesterol and ganglioside GM121[314-316]. Additionally, they contain heat shock proteins HSP60, HSP70, HSPA5, CCT2 and HSP90[317]. Tetraspanins, such as CD9, CD63 and CD81 are the most frequently identified proteins and by now, they are considered good general markers of exosomes[318]. However, they may be expressed by other subtypes of EVs emerging from the plasma membrane due to their presence on the surface of various cell types and have already been identified on the surface of MVs as well[319].

Exosomes contain more sphingomyelins, gangliosides, and desaturated lipids, while their phosphatidylcholine and diacylglycerol proportion is decreased when compared to the membranes of the cells from which they originate[302]. Furthermore, they are enriched with nucleic acids, such as messenger RNA (mRNA) and micro RNA (miRNA), further supporting the hypothesis that they are a biological vehicle with the ability to modulate the target cell's protein synthesis and can confer new functions to the target cell[305]. They have been identified in several body fluids, such as plasma, urine, saliva, bile, breast milk, sperm, amniotic fluid, cerebrospinal fluid, ascites fluid[303, 320-325], and most recently, in lymph[303]. They can be released by practically any cell type following activation or apoptosis, including ECs, platelets, RBCs, SMCs, DCs, monocytes and macrophages, and cardiomyocytes[326]. Importantly, the microenvironment features, such as hypoxia, also exert important effects on the properties of the origin cell-derived exosomes[319]. For example, exosomes produced by cells exposed to oxidative stress can mediate protective signals, thus reducing oxidative stress and cell death in recipient cells[327]. Exosomes can also contain cytokines that induce inflammation via numerous different pathways[328], and can contribute to cell aggregation following neutrophil and leukocyte recruitment[329]. Therefore, exosomes, and especially their cargoes, play different key roles in various normal physiological instances and pathological responses to disease.

2.1.2 Microvesicles

MVs bud directly from the plasma membrane, measure approximately 100 nm to 1 micrometer (μm) in size and contain cytoplasmic cargo[308]. They are formed by the

remodeling of the cytoskeleton, and their release is increased under inflammatory conditions, hypoxia or activation[330, 331]. Following cell stimulation, a cytosolic influx of Ca^{2+} may disrupt the asymmetric distribution of phospholipids in the membrane bilayer by activation of the scramblase involved in the translocation of membrane phospholipids. This results in redistribution of phospholipids. Subsequently, this leads to phosphatidylserine (PS) translocation, creating an imbalance between the internal and external leaflets that leads to budding of the plasma membrane and MVs release. The cytoskeleton degradation caused by the Ca^{2+}-dependent proteolysis promotes the budding of these vesicles which can then express at their surface phospholipids such as PS, that normally constitute the inner membrane layer[332]. This translocation can then be used to identify them[312]. However, it has already been observed in plasma that not all MVs externalize PS[330, 333], but they contain flotillin-2, selectins, integrins and metalloproteinases[314].

Highly discussed is the fact that circulating vesicles seem to be composed of both exosomes and MVs. Therefore, currently available purification methods, as will be later discussed, do not yet allow to fully discriminate beyond a reasonable doubt between these two entities. Several cell types however, such as platelets[334], ECs[335], and breast cancer cells[336] have been shown to release both subtypes as characterized by a lipid bilayer.

2.1.3 Apoptotic bodies

Apoptotic bodies are released as a result of apoptotic cell disassembly and consist of apoptotic material surrounded by a permeable membrane. Apoptotic bodies typically range in size between 1 – 5 μm[337], under certain conditions can become more abundant than exosomes and MVs, and greatly vary in content between biofluids[338].

Several changes affect cell morphology during apoptosis, such as cell contraction, membrane budding, and DNA fragmentation[339]. Contractive forces mediated by actin and myosin filaments leading to the formation of the apoptotic bodies are then produced. They can contain intact organelles, DNA and histones, and can therefore be labeled with propidium iodide intercalated in the DNA[340]. The rapid clearance of apoptotic body fluids by phagocytic cells complicates their characterization[341].

Although EVs originating from healthy cells, such as exosomes and MVs have been more thoroughly studied to date, as they are significantly and differentially involved in diverse

pathologies, apoptotic bodies have similar functional importance with respect to immunomodulatory effects. Exosomes in particular represent an attractive mean of cargo transportation, so many studies to date focused on understanding the precise functions of these smaller entities. Interestingly, apoptotic cells are also suspected to release exosomes, but it remains to be confirmed[342]. Apoptotic bodies are created to aid in apoptotic cell clearance, as well as a means of intercellular communication. They are involved in the horizontal transfer of DNA including tumor DNA that can result in the induction of a tumorigenic phenotype, in the presentation of epitopes to T cells via internalization by phagocytic cells and in the presentation of autoantigens to B lymphocytes[340, 343]. Despite healthy cells undergoing apoptosis for turnover, apoptosis is just as important in different immunological disease settings such as inflammation, infection, autoimmunity, and cancer[344-346].

2.2 Internalization

An abundance of mechanisms can be involved in the internalization of EVs inside target cells[314]. Evidence supports EVs internalization, such as mRNA and miRNA transferred from mouse to human mast cells that led to identification of the respective proteins in these cells[305], small interfering RNA (siRNA) delivery that knocked down target gene expression[347], and production of bioluminescence following administration of EVs loaded with luciferin substrate to luciferase expressing cells[348]. One of the most widely used methods to detect EVs uptake is through the use of fluorescent lipid membrane dyes to stain EVs membranes, such as PKH67, PKH26, rhodamine B, DiI and DiD[349]. Importantly, some downside to lipid-anchored fluorophores is that they can be non-specific due to the contamination of lipoproteins within the samples and they can even aggregate and form particles similar in size to EVs[350]. Popular alternatives include membrane permeable chemical compounds like carboxy- fluorescein succinimidyl ester (CFSE)[351] and 5(6)-carboxyfluorescein diacetate (CFDA)[352]. A third dye has recently emerged as well, the Cell Trace Violet (CTV) and allows for use of the 488nm laser since it is excited by blue laser light[353]. The markers locate to the cytosol and emit fluorescence following esterification. Using methods such as flow cytometry and confocal microscopy, subsequent entry of EVs into recipient cells can be measured. Importantly, differentiation between EVs cellular internalization and cell adhesion is not always clear, but remains indispensable, so stripping of the cell surface with acid[354] or trypsin[355], or

quenching of the surface fluorescence[356] can be performed. Impressively, up to ten different markers on the same EV can be detected with this last technique. Single EV analysis, a multiplexed biomarkers technique that can assess several different markers on individual EVs, was also developed and allows for specific characterization using fluorescent microscopy[357]. Even an enzyme-linked immunosorbent assays (ELISA) kit can now be used to detect exosomes, as it contains specific antibodies able to bind these vesicles in a variety of biological fluids[358].

Different surface proteins expressed by EVs can interact with receptors on the surface of target cells[349]. Currently, an increasing number of specific protein-protein interactions that seem to differentially mediate EVs uptake/internalization are on the rise[349]. Tetraspanins, often found in clusters along with adhesion molecules and transmembrane receptor proteins, are abundantly expressed by EVs in raft-like structures in their plasma membrane and are generally involved in adhesion, displacement, fusion, activation and proliferation[318, 359]. Inhibition of tetraspanin expression with antibodies was shown to decrease the internalization of EVs in target cells[360]. Integrins and immunoglobulins, involved in leukocyte adhesion and transmigration, as well as intracellular signaling, also seem to be involved in EVs uptake[349]. Blockade of CD11a, its ligand ICAM-1, CD51 and CD61 each caused a decrease in the internalization of EVs by dendritic cells[349, 361]. Proteoglycans are entities that contain a significant amount of carbohydrate components, such as heparin sulphate proteoglycans. Treating bladder cancer cells with a heparin sulphate mimetic reduced cancer cell exosomes uptake thus showcasing their role as key receptors of macromolecular cargo[355, 362]. Lectins such as DC-SIGN, DEC-205 and Galectin-5, which can trigger phagocytic entry, have also been associated with the internalization of EVs through inhibition or competition experiments[363-365].

Endocytosis is another mechanism largely involved in the internalization of EVs. Uptake of EVs within the endosome can occur in a matter of minutes and this internalization was significantly reduced at 4°C, which demonstrates that endocytosis of EVs is an active process that requires energy[349]. Cytochalasin D, an actin depolymerizing agent that alters endocytosis in mammalian cells, has also contributed to a decrease in EVs internalization in several cell types[349].

Clathrin-mediated endocytosis involves the progressive formation of clathrin-coated vesicles expressing several transmembrane receptors, as well as their ligands. This allows them

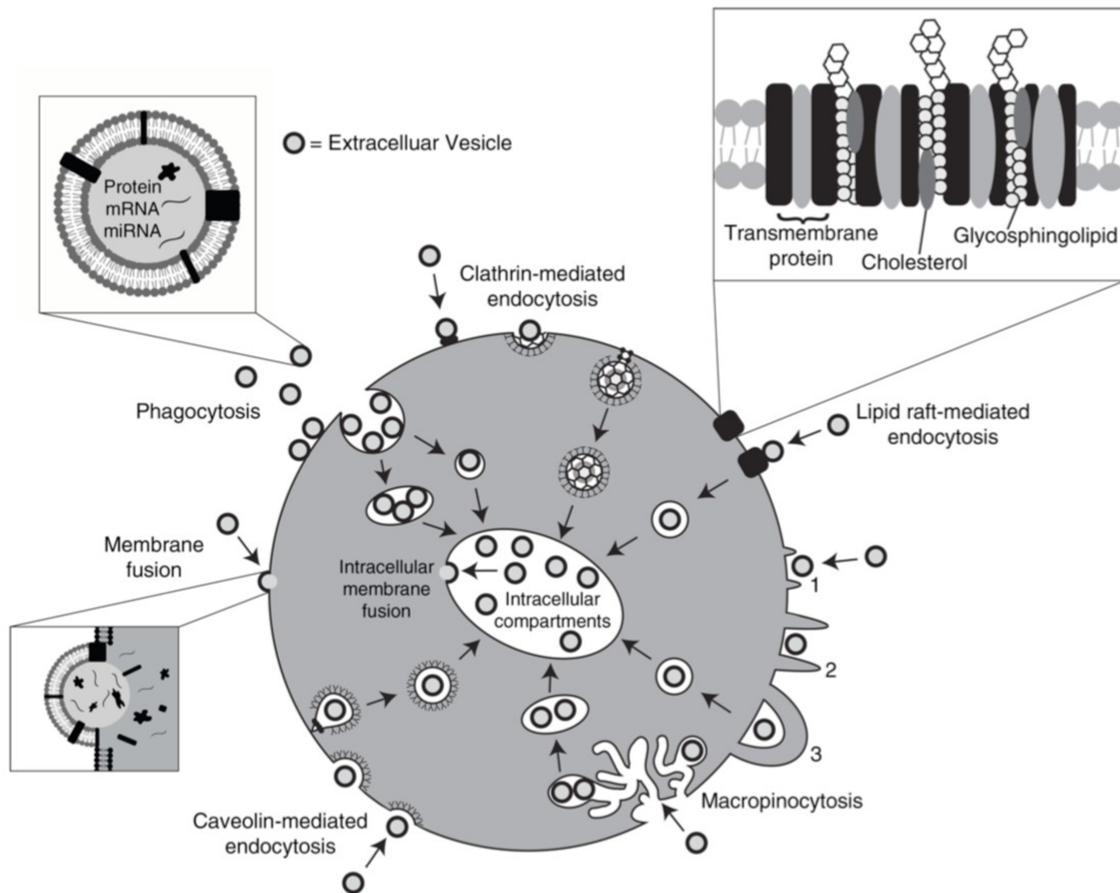
to integrate the target cell, undergo clathrin un-coating and fuse with the endosome where it can release its contents[366]. Alternatively, caveolin-dependent endocytosis involves the invagination of caveolae, which are subdomains of lipid rafts, formed by the action of caveolin[367]. Contrarily to clathrin-mediated endocytosis, caveolin-dependent endocytosis showed sensitivity to cholesterol depletion[368]. Several studies demonstrate that both types of endocytosis are involved in the internalization of EVs. Blocking dynamin2, a highly conserved GTPase involved in endocytosis and vesicle transport[369], significantly impaired exosome entry into cells[370]. Since dynamin2 is relied upon by both types of endocytosis, further confirmation of caveolin-dependent endocytosis -specific implication requires knockdown of the *Cav1* gene which results in significantly impaired EVs uptake[371]. EVs can also be internalized via macropinocytosis that involves the invagination of the cell membrane ruffles that contain an extracellular fluid zone, and then pinches off into the intracellular compartment of the cell. This pathway is rac1-, actin- and cholesterol-dependent[349]. Na^+/H^+ exchange activity is also required[349, 372]. Inhibition of the Na^+/H^+ exchanger activity with amiloride, and of rac1 with NSC23766, greatly reduced EV uptake in microglia[349, 373]. Recently, EVs were shown to depend mainly on macropinocytosis and clathrin-independent endocytosis to enter cells[374]. Phagocytosis is induced by physical contact with receptors on the surface of specialized phagocytic cells, such as macrophages, having previously formed invaginations intended to internalize given agents[375]. The use of wortmannin and LY294002, PI3-kinase inhibitors that prevent phagosome formation, caused dose-dependent inhibition of the internalization of exosomes within macrophages[349, 354]. PS, important in phagocytosis of apoptotic bodies, is frequently externalized on EVs outer membrane[376] and seems to be involved in their internalization through phagocytosis or macropynocitosis. Treatment with inhibitors that bind TIM4, present on macrophages and involved in PS-dependent phagocytosis, or that directly bind PS, such as annexinV, significantly reduced EVs uptake within macrophages and natural killer cells[354, 377, 378]. Clathrin-independent endocytosis, alternatively called raft-dependant endocytosis, requires functional lipid rafts within the plasma membrane and are dependent on cholesterol[379]. Lipid rafts are found within invaginations formed by caveolin-1 or in planar regions of the plasma membrane that associate with flotillins[380]. As cholesterol reducing agents like filipin and simvastatin have been shown to prevent EVs uptake, lipid rafts are suspected to play a role in EVs internalization[374, 381].

Furthermore, inhibition of lipid rafts prevented the release of platelet-derived EVs (pdEVs) that expose PS at their surface[349, 382].

Lastly, EVs membranes can directly fusion with the cell plasma membrane. Increased exosome uptake at low pH by fusion with melanoma cells was observed *in vitro* potentially due to EVs lipid content and ionic charge, as assessed by pre-treatment with proton pump inhibitors [383].

Since the precise proportions of each mechanism involved in EVs internalization are incompletely defined, the general consensus is that endocytosis is primarily involved through surface binding. Overall, studies suggest that despite the fact that EVs can be uptaken by virtually every cell, through a variety of mechanisms (Figure 8), specificity to certain target cells is not to be neglected, as the uptake of certain EVs is more effective in one cell type than another[349].

Figure 8. Internalization pathways of extracellular vesicles within target cells.



Mulcahy *et al.* illustrate different mechanisms of EVs uptake within a cell, discussed in detail in the section above[349]. Open Access article distributed under the terms of the Creative Commons Attribution-Noncommercial 3.0 Unported License.

2.3 Intercellular communication

Beyond classical signaling through cell-cell contact and soluble factors, such as cytokines, inflammatory mediators, metabolites, and hormones, EVs are now recognized by the scientific community as important mediators of both local and systemic cellular communication[31, 384]. EVs have several functions depending on the number of bioactive molecules, surface receptors, and genetic information they carry, as well as the cell type of origin and the particular physiological and pathological condition at the time of their packaging

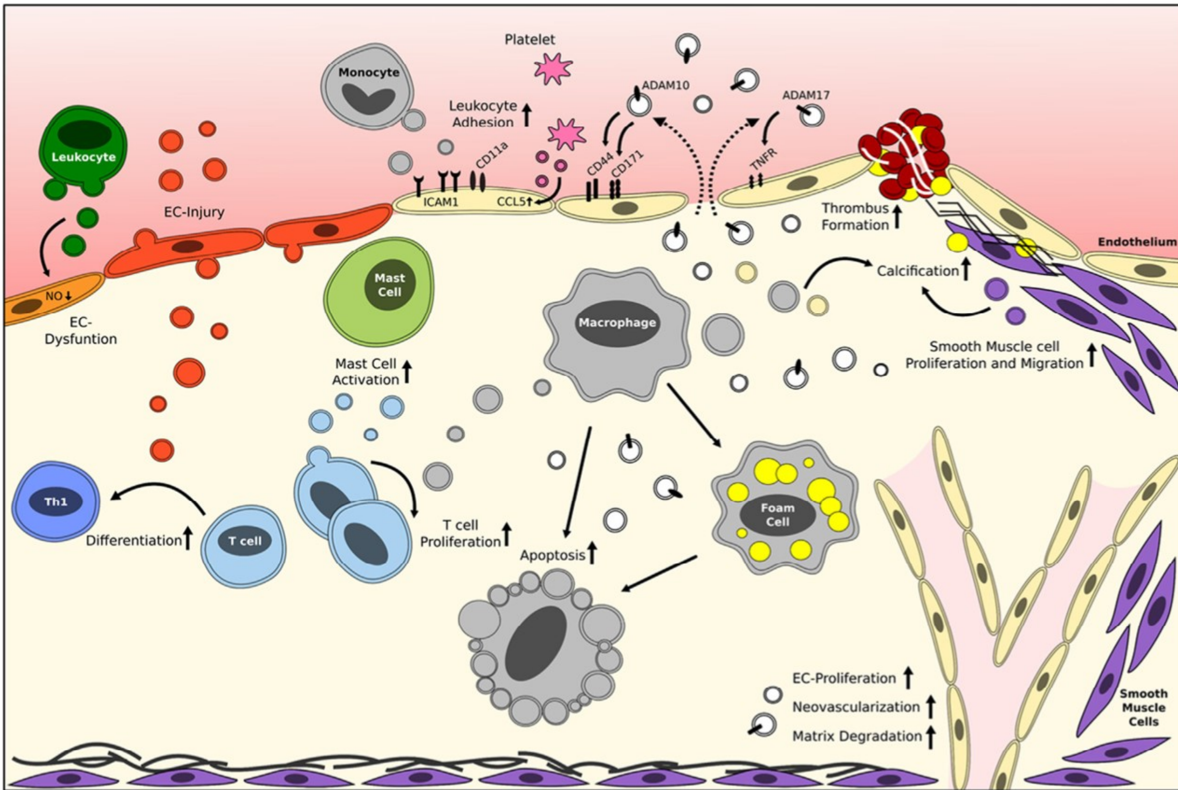
and secretion[348]. Their membrane bilayer gives them the ability to protect their cargo from the enzymes that could degrade them, such as ribonuclease or trypsin[385]. In fact, it has been demonstrated that the mRNAs and miRNAs contained within EVs, once transferred to target cells, are translated into proteins, or regulate gene expression via *de novo* translation or post-transcriptional regulation[305], and may even favor specific signaling cascades in order to induce phenotypic changes[386].

Several examples showcase how EVs can differentially control the function of specific cell types, including types distinct of those from which they originated[314]. Exosomes may exert local paracrine communication, but may also enter systemic circulation to exert more endocrine communication[314]. EVs participate in the maintenance of cardiovascular homeostasis and remodeling, in part by influencing ECM remodeling in response to the environment[31].

2.4 EVs and atherosclerosis

Extracellular vesicles have been associated with various stages of atherosclerosis[330], and the atherosclerotic lesions contain and release EVs derived from leukocytes, platelets, SMCs, and endothelial cells (Figure 9)[387, 388]. Consequently, patients with CVD often show increased levels of circulating cell-derived EVs[39, 389-391]. Furthermore, several risk factors for atherosclerosis are associated with increased levels of circulating EVs in the blood, including diabetes, hypertension, dyslipidemia, and smoking[330]. When it comes to plaque rupture and thrombosis, EVs carry various proteolytic factors that promote matrix degradation and as a result are believed to play an active role in plaque destabilization[392]. Furthermore, presence of MVs in human plaques are associated with thrombogenic effects[388]. With the help of tissue factor (TF) and exposure of PS on the outer membrane layer, MVs can contribute to the coagulation pathway [330, 393]. Contrarily to MVs, exosomes seem to dominate with antithrombotic effects[392]. Several cell types may release extracellular vesicles that may prove to be important players in the progression or regression of atherosclerosis. In the blood stream, approximately 70–90% of total EVs emerge from platelets, 10% stem from granulocytes and only 5% are believed to originate from ECs, RBCs and monocytes[394, 395]. A better understanding of each subtype will allow for not only discovery of novel biomarkers, but also their therapeutic potential.

Figure 9. Extracellular vesicles in vascular inflammation and atherosclerosis.



Van der Vorst *et al.* provided a summarized representation of the multitude of effects that both circulating- and plaque- derived EVs exert[392]. Open-access article distributed under the terms of the Creative Commons Attribution License (CC BY).

2.4.1 EVs released by platelets

Platelet-derived EVs are the most abundant population in the blood[396, 397], despite the fact that erythrocytes are around 30 times greater in number than platelets[398], and range in size between 100 to 1000 nm. Exosomes being released via the exocytosis of multivesicular bodies or α granules, the most abundant organelle in platelets, measure 50 to 150 nm[301]. The megakaryocytes from which the platelets originate can also produce EVs[396], however they will not express surface molecules such as CD62P, LAMP-1, CLEC-2 and GPVI[395]. Nonetheless, megakaryoctye-derived EVs are found in large quantities in circulation[399].

PdEVs are commonly characterized by the expression of surface platelet antigens, including TF, selectins, coagulation factors V and VIII, surface proteins involved in adhesion

such as CD31, GPIb, GpIIb/IIIa and P-selectin, cytokines and chemokines such as IL-1β, CXCL4, CXCL7 and CCL5, and glycoproteins[400-403]. Transfusion of platelet concentrates was shown to cause adverse reactions in recipients, which can be explained by the fact that pdEVs are rich in inflammatory molecules, so they can adhere to leukocytes by CD62P-PSGL-1 interactions and transport proinflammatory signals[404]. They also contain PS, however only a fraction harbor it, and solely a distinct subset contains respiratory-competent mitochondria[301, 405]. The phospholipid composition of pdEVs is intermediate to that of plasma and platelet granule membranes, but exhibits higher cholesterol levels compared to platelets, possibly due to lipid raft enrichment[406]. Since cholesterol enrichment in membranes is critical for the secretion efficiency of platelets[407], its abundance in pdEVs must be particularly necessary to allow them to deliver their cargo from one cell to another.

Platelets can release EVs in response to activation by various agonists such as collagen[408], thrombin[409], C5b-9 membrane attack complex[401, 410], lipopolysaccharides[411], some immune complexes and viruses[412], but also in response to shear stresses[413, 414] and when triggering the GPIIb/IIIa glycoprotein signaling pathway[400]. Interestingly, most agonists trigger the release of a similar miRNA profile while the procoagulant activity of the pdEVs was agonist dependent[415].

PdEVs represent markers of platelet activation in CVD[416]. An elevated number was reported in circulation in various proinflammatory and prothrombotic conditions including atherosclerosis[393, 417-419]. Of note, the number of circulating pdEVs is influenced by cardiovascular medication, such as antiplatelet agents, antihypertensive agents and cholesterol-lowering drugs, which inhibit their release from platelets[395, 420, 421]. PdEVs have been associated with increased blood endothelial permeability[330, 422] and were shown to attach to activated ECs and recruit activated platelets to the sites of vascular damage[423]. This is due to induction of endothelial production of ICAM-1 followed by secretion of inflammatory cytokines such as IL-1b, IL-6, and IL-8 by activated ECs[424]. In MI, platelets were reported to release different functional miRNAs such as miR-191 that are uptaken by ECs and induce ICAM-1 expression in ECs[425].

Platelet-derived exosomes and MVs exhibit diverse effects on inflammation. Contrarily to MVs, exosomes contain P-selectin on their surface[426], and were shown to possess proinflammatory properties and promote immune reactions, such as activation of ECs and

recruitment of monocytes[427]. Furthermore, exosomes produced by proinflammatory activated platelets carry the CD40L, a costimulatory molecule that is involved in activation of antigen-presenting cells such as DCs[428]. Platelet-derived MVs however, also contribute to atherogenesis by inducing hyperplasia of vascular SMCs[429], proinflammatory activation of ECs via induction of NF-κB and increased adhesion of monocytes to ECs[430]. Alternatively, MVs shed by stored human platelets suppressed proinflammatory differentiation of monocytes to macrophages, as well as maturation of DCs[426]. Similarly, platelets exposed to a higher shear stress increased IL-10 production and induced DCs maturation capable to promote less proinflammatory T cells from naïve T cells *in vitro*[431].

Indistinctly, pdEVs were reported to harbor caspase 3 and cause apoptosis of macrophages, which then contributed to the accumulation of extracellular lipids in the atherosclerotic plaque[432]. PdEVs, along with other EVs derived from other cell sources such as ECs, erythrocytes, and leukocytes are able to stimulate thrombosis by binding to TF and delivering it to platelet outer membrane, which in turn promotes thrombus enlargement[433]. While platelets and vessel wall interactions are numerous, implicating numerous cell surface receptors and diverse chemical substances[434], pdEVs are released when binding occurs[435] and it remains to be confirmed whether these vesicles get incorporated, at least partially, within the artery wall.

2.4.2 EVs released by red blood cells

Red blood cells, or erythrocytes, are found in the largest quantity in the blood, accounting for nearly 83% of total cells[436]. Their main functions are the transport of oxygen from the lungs to body tissues, and of CO_2 as a waste product, away from tissues and back to the lungs[437]. RBCs are also recognized to supply ATP, which in turn contributes to vessel dilation by stimulating NO production in the endothelium[438]. Generally, RBCs contain enzymes and molecules with antioxidant activities[437]. EVs derived from circulating erythrocytes generally measure between 100 and 300nm, acquire a spherical form[333], and are surrounded by a lipid bilayer rich in phospholipids, proteins, cholesterol, lipid raft, hemoglobin and acetylcholinesterase[437].

Contrarily to intact RBCs, EVs derived from erythrocytes are associated with increased oxidative stress, including free heme transfer to endothelial cells[439], and thus they can

interfere with NO signaling and promote initiation of endothelial dysfunction[330]. RBC-derived EVs were reported to scavenge NO faster than intact erythrocytes[440], making them important vasoconstrictors. This also increased erythrocyte adhesion, enhanced endothelial damage and caused vaso-occlusions[437, 439].

Furthermore, EVs derived from RBCs exert a procoagulant activity as they can generate thrombin by a factor XIIa126-dependent mechanism and stimulate thrombus formation or erosion of the atherosclerotic plaque in proportion to their exposure to TF[388, 433]. The presence of PS on the surface of RBC-derived EVs provides a site for the assembly of prothrombinase promoting a thrombin clot. Additionally, PS-exposed RBC-derived EVs provide sites for adhesion of platelets and neutrophils localized in the subendothelium, further aggravating cardiovascular disease progression[441].

2.4.3 EVs released by endothelial cells

Vascular ECs were shown to be important EVs producers, involved in crosstalk between ECs, between ECs and SMCs, as well as between ECs and immune cells in both normal and atherosclerotic conditions, in response to extracellular stimuli that trigger changes in phenotype and tissue remodeling[442]. Similar to other cell types, EC-derived EVs contain a multitude of proteins and RNAs[443].

Various stressful conditions such as hypoxia or TNF-α, both simulating an inflammatory environment as observed in atherosclerosis, affected the proteome and transcriptome of EVs secreted by cultured ECs[427, 443]. In hypoxic EVs, proteins involved in stress response and proapoptotic function were observed[444]. In TNF-α EVs significant changes in the amount of mRNA were observed, especially proinflammatory ones such as IL-8, MCP-1, IL-32 and VCAM-1[427]. In another study, EVs released by cultured serum-starved human ECs that underwent advanced apoptosis and autophagy were shown to contain an autophagosomes, as well as mitochondria, and delivered various danger signals including ATP release which is involved in autophagy regulation[445]. As such, since apoptosis of the vascular epithelium is a common event in atherosclerosis, detection of these EC-derived apoptotic EVs in blood may suggest endothelial dysfunction[427].

High serum concentrations of homocysteine and oxLDL are predisposing factors of early atherosclerosis development[446]. In cultured rat aortic ECs, both these factors contributed to

release of HSP-70 containing exosomes[447], which may contribute to environment toxicity[448]. Furthermore, an increased level of HSP-70 can cause hyperactivation of monocytes through proinflammatory differentiation to macrophages in a TLR-4-dependent manner[449].

Exposure to higher shear stress is another factor that predisposes to atherosclerosis, and in human umbilical vein endothelial cells, leads to formation of EC-derived EVs that contain miR-143/145 clusters[450]. The latter were shown to prevent hyperplasia and maintain the contractile phenotype of co-cultured SMCs[451, 452].

Since the emergence of the lymphatic system as a key prerequisite player in mRCT, lymphatic endothelial cells (LECs) derived EVs are an attractive new tool to assess lymphatic vessel function. Recently, LECs were shown to release exosomes that enhanced the migratory capacity of DCs in particularly challenging environments such as during inflammation[453]. While lymphatic exosomes appear to aid with cellular transport through the lymphatics, all types of LEC-derived EVs can also be used as biological particles reflective of lymphatic integrity, an important aspect of this thesis.

2.4.4 EVs released by immune cells

In proinflammatory conditions, macrophages and monocytes produce large amounts of EVs whose levels may at times exceed those of pdEVs in atherosclerotic lesions[393]. *In vitro*, cultured monocytes in cholesterol depleted medium led to production of highly procoagulant EVs that when injected in rats, induced an increased leukocyte adhesion to ECs. Alternatively, exposing cultured human ECs to cholesterol-induced monocyte-derived EVs caused proinflammatory activation of ECs and upregulated the expression of ICAM-1 and NF-κB[430]. Another study reinforced these results by showing that EVs released following starvation of cultured monocytes enhanced atherogenesis in *apoE*$^{-/-}$ mice by enhancing IL-6 release by macrophages and ICAM-1 expression in ECs[454]. The authors thereby suggest that biological functions of monocyte derived EVs are destructive due to the fact that they exhibit proinflammatory effects. In human lipid-rich atherosclerotic carotid arteries, circulating monocyte-derived EVs were shown to promote high thrombotic risk by disbalancing the content of TF and TF pathway inhibitor in affected vessels[455]. In 2016, a study assessing circulating EVs in atherosclerotic patients versus healthy subjects, came to the conclusion that patients had

significantly higher amounts of leukocyte derived EVs that promoted SMC adhesion and migration. In particular, it was foam cell–derived EVs that were shown to bind to SMCs and transfer integrins to the surface of these cells[456]. Furthermore, leukocyte-derived EVs that contain both TF and PSGL-1 were reported to act with P-selectin on the surface of platelets and induce blood coagulation that leads to thrombus formation[457, 458].

Interestingly, *in vitro*, EVs containing cholesterol promoted foam cell formation by increasing cholesterol uptake in macrophages in proportion to their cholesterol content, an event that was enhanced by TLR stimulation or IFN-γ. Interestingly, these same EVs suppressed TNF-α and T cell activation, so would otherwise be considered anti-inflammatory[459]. Exosomes released by activated T cells also favored cholesterol uptake by cultured human macrophages and stimulated their production of TNF-α following PS receptor-dependent internalization[460].

Studies have found MMP enrichment in macrophage-derived, as well as EC-derived EVs, suggesting that they also play a role in degradation of ECM collagen and atherosclerotic plaque fragility[461, 462]. Furthermore, since lymphatic vessels are present in the adventitia of the artery wall, where T cells are particularly present[463], proinflammatory T cell-derived EVs interactions with these vessels deserves further attention.

Circulating exosomes that express on their surface major histocompatibility complexes (MHCs) can be engulphed by immature DCs[361], and can then be presented to naïve T cells, which results in induction of T cell-mediated autoimmune response associated with atherosclerosis[464]. Furthermore, caspase transport and cholesterol accumulation in the clearance of EVs by macrophages and foam cells can induce apoptotic death and lead to the accumulation of extracellular lipids in the lipid heart of the atherosclerotic lesion[432, 465].

When it comes to EVs shed by apoptotic Tregs, these showed anti-oxidant effects by decreasing ROS production, due to their capacity to transfer antioxidant enzymes such as catalase and superoxide dismutase, and apoptosis of ECs[466].

2.5 Methods and challenges in studying EVs

EVs research is on the rise, however, some challenges remain considerable due to their complexity. One major issue in the field is to improve and standardize methods for EVs isolation

and analysis[467]. Due to their small size, it is still a challenge to properly identify the specific EVs subtypes with the imaging tools available to date.

2.5.1 Isolation

A lot of debate has emerged in recent years concerning EVs isolation techniques. Since they represent a very diversified population, with respect to size, composition and cell-specific function, it has led to a broad nomenclature, thus causing further confusion. With emerging research, some consensus has been achieved and continues to[392]. Currently, a variety of techniques and methods help identify and isolate different EVs subtypes, such as differential ultracentrifugation, density gradient centrifugation and size exclusion chromatography, among many others emerging[392]. As a result, these methods allow for the preparation of EVs of different composition and purity.

The effectiveness of one of the most used EVs isolation methods, the centrifugation, depends on many factors, such as the acceleration speed, the type of rotor and its diameter, as well as the viscosity of the sample[468]. This method uses the buoyant density of the particles to separate them accordingly and uses several subsets to avoid contamination of cell lysis and cosedimentation. At first, particles with the highest buoyancy are sedimented, meaning that the densest particles will tend to remain floating on top, such as cells at 300–400 ×g for 10 min, followed by another spin at 2000 ×g to remove cell debris. The supernatant, containing the EVs will be ultracentrifuged at >100,000 ×g for 2 h. Still a subject of debate, ultracentrifugation appears to sediment EVs together with protein aggregates, therefore lipoproteins with a similar size and density to EVs are often co-isolated[469]. To further distinguish EVs, ultracentrifugation can be used in conjunction with density gradients and sucrose cushions to further separate EV subpopulations based on their density and therefore aid in the removal of contaminating proteins, apoptotic bodies, and other unwanted particles [470].

Although the golden standard for EVs isolation has been ultracentrifugation, in recent years, size exclusion chromatography has also gained a lot of attention as it is a quick and efficient single-step method for EVs isolation, particularly useful when the quantity of biological fluids is limited[471]. A column matrix filters larger compounds and allows EVs to pass directly through[472]. Importantly, EVs isolated using chromatography are more intact than EVs isolated using ultracentrifugation, possibly due to the absence of centrifugation at high

speeds[473]. As size exclusion chromatography confers more functional EVs compared to ultracentrifugation, it is especially crucial to keep in mind when developing EVs as therapeutics[473].

A multitude of isolation protocols that produce distinct populations of EVs do exist, and new ones emerge relatively often, which makes data comparability difficult. While some methods were shown to induce cellular stress and affect both the quantity and function of the EVs produced, others require the use of expensive reagents, unrealistic time delays for a clinical setting, or equipment unavailable to most laboratories[474]. Thus, there is need for a standardized, feasible and cost-effective method to isolate EVs properly, and currently this is greatly acknowledged within the EVs community. The International Society for Extracellular Vesicles attempts to provide a specific guideline so as to avoid inaccurate reporting of the specific methods used[475]. To further improve reliability, the EV-TRACK (trans- parent reporting and centralizing knowledge in EV research) platform was developed[392, 476]. Its purpose is to encourage scientists to integrate all collected data in a uniform matter with associated details so that studies can be replicated accurately and can also be compared[392]. In addition, a recent position statement of the International Society for Extracellular Vesicles updated the Minimal information for studies of extracellular vesicles guidelines, all in the efforts to clearly standardize EVs procedures[477].

2.5.2 Quantification

Once EVs have been isolated from their respective media, proper quantification is no easy task. The biggest problem to date remains EVs purity, which is crucial when evaluating EVs dosage for functional studies and efficient therapies[478]. Currently there is not one method that allows for entirely accurate phenotyping, characterization and sizing of all types of EVs. It is however possible to measure EV specific activity by measuring the expressed concentration of a specific surface-marker antigen using an ELISA assay, Western blot (WB), flow cytometry or EV arrays, and can even assess EV protein concentration using proteomics[478, 479]. Furthermore, there are numerous methods for measuring EVs numbers, including optical methods, such as nanoparticle tracking analyses, dynamic light scattering, and standard flow cytometry for larger EVs or high resolution flow cytometry for smaller EVs, as well as nonoptical methods, such as cryo-electron microscopy (cryo-EM)[477, 480, 481]. Of notice,

light scattering and similar techniques can lead to overestimation of EV counts since the techniques are not yet entirely optimized for EVs and can take into account contaminating lipoproteins and protein aggregates that may also be present in the sample[477]. Contrarily to the other techniques, cryo-EM provides a unique means of analyzing particle diversity in native biological fluids at nanometer resolution, thus allowing for clear visualization of EV natural morphology and even contents [482]. It was this technique that allowed for clear identification of EVs that possess PS at the surface, by labelling them with annexinV gold nanobeads[301].

In order to better understand how to identify EVs, it is important to thoroughly understand their morphology and possible changes they may undergo. The exterior layer of the EV surface is the lipid bilayer that contains rare lipids, like ceramide and aminophospholipids. As such, these can serve as ligands[483]. A broad variety of proteins are present in the membrane, including random coils and other complex secondary structures[484]. EVs membrane is composed of three distinct regions: the hydrophobic core, the electrostatically charged phosphate and lipid head groups, and the hydrophilic solvent[485]. The interaction with proteins relies on a lock and key like mechanism and additionally, divalent cations like Ca^{2+} or Zn^{2+} play an important role in any membrane changes[486, 487]. An example is annexinV which recognizes PS translocated at the surface of the membrane and binds to the lipid head group with the help of Ca^{2+} ions. PS carries an uncommon net negative charge and is used to identify EVs of apoptotic origin[302, 484]. There are also proteins that can sense the membrane curvature, so they bind with varying affinity depending on the shape of the membrane[488].

Dyes that are activated by intracellular components can also be used to label EVs. Calcein and CFSE are popular examples of cell permeant, non-fluorescent pro-dyes that are cleaved by intracytoplasmic enzymes, thereby leading to an impermeant fluorescent molecule[351, 489]. This labelling allows to differentiate intact EVs from membrane fragments, or debris, as long as the required enzymes are present in the EVs. Although the presence of components other than EVs but that have comparable features, such as lipoproteins, can contribute to biased results in the preparations of EVs[490], it is not the case for dyes such as CFSE. It is however a major pitfall in the use of lipophilic fluorophores[350]. Nonetheless, dyes may still affect the normal behaviour of EVs, and although uptake of EVs did not seem to be affected, further studies still need to be performed to confirm with certainty[349].

Especially important when quantifying EVs in a clinical setting, is to take into consideration the influence of medication. Several antiplatelet agents, like aspirin, inhibit platelet activation which decreases EVs release[330]. Similarly, antihypertensives reduced circulating platelet- and monocyte- derived EVs[330]. Importantly, some therapies, such as statins, were even shown to affect the composition of EC-derived EVs[491].

With the help of emerging technologies and dedicated scientists, an in-depth appreciation of EVs diversity through detection refinement and standardization can shed light on the specific roles played by each subtype in health and disease, and ultimately, use them as a trusted biomarker for disease and a promising therapeutic target.

3 THE LYMPHATIC SYSTEM

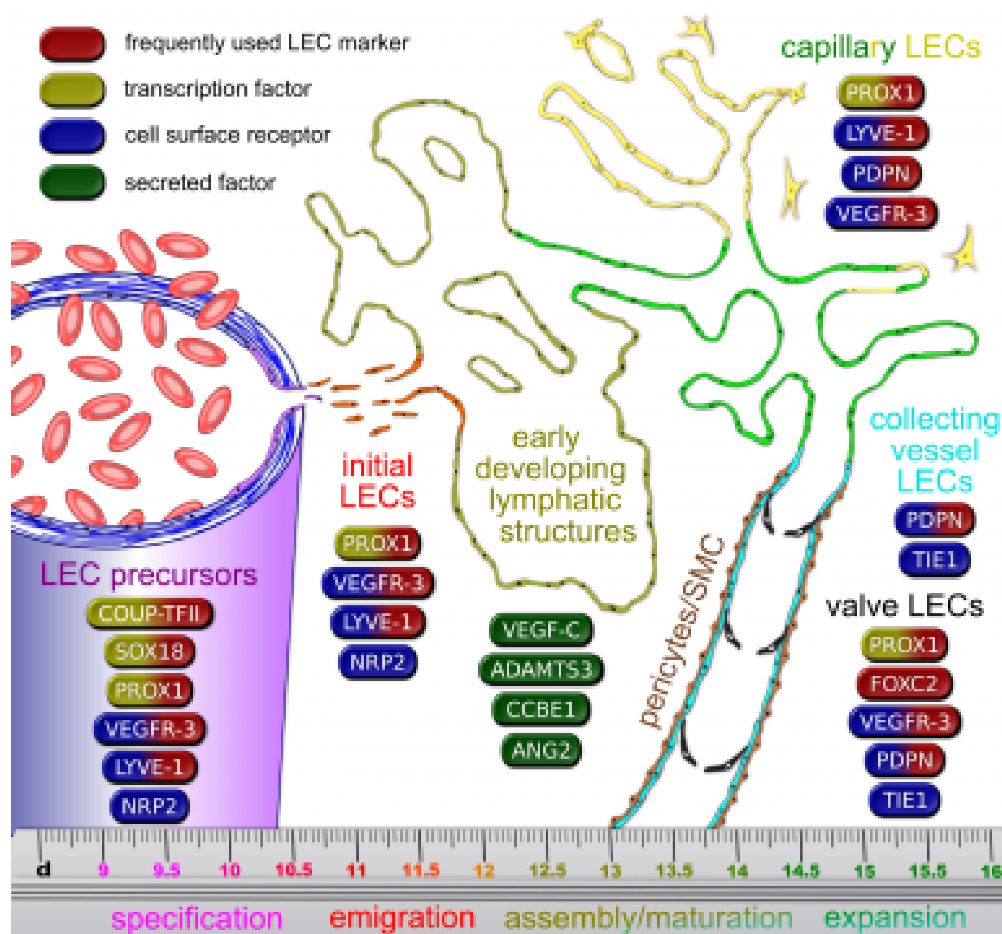
As far back as the 1620s, two important networks of vessels were discovered: the blood and the lymphatic circulation[492]. Although interdependent, these two represent separate circulatory systems[493]. Although considered as simple milky-white vessels in the early beginnings, the lymphatic system is now recognized as one of the most crucial supporters of the immune system as it is responsible to protect the body from diseases and infections[494]. It is a network composed of lymphatic vessels that allow lymph to flow throughout the body, LNs which act as filters for lymph and lymphocytes, as well as organs in all parts of the body. Hundreds of LNs are found in almost every part of the body, including the elbows, groin, neck and armpits[495]. It is only recently however that the existence of meningeal LVs in human and nonhuman primates, as well as the feasibility of noninvasively imaging and mapping them *in vivo*, was reported[496]. Among the organs considered part of the lymphatic system are primary lymphoid organs such as the bone marrow and the thymus, as well as secondary lymphoid organs like the tonsils, the spleen, and the lymphocyte accumulations inside the intestinal, respiratory, genital and urinary tract[495].

The lymphatic system is generally known to perform three main functions[497]. First, it helps with fluid circulation throughout the body and homeostasis. Any excess fluid that escapes from the bloodstream is collected by the lymphatic system and returned, thus preventing the formation of edema[498]. Second, as lymphatic vessels are located within the digestive tract, it helps absorb fat-soluble vitamins and fat from foods. The fats are then transported to the bloodstream and used as needed[499]. Lastly, the lymphatic system defends the body against infections. The vessels displace lymphatic fluid and lymphocytes throughout the body, and the lymph nodes act as a filter to get rid of any debris, bacteria, viruses and other foreign bodies[500].

In recent years, impressive progress has been made to allow for in-depth characterization of the interplay between the lymphatic system and chronic inflammatory diseases. With the development of genetic mouse models and imaging tools adapted to fit both animals and humans, new functions have been brought forward, including the central role of functional lymphatic vessels in the onset and progression of atherosclerosis. Furthermore, the characterization of a vast array of key molecules in lymphatic function and identification

allowed for a better understanding of their role at each stage of lymphatic development, including lymphatic specification, proliferation and maturation (Figure 10), and will be elaborated upon in the following sections[494].

Figure 10. Brief introduction to the molecules that are most central to the molecular biology of the lymphatic system.



Jha *et al.* provide a mini-review of the main proteins linked to the lymphatic vasculature with implications throughout development and predominant at different structural levels of the network. Open access article available under the terms of the Creative Commons Attribution License (CC BY).

3.1 Development

New awareness regarding the molecular regulation of lymphatic endothelial specification and development has emerged recently. The origin of LECs has been highly debated, but until now, two different models are believed to explain lymphatic vessel origins[501].

3.1.1 Origin and specification

For the most part, the molecular mechanisms that frame the development and growth of lymphatic vessels differ from those observed in blood vessels. Sabin *et al.* brought forth the first insight on lymphatic development. She proposed that the blood vasculature gave rise to the lymphatic vasculature. More precisely, by experimenting with ink-injections in pigs, she proposed that lymph sacs originate from ECs that outgrow from the cardinal veins during early development, and migrate toward the periphery to form LVs that spread throughout the body[500].

Just a few years later, Huntington and McClure proposed that lymphangioblasts form the original lymph sacs and that they later establish venous connections[502]. Since then, additional molecular support has been produced for Sabin's model, and several lineage tracing experiments helped confirm that some lymphatic vessels are derived from the venous system[502, 503]. However, in more recent years, using quail-chick-chimeras and DiI-conjugated LDL labeling of blood vascular endothelium, a subsequent study suggested that deeper parts of the lymph sac arise from adjacent veins, while the superficial parts and the dermal lymphatics are derived from lymphangioblasts, thus proving a dual origin[504]. Klotz *et al.* found that LECs that form lymphatic vessels in the heart originate both from embryonic veins and from other non-venous sources, like the yolk sac[505]. Analysis of the expression of the Prospero-related homeobox gene (Prox1) in *Xenopus* tadpoles supported the hypothesis that LECs derive from both veins and the mesenchyme[500, 506]. Experiments with lineage-tracing in mice using the venous/endothelial cell marker Tie2 showed that a significant portion of dermal LECs are not labeled by the transgene, supporting once again their origin from alternative sources[507]. Another recent study, in mice, characterized the origins of mesenteric lymphatic vessels and similarly identified a non-venous origin for these vessels[508]. Therefore, contrary to previous

belief, a multitude of evidence shows that a significant part of the dermal lymphatic vasculature forms independently of sprouting from veins[502, 507].

With respect to the regulation of LVs specification, the use of LEC-specific markers has allowed for the characterization of the initial LVs in mouse embryos. The gene *lymphatic vessel endothelial hyaluronan receptor 1* (*Lyve1)* is expressed on LECs and is the first indication that lymphatic development has started[509]. Interestingly, although apparent at the very early stages of development in LEC progenitor cells, the lymphatic vasculature appears to form independent of *Lyve1* expressio*n*, thus excluding its involvement in the establishment of cell identity[510]. Another study further confirmed that *Lyve1* is not essential for LEC specification and lymphangiogenesis, which is the growth of new LVs, despite the absence of *Lyve1* [511]. Another indication that lymphatic vascular development has commenced is the presence of Prox1^{+} ECs within the cardinal veins, that appear at embryonic day (E) 9.5 and are considered lymphatic endothelial progenitor cells[512]. These cells also migrate to form a primitive lymphatic plexus and lymph sacs, that lie close to the cardinal veins[513-515]. Some studies show that *Sox18,* an SRY-related HMG domain transcription factor, and *COUP-TFII* are required for activation of *Prox1* expression[516, 517]. In the cardinal vein, COUP-TFII also represses *Notch* activity, a negative regulator of LEC specification[518]. Sox-18 is expressed in the cardinal vein at E9 in a subpopulation of endothelial cells, and can then bind to the promoter of *Prox1*, to initiate lymphatic specification[519]. The transcription factor *Myc* was also shown to be required for Prox1 expression in *Xenopus*[520]. As well, during lymphatic network formation, TGF-β was shown to inhibit *Prox1* expression and was shown to enhance LEC sprouting and branching, while it attenuated LEC proliferation[521]. Contrarily to *Lyve1*, *Prox1* is required for LEC specification and its importance was first demonstrated in *Prox1*$^{-/-}$ mice embryos, where LVs were inexistent, despite vasculogenesis and angiogenesis of the vascular system remaining unaffected[512]. *Prox1*$^{-/-}$ embryos did not co-express any lymphatic markers such as vascular endothelial growth factor receptor 3 (VEGFR-3), LYVE-1 or secondary lymphoid-tissue chemokine (SLC). Instead, they appeared to have a blood vascular phenotype, by their expression of laminin and CD34[522]. This showed that originally, embryonic EC budding is of blood vasculature phenotype, and that *Prox1* activity is implicated in both venous EC budding and the differentiation into LVs[522]. *Prox1* is not only required for the initial specification of LEC identity, but it is also necessary to maintain its expression in

mature LECs[523]. Temporal inactivation of *Prox1* during postnatal lymphangiogenesis led to loss of LEC identity and reversion to BEC identity[523]. As *Prox1* is also required in mice, zebrafish, frogs and even chicks for LEC specification, it is believed to have been conserved throughout evolution[500, 506, 524]. Cells that are Prox1$^+$ depend on a paracrine factor called vascular endothelial growth factor C (VEGF-C) to be able to spread away from the embryonic veins. A thorough description of this growth factor and its associated functions can be found in study #1 and chapter 7.1 below.

3.1.2 Formation of lymph sacs

Once PROX1 expressing LEC progenitors have undergone specification in the embryonic veins, they bud into the surrounding mesenchyme and are held interconnected by adhesion junctions that express high levels of vascular endothelial cadherin (VE-cadherin)[525]. These junctions act as gatekeepers of endothelial integrity during budding. In fact, previous studies report that PROX1$^+$ cells that bud from the cardinal vein at E12.5 are characterized by zipper-like junctions that express VE-cadherin[526], and when these cell-cell junctions were disrupted, edema developed due to lymphatic development impairment[527]. Budding of LEC progenitors leads not only to cell and nuclear shape changes[515], but also different LEC marker expression. PDPN is an important differentiator[528], as it is only expressed by PROX1$^+$ LECs when they have exited the embryonic veins and become contributors to lymph sac formation[514]. The latter represent the main source of LECs that are required for the formation of the entire lymphatic vasculature, but interestingly, are not required for LN formation. Their study did show that LN formation is impaired in Prox1+/- mice, possibly due to unavailability of CCL21$^+$ LECs [529].

A few factors affect lymph sac formation in mammals. Loss of any of the components of adrenomedullin signaling, leads to underdeveloped lymph sacs and accumulation of subcutaneous edema[530]. *Tie1*$^{-/-}$ embryos were characterized with abnormally large lymph sacs and edema[531]. On the contrary, lymph sacs were significantly small in *Gata2*$^{-/-}$ embryos[500, 532]. Furthermore, GATA2 is also expressed in lymphatic valves [533] and loss of function was shown to make cancer patients more vulnerable to primary lymphedema[533].

While Prox1 and VEGFR-3, along with other factors previously mentioned help define LEC fate and expansion in all organs, similarly to the cardinal vein, downstream effector

influence was shown to differ between mesenteric and dermal lymphatics for example, since blocking phosphatidylinositol 3-kinase (PI3K) only impaired mesenteric development[534].

3.1.3 Proliferation and Migration

Once LECs have been specified from primitive veins, migration and proliferation are the subsequent steps in order to establish the initial lymph sacs, as well as some of the earliest formed LVs, namely the thoracic duct[500]. VEGF-C/VEGFR-3 signaling is implicated throughout the stages of lymphatic vascular development. Although VEGFR-3 is at first a constituent of both blood and lymphatic ECs, at E12.5, its expression remains solely on LECs[535, 536]. Importantly, the exit of lymphatic endothelial progenitor cells from the embryonic veins is dependent on VEGF-C, since LEC budding and formation of jugular lymph sacs is inhibited in *Vegf-c*$^{-/-}$ mice[537, 538]. In the absence of VEGF-C, PROX1^{+} cells are specified, and the expression of LYVE1, PROX1, and VEGFR-3, is unaltered. However, these cells are not able to exit the veins[515, 537]. Contrarily, no defects in lymphatic endothelial progenitor cell migration from the embryonic veins have been observed in VEGF-D deficient mice[512, 539]. Studies also attributed a role to PROX1 in the budding of LECs from the cardinal vein[525], attributing it a central role in the specification of lymphatic fate and organ-specific LV development[540].

Integrins, which are heterodimeric transmembrane receptors that interact with ECM ligands, play a role in VEGF-C/-D/VEGFR-3 signaling. *In vitro*, adding integrin α5β1 antibody to cultured LECs, blocked the increase in VEGFR-3 phosphorylation and reduced cell death when VEGF-C was added[541]. Furthermore, VEGF-C and -D are also bound by integrin α9β1 and were shown to activate the phosphorylation of ERK1/2 and Paxillin in cultured LECs, further reinforcing the role of integrin activation in LEC migration[542].

Studying the specific effects of VEGFR-3 in lymphatic endothelial progenitor cell migration has proven more difficult as *Vegfr-3*$^{-/-}$ embryos develop deadly cardiovascular defects[543]. In mice containing an inactivating mutation in the VEGFR-3 tyrosine kinase domain, known as Chy mice[544], or deficient in the ligand binding domain of VEGFR-3[545], the dermal lymphatic vessel plexus is absent. Interestingly, lymph sacs formed in VEGF-C binding domain mutants, but not in kinase domain mutants suggesting that VEGFR-3 kinase

activity is necessary for the migration from veins, but in the absence of VEGF-C binding to VEGFR-3, VEGF-C signals can still be transduced through alternative receptors[513].

A likely suspect is the receptor neuropilin2 (Nrp2), which has been shown to bind VEGF-C[544], and induce Nrp2 interaction with VEGFR-3, thereby promoting lymphatic vessel sprouting and migration[546]. Nrp2 is mainly present on LECs that have exited the veins and are migrating, but some is also expressed on cells within the lymph sacs that remain close to the cardinal veins[514]. *Nrp2*$^{-/-}$ mice do not form normal initial and small LVs, but collecting LVs developed normally[547]. Integrin α9β1 is another receptor that can bind VEGF-C[542] and its Prox1-mediated regulation has been shown to regulate lymphatic endothelial cell migration following VEGF-C binding[548].

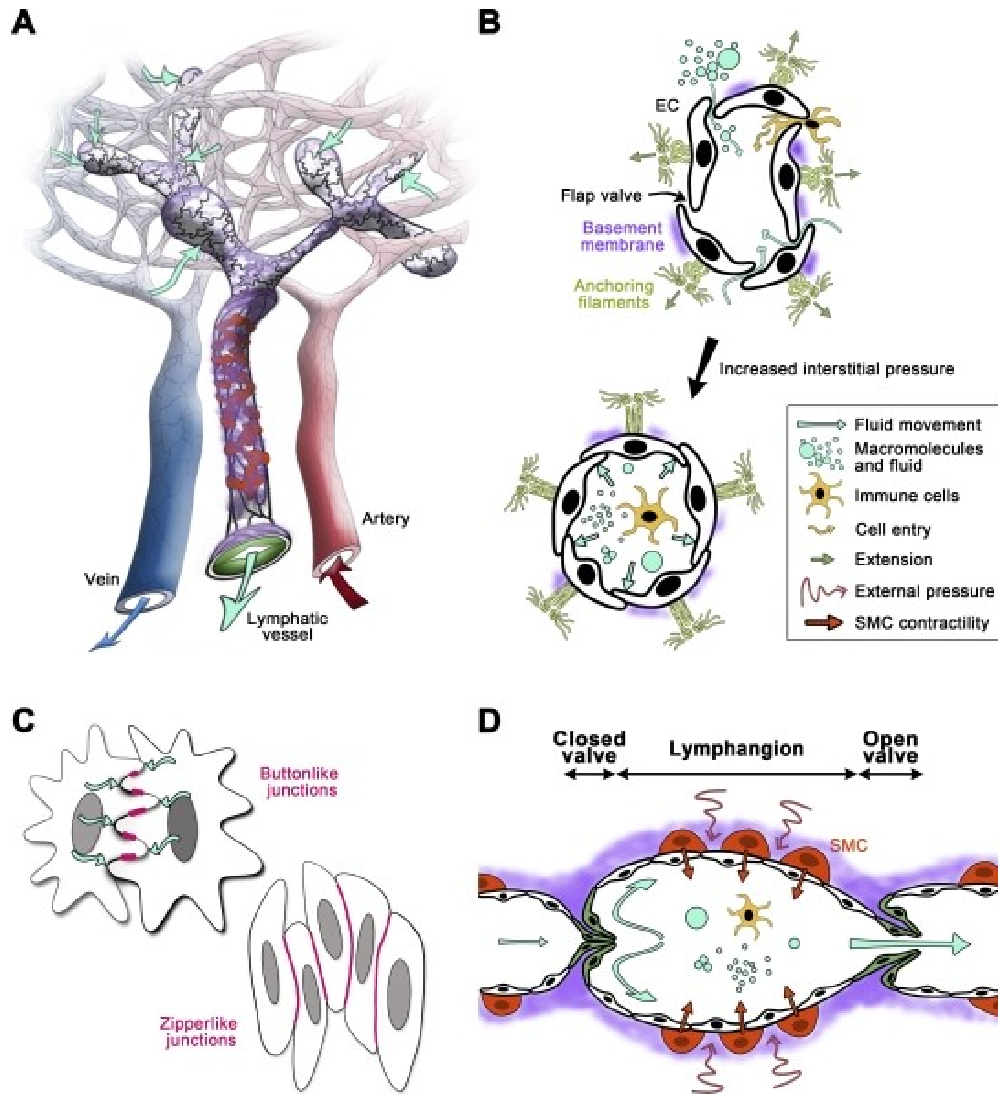
Recently, Zhang *et al.* showed that although *Vegfr-3* deletion led to abnormal LVs in mouse embryos, incomplete deletion during postnatal development alternatively causes aberrant lymphangiogenesis[549]. Novel work has also emphasised a VEGF-C-independent phosphorylation of VEGFR-3 following elevation in interstitial pressure in the mid-gestation mouse embryo. This mechanosensitive activation of VEGFR-3 is suspected to depend on β1 integrin activation[550] and likely mediated by Src family kinases[551]. This interesting data emphasizes the complexity and importance of different mechanical and environmental signals that drive expansion of the embryonic lymphatic vasculature and reinforce that a precise combination of mechanical and ligand-based signals is required for optimal development of the lymphatic vascular network[513].

3.1.4 Formation of lymphatic vessels and valves

Once the lymph sacs form, LECs pursue their path to proliferate and migrate into the mesenchymal tissue, whilst the primitive lymphatic plexus differentiates and gives rise to two distinct lymphatic vessel portions, which are the collecting and initial LVs[552]. Although they each have unique morphological features, both entities share the expression of common genes that help distinguish them from the blood vasculature such as *Prox1, Pdpn, Vegfr-3* and *Nrp2*[553]. The expression of junctional proteins VE-cadherin, Claudin5, and PECAM-1, is also shared and the only differences are in the way these junctions are organized to allow each entity different functional abilities[553, 554].

Collecting vessels are contractile lymphatics that propel lymph in a unidirectional manner, with the help of intraluminal bi-leaflet valves, as seen in the great veins[555], present to prevent the backflow of lymph[556]. Endothelial cells near the valves, which form the valve leafs, also express high levels of the transcription factors *Forkhead Box C2 (Foxc2*)[557], *GATA-binding protein 2 (Gata2*)[558], and Integrin-α9[559], similar to their valve counterparts[553]. Valve ECs further express high levels of PROX1, Connexin-37(Cx-37), and calcineurin/NFATc1 signalling, while levels of LYVE1 and NRP2 are low or inexistent. Additionally, Cx-37 proved to be required for the formation of the ring-like valve territory and lymphatic valves were nearly absent in *Cx-37*$^{-/-}$ mice, showing similarity to Foxc2 in its necessity[560]. There is no clear delimitation between capillary and collecting vessels, which is why the term precollecting vessel is used to describe this particular region of the lymphatic network that has features from both initial and collecting LVs[561]. Figure 11 resumes the information in this section to date.

Figure 11. Organization of the lymphatic vasculature.



Schulte-Merker *et al.* clarify broadly the morphology of the lymphatic vessels and their propelling capacity. (A) The lymphatic vasculature runs in parallel to the blood circulation and is responsible for the absorption of fluid, macromolecules, and cells from the interstitium. (B) Interstitial components penetrate lymphatic capillaries via openings between LECs, which are held open by anchoring filaments to prevent vessel collapse. (C) Both button-like and zipper-like junctions share a similar array of adherens and tight junction proteins, with differences residing solely on their organization. (D) Coordinated opening and closure of lymphatic valves allows forward flow and is required for efficient lymph transport. Lymphatic muscle cells (LMCs) cover sporadically the functional pumping unit of a collecting lymphatic vessel, the lymphangion, and possess intrinsic contractile activity[562]. Figure used with permission from ROCKEFELLER UNIVERSITY PRESS.

Studies using mouse models have clarified diverse molecular mechanisms that regulate the development of the embryonic lymphatic vasculature into a fully functional network of initial and collecting LVs[553]. A few examples will be mentioned herein.

Collecting LVs and their valves develop in parallel. While collecting LVs mature, PROX1 and LYVE1 are downregulated in most LECs and the ECM starts surrounding the vessels followed closely by sporadic LMCs deposition around the LVs[563]. Importantly, in LECs that form the valves, expression of PROX1 and FOXC2 remains elevated[564]. The best-characterized signaling pathway for the formation of collecting LVs is FOXC2/calcineurin/NFATC1 signaling, which plays a critical role in their maturation, formation and valve integrity[561, 565, 566]. During maturation of collecting LVs, the transcription factor *Foxc2* is required for proper lymphatic patterning, as well as mural cell recruitment [567]. In *Foxc2*$^{-/-}$ mice the primitive lymphatic plexus maintained high expression of capillary markers and did not differentiate to functional collecting lymphatics and valves, which led to lymph backflow[557]. FOXC2 was also shown to cooperate with the cardiac valve development transcription factor calcineurin/NFATC1, as pharmacological inhibition of calcineurin, which is an upstream regulator of NFAT activation, impaired lymphatic valve formation[557]. Furthermore, *FoxC2* knockout mice treated with the NFATC1 inhibitor cyclosporin A display increased expression of angiopoietin-2 (Ang2) in the lymphatic endothelium, suggesting that Ang2 stimulates SMC recruitment to the collecting vessels downstream of NFATC1 and FOXC2[493, 510, 557].

Developmental lymphangiogenesis is also modulated by gap junction proteins that are part of the connexin family, CX26, CX37, and CX43[568, 569]. Leaky valves, impaired lymph transport and an increase in the incidence of lethal chylothorax, were all associated with *Cx43* absence[566]. *Cx37*$^{-/-}$ mice do not have any cells to grow the lymphatic valves, so none are formed as a result. Furthermore, mutations in *Cx47* lead to primary lymphedema in humans. Interestingly, *in vitro*, absence of *Cx37* reduces calcineurin/NFATC1 activation. On the other hand, PROX1, FOXC2, and oscillatory shear stress were shown to upregulate *Cx37* expression*[561].*

3.1.5 Lymphangiogenesis in the adult

Generally, in the adult, lymphangiogenesis occurs mainly under pathological conditions such as tissue repair, inflammation and tumor growth. Consequently, the possibility of administrating lymphatic growth factors or their antagonists is a promising venue to target lymphatic vessels in human disease and can act as potential therapy [493]. These concepts are further elaborated upon in the following chapters and study #1.

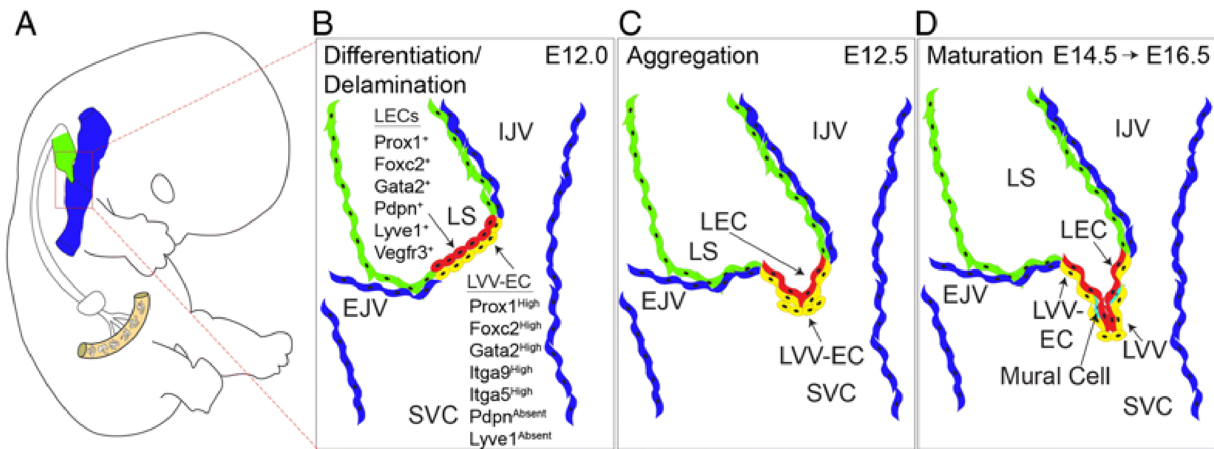
3.1.6 Separation of the venous and lymphatic vasculature

The lymphatic vasculature transports lymph to the blood vasculature, by flowing through the lymphovenous junction. Accumulated lymph from the right side of the body on top of the diaphragm flows towards the right lymphatic duct and drains into the right subclavian vein. Alternatively, the left lymphatic duct collects lymph from both sides of the body under the diaphragm, as well as from the left side of the body superior to the diaphragm, all the way into the left subclavian vein[570]. In healthy adults, around 2 to 4 liters (L) of interstitial fluid that contain 20–30 g/L of proteins return through the lymphatic system into the venous side daily[493].

The lymphovenous valve, which represents the barrier between the two systems, is composed of bicuspid leaflets that open obliquely into the central venous system[571]. At the lymphovenous junction, the thoracic duct is narrow with a thin endothelial layer and a sub-endothelial layer composed of smooth muscle, elastic, and collagenous fibers. The valve leaflets are made of two distinct layers of ECs. The outer layer of lymphovenous valve -ECs is oriented towards the lumen of the superior vena cava in continuity with the endothelial lining of the jugular or subclavian vein. The inner layer of LECs is oriented towards the lumen of the lymph sac[572, 573]. Lymphovenous valve-ECs are characterized by high expression of PROX1, GATA2, FOXC2, Integrin subunit $\alpha9$ and Integrin subunit $\alpha5$, low expression of VEGFR-3, and no expression of PDPN or LYVE1. Contrarily, LECs express reduced levels of Prox1, Gata2, and Foxc2 but express typical lymphatic markers such as VEGFR-3, LYVE-1, and PDPN[572, 574]. Murine lymphovenous valve development begins at E12 with the specification of lymphovenous valve-ECs from venous endothelial cells. Following, during the next 12h, lymphovenous valve-ECs undergo delamination, elongation, and perpendicular alignment to

form an immature valve-like structure. This structure then continues to develop until birth through EC elongation, ECM deposition, and recruitment of a variety of mural cells to the valve leaflets (Figure 12)[572].

Figure 12. Model of the development of the lymphovenous valve in the murine embryo.

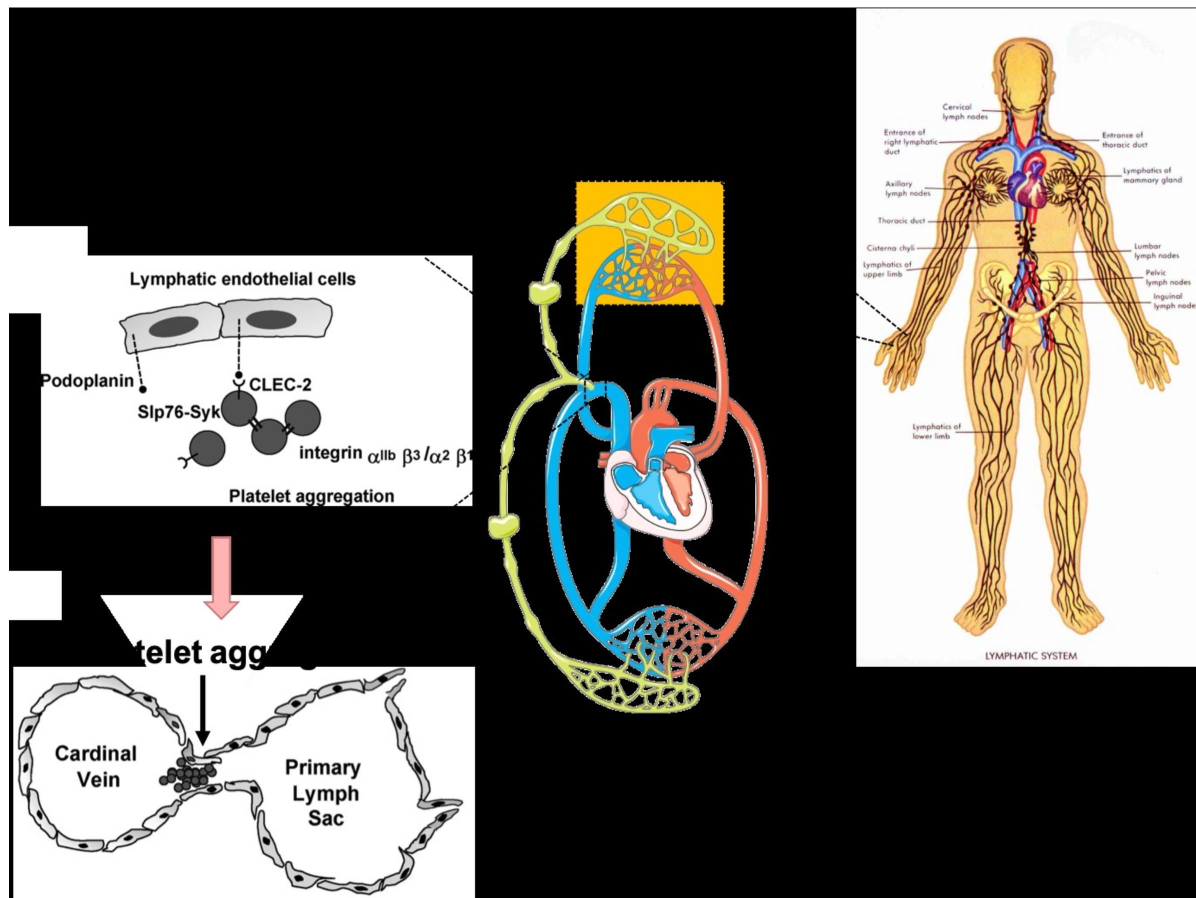


Janardhan *et al.* illustrated sequentially the establishment and maintenance of blood and lymph separation. (A) The lymphovenous valve-ECs differentiate at E12 and undergo (B) delamination, (C) aggregation and (D) maturation to form functional lymphovenous valves at the lymphovenous junction. Lymph sac (LS, green), external jugular vein (EJV, blue), internal jugular vein (IJV, blue), superior vena cava (SVC, blue), lymphovenous valve endothelial cells (LVV-EC, yellow), and lymphatic endothelial cells (LECs, red)[572]. Figure used with permission from Copyright © 2019, Springer Nature Switzerland AG.

Additionally to their established protective role in wound healing, platelets are also important players during embryonic lymphangiogenesis and allow proper delimitation of the nascent lymphatic vasculature from blood vessels[575]. More precisely, PDPN allows aggregation of platelets through interaction with CLEC-2 on platelets[576]. This interaction activates platelets, resulting in a platelet plug and thrombus formation specifically at the lymphovenous junction, that prevents backflow of the venous blood into the lymphatic circulation throughout life[577]. Sialylated *O*-glycans protect PDPN from degradation by various MMPs in lymph and are essential components that allow for platelet binding[578].

Studies identified that binding of CLEC-2 on platelets to PDPN on LECs activates Slp76 signaling to regulate embryonic vascular development[579]. Experiments in mutant mice provided further evidence of different mechanisms that alter the separation between the lymphatic and blood vasculature. In both *Slp76*$^{-/-}$ and *Syk*$^{-/-}$, there was evidence of blood-filled jugular lymph sacs, chylothorax, and chylous ascites, due to abnormal connections between the two systems, as early as E11.5[580, 581]. Disruption of platelet aggregation with an anti-PDPN antibody in mice resulted in blood-filled lymphatics, furthering the importance of platelet-mediated blood–lymph separation[582]. Similarly, genetic experiments in mice showed that megakaryocytes or platelets C-Type Lectin Domain Family 1 Member B (*Clec1b)* and Lymphocyte Cytosolic Protein 2 (*Lcp2)* are critical for maintenance of blood–lymph separation[572]. Interestingly, Clec1b function in other hematopoietic lineages, such as neutrophils and macrophages, seems to be dispensable for lymphovenous junction integrity[572, 583]. Of interest, *Pdpn*$^{-/-}$ mice exhibit a partially penetrant blood–lymph separation phenotype at postnatal stages, suggesting that additional ligands for Clec1b are present on LECs[582]. Nonetheless, a recent study reinforces the importance of PDPN in the maintenance of blood–lymph separation throughout postnatal life (Figure 13)[584].

Figure 13. Platelets are required throughout life to maintain proper blood-lymphatic separation.

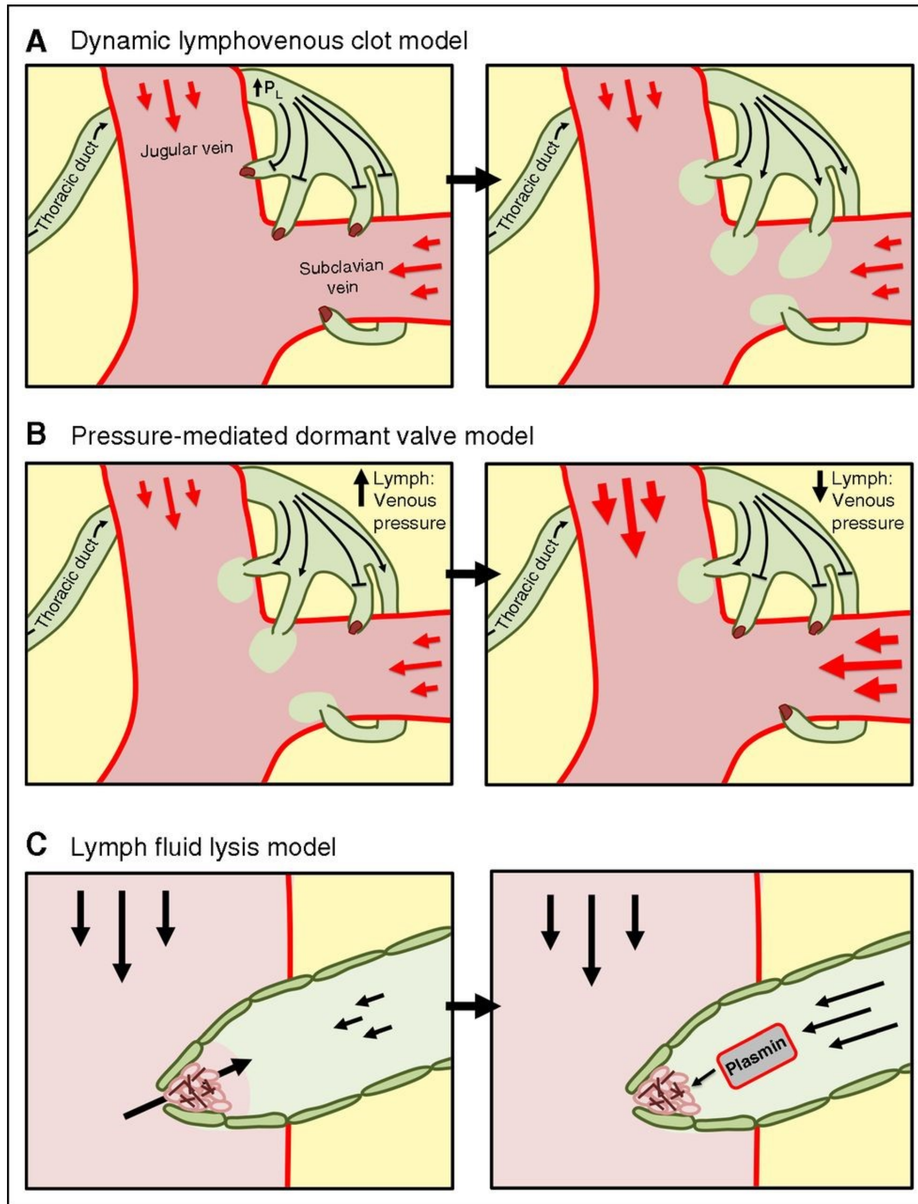


Platelets are essential in proper lymphatic function as the interaction between CLEC-2 on platelets and PDPN on LECs is crucial for the formation of the lymphovenous junction and for the lymphatic vessel integrity, both during development and throughout life. Adapted from *Kim et al*.[585] (with permission from Copyright © 2015, Springer-Verlag Berlin Heidelberg) and Servier Medical Art http://www.servier.com/slidekit/?item=16

A fascinating mechanism allows the thrombus at the lymphovenous junction to prevent backflow of venous blood yet permits unimpeded anterograde lymph flow. LEC-specific ablation of *Chd4,* a component of the NuRD chromatin repressive complex[586], in mice increases expression of Plasminogen activator urokinase (Plau), an anticoagulant enzyme, thereby leading to dissolution of the thrombus at the lymphovenous junction[572]. Currently, three models are believed to explain how the lymphovenous junction could both maintain

forward lymph drainage and prevent blood reflux (Figure 14)[572, 587]. The dynamic lymphovenous clot model proposes that thrombi at the lymphovenous junction block retrograde blood flow while allowing lymphatic pressure to rise, and once it crosses a threshold, this dislodges the thrombus, and allows anterograde lymph flow[587]. The second model is a pressure-mediated dormant valve model. Based on recent data that shows that the thoracic duct separates into smaller vessels at the lymphovenous junction, clots are believed to form solely near valves where retrograde blood flow takes place, while valves that promote forward flow would remain open[587]. Lastly, the lymph fluid lysis model suggests that forward lymph flow occurs due to lymph fluid being biochemically more thrombolytic than blood[588], thereby mediating clot resolution when clot lysis factors, such as Plau, come in close proximity to the LECs. In contrast, venous blood contact with the LECs abrogates this local accumulation of lytic factors, and restores clot formation which re-establishes blood-lymph segregation[587].

Figure 14. Models of lymphovenous clot dynamics and lymph flow.



Welsh *et al.* outline three main mechanisms that demonstrate how a lymphovenous clot has a dual role and is able to permit forward lymph flow, while blocking retrograde blood flow[587]. Figure was used with permission from AMERICAN SOCIETY OF HEMATOLOGY (ASH).

3.1.7 Lymph formation and ultrafiltrates

Lymph derives from interstitial fluid and its composition varies depending on its interaction with the surrounding cells and blood. Human lymph analyses have confirmed that lymph composition differs to that of plasma or serum[589]. Nanjee *et al*. demonstrated that the concentration of small pre-βHDLs in human tissue fluids is determined only in part by their transfer across capillary endothelium from plasma. They showed that the local production of pre-βHDL in the periphery, by remodelling of spheroidal HDLs in tissue fluids, is just as important[590]. Furthermore, Nanjee *et al.* examined the composition, as well as the ultrastructures of different subclasses of normal human peripheral lymph lipoproteins. Total cholesterol concentration in lymph HDL was 30% more prominent in lymph than what transendothelial transfer of HDL from plasma could account for, which provided direct confirmation that HDL acquire cholesterol in the extravascular compartment[591, 592].

Lymph formation is believed to rely on fluid exchange in tissues that is based on two antagonistic pressures[593]. The hydrostatic pressure pushes the plasma out of the blood capillaries and contributes fluid to the interstitium. The oncotic pressure is developed by the plasma proteins that are not passing through the capillaries[594]. The balance between the hydrostatic and oncotic pressures determines in which direction water and solute movement alternates between the blood, tissue spaces and LVs. This is the principle of the Starling's Law of fluid filtration, when the oncotic pressure exceeds that of the hydrostatic pressure, fluid reabsorption occurs[595]. At the tip of a capillary, when the hydrostatic pressure is higher than the oncotic pressure, there will be lymph formation[596]. Contrarily, at the venous tip, the hydrostatic pressure is lower than the oncotic pressure leading to lymph reabsorption[597]. Generally, LVs are compliant at low intraluminal pressures between 1 to 5cmH_2O. However, at pressures above 5cmH_2O, a sharp stiffness transition occurs[598]. The few studies on interstitial pressure gradients propose that as low as 0.12cmH_2O is enough to drive the capillary filtrates into the low resistance lymphatic initial vessels[599]. Furthermore, even a positive pressure gradient allows for fluid to be uptaken in the initial LVs even when there is a negative interstitial pressure[600].

Interstitial fluid flow and lymph formation are crucial compensatory mechanisms against edema formation in different tissues. Importantly, Scallan *et al.* affirm that no matter the cause

of lymphatic dysfunction, it is believed that edema does not take place until lymph flow is reduced by half[601]. The lymphatic system is an intricate system that is able to adjust by elevating lymph flow to help return the interstitial volume back to normal levels, but only as long as it does not get overwhelmed by a surplus of volume[602].

Interestingly, lymph composition is changed during its flow from the periphery, with its protein concentration increasing along the lymphatic vessels[603]. A vast number of cytokines, proteins, growth factors and lipoproteins are present in lymph, and play important and widespread roles in metabolism, proliferation, as well as immunoregulation[604]. Considering how crucial the lymphatic system is to help with the clearance of cells and molecules from peripheral tissues, it is not surprising that circulating EVs can also be found in lymph[303]. From an immunology perspective, it was reported that exosomes can be transported by the lymphatic vessels from peripheral tissues to draining LNs where they can be observed for up to two days. The internalization of exosomes by macrophages and B cells plays an important role in this process[605]. Exosomes derived from melanoma can also travel through the lymphatic vessels to accumulate in the lymph nodes and promote tumor metastasis[606].

3.2 Physiological functions

The lymphatic system plays a crucial role in immune function, fat absorption, and uptake of excess fluid from peripheral tissues[607].

3.2.1 Tissue fluid homeostasis

Blood plasma is continuously filtered to the extracellular space by a semipermeable layer of BEC. Although the blood vasculature was suspected to reabsorb these ultrafiltrates, Michael & Philips demonstrated that fluid reabsorption cannot occur across blood vessels due to their steady-state properties, such as constant pressure and flow produced by upstream arterioles[594]. It is now confirmed that the majority of the extravasated interstitial fluid and macromolecules are reabsorbed by the LVs, and the venules only play a minor role[595]. Importantly, some areas are exceptions, like encapsulated organs such as the kidney and the intestinal mucosa, since venous fluid absorption is mainly covered by local epithelial secretions[608]. The heart also differs as pressure changes are often and rapid, allowing the

blood vasculature to compensate for some fluid absorption[594]. Nonetheless, the plasma volume of the human body, close to 3L, extravasates in its entirety from the blood circulation every 9 hours and is returned in its majority to the systemic circulation by the lymphatic system[595]. Failure to deliver appropriate plasma volumes back in circulation, as seen in mice with severe lymphatic defects, leads to lymphedema and lethality[608].

In cases of primary lymphedema, such as the widely used model of Milroy disease due to mutations in the VEGFR-3, there is increased infection in the limbs[609]. Mouse models such as K14–VEGFR-3–Ig, which lack dermal lymphatic capillaries, and Chy mice, which have an inactivating *Vegfr-3* mutation[544], have helped study the cause for infection and helped demonstrate the importance of lymph draining for the organization and function of B cells that reside within the draining LN, crucial for proper antibody production[610]. Furthermore, impaired lymphatic drainage to a sentinel LN leads to impaired peripheral tolerance[610], contributing to autoimmune disease and organ graft rejection. Clinically, patients with secondary lymphedema post-masectomy have been correlated to obesity and weight gain[611]. Congenital malformations of the intestinal lymphatics manifest in a variety of pathologies, and have been associated with decreased lipid transport even before disease onset[612]. Primary intestinal lymphangiectasia, characterized by abnormal dilation of the LVs, exhibit lymphedema due to low levels of serum albumin, and were also shown to exhibit chylous reflux in the skin, as briefly mentioned above[613]. Animal models such as the *Prox1*$^{-/-}$ mouse whose lymphatic system is dysfunctional, but they survive past birth, prove crucial to better understand the precise mechanisms of interplay between LVs and metabolism. In fact, these mice are characterized by leaky LVs and since the return of lipoproteins to the blood relies on functional LVs, their loss to the interstitium leads to fat deposition, impaired immune response and obesity[612, 614].

Clinical trials to improve lymphedema have been underway for several years. Undesired effects remain a major concern for most drugs undergoing trials as responses to different factors still remain largely untargeted[615]. In recent years, to simplify delivery, growth factors can be administered after being engineered with more specific clinically approved substances or even with endogenous ECM that can easily incorporate at the selected delivery site[616, 617]. Nonetheless, pro-lymphangiogenic clinical research still has to be further verified and safety results taken into account. As of 2019, the first and only clinical trial using Lymfactin® therapy, that contains VEGF-C and aims to restore the growth of new functional LVs in injured areas,

has started showing promising results[615]. To date, the treatment appears safe and well tolerated in all the patients, and no severe adverse events have been reported (Herantis Pharma). Additionally, there are no molecules currently available for patients to treat specifically the condition, but several others are attempting to find relief, such as ketoprofen treatment that reduces inflammation and Ubenimex which promotes lymphangiogenesis but only if healing is biologically possible and the treatment is given at a precise time and specific dosage[618]. Alternatively, more manual techniques were also being tested, such as low frequency sound waves. The results showed that although it did not reduce lymphedema volume, significant effects were observed with respect to the pain and tightness of the limbs[619]. Since lymphedema is a terribly detrimental disease, even the smallest improvements can make a difference in the quality of life of the patient and should not be taken for granted.

3.2.2 Immune surveillance

Lymphatic vessels greatly contribute to immune responses by providing the structural and functional support for the delivery of antigens and antigen presenting cells to the draining LNs[620]. The first reaction is innate and exhibits general or limited specificity. The innate immune system is comprised of a variety of cells, such as neutrophils, macrophages and DCs[621]. The latter two play a central role in the innate system. The adaptive immune response is highly specific and leads to a memory component which ensures that on a second encounter with a specific pathogen, a more immediate response is seen[621]. The main cells of the adaptive immune system are B and T lymphocytes[621]. Different mechanisms allow for the lymphatic system to control an immune response by guiding antigen/DC entry into the initial lymphatics from the periphery, all the way to the bloodstream[622].

During tissue steady-state, peripheral DCs constantly migrate to the corresponding draining LNs to maintain peripheral tolerance[623]. Upon activation during innate immunity, DCs sample the foreign antigen and were shown to migrate through the ECM of the tissue and exert amoeboid movement by pushing against matrix fibers to reach the LV[167]. With the help of C-C chemokine receptor type 7 (CCR7), DCs are also directed towards the initial LVs in order to reach the LNs and elicit the adaptive immunity[624]. Additionally, CCR7 is responsible for significantly increased DC migration speed towards the appropriate lymphatic vessels[625]. Chemokine (C-C motif) ligand 19 and 21 (CCL19 and CCL21), both which are CCR7 ligands,

mediate chemotaxis responsible for DC migration to the lymphatic vessels[626]. In fact, CCL21 seems to be a more potent directional cue for DC migration than CCL19 and exhibits cluster patterns on LECs[626]. DCs seem to enter initial LVs by docking on LECs through their interaction with CCL21 and following, they are transported to the lumen through hyaluronan-mediated interactions with LYVE1, in dynamic transmigratory-cup-like structures[627]. Alternatively, not only platelets but also leukocytes, including DCs, express CLEC-2, and a deficiency of CLEC-2 in DCs diminishes their entry into lymphatic vessels, as well as their migration to and within lymph nodes[628]. Nonetheless, initial LVs are essential in the migration of DCs to the draining LN, and their impairment leads to devastating effects and altered immune responses. Interestingly, just a few initial lymphatic vessels are enough to properly traffic DCs[629], that is assuming that the upstream collecting LVs are unaltered.

Unlike most other molecules and cells, once in the lymphatic vessel lumen, DCs were shown to actively crawl along the initial LV and once they have reached the lymphatic collectors DCs are assumed to switch from an active to a passive mode of movement, thereby letting the lymphatic flow carry them to the draining LN[630]. This may be due to the fact that collecting LVs express different adhesion molecules or chemokines, such as CCL27[631], thereby favoring DC detachment[629]. Following their journey through the afferent lymphatic vessel, DCs will reach the LN through the subcapsular sinus, and initiate the required steps in order to activate the adaptive immunity[629]. In order to reach the T cell zone, DCs transmigrate across the lymphatic endothelial layer, known to express CCL1 which binds to CCR8 on DCs[629]. CCL1 also regulates CCL19 and CCL21 involved with helping DC migration in the LN[632]. Through the collecting LV, DCs are carried in a one-way direction as the lymph flow is aided by intraluminal valves present along the vessel. The valves accommodate flow depending of the pressure, with increased pressure downstream leading to valve leaflet opening and lymph propulsion upstream[598].

Once a contact is established between DCs and T cells, antigen recognition occurs through T-cell receptor interactions with peptide MHCs complexes that are present at the DC surface[633]. Effective activation by DCs leads to differentiation into effector and memory T cells[634]. The egress of these cells from the LN is highly dependent on sphingosine-1-phosphate receptor 1 (S1PR1) expressed by T cells, whose ligand is S1P, an endogenous sphingolipid that mediates cellular chemotaxis, and is secreted by LECs in lymph[635]. In fact,

a lower level of S1P in lymphoid tissues compared to lymph is believed to create a sufficient gradient across LECs that allows for transmigration of S1PR1-expressing T cells into the efferent collecting LVs[635]. The C-type lectin Cluster of Differentiation 69 (CD69) has also been reported to regulate S1PR1 surface expression, leading to its internalization[636]. CD69 is an early T cell activation marker, responsible for T cell retention in LNs until they are fully activated and ready to leave the LN[637]. T cells are known to sequester to the paracortex of the LN[638], whereas B cells locate to lymphoid follicles[639]. In order to activate B cells, other activated T cells remain in the LN and administer the antigens[640]. The activated B cells then secrete antibodies that circulate through the LVs to the body for efficient phagocytosis[641]. If the necessary antigen has not been encountered, B cells are transported back to the bloodstream through the efferent LVs, in an S1PR1-dependent manner similar to T cells[642]. Although most B and T cells enter the LN through high endothelial venules, effector-memory T cells, which represent the majority of lymphocytes in the body, also seem to circulate from peripheral tissues to the draining LNs[635]. While tissue resident memory T cells lack CCR7 expression[643], these migrating memory T cells express it and are believed to enter the LVs by using similar cues to those discussed above pertaining to DCs[644]. As mediators of inflammation are known to increase vascular permeability, which in turn leads to increased passage of important effector molecules, T cells were shown to help deliver a proper immune response in an IFN-γ dependent manner[645].

For a long time, it was believed that neutrophils are not present within the lymphatic vessels, as they are believed to die at the site of injury and solely contribute to innate immunity. Neutrophils do localize to the LNs, and they were also discovered in the afferent lymph of sheep[646]. In fact, analysis of the migration of neutrophils in response to *S. aureus* showed that they are the first immune cell subset to reach LNs from the site of inflammation[647]. Furthermore, they were seen to carry *S. aureus* particles to the LNs and in a dog model, they were seen to carry fluorescent microspheres from the lung to the tracheobronchial LN[648]. Neutrophils turned out to be a lot more complex than initially thought, and they seem to be able to enter LVs through different mechanisms than DCs. Studies have shown that neutrophils egress from skin via LVs, a phenomenon dependent on CD11b and CXCR4 but not CCR7[646]. However, CCR7 is implicated in the migration of neutrophils to the LNs[649]. Recently, neutrophils are at the core of an important neuro-immune axis that links sleep to

haematopoiesis and atherosclerosis. McAlpine *et al.* demonstrated that mice subjected to sleep fragmentation produce more Ly-6C^{high} monocytes, develop larger atherosclerotic lesions and produce less hypocretin. The latter is a stimulatory and wake-promoting neuropeptide originating in the hypothalamus and travelling through the lymphatic system to reach the bone marrow where it controls myelopoiesis by restricting the production of CSF1 by hypocretin-receptor-expressing pre-neutrophils[650].

Monocytes and macrophages are one of the first responders to tissue injury[651]. Although macrophages are suspected to die *in situ* due to apoptosis, they can be induced to migrate to draining LNs through the afferent lymph[652]. Interestingly, studies have shown that F4/80$^+$ cells, indicative of macrophages, can be seen spread out in the wall of the subcapsular sinus of the LN[652]. These macrophages engulf foreign particles and pathogens and filter the lymph. Macrophages are present in all tissues of the body and have tissue-specific functions, so the ones present in the LN can support LN-specific function[652]. Concerning tissue specific macrophages, similar to DCs, although their presence is rare in the efferent lymph, they are not absent[652]. In fact, under non-physiological conditions, a significant number of macrophages and DCs were seen entering the efferent lymph, even while the lymph node itself underwent considerable remodeling, such as a fibrotic response[653]. To resolve an inflammatory reaction, monocytes need to be mobilized and transported where needed through nearby LVs[654]. By converting to cells resembling DCs, monocyte-derived cells enhance clearance from inflammatory sites such as the atherosclerotic plaque, by getting uptaken through the lymphatic vessels and reaching the draining LNs[654]. Lipid-derived signals are believed to impair migration, and oxidized phospholipids as well as lysophosphatidic acid are disease-prone mediators that shift the fate of monocyte-derived cells to a more immobile phenotype and promote retention within the plaque, thereby impairing their proper uptake by the lymphatic vessels of the adventitia in a DC-like manner[654]. The expression of CX3CL1 by plaque smooth muscle cells was also associated to CX3C Receptor 1$^+$ macrophages retention in plaque[655].

Understanding how all immune cells interact with the lymphatic system is crucial for many pathologies. Although a lot of work is left in order to better grasp all the interactions between the cells and the complex lymphatic network, a multitude of studies have helped

elucidate numerous migratory pathways that have led to a better understanding of how cells constantly adapt to their environment and the stimuli they are given.

3.2.3 Uptake of dietary lipids

Throughout the body LVs exist parallel to the vasculature and are required for lipid transport, such as in the liver, the skin, and the intestine[612]. Consumption of foods rich in lipids have been shown to increase lymph flow[656]. Although lacteals resemble the function of capillaries, a recent study revealed that lacteals are surrounded longitudinally by bundles of smooth muscle cells, indicative of spontaneous contractile dynamics[657]. This indicates that the lacteal has unique tissue-specific lymphatic properties, that enhances lipid transport by active pumping[58]. Furthermore, lacteals do not have button-like junctions similar to those of dermal initial LVs but rather, a combination of both continuous zipper junctions and discontinuous button-like junctions[658].

CM uptake in the lacteals has been the subject of controversy. Paracellular transport was believed to be the only mechanism through which CMs enter the lumen[58]. With the help of transmission electron microscopy images of CMs inside a lacteal, as well as several others entering between two overlapping junctions were seen, showcasing a potential for transcytosis[58]. Once inside the lacteal, CMs are propelled towards the collecting LVs by the pumping activity of the surrounding muscle, as well as by the peristaltic movement of the intestinal wall[659]. Although chylomicron uptake by the lymphatics still needs to be further elucidated, several disease models do attribute a critical role to the lymphatic system in lipid absorption in the gut. Leaky lymphatics were characterized in *Prox1*$^{+/-}$ mice and proved important for lymphatic lineage commitment[540], thereby resulting in adult onset obesity attributed to the adipogenicity of chyle, the milky fluid composed of fat droplets and lymph during digestion, leaking out of the lacteals[660]. Although other genes important for LV integrity have been implicated in chyle leakage, abnormal lipid transport and metabolism, it is interesting to note that lacteals greatly vary with dietary intake, as lacteals were shown to drastically retract into the lamina propria during fasting, but can quickly be restored upon refeeding, within 3 days[661].

In the intestine, VLDLs are synthesized through a different pathway than CMs and are more often seen in fasting states. Similar mechanisms of transport were observed for VLDL

particles in the lacteals. Intestinal VLDLs seem to contribute to plasma VLDLs from which they appear to be indistinguishable[662].

Mechanisms of LDL uptake by the lymphatics are scarce, probably due to the fact that most LDL gets degraded by peripheral cells or the liver and a significant quantity might not reach the lymph. Importantly, new research has shown that LDLR, important for internalization of LDL in the liver which leads to its excretion, is present at the surface of LECs[230]. Whether LDL is uptaken in the lymphatics through this receptor, although highly probable, remains to be proven. At the very least, LDLR modulation seems to be associated with lymphatic dysfunction in early atherosclerosis[230], but unlikely to be solely dependent on LDL binding.

Alongside the liver, intestinal lymph contains a high amount of apoA-I as a component of nascent CMs and in combination with phospholipid and cholesterol as discoidal nascent HDL[591]. From extrahepatic tissues, HDL transports excess cholesterol to the liver by mRCT, as previously described[80]. Opposing the concept of receptor-independent entry, as seen in CMs, SR-B1 is suspected to be one receptor responsible of HDL uptake by the initial LVs[663]. However, it is suggested that the lymphatic vasculature in the skin may just be abnormal in SR-B1 deficient mice, which would negatively impact mRCT due to general impairment of LVs, therefore indirectly of SR-B1 mediated uptake of HDL into the lymphatics[664]. The quest for further clarification of the complex uptake of HDLs by the lymphatics is ongoing, but nonetheless LVs are highly promising targets for lipid-related diseases, such as atherosclerosis.

Recently, a novel pathway called trans-intestinal excretion of cholesterol (TICE) has been proposed to play an important role in cholesterol metabolism. TICE is believed to be inducible and is complementary to the hepatobiliary pathway, thus allowing the elimination of plasma cholesterol directly into the intestine lumen through the enterocytes[665]. An estimated 30% neutral sterols found in feces are due to the TICE pathway, which makes it an attractive target for therapy modulation[666, 667]. To date, HFD, plant sterols, and liver X receptor activation as well as PPARδ ligands have been identified as activators[668]. However, despite its involvement in cholesterol clearance, HDL had no significant role in TICE in mice[669, 670].

In conclusion, lipoproteins seem to enter lymph, through lacteals or initial LVs, by different mechanisms that may depend on specific receptors or alternative paths. Certainly, there is an increased need for research on lymphatic function and lipid metabolism.

3.3 Mechanobiology of lymphatic contraction

Lymph flow is much slower than blood flow, and even a short interruption leads to rapid swelling of the LVs and the interstitial space[671]. Mainly aqueous with relatively low concentrations of proteins and cells compared to blood, lymph is driven through the collecting LVs via two primary mechanisms. Intrinsic pumping is characterized by muscle cell contraction, the lymphangions and the valves, whereas extrinsic pumping involves compression mechanisms such as the movement of skeletal muscle or other tissues surrounding the lymphatics, as well as respiration[672]. The opening and closure of lymphatic valves is highly coordinated and is required for efficient lymph transport. When there is a change in surrounding interstitial pressure, LVs will either expand in order to fill with lymph or rather, they will contract and push lymph forward[673]. One of the major driving forces of lymphatic pumping is the surrounding LMCs whose activity largely depends on intracellular Ca^{2+} levels which are modulated by NO levels produced by the lymphatic endothelial cells[674]. Specialized tools are increasingly available and can aid the study of lymphatic vessels in patients and genetically modified mice. This offers unprecedented hope for the development of therapeutic strategies that target lymphatic dysfunction even in certain diseases that were previously unknown to contain a lymphatic component[601].

3.3.1 Intrinsic lymphatic pump

The intrinsic lymphatic pump represents the necessary machinery for contraction found within the lymphatic wall. The contractile unit of the collecting LVs is the lymphangion. As the force generated by one lymphangion is not enough to propel lymph throughout the lymphatic network, they are arranged in series and separated by valves[598]. Therefore, each lymphangion acts as a local pump chamber and drives a bolus of lymph along to the next couple of lymphangions to ensure long-distance lymph transport[674]. Lymphatic valves operate in an open–close manner, as their function is passive and responds to differential pressure between pre- and post-valve lymphagions[675]. Unlike blood vascular smooth muscle, lymphatic muscle is characterized by rapid, phasic contractile activity that drives the intrinsic lymphatic pumping, as well as a slower, tonic form of contractions as seen in blood SMCs[676]. It is the responsibility of the lymphatic muscle to regulate the lymphatic diameter, as well as its

compliance and resistance, in order to be able to modulate lymph flow[674]. The uniqueness of lymphatic contractility is due in part to the fact that lymphatic muscle possesses both smooth and striated muscle features[677]. The lymphangion contraction is an active process that depends on force being generated by LMCs, in turn dependant on myocyte intracellular Ca^{2+} levels. In this regard, a subset of muscle cells is hypothesized to act as pacemaker cells, and the propagation of the action potential that causes synchronized contractions of lymphangions downstream, is believed to vary with LMC density which is lowest at valve sites. Supporting action potential generation is the heterogeneity in the electrical activity of LMCs, which in turn, when they slightly overlap, protects against action potential decoupling[678, 679].

Phasic contractions of lymphangions can also be spontaneous since they can take place without any stimulation. Therefore, contraction by the lymphangions is an alternate process, and simultaneously, two or more adjacent lymphangions can contract or relax[680]. Nonetheless, even if each lymphangion contraction dynamic is independent, stroke efficiency is improved by interlymphangions electrical communication which allows them to synchronize[672]. Intercellular communication is dependent upon expression of gap junction channels and connexins, which are well characterized in lymphatic and blood vessels[681, 682].

The intraluminal pressure of a lymphangion is modulated by the upstream lymph flux, thus leading to expansion of the vessel wall. Such stretch will trigger a myogenic response from the surrounding LMCs which will ensue thus increasing intraluminal pressure that leads to proper lymph propulsion[683]. Several mechanisms trigger and modulate vessel phasic or tonic contraction by influencing the contractile and accessory proteins characteristic of smooth and striated muscle cells expressed in LMCs[684]. Modulating the myosin light chain 20 (MLC_{20}) is important for lymphatic contractile strength. MLC_{20} is controlled by the activities of myosin light-chain kinase (MLCK) and myosin light-chain kinase phosphatase (MLCP). Following an increase in cytosolic Ca^{2+} levels, the lymphatic muscle contraction begins by binding to the intracellular Ca^{2+} receptor protein, calmodulin, and activates MLCK. Sequentially, MLCK phosphorylates MLC_{20} and the lymphatic contraction takes place. To induce muscle relaxation, the MLC_{20} phosphorylation can be reversed by MLCP[676, 685].

The intrinsic lymph pump is very similar to a cardiac cycle as the phasic contractile cycle can be separated into lymphatic diastole and systole[674]. Due to this, lymphatic function can

be evaluated using different parameters such as frequency, stroke volume, ejection fraction and lymph pump flow[686]. Complementary to these, four major lymphatic regulators can be calculated[601]. The preload is based on the end diastolic volume and can be modulated as an increase in filling pressure that leads to an increased pump output as per the Frank-Sterling relationship. The afterload is the force against which the pump must eject. The contraction frequency is very sensitive to pressure, it was shown to increase proportionally with the magnitude of step increases of the input pressure, demonstrating a fivefold increase over control at 16 cmH_2O. Alternatively, at 8 cmH_2O and lower, the frequency of contraction was only twofold higher than control[687]. Interestingly, similar to the heart, a high frequency can limit filling and have a negative impact on the amplitude of contraction. Lastly, the contractility is generally used to assess both the frequency and amplitude of contraction in response to pressure modulation or agonist addition[688]. Regulation of the intrinsic lymph pump can be observed using local, neural and humoral factors, like adrenergic agonists, prostanoids, bradykinin, substance P, natriuretic factors, among others[689-693]. In a concentration-dependent manner, prostaglandin E2 and prostacyclin decreased lymphatic frequency of contraction, with no effects on the amplitude in guinea pig mesentery[694]. Contrarily, bradykinin increases lymphatic contraction frequency through stimulation of B1 receptors, by enhancing the lymphatic pacemaker activity independent of LMC stretch since the diameter of the lymphatic vessels was not altered[691]. Substance P alternatively, enhances the lymphatic pump efficiency by acting mainly on LMCs[695]. The atrial natriuretic peptide significantly inhibited any lymph pumping activity in isolated bovine LECs[696]. More physical modulations, such as stretching and shear stress, also should be taken into consideration as they play a crucial role in modulating lymphatic tone and pump function[697].

Aging is well known to have adverse effects on the body, lymphatics not excluded, as increased chances of edema and impaired immune functions were reported[698]. As far back as 1983, it was reported that the human thoracic duct reaches a maximum number of LMCs per unit area at about 30 years of age, and as of 65 years of age, atrophy of these cells begins. A destruction of the lymphatic elastic matrix paralleled this change and formation of tiny sacs containing only ECs were identified downstream of the lymphatic valves[697]. Furthermore, the number of lymphatic vessels and their connections were also significantly decreased in the human mesentery, after 65 years of age. A decrease in the depth of the LEC glycocalyx and

significant reduction of gap junction proteins in aged lymphatic collectors isolated from rats, were also observed[698]. These changes lead to impaired lymphatic vessel integrity, that leads to hyperpermeability with pathogens escaping from the collectors into the surrounding tissue and a decreased ability to control tissue fluid homeostasis. Other experiments on aged rats indicated that both the pacemaking and contractile machinery are altered. The phasic lymphatic contraction frequency was decreased, as well as the tone and the amplitude, pointing out an age-dependant weakening of lymphatic muscle resulting in a diminished ability to create the force needed[697]. In human legs, there was a significantly inverse correlation between lymphatic pumping and age. As well, comparison between the sexes led to the conclusion that lymphatic pumping is significantly impaired in postmenopausal women[699]. Noteworthy, further investigations are longed for to get a better grasp of the mechanisms that modulate the lymphatic vessel dynamics during aging.

3.3.2 The extrinsic lymphatic pump

The extrinsic lymph pump is composed of all of the forces that are generated outside of the lymphatic itself which can generate supplementary lymph pressures favorable to advance lymph flow[697]. These outside forces originate from tissues surrounding the lymphatic vessels, most commonly through the contraction of the muscles contained in those tissues, which leads to compression of the lymphatic vessels. Such forces include the pressure generated by lymph formation, cardiac contractions, respiration motions, skeletal muscle contractions, arterial and venous pulsations and GI peristalsis[697]. For example, at the level of the thoracic duct, both cardiac and respiratory tissues contribute rhythmic compressions and expansions of the lymphatic, thus enabling lymph propulsion[700, 701].

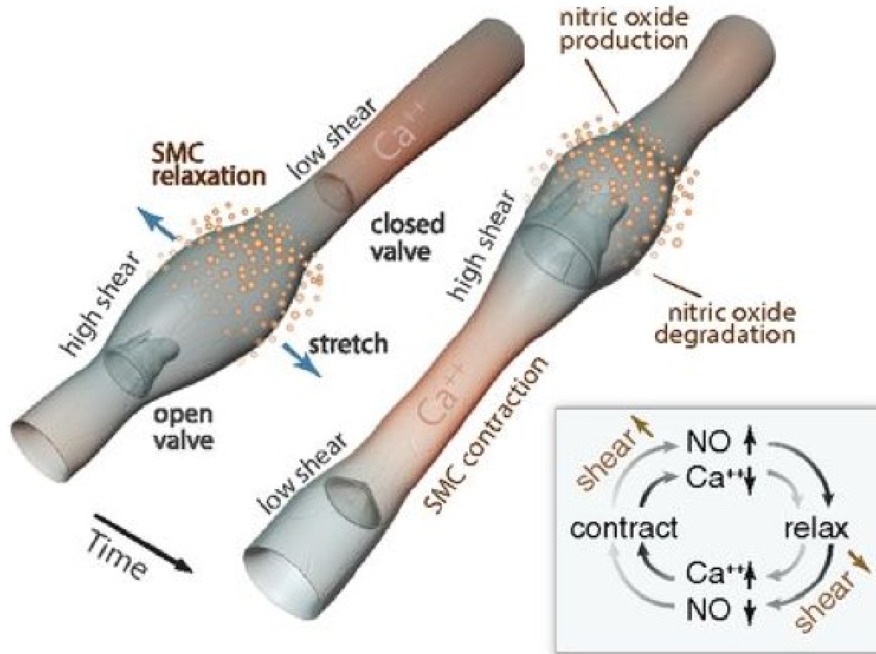
Exercise plays an important role, as the contraction of skeletal muscles causes significant compression and expansion of adjacent lymphatic vessels. Importantly, they supply the needed force to empty and refill those lymphatics. In human legs, during exercise, lymph pressure and flow correlate with the intensity of the skeletal muscle activity[702]. In sheep, short duration exercise showed an increase in frequency of lymphatic vessels contractions and lymph flow, but no effects on the amplitude of contraction were reported[703]. Furthermore, it is believed that during exercise both intrinsic and extrinsic pumping mechanisms are strengthened[704]. The extrinsic pump is modulated by the activity of muscles in motion employed during exercise,

whereas the intrinsic pump is favored by a decrease in perilymphatic accumulation of proinflammatory T cells and macrophages, and reduction of inflammatory cytokines like TNF-α and IFN-γ[705, 706]. In pathological conditions, such as breast cancer, lymphedema is the most common physical complication, that develops any time after surgery[707]. It was shown that regular exercise not only promotes lymphangiogenesis, which reduces the damage caused by dissection and radiotherapy[708], but exercising for just two minutes can normalize lymphatic pressure and accelerate lymphatic drainage as demonstrated in lymphoedema[709, 710]. Additionally, Hespe *et al.* observed that exercise decreased inflammation that led to lower levels of iNOS and thus a decrease in immune cell accumulation, and it also upregulated the expression of crucial lymphatic genes like *Vegfr-3* and *Prox1* [705].

3.3.3 Lymphatic relaxation: endothelial derived relaxing factors and fluid shear stress

Shear stress is the most important physiological stimulus of NO production and phosphorylation of eNOS by protein kinase B (Akt) represents a Ca^{2+}-independent regulatory mechanism for its activation (Figure 14).

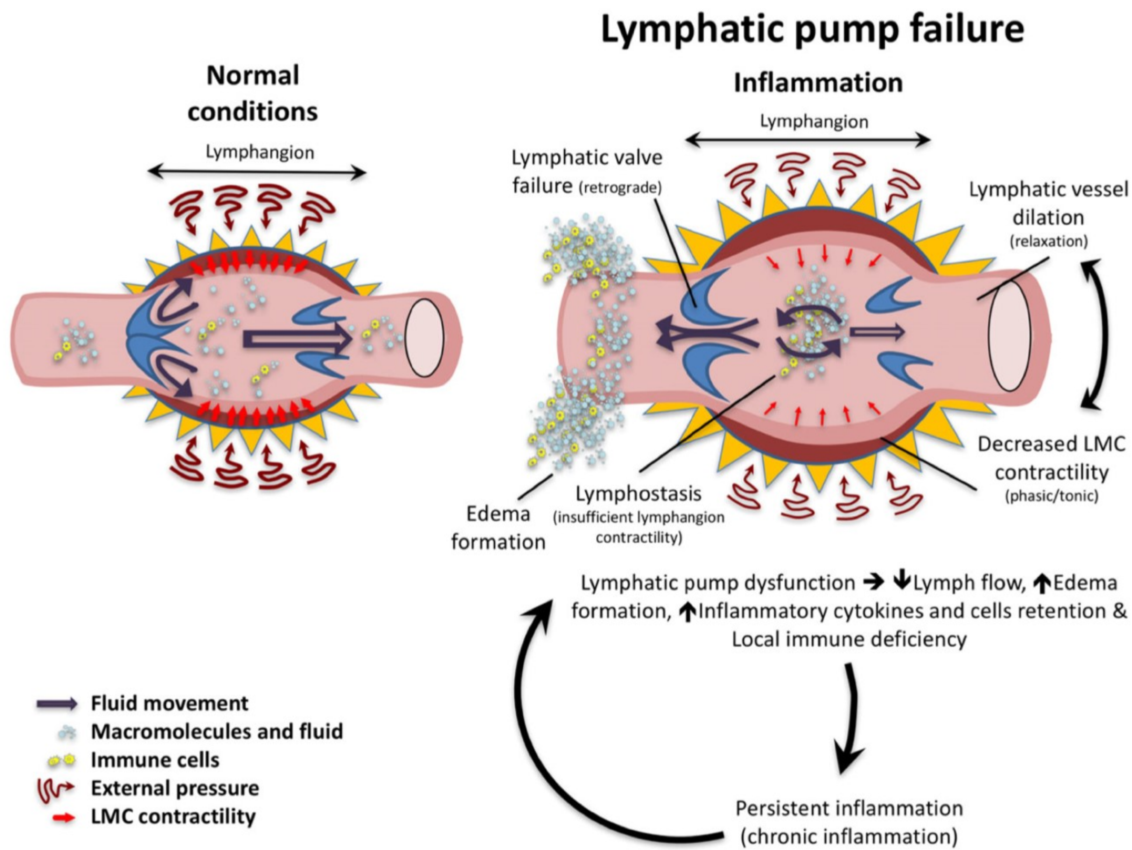
Figure 14. Effect of fluid sheer stress on lymphatic function.



When it comes to the lymphatic system, once it is released, NO can diffuse to the muscle cells enveloping the lymphatic vessels and affect pumping. It works by modulating Ca^{2+} release and uptake[711], as well as the enzymes responsible for force production[712, 713]. NO decreases vascular tone through a cGMP-dependent mechanism, that leads to reduced intracellular Ca^{2+}, by inhibiting its entry from internal stores. Conversely, it can activate several other channels to increase Ca^{2+} outflux[714]. Figure adapted from Kunert *et al*[715].

Overall, NO is a vasodilator that opposes the Ca^{2+} response of the lymphatic vessels, leading to dilatation and decreased contraction frequency[716]. Removing NO, reduced the contraction strength and increased lymphatic diameter, in both eNOS$^{-/-}$ mice and wild type mice that were applied N(omega)-nitro-L-arginine methyl ester (L-NAME), a NOS inhibitor[717]. Furthermore, NOS blockade caused the lymphatic vessels to contract at a higher frequency to be able to maintain the pump flow, due to impairment in filling and low phasic contraction strength[697]. As observed, the lymphatic function is under ongoing and thorough influence of a variety of factors that modulate its unique contractile ability. Under inflammatory conditions, a disbalance occurs and eNOS is not able to maintain robust lymphatic contractions (Figure 15).

Figure 15. Impairment in the collecting lymphatic vessel pumping capacity under inflammatory conditions.



Physiologically, basal levels of endogenously released NO by eNOS maintain proper lymphatic contraction strength by lowering their frequency[718]. In inflammation, iNOS releases abundant levels of NO and floods the already present flow-dependant NO released by eNOS, thereby inhibiting autonomous lymphatic vessel contractions[719]. This leads to detrimental effects such as edema and reduction of antigen delivery to the lymph nodes, and thereby reduces antigen-presenting cells and T cell activation. Figure from Al-Kofahi *et al*. illustrated with permission from © 2017 Japanese Society for Neuroimmunology.

The surplus of NO originates from proinflammatory cells, such as CD11b^{+} myeloid cells, that express iNOS and infiltrate the tissue surrounding the contractile lymphatics during inflammation [717]. Experiments with WT mice with *iNOS*$^{-/-}$ bone marrow transplants showed that they were able to maintain robust contractions[717]. Similarly, removing the excess NO produced by iNOS in bone marrow-derived DCs increases contraction strength and augments

lymph flow rates[720]. Additionally, Scallan *et al.* demonstrated that while basal NO is needed physiologically to decrease an exaggerated contraction amplitude and frequency, greater NO production inhibits all aspects of contractile function and impairs transport through the LVs. As such, in the peripheral lymphatic vasculature, NO production depresses the contractile function as much as deemed necessary to influence the lymph flow needed for fluid regulation, humoral immunity and cancer metastasis[718]. Other factors, such as cytokines like TNF-α[721] and growth factors like VEGF-C[722] are also likely to affect collecting lymphatic contraction, and even other gases, like carbon monoxide, can regulate LMC activity[723]. Moreover, some critique the evidence that NO plays as much of a significant role in enhancing lymphatic pumping as previously reported *in vivo*. Michael J. Davis believes that further studies need to test the precise role of NO under conditions of well controlled pressure and specific flow, to account for possible adjacent changes systemically following eNOS activation[724]. In response, Munn *et al.* defend their *in vivo* model, catered to study NO and Ca^{2+} dynamics rather than lymphatic basal tone, and suggest that experimental studies performed in excised vessels, on the contrary, may not take into account the entire environment surrounding the LVs[725]. While some of the models used to date to study the modulation of contractility by NO can incite debate, quite a few studies can at least agree that forced flow decreases both the contraction amplitude and the frequency of LVs[726-729].

Immune cell and lipoprotein transport through the lymphatic network is now under grand investigation, especially with the emerging technology and varied animal models. Therapeutic targets based on the interplay between lipid metabolism and the functional properties of the immune cells, would greatly aid in atherosclerosis, as well as other chronic inflammatory disorders. Nonetheless, lymphatic function is impaired in mouse models of hypercholesterolemia[215, 230, 730] and improper function of the lymphatic network contributes to atherosclerosis exacerbation.

RESULTS

4 GENERAL THESIS OBJECTIVES

Similar to the blood circulation, the lymphatic system is essential for the proper functioning of the body. It is composed of specific organs and a large network of lymphatic vessels that carry lymph. While intricate and with a multitude of roles, the lymphatic vasculature has recently emerged as a critical participant in proper excretion of cholesterol from tissues, specifically the atherosclerotic plaque. Based on published data by Dr. Martel[215] and the lab[230], several objectives were brought forward and allowed us to hypothesize that lymphatic transport is impaired in young, non-hypercholesterolemic atherosclerosis-prone mice, by first being associated with a defect in the collecting LVs.

We herein sought to further understand the lymphatic vessel dysfunction associated with atherosclerosis and assess from a potentially therapeutic point of view whether we can improve and/or preserve lymphatic vessel function. Our aim is to redefine current therapies and emphasize the relevance of preserving this network to limit the progression of atherosclerosis.

Specific objectives were elaborated, and we first aimed to assess whether lesion onset could be prevented if we rescue the lymphatic dysfunction early on in the development of the disease. We questioned whether collecting lymphatic vessel impairment can be abolished by enhancing VEGF-C/VEGFR-3 specific binding, by administering therapy with the lymphatic growth factor VEGF-C that was specifically tailored to act solely upon the lymphatic vasculature. Conversely, we acknowledge that clinically, patients are diagnosed when the onset of the lesion is already commenced. As such, plaque regression is especially important and therapeutically relevant since most CVD patients are treated after advanced plaques are established. Moreover, patients having survived an MI are at significantly higher risk of recurrent infarctions and even stroke[731]. Consequently, we also aimed to investigate if an established atherosclerotic lesion could benefit from apoA-I therapy, which in addition to its pleiotropic anti-atherogenic roles already uncovered, would enhance lymphatic transport and maintain CLEC-2 mediated platelet activity. Due to the fact that platelet presence is scarce in lymph, this led us to acknowledge the importance of diverse cell releasants that had previously been identified in most fluids and tissues of the body, with the exception of lymph. We discovered novel particles with high potential as biomarkers of atherosclerosis, namely EVs of

heterogeneous origin, in the lymph of mice with an atherosclerotic background. Three different manuscripts have been published and will be presented in sections 4.1 to 4.3.

4.1 Presentation of the first article

We had previously reported that lymphatic dysfunction following treatment with VEGF-C 152s, a mutant form of VEGF-C that binds solely to VEGFR-3, can be rescued in pre-atherosclerotic mice, but not due to improved absorption through the lymphatic capillaries nor through a cholesterol-dependent mechanism. Collecting lymphatic vessels were shown to be potentially responsible for the impairment in lymphatic function of *Ldlr*$^{-/-}$*; hApoB100*$^{+/+}$ mice. We herein aimed to specifically target the lymphatic contraction capacity prior to lesion formation as a novel therapeutic approach for the prevention and treatment of atherosclerosis.

Our results suggest that early treatment with VEGF-C 152s:

- Prevents lymphatic dysfunction and maintains the contractile capacity of the collecting lymphatic vessels throughout the whole atherosclerotic process;
- Limits plaque buildup, then stabilizes plaque progression in *Ldlr*$^{-/-}$ mice.

Participation of each author of the article:

AM: Project conceptualisation, troubleshooting, methods validation, experiments, results analysis and writing of the manuscript.

AS: Experiment assistance and reading of the manuscript.

CM: Project conceptualisation, resources, troubleshooting, methods validation, experiments, results analysis and writing of the manuscript.

4.1.1 First article

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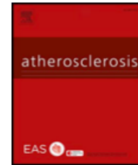

Early rescue of lymphatic function limits atherosclerosis progression in *Ldlr*$^{-/-}$ mice

Andreea Milasan[a,b], Ali Smaani[a,b], Catherine Martel[a,b,*]

[a] Department of Medicine, Faculty of Medicine, Université de Montréal, Montreal, Quebec, Canada
[b] Montreal Heart Institute, Montreal, Quebec, Canada

HIGHLIGHTS

Early treatment with VEGF-C 152s:

- Prevents lymphatic dysfunction and maintains the contractile capacity of the collecting lymphatic vessels throughout the whole atherosclerotic process;
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Early rescue of lymphatic function limits atherosclerosis progression in *Ldlr*$^{-/-}$ mice

Andreea Milasan[1, 2], Ali Smaani[1, 2] and Catherine Martel[1, 2]

[1] Department of Medicine, Faculty of Medicine, Université de Montréal, Montreal, Quebec, Canada,

[2] Montreal Heart Institute, Montreal, Quebec, Canada,

Corresponding author:

Catherine Martel, PhD

Faculty of Medicine

Université de Montréal

Montreal Heart Institute

5000, Belanger street, Room S5100

Montreal, Quebec (Canada)

H1T 1C8

Telephone: (514) 376-3330 #2977

E-mail: catherine.martel@icm-mhi.org

ABSTRACT

Background and aims. Our previous data showed that lymphatic function impairment occurs before the onset of atherosclerosis in mice and is precociously associated with a defect in the propelling capacity of the collecting lymphatic vessels. Concomitantly, we found that lymphatic transport can be restored in mice by systemic injections of a mutant form of VEGF-C (VEGF-C 152s), a growth factor known to increase mesenteric collecting lymphatic vessel pumping through a VEGFR-3-dependent mechanism in rats. In the present study, we aimed to determine whether and how early modulation of collecting lymphatic vessel function could restrain atherosclerosis onset and limit its progression.

Methods. Before the administration of a pro-atherosclerotic regimen, *Ldlr*$^{-/-}$ mice at 6 weeks of age were injected intraperitoneally with VEGF-C 152s or with PBS three times per week for 4 weeks, fed on high fat diet (HFD) for an additional 8 weeks to promote plaque progression, and switched back on chow diet for 4 more weeks to stabilize the lesion.

Results. Early treatment with VEGF-C first improved lymphatic molecular transport in 6-week-old *Ldlr*$^{-/-}$ mice and subsequently limited plaque formation and macrophage accumulation, while improving inflammatory cell migration through the lymphatics in HFD-fed mice. The contraction frequency of the collecting lymphatic vessels was significantly increased following treatment throughout the whole atherosclerotic process and resulted in enhanced plaque stabilization. This early and maintained rescue of the lymphatic dysfunction was associated with an upregulation of VEGFR3 and FOXC2 expression on lymphatic endothelial cells.

Conclusions. These results suggest that early treatments that specifically target the lymphatic contraction capacity prior to the lesion formation might be a novel therapeutic approach for the prevention and treatment of atherosclerosis.

INTRODUCTION

Atherosclerosis, characterized by the over accumulation of cholesterol and immune cells within the arterial wall, is the principal cause of mortality worldwide and is at the origin of most cardiovascular diseases[1]. The lymphatic system is now increasingly emerging as a potential contributor to cardiovascular disease. Although it has been well known for many years that lymphatic vessels are present within the adventitia of blood vessels[2], it is only recently that Martel *et al.* introduced a new integrated model of reverse cholesterol transport (RCT) in which they clearly showed that without a functional lymphatic network, cholesterol cannot be properly conducted out of the artery wall[3]. As lymphatic vessels are responsible for the transport of immune cells, their functional properties have become of great interest in many pathological settings. In contrast to the blood vascular network, the lymphatic vasculature is an open, unidirectional and low-pressure vascular system[4]. The lymphatic vessels (LVs) are composed of two different entities, bearing distinct but complementary roles. Firstly, initial LVs, also called lymphatic capillaries, help absorb plasma ultrafiltrates from the periphery, they are highly permeable and they specifically express lymphatic vessel endothelial hyaluronan receptor (LYVE-1) at the surface[5]. Secondly, collecting LVs are contractile entities that propel lymph in a unidirectional manner with the help of intraluminal bi-leaflet valves. They are covered sparsely with smooth muscle cells (SMCs)[5].

Different transcription factors regulate lymphatic development, some of the most crucial ones being vascular endothelial growth factors (VEGFs). The vascular endothelial growth factor receptor-3 (VEGFR-3) serves as a receptor for LV-specific VEGFs, such as VEGF-C which is important for normal development of lymphatic vessels and is critical in the process of lymphangiogenesis[6]. In addition to this crucial role, VEGF-C was also shown to stimulate lymphatic pumping[7]. VEGF-C 156s is a point mutant that only binds to and activates signaling through VEGFR-3, which unlike wild type VEGF-C, is unable to bind VEGFR-2, a well-known receptor that contributes to angiogenesis[8]. Supporting data from the literature suggests that this mutant form of VEGF- C increases lymphatic contraction frequency, dilation, and pump flow through its action on VEGFR-3[7, 9]. Interestingly, VEGF-C also reversed hypercholesterolemia-associated lymphatic dysfunction in *apoE*$^{-/-}$ mice[3]. VEGF-C 152S is analog to the human VEGF-C 156S mutant and also a selective VEGFR-3 agonist. Of interest,

the rat VEGF-C cDNA encodes a pre-pro-protein of 416 amino acids residues making it almost identical to the mouse VEGF-C protein. When treating young *Ldlr*$^{-/-}$*; hApoB100*$^{+/+}$ mice systemically with VEGF-C 152s, this rescued the lymphatic impairment apparent before atherosclerosis onset, in a cholesterol-independent manner[10]. In another inflammatory model, VEGF-C-dependent stimulation of lymphatic function ameliorated experimental inflammatory bowel disease[11]. To date, no study addressed whether VEGF-C-dependant stimulation of lymphatic function before the onset of atherosclerosis had an impact on subsequent atherosclerotic plaque buildup and characteristics of lesion stability.

This present study suggests that early treatment with VEGF-C 152s instigates and maintains rescue of the lymphatic dysfunction throughout the whole atherosclerotic process, restraining atherosclerotic plaque size and CD68^{+} macrophages content, and stabilizing plaque progression.

MATERIALS AND METHODS

Sex of mice. This study was conducted on *Ldlr*$^{-/-}$ female mice on a C57BL/6 background that were purchased from Jackson Laboratories. As the most widely reported sex effect on atherosclerosis is that female mice have larger aortic root lesion areas than male mice[12], we deemed fit to start our present study on this one gender with exacerbated plaques.

Experimental model. One of the most commonly used atherosclerotic model is the LDL receptor–deficient mouse[13]. The normal diet–fed *Ldlr*$^{-/-}$ mice only develop small atherosclerotic lesions, even at an advanced age (beyond 6 months) and therefore, this mouse strain is fed a Western diet (HFD—adjusted calories diet, 0.2% total cholesterol and 42% from fat, Harlan 88137) to accelerate atherosclerosis. To our knowledge, no systematic pathological analysis of lesion development and lymphatic function has been reported in normal diet–fed *Ldlr*$^{-/-}$ mice that are not yet baring an atherosclerotic plaque, but prone to develop atherosclerosis under a pro-atherogenic regimen.

Experimental design. Animals were housed in a pathogen- free environment under 12-hour light–dark cycles with free access to water. The experimental design of our study is illustrated

in Supplementary Figure 1. At 6 weeks of age, mice were injected intraperitoneally with VEGF-C 152s or control (phosphate buffer saline, PBS), 50ng/25g of body weight. Following, for 8 weeks, mice were fed a high-fat diet (HFD - adjusted calories diet, 0.2% total cholesterol and 42% from fat, Harlan 88137) to induce plaque progression. Contrarily to a 10-12-week diet that will lead to a well-established lesion, we aimed to assess an early -stage evaluation of the plaque, which is the main reason why our time lapse is shorter. Afterwards, mice were switched back to a chow diet for a remainder of 4 weeks in order to stop plaque growth. At the end of each milestone, 4 weeks of VEGF-C 152s treatment, 8 more weeks of HFD and 4 more weeks of chow diet, lymphatic function assays were performed, and blood and lymph were collected under anesthetic conditions. Following this, mice were euthanized by cardiac puncture while on isoflurane administration (4% for induction, 2–3% for maintenance), or by carbon dioxide (CO_2), and were perfused with 15 mL PBS. Finally, organs were collected and stored until batch analysis. Lymph nodes (LNs), ears, dermal back skin sections, aortas, hearts, and popliteal collecting lymphatic vessels were harvested and either freshly processed for flow cytometry analysis, or fixed in 4% paraformaldehyde, 10% formalin or radioimmunoprecipitation assay buffer (RIPA buffer) for future analysis. All animal studies were performed in accordance with the Canadian Council on Animal Care guidelines and approved by the Montreal Heart Institute Animal Care Committee.

Atherosclerotic lesion assessment. The heart and aorta were removed and fixed in 4% paraformaldehyde. The heart was transferred into PBS containing 30% sucrose (wt/vol) overnight at 4°C before being immersed in optimal cutting temperature compound (OCT) and stored at -80°C. Eight-micrometer-thick cryosections of the aortic sinus were prepared. Aortas were cleaned by removing the surrounding fat and were then split along their outer curvature. Neutral lipid assessment in atherosclerotic lesions in the aortic sinus and *en face* aorta was performed by Oil-red-O (ORO) staining (Sigma, O-0625).

Total circulating cholesterol, lipoprotein profile, triglycerides and apolipoprotein B levels. Lymph was collected as previously described[14]. Blood was collected on ethylenediamine tetraacetic acid (EDTA) by cardiac puncture and plasma was obtained following centrifugation at 2400 g for 10 minutes, after which sucrose was added before samples were stored at −80°C.

Mouse lipoproteins were separated from plasma by size exclusion chromatography [fast protein LC (FPLC)] using a Superose 6 column on a FPLC system with a Model 500 pump from Waters (Milford, MA). In short, a 100 µl aliquot of mouse plasma pooled equally from five (5) different mice was injected into a 1.0-ml sample loop and separated with a buffer (0.15 M NaCl, 0.01 M Na_2HPO_4, and 0.1 mM EDTA) at a flow rate of 0.5 ml/min. Forty-three fractions of 0.3 ml each were collected with the lipoproteins being contained within. Batch analysis was performed to measure circulating total cholesterol (Wako) in lymph, plasma and plasma lipoprotein fractions according to the manufacturer's protocol. Due to technical limitations for lymph volume, triglyceride (#10010303, Cayman Chemicals) and apolipoprotein B (apoB, ab230932, Abcam) circulating levels were measured solely in plasma according to manufacturer's protocols.

Initial Lymphatic Density Quantification and immune cell accumulation. Cross-sections of the aortic sinus were stained with anti-LYVE-1 (Abcam, ab14917) and anti-CD68 (Biolegend, 137001) antibodies, and then incubated with the appropriate secondary antibodies. As macrophages can also be positive for LYVE-1, adventitial initial lymphatics were identified as LYVE-1^+CD68^- cells forming vessel-like shapes. Whole- mount immunohistochemical analysis of the ear dermis to visualize lymphatic vessels was performed as previously described. Ear dermis was stained for lymphatic capillaries (anti-LYVE-1) at 4°C, and then sections were incubated with Alexa Fluor 647-conjugated donkey anti- rabbit antibody (Abcam, ab150075) and Cy3 donkey anti-rat (Jackson ImmunoResearch, 712-165-150). All imaging was performed on a Fluoview FV10i (Olympus). The relative quantification of the number of initial lymphatics (LYVE-1^+ vessels) and the total surface area they occupy was determined by computer-assisted morphometric analysis.

Atherosclerotic lesion calcium and collagen content. Histological visualization of calcium deposits (ab150687) and collagenous connective tissue fibers (ab150686) in 8µm aortic sinus frozen sections was performed as per manufacturers' instructions. Briefly, for calcium staining, frozen sections were treated with a silver nitrate solution and the silver is deposited by replacing the calcium reduced by the strong light. Calcium in mass deposits will show as black, whereas dispersed deposits will be visualized as metallic silver.

Lymphatic Functional Assessment. Lymphatic function was analyzed by four complementary methods.

1) **Cellular transport -** The propensity of dendritic cells to migrate through the lymphatic vessels from the periphery to draining LNs was measured as described previously[15]. The animals were euthanized 18 hours after the application of a solution that contains fluorescein isothiocyanate (FITC), dibutyl phthalate, and acetone solution, in order to instigate an immune response and thus dendritic cell trafficking. The corresponding skin-draining LNs were recovered and enzymatically digested in collagenase D for 25 minutes at 37°C. Cells were then passed through a 70-μm cell strainer, washed, counted, and stained for analysis by flow cytometry (BD Biosciences LSR II). Conjugated antibodies CD11b PerCp-Cy5.5 (BioLegend, CA101227-BL), CD11c PeCy7 (Tonbo Biosciences, 60-0114), MHCII-VioletFluor 450 (Tonbo Biosciences, 75-5321), and CD45-APC (Tonbo Bio- sciences, 20-0451) were used. The number of dendritic cells that uptake FITC and traveled to the corresponding skin-draining LN was then counted based on the total LN cellularity (% of $FITC^+$ cells/# of cells/LN).
2) **Lymphatic vessel permeability -** Lymphatic vessel permeability was assessed using Evans Blue dye for tracing the path of lymph through popliteal lymphatic vessels. Mice were anesthetized with isoflurane and skin was carefully removed from the legs. Following Evans Blue intradermal injection in the footpad, popliteal collecting lymphatic vessels were visualized using a Stereo Discovery V8 (Zeiss). The effusion of Evans Blue around the vessel marks the area the leakage covers and was analyzed using ImageJ software.
3) **Lymphatic vessel contractility -** Lymphatic vessel contraction capacity was assessed following injection of ovalbumin, coupled to Alexa Fluor 488 (Fisher, catalog # O34781) in the dermis of the footpad of the mouse to enable proper visualization of collecting lymphatic vessels[9]. Briefly, the mouse was placed on his abdomen on a heat pad at 40°C and an incision was made all around his upper hind limb. Following, skin was removed ensuring no blood vessels were severed while pulling the skin away. Warm saline was administered to keep the limb tissue hydrated at all times. 10uL of Alexa Fluor 488 (2mg/mL) were injected in the footpad, following which the foot was moved three

times for the vessels to appear on the screen. Once adequate perfusion of tracer was confirmed, the vessel was observed for 5 minutes to ensure that the lymphatic contractility was active and that no obstructions had occurred or tearing of vessels. Timing was cautiously kept equal for all subjects. The *in vivo* contraction imaging was performed for a duration of 10 minutes with photos being taken at intervals of 1 second with an Axiozoom V.16 microscope. It has been proposed that alterations in lymphatic smooth muscle activity significantly impact lymphatic function. Numerous endogenous vasoactive agents are known to constrict lymph vessels. We used 10uL of phenylephrine (1.0 µM, Sigma-Aldrich), which acts upon alpha-adrenoceptors on lymphatic smooth muscle[16], and injected in the footpad of the mouse. Thereafter, the foot was moved three times after which it was left to rest for 5 minutes. Acquisition was performed for 10 minutes and photos taken at intervals of 1 second, as mentioned above.

4) **Lymphatic molecular transport** – Following the aforementioned analysis of lymphatic vessel contractility, blood was collected, and presence of fluorescent ovalbumin was quantified using a fluorescence plate reader to assess the efficiency of lymphatic vessels to propel lymph into the bloodstream.

Lymphatic contraction frequency and amplitude analysis. Videos of the collecting lymphatic vessel contraction capacity were analyzed using ImageJ. We traced a line of equivalent length at 3 different regions of interest (ROI) along the vessels. The raw integrated density (RawIntDen) was assessed for each and a graph was computed. Appropriate peaks and valleys were manually identified and counted by two different observers. The frequency and amplitude of each ROI were assessed and an average per mouse was computed[17, 18].

Immunofluorescence of the collecting lymphatic vessel. Popliteal collecting lymphatic vessels were identified following Evans Blue dye intradermal injection, as described above and harvested. For analysis of lymphatic vessel integrity, whole-mount immunofluorescence analysis following incubation with anti -smooth muscle actin already coupled to FITC (Sigma), -FOXC2 (R&D Systems, AF6989), -phosphorylated eNOS, -VE-cadherin, -podoplanin and -VEGFR3 antibodies was performed on isolated popliteal lymphatics of mice. Secondary antibodies donkey anti-rat 488(Jackson ImmunoResearch, 712-546-153), donkey anti-sheep

555 (Jackson ImmunoResearch, 713-165- 147), donkey anti-hamster 555 (Abcam, ab150106), donkey anti-rabbit 647 (Abcam, ab150075) and DAPI were then added. Images were acquired with an LSM 710 Confocal Microscope (Zeiss) equipped with a 63/1.4 oil dic objective. All image handling was performed using ImageJ software.

Cytokine analysis. A BD Cytometric Bead Array (CBA) Mouse Inflammatory Cytokines Kit was performed in plasma to measure the circulating levels of IL-10, MCP-1 and TNF-α proteins. No results were obtained in lymph as the quantity is inferior to the detection threshold of the kit.

Cell culture. Primary human dermal lymphatic microvascular endothelial cells-adult (HMVEC-dLyAd) were cultured according to the manufacturer's protocol (Lonza) in EBM-2 medium containing the EGM-2 MV SingleQuots. Cells were seeded in culture dishes at 80% confluence and were treated with VEGF-C 152s (50ng/mL) or concentration matched PBS for 24 hours.

Immunoblotting of Human Lymphatic Endothelial Cells. Proteins were extracted using radioimmunoprecipitation assay buffer and the protein concentration was established using the Bradford Protein Assay Kit (Bio Basic). Protein samples were diluted in 4X Laemmli buffer, then heated at 95°C for 5 minutes. Proteins were separated by electrophoresis on a 12% SDS-PAGE, then transferred on polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% non fat dry milk in Tris-buffered saline (TBST, 0.1% Tween 20) for 1 hour at room temperature, then incubated with an anti-phosphorylated-eNOS (Cell Signaling, #9275), an anti-vascular endothelial growth factor receptor (VEGFR)-3 (Abcam, AB27278), and anti-phosphorylated-AKT (Cell Signaling, #9570) or an anti-beta-actin (Abcam, AB8227) overnight at 4°C. The membranes were washed with TBST and incubated with horseradish peroxidase–conjugated secondary antibodies (Abcam, AB6721 and AB6721) for 1 hour at room temperature. Clarity Max Western ECL Blotting Substrates (BioRad) was used for detection. Each sample was normalized with its respective beta-actin expression.

Statistics. Data are expressed as the mean ± SEM. Statistical significance was evaluated by unpaired t test or, for multiple comparisons, one-way ANOVA using appropriate corrections when data were not normally distributed, or for unequal variances. All calculations were done

with GraphPad Prism v6c software (GraphPad Software, La Jolla, CA, USA), and p values <0.05 were considered statistically significant.

RESULTS

1. Early treatment with VEGF-C 152s before the onset of atherosclerosis first boosted, then sustained, lymphatic transport in *Ldlr*$^{-/-}$ female mice, while modulating the inflammatory response throughout development of the disease

Our previous studies show that lymphatic function is impaired even before the development of atherosclerosis in 3-month-old *Ldlr*$^{-/-}$*; apoB100*$^{+/+}$ mice that, unlike the *Ldlr*$^{-/-}$ mice, are prone to develop atherosclerosis on a regular chow diet[19]. This dysfunction is presumably associated with a defect in the collecting lymphatic vessels, first in a non-specific cholesterol- but LDLR-dependent manner, as chow-fed *Ldlr*$^{-/-}$ mice also had a significant defect in dendritic cell migration through the lymphatics, without having as much total circulating cholesterol in plasma[10]. Therefore, we subsequently aim to test whether specifically targeting lymphatic function in *Ldlr*$^{-/-}$ mice before the onset of the risk factors, such as an atherosclerotic regimen, could restrain the development of the atherosclerotic plaque and limit its progression. To test this, we first verified whether lymphatic function can be efficiently improved early on in the disease process. We measured both molecular and cellular lymphatic transport before plaque onset in 3-month-old *Ldlr*$^{-/-}$ mice that experienced a VEGF-C 152s treatment while fed on a chow diet. Following intradermal injection of ovalbumin 488 in the footpad of mice to assess the capacity of large molecules to travel within the lymphatics up to the blood circulation, a higher fluorescence was observed in the plasma of the mice treated with VEGF-C 152s before the administration of the pro-atherogenic regimen (Fig. 1A). Whereas chemoattractant protein monocyte chemoattractant protein 1 (MCP-1) was significantly downregulated (Fig. 1B immediately following VEGF-C treatment, no differences were yet observed with respect to cellular transport by the lymphatic vessels (Fig. 1C). Hypercholesterolemia has been reported as a risk factor for lymphatic dysfunction[20], and VEGF-C proved to be effective in restoring lymphatic function in hypercholesterolemic mice[21]. We thus next sought to confirm that VEGF-C 152s, even if applied before the atherosclerosis regimen, would be as effective to improve lymphatic function during hypercholesterolemia. Therefore, *Ldlr*$^{-/-}$ mice that were pre-

treated with VEGF-C 152s (or control) were fed an HFD for an additional 8 weeks to induce atherosclerotic lesion formation. We observed that the effect of VEGF-C treatment on dendritic cell migration through the lymphatics actually appeared to kick in when mice were switched to a HFD (Fig. 1C), as it almost doubled the amount of cells that travelled up to the lymph nodes. Therefore, we show that VEGF-C 152s palliates to the hypercholesterolemic status to improve lymphatic function when atherosclerotic plaque build-up should be at its apogee, at least in our protocol. This effect is reflective of the observation made when a chow diet is given to PBS-treated mice to reverse the hypercholesterolemic phenotype. Interestingly, in hypercholesterolemic plasma, associated proinflammatory cytokine tumor necrosis factor alpha (TNF-α) was significantly lower in VEGF-C treated-mice than control (Fig. 1D), thus preventing systemic inflammation, while MCP-1 levels were comparable between groups (Fig. 1B). While during the early stages of disease development IL-10, an anti-inflammatory cytokine, was not different between groups, early treatment with VEGF-C 152s significantly increased circulating levels of IL-10 in *Ldlr*$^{-/-}$ mice following a switch to a chow diet (Fig. 1E).

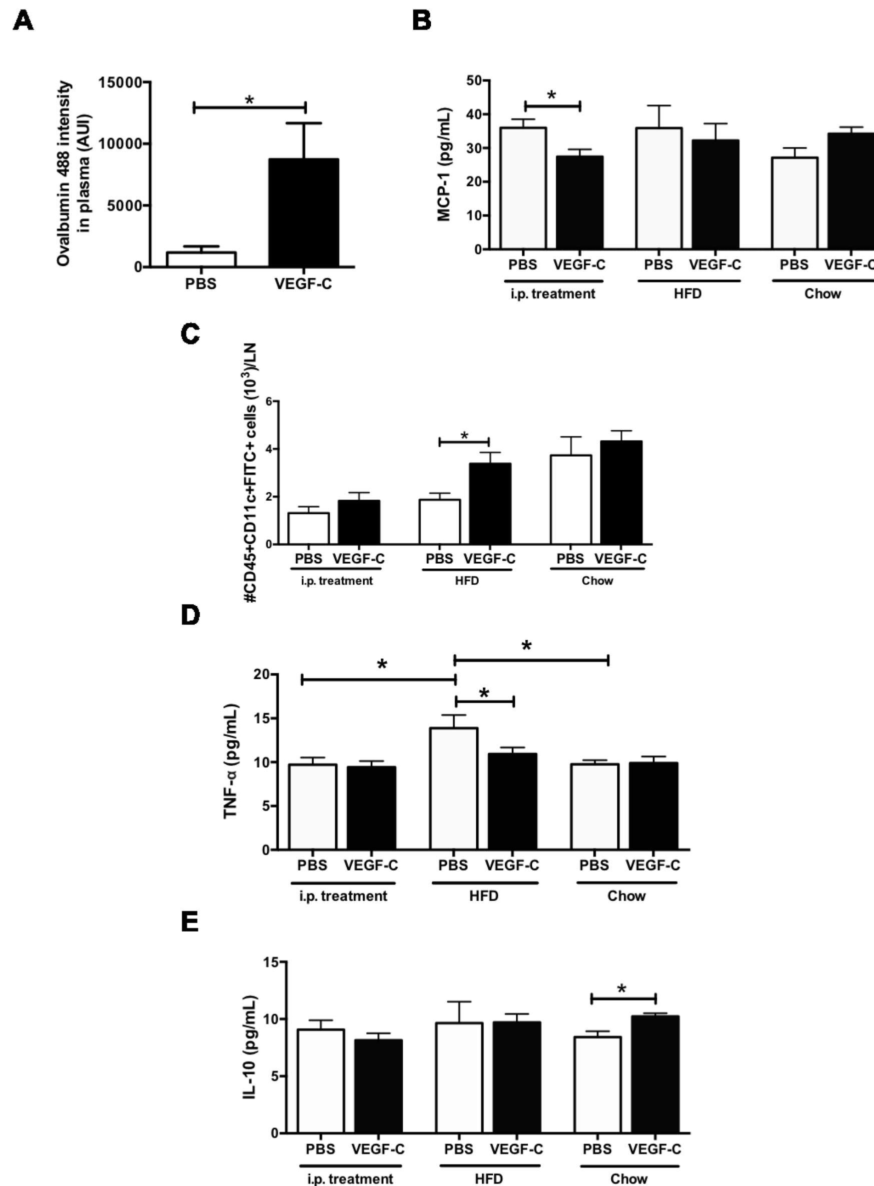

Figure 1. Early treatment with VEGF-C 152s before the onset of atherosclerosis first boosted, then sustained, lymphatic transport, and modulated inflammatory cytokines in *Ldlr*$^{-/-}$ female mice. Following i.p. treatment with VEGF-C 152s or control (PBS) over a period of 4 weeks, **(A)** molecular transport from the dermis of the mouse footpad to the plasma was assessed after mice were injected intradermally with 488-coupled albumin. Dye intensity in plasma was measured and reported in arbitrary units of intensity (AUI). Mann-Whitney t-test. **(B, D, E)** Quantification of monocyte chemoattractant protein 1 (MCP-1), tumor necrosis factor alpha (TNF-α) and interleukin-10 (IL-10) were assessed in mouse plasma by a Cytometric Bead Assay (CBA) kit. **(C)** Skin-draining lymph nodes were harvested after 18 hours and the number of dendritic cells ($CD45^+CD11c^+FITC^+$) that migrated from the skin to the lymph node was reported. ANOVA with Bonferroni post hoc test. Experiments were performed with 4-5 mice per experimental group. *$*p<0.05$*

2. Early treatment with VEGF-C 152s restrains buildup of atherosclerotic plaque

As our results presage that early treatment with VEGF-C 152s would prepare the lymphatic vessels for subsequent harmful situations by enhancing its clearance capacity, we then sought to investigate whether this pre-treatment with VEGF-C 152s would eventually protect mice from subsequent plaque accumulation in the presence of excess circulating cholesterol. The atherosclerotic lesion size and composition were examined in the aortic sinus and *en face* aorta using Oil Red O (ORO) staining. A decrease in ORO^+ staining of 30% in the aortic sinus (Fig. 2A) and 40% in *en face* aorta (Supplementary Fig. 2A) was observed following a HFD in mice pretreated with the VEGF-C 152s treatment. No effect on ORO^+ staining is observed in the aortic sinus, nor in *en face* aorta lesion composition in mice that were switched back to a chow diet. The results of neutral lipid quantification are expressed as percent of control based on percentages of ORO^+ total surface.

To test whether this reduction in plaque is due to an enhanced reduction in plasma cholesterol, we have measured total cholesterol in lymph, plasma and plasma lipoprotein fractions. Albeit total circulating cholesterol levels were not affected by VEGF-C 152s treatment before the onset of the atherosclerotic regimen, a significant reduction was observed thereafter in both lymph and plasma compared to control when mice were given a HFD (Fig. 2B). Furthermore, when mice where switched back to a regular chow diet, VEGF-C 152s and PBS equally reduced circulating plasma cholesterol levels, while lymph cholesterol remained at the same low level in the VEGF-C-treated group, whereas it drastically reversed the hypercholesterolemic phenotype in the PBS-treated group (Fig. 2C).

Lacteals are lymphatic vessels that reside in the small intestines and are responsible for dietary lipid absorption[22]. Without a HFD, *Ldlr*$^{-/-}$ mice are not hypercholesterolemic[23], and it is not surprising that, despite a positive effect on lymphatic function as described above, systemic injections of VEGF-C 152s had no effect on the lipoprotein profile (Fig. 2D) at this point. When the pro-atherogenic regimen was applied, however, VEGF-C 152s improved the absorptive capacity of the lacteals, as reflected by the presence of plasma chylomicron (CM) remnants. This observation was paralleled with lower levels of plasma VLDL and LDL/IDL as compared to the PBS control group, whereas TG (Supplementary Fig. 3A) or total apoB (Supplementary Fig. 3B) levels were undistinguishable between the PBS- and the VEGF-C-

treated groups. Once the mice were switched back to a chow diet, TG concentrations went down in both groups, while total apoB levels remained high. VLDL and IDL/LDL greatly diminished in the control group, whereas the reduction in the VEGF-C-treated group was more modest. Throughout the whole experimental design, VEGF-C 152s did not significantly modulate HDL levels.

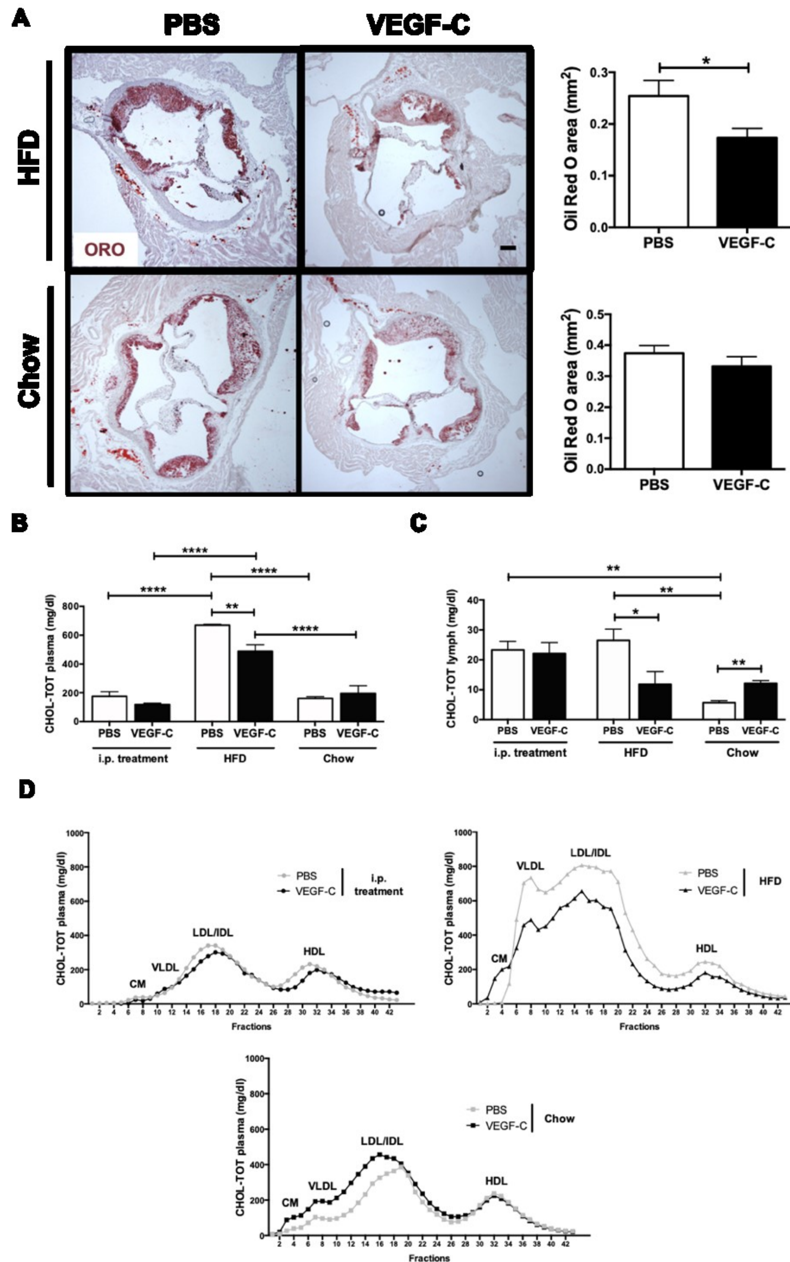

Figure 2. Early treatment with VEGF-C 152s enhances total cholesterol mobilization through lymphatic vessels and restrains buildup of atherosclerotic plaque. Oil Red O (ORO) percentage was quantified in the two groups of mice, **(A)** in 8 μm-thick aortic sinus sections, parametric t-test with Welch correction. Experiments were performed with 8-10 mice per experimental group. Total cholesterol was measured in **(B)** plasma and **(C)** lymph of *Ldlr*$^{-/-}$ mice treated with VEGF-C 152s or PBS, at all timepoints. ANOVA with Bonferroni post hoc test. **(D)** Plasma from mice at all timepoints was pooled and subjected to gel filtration by FPLC. The concentration of lipoproteins in each fraction (total of 43) was measured. Experiments were performed with 5 mice per experimental group. Scale bar = 100μm. *$*p<0.05$, $**p<0.01$* and *$****p<0.0001$*

3. Early treatment with VEGF-C 152s allows the stabilization of the atherosclerotic lesion following cessation of high fat diet

The atherosclerotic plaque is characterized by accumulation of lipids and immune cells, such as macrophages, which produce proinflammatory mediators in the artery wall, and the formation by vascular smooth muscle cells (SMC) of a fibrous cap composed mostly of collagen[24]. Furthermore, the majority of coronary thrombi are caused by *plaque rupture[25]*. Inflammation modulates atherogenesis and plaque destabilization. Thus, inflammatory cytokines may attenuate interstitial collagen synthesis, increase matrix degradation, and promote apoptosis in several atheroma-associated cell types, and all these cellular events may enhance plaque vulnerability[26]. Particularly of interest are the mechanisms leading to plaque instability which includes the proteolysis of collagen by metalloproteinases released by activated macrophages, and apoptosis of intimal SMCs which impedes collagen synthesis[27, 28]. We assessed $CD68^+$ macrophages within the atherosclerotic lesion (Fig. 3A). As expected, the reversal of the hypercholesterolemic phenotype with the chow diet significantly decreased the accumulation of macrophages, notwithstanding of the pre-treatment applied. While on HFD, however, the sole impact of VEGF-C was as important as the one mediated by the diet reversal in the PBS-treated group. The chow diet further reduced macrophage content by half in the VEGF-C treated group. The macrophage area/smooth muscle cell area ratio was significantly lower in the VEGF-C treated-animals when compared to control mice (Fig. 3B), notwithstanding of the diet. Although plaque calcification was not significantly different between groups or dietary conditions (Supplementary Fig. 2B), we observe a marked increase in collagen content during atherosclerosis progression in VEGF-C pre-treated $Ldlr^{-/-}$ mice, suggesting a less pronounced proinflammatory phenotype of the lesions (Fig. 3C).

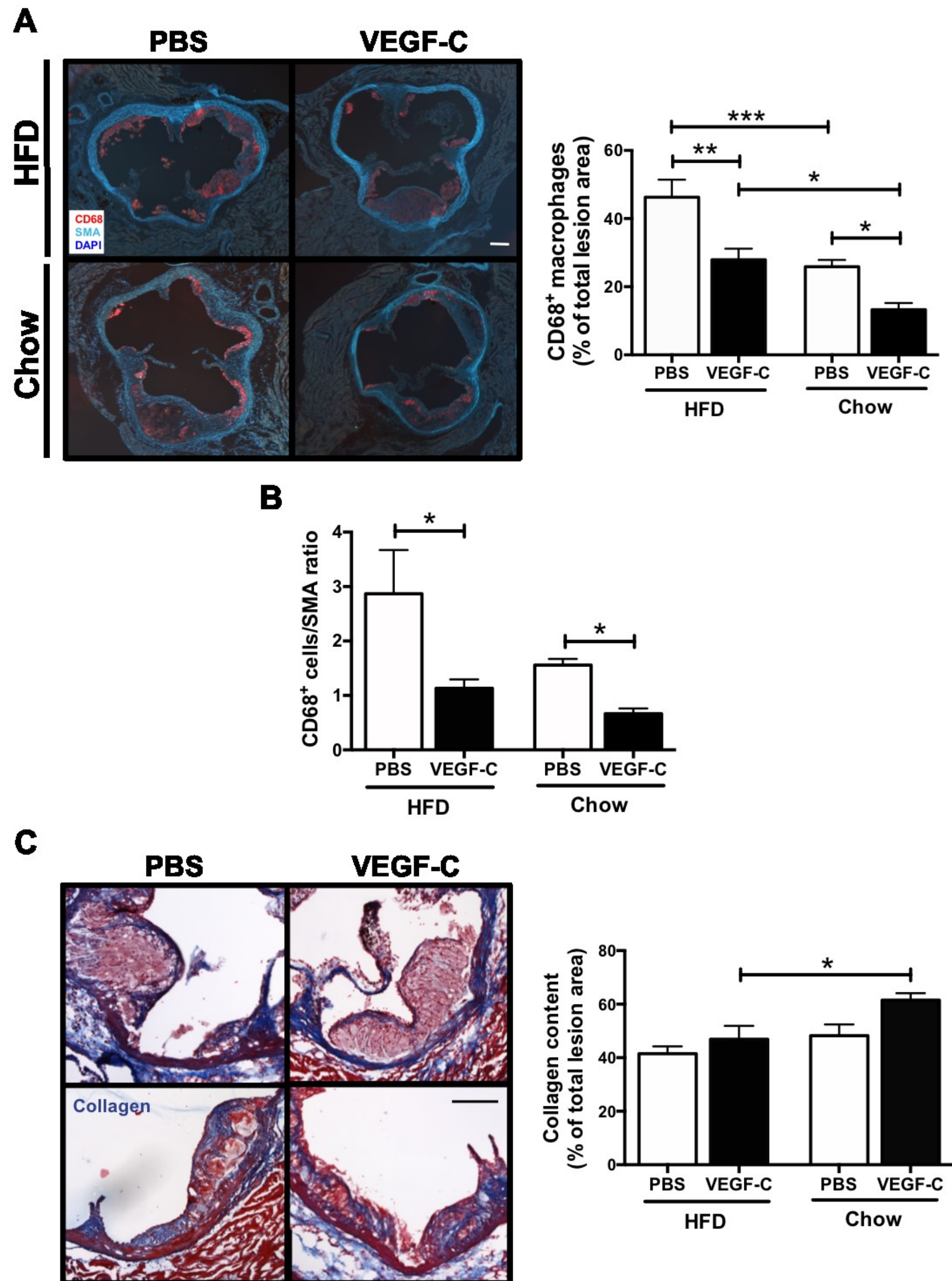

Figure 3. Early treatment with VEGF-C 152s modulates atherosclerotic plaque development in HFD- and chow-fed *Ldlr*$^{-/-}$ female mice. The percentage of **(A)** CD68^{+}DAPI^{+} macrophages and **(B)** CD68^{+} cells/SMA area were quantified in 8-µm-thick aortic sinus sections using ImageJ software in HFD- and chow-fed *Ldlr*$^{-/-}$ female mice. **(C)** The percentage of collagen content using Masson Trichrome staining (blue fibres) was quantified using ImagePro software. Experiments were performed with 7-10 mice per experimental group and 3 to 6 different sinuses per mouse were averaged for the final result. Images were quantified using ImageJ software. Scale bar = 100µm *$p<0.05$, **$p<0.01$ and ***$p<0.001$; DAPI, 4′,6-diamidino-2-phenylindole.

4. VEGF-C 152s treatment modulates atherosclerosis development and progression independent of lymphatic collecting vessel permeability

Next, we sought to investigate the mechanisms by which atherosclerosis-related lymphatic function is rescued by VEGF-C 152s throughout the disease process. Lymphatic vessels are known to absorb interstitial fluid from the periphery through the initial lymphatics, and since VEGF-C is a well-known factor of lymphangiogenesis, we sought to characterize lymphatic vessel growth in the aortic sinus, as well as the ear dermis. As reported by others[11], VEGF-C has a positive effect on lymphangiogenesis in hypercholesterolemic mice, as reflected by the quantification of the LYVE-1-positive vessels both in skin dermis and the aortic sinus of atherosclerotic mice. Whereas both the number of vessels per se (Supplementary Fig. 4A) and the number of branching (Supplementary Fig. 4B) were higher in the VEGF-C treated group immediately following the treatment, the number of LYVE-1-positive vessels was not elevated at this early stage in the aortic sinus (Supplementary Fig. 4C). Twelve weeks following the interruption of the treatment, when mice were no longer on the pro-atherogenic regimen, VEGF-C 152s seems to lose its lymphangiogenic potential. The effect of increased density in lymphatic capillaries could not account for the sustained lymphatic function improvement observed at this stage.

We have previously reported that collecting lymphatic vessels are impaired in mice with an already well-established plaque and therefore, transport is less efficient as there is significant leakage surrounding the vessels[29]. To assess whether the improved lymphatic transport we observed following VEGF-C 152s treatment throughout the atherosclerotic process was mediated through mechanisms that first affect lymphatic vessel permeability, we injected Evans Blue in the footpad of the mouse and quantified any blue dye that may have leaked surrounding the vessel. Our results revealed that VEGF-C 152s had no significant effect on the leakage (Supplementary Fig. 5A). Concomitantly, VE-cadherin expression throughout the atherosclerotic process was also unchanged (Supplementary Fig. 5B).

5. Early treatment with VEGF-C 152s increases collecting lymphatic vessel capacity to contract throughout the entire atherosclerotic process

As the results stated above suggest that VEGF-C 152s does not act through permeability-related mechanisms to maintain proper integrity of the lymphatic vasculature from the initiation to the progression of the atherosclerotic process, we turned our attention to its contraction inducing capabilities. The VEGF-C/VEGFR3 axis has previously been shown to modulate the contraction capacity of rat mesenteric lymphatic vessels, so it is with great interest that we observed *in vivo* the effect of baseline, as well as stimulated contractions in our different groups. At rest, approximately 1/3 of lymph transport in the human lower extremities results from compression by skeletal muscle contractions and 2/ 3 to active pumping of the collecting vessel network[30]. We wanted to evaluate the effects of VEGF-C 152s on lymphatic endothelial cells and following injection with phenylephrine, we sought to assess the response to adrenergic stimulation of the smooth muscle cells surrounding the vessels. Previous studies support the existence of alpha-adrenoceptors on lymphatic smooth muscle[16, 31]. It has been concluded that conditions characterized by increased sympathetic outflow may augment lymphatic function through alpha 1- but not alpha 2-adrenoceptors[32]. Phenylephrine (PE, 0.1-1.0 μM) was shown to produce dose-dependent increases in frequency and decreases in diameter[16]. In our experimental design, lymphatic contraction frequency was assessed under basal conditions and significantly more contractions were observed in mice pretreated with VEGF-C 152s throughout atherosclerosis onset and progression (Fig. 4A). The amplitude of contraction remained unchanged (Fig. 4B). Following PE addition, the same trend as baseline was observed with the exception of the period of time that mice were fed on an HFD (Fig. 4C). This time, the amplitude of the contraction seems to have been favored (Fig. 4D). A possible mechanism that seemed plausible for the modulation of the contraction capacity is nitric oxide (NO) produced by the endothelial NO synthase (eNOS). In fact, eNOS affects the function of the whole lymphatic system and is regulated via the collecting lymphatics[33]. VEGF receptor-2 and -3 stimulation in lymphatic endothelial cells activates downstream effectors such as Akt and increases the phosphorylation of eNOS[34]. We show that eNOS expression in the collecting lymphatic vessels of our different groups of mice (Supplementary Fig. 6A, B) is not modulated in mice that undergo early treatment with VEGF-C 152s. *In vitro*, lymphatic endothelial cells treated with VEGF-C 152s had no significant changes in the phosphorylation of Akt (Supplementary Fig. 6C) or eNOS (Supplementary Fig. 6D).

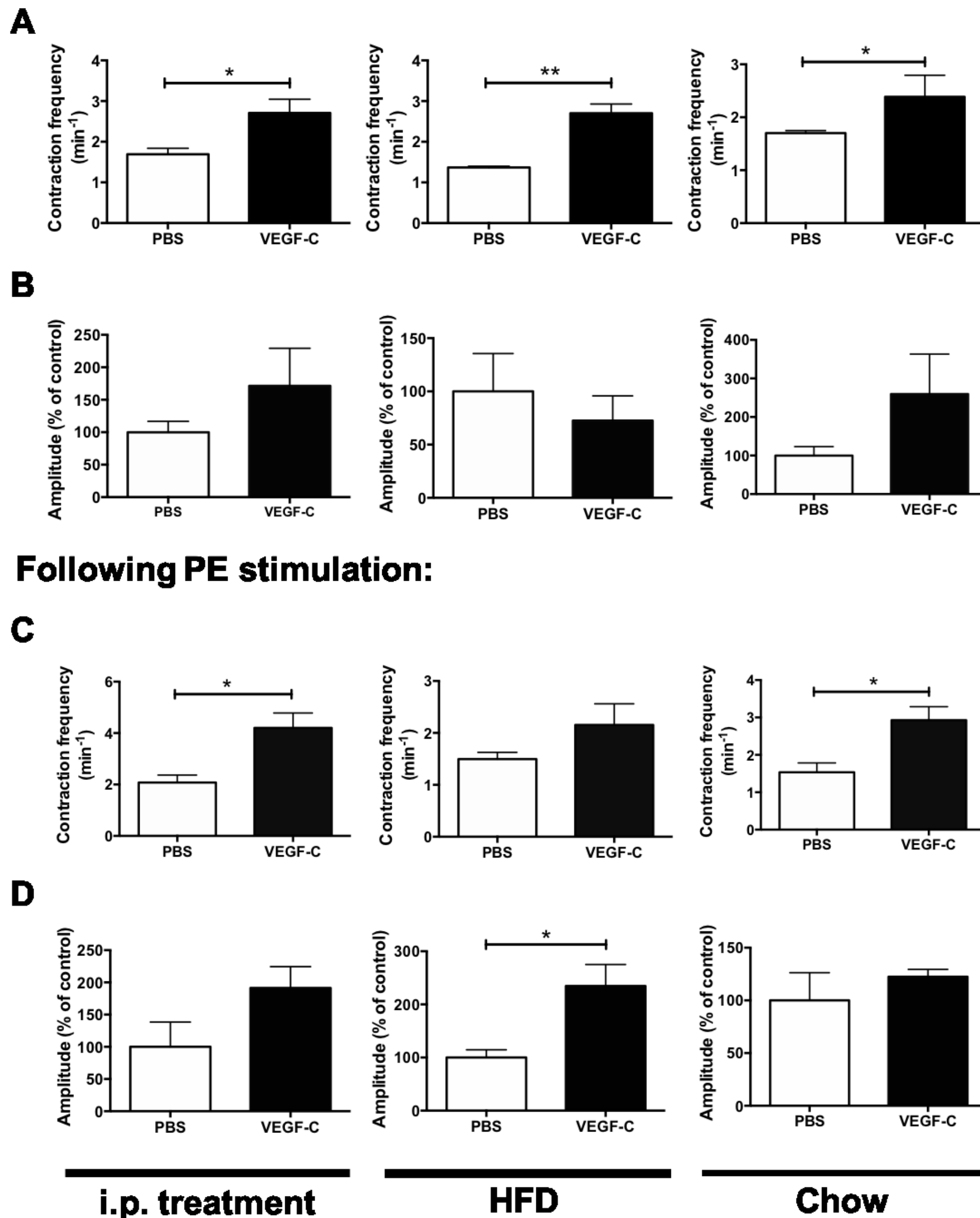

Figure 4. Early treatment with VEGF-C 152s increases collecting lymphatic vessel capacity to contract throughout the whole atherosclerotic process. Popliteal lymphatic vessels exhibit consistent pumping activity *in vivo*. Representative lymphatic contraction frequency curves at **(A)** baseline and **(C)** following phenylephrine (PE) stimulation show reduced contraction frequency in control compared to VEGF-C 152s treated *Ldlr*$^{-/-}$ mice. Lymphatic **(B)** baseline and **(D)** following PE analyses of amplitude, respectively. Experiments were performed using 5 mice per experimental group and three different regions of interest were analysed at random. Parametric t-test with Welch correction. *$p<0.05$ and **$p<0.01$

6. Pre-treatment with VEGF-C 152s instigates and maintains high levels of VEGFR3 expression on lymphatic endothelial cells

The VEGF-C/VEGFR3 signaling pathway contributes to resolving chronic skin inflammation by activating lymphatic vessel function[35]. Of particular interest, VEGF-C stimulates the lymphatic pump by a VEGF receptor-3-dependent mechanism[7]. We wanted to see if VEGFR3 is modulated in an atherosclerotic setting in mice pre-treated with VEGF-C 152s. We observed that VEGFR3 expression at the surface of collecting lymphatic vessels is higher compared to the control group before atherosclerosis onset and maintained all throughout the atherosclerotic process (Fig. 5A). Control mice had a significantly lower VEGFR3 expression at the surface, but this significantly caught up with treated-mice levels once the atherosclerotic disease progressed and a lesion was formed, an effect maintained even after mice were switched to a normal diet. Podoplanin is also expressed on lymphatic endothelial cells and facilitates blood/lymphatic vessel separation[36]. No changes in podoplanin expression were observed at any time point (Supplementary Fig. 7A)

7. FOXC2 might contribute to the maintenance of collecting lymphatic vessel phenotype following onset of atherosclerosis.

Previous studies indicate that flow is required to initiate valve formation, to direct the vascular remodeling that converts a primary mesenteric plexus into a hierarchical drainage system, and to control the extent of SMC coverage[37]. FOXC2-deficient animals exhibit defects in both valve formation and SMC recruitment in lymphatic collecting vessels[38]. As valves play a crucial role in proper lymphangion contraction[39], we sought to investigate if VEGF-C 152s treatment might affect FOXC2 expression on the collecting lymphatic vessels. We have shown in a recent study that FOXC2 is downregulated while mice are on a high fat diet[29], and have reproduced this finding here. The results we report herein suggest that VEGF-C 152s prevents against this decrease mediated by a HFD (Fig. 5B). This may showcase a potential mechanism of valve rescue and overall collecting lymphatic vessel integrity that allows for a good contraction capacity despite the onset of atherosclerosis. As FOXC2 is known to recruit SMCs on the collecting vessels, which play a thorough role in the muscle stimulated

contraction of the vessel, we also looked at SMC coverage of collecting lymphatic vessels but saw no differences in our treated mice compared to control (Supplementary Fig. 7B). This indicates that FOXC2 may not be a primary mechanism of action in this case and is responsible at the very least in maintaining proper lymphatic remodeling, and we assume proper valve function as well, in mice treated with VEGF-C 152s.

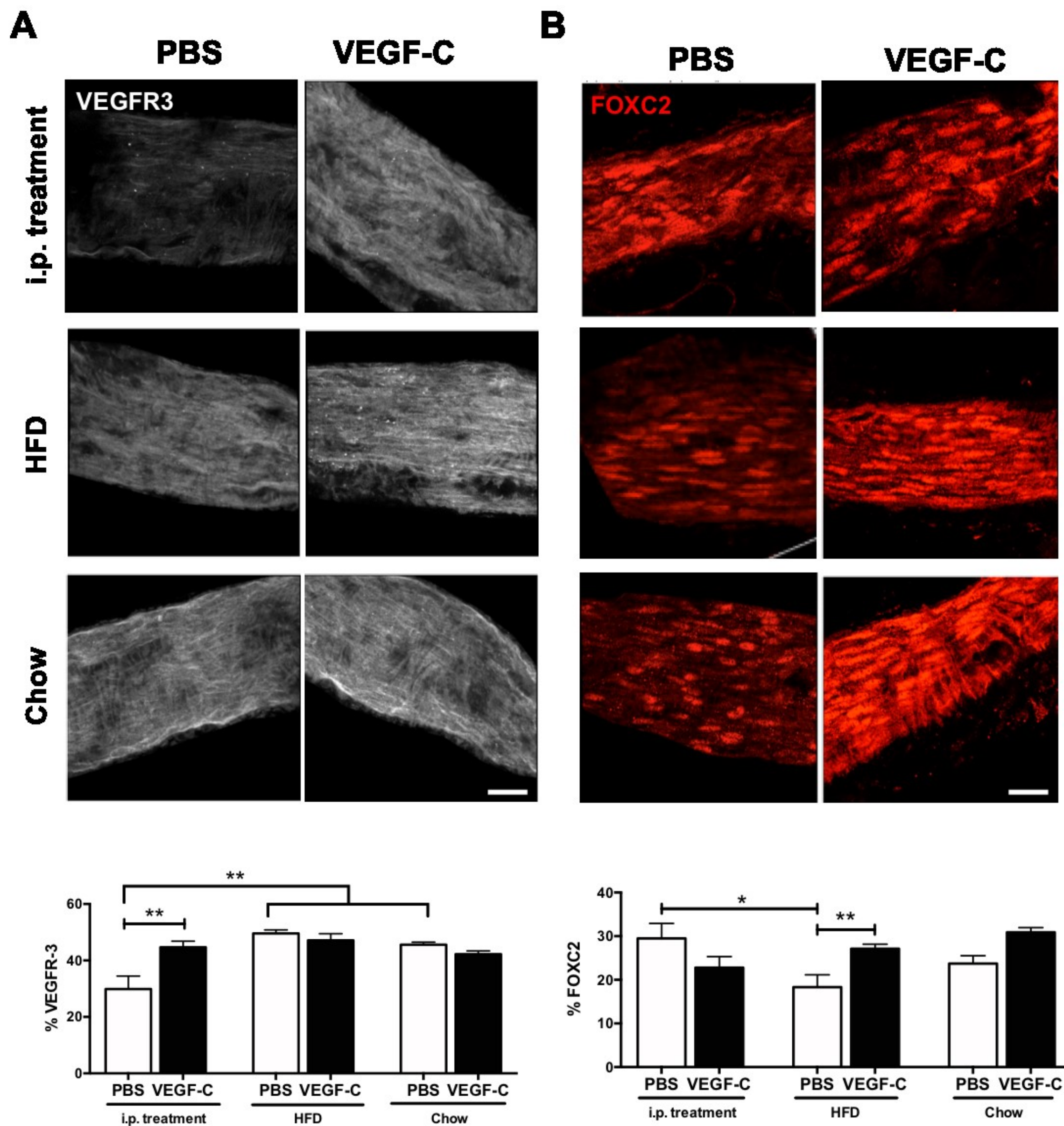

Figure 5. Modulation of the expression of different lymphatic endothelial cell markers maintains collecting lymphatic vessel characteristics at different stages of the atherosclerotic process. Popliteal collecting lymphatic vessels were harvested and processed for immunofluorescence detection. Representative images of **(A)** VEGFR-3 and **(B)** FOXC2 expression and quantification on collecting lymphatic vessels of PBS and VEGF-C 152s treated *Ldlr*$^{-/-}$ mice throughout the entire atherosclerotic process are illustrated. Longitudinally imaged vessels were acquired with an LSM 710 Confocal Microscope (Zeiss) equipped with a 63X/1.4 oil dic objective. Experiments were performed with 5 mice per experimental group and three different regions of interest were analysed at random. ANOVA with Bonferroni post hoc test. Scale bars=10µm. *$p<0.05$ and **$p<0.01$

DISCUSSION

As atherosclerosis develops from an early stage, depending on numerous genetic and environmental risk factors, prevention would be the best treatment of all. Therapies that could prevent or at least diminish atherosclerotic plaque buildup should be the ultimate goal. Systemic inflammation is a critical part of the atherosclerotic process, and studies of VEGF-C in inflammatory bowel disease (IBD), a chronic inflammatory disease, proved to be useful in better understanding atherosclerosis. Crohn's disease, for example, is associated with an aberrant mucosal immune response and, furthermore, D'Alessio *et al.* demonstrated that adenoviral induction of VEGF-C expression provides marked protection against the development of acute and chronic colitis in two different animal models[11]. These authors believe that VEGF-C offers protection mediated by decreasing the production of M1 macrophages (pro-inflammatory), thus allowing the alternative activation of repair macrophages, M2 (anti-inflammatory). This all leads to a decrease in pathological inflammation, once more suggesting that treatment with VEGF-C may serve to delay the development and/or progression of atherosclerosis.

Lymphatic vessels are now recognized as prerequisite players in the modulation of cholesterol removal from the artery wall[3]. Previous studies have demonstrated a rescue in lymphatic dysfunction associated with the early stages of atherosclerosis development[3, 10, 29, 40]. In this study we aimed to investigate if by administering lymphatic vessel specific treatment before the onset of atherosclerosis, we would be able to modulate the initiation of atherosclerosis and its progression. VEGF-C, an important growth factor for the lymphatic vasculature, has been under thorough investigation with respect to his abilities to induce the formation of new lymphatic vessels. However, despite VEGF-C being often associated with lymphangiogenesis, we herein evaluated its effect on the contractile capacity of the collecting lymphatic vessels, the main subunits dysfunctional in the early stages of atherosclerosis development[10]. Specific mutants of VEGF-C that solely bind VEGFR3 have proven efficient in the past, both by rescuing lymphedema through mechanisms of lymphangiogenesis, but most importantly, by increasing the pumping capacity of the collecting lymphatic vessels in the rat mesentery with a different isoform than the VEGF-C we used[7].

Pre-treatment with VEGF-C 152s before mice were fed a pro-atherosclerotic regimen led to promising results in the quest of new treatments that may one day prevent altogether any signs of cardiovascular disease[10, 41]. Following treatment administration, before the development of the atherosclerotic lesion, the molecular transport capacity was already higher, an effect that may be mirroring an increase in cholesterol transport by the lymphatic vessels. Enhanced expression of MCP-1 was demonstrated in a variety of pathologic conditions associated with inflammation and mononuclear cell accumulation. Extensive experimental evidence suggests that MCP-1 is highly expressed in atherosclerotic plaques and mediates macrophage recruitment in the atheromatous lesion[42]. In our study, immediately following VEGF-C 152s treatment, MCP-1 is significantly downregulated, as a possible pre-emptive move to prevent macrophage recruitment, which might be at least partially responsible for a limited macrophage accumulation, once mice are fed on an HFD to promote atherosclerotic lesion formation, as compared to control. This prevention is important, as newly differentiated macrophages fail to penetrate significantly deeper than the limited depth they reside on initial entry, regardless of plaque progression, or regression[43].

In this present study, cholesterol levels are similar between groups before the administration of the pro-atherogenic regimen, notwithstanding of the pre-treatment applied. However, once these mice are switched to a HFD they have drastically increased total circulating cholesterol levels in plasma, and in the control mice the concentration is significantly higher than VEGF-C 152s treated mice. Contrarily, in previous studies[10], *Ldlr*$^{-/-}$*/hapoB100*$^{+/+}$, a different strain of mice that do not require a HFD for plaque to be induced, had increased cholesterol levels following early treatment with VEGF-C 152s despite restauration of the lymphatic function. The *Ldlr*$^{-/-}$*/hapoB100*$^{+/+}$ mice genetically overexpress human apoB100, which is synthesized by the liver and secreted within VLDLs that are metabolized in plasma to form LDL[44]. Of notice is that chylomicrons produced by the intestine contain apoB48, whereas VLDLs made by the liver contain apoB100. Our values do not differentiate between these two entities, as the total apoB concentrations were measured. As observed, systemic injections of VEGF-C 152s had no effect on the lipoprotein profile immediately following the VEGF-C 152s treatment. When the pro-atherogenic regimen was applied, however, pre-treatment with VEGF-C 152s improved the absorptive capacity of the lacteals, as reflected by the presence of plasma chylomicron remnants. This observation was paralleled with lower levels

of plasma VLDL and LDL/IDL as compared to the PBS control group, whereas TG or total apoB levels were undistinguishable between the PBS- and the VEGF-C- treated groups. Once the mice were switched back to a chow diet, TG concentrations went down in both groups, while total apoB levels remained high. VLDL and IDL/LDL greatly diminished in the control group, whereas the reduction in the VEGF-C-treated group was more modest. This is probably due to the fact that chylomicron remnants are left over due to the increase of lacteal function and supposed absorption. The chylomicron remnants are taken up by the liver and processed to VLDL/IDL/LDL[45]. Throughout the whole experimental design, VEGF-C 152s did not significantly modulate HDL levels. Interestingly, cholesterol was increased in lymph compared to control, meaning that instead of being deposited into the plaque like it does in control, VEGF-C 152s treated mice have proper and constant cholesterol mobilization out of peripheral tissues. This supports the results of significantly higher collecting lymphatic vessel contractions when mice undergo atherosclerosis development and are fed on an HFD or chow diet.

As soon as atherosclerosis development progressed, cellular transport was rescued in comparison to control. In parallel to lower circulating cholesterol levels, in both lymph and plasma, mice fed on an HFD also showed significant signs of restrained accumulation of atherosclerotic plaques, as well as decreased immune cell accumulation. Interestingly, once the HFD was removed and replaced by a chow diet to diminish plaque burden, and assess if the lesion was further stabilized, we observed that mice having been pre-treated with VEGF-C 152s had plaques that contained a higher level of collagen, decreased $CD68^+$ cells accumulation and a lower macrophage/smooth muscle cell ratio. Further supporting these data was an augmented concentration of circulating IL-10, a known atheroprotective cytokine that reduces immune cell accumulation and maintains collagen levels[46]. One possible mechanism for these beneficial effects could be the modulation of systemic circulatory levels of this atheroprotective cytokine. IL-10 plays critical roles in both atherosclerotic lesion formation and stability. Atherosclerotic lesions of IL-10–deficient mice showed increased immune cell accumulation and decreased collagen content[47].

The integrity of lymphatic vessels in inflammatory settings has quickly emerged in recent years as a crucial factor in the severity of the atherosclerotic process. Previous studies have shown that the lymphatic dysfunction, which we attributed to occur at the level of the collecting lymphatic vessels, could be rescued in mice with already established atherosclerosis.

Although VEGF-C 152s does promote lymphangiogenesis, as observed in both the aortic sinus and systemically, we do not attribute any significant role to these supplementary vessels in combatting atherosclerosis. We cannot eliminate the fact that the initial lymphatic vessels may compensate in cases of increased interstitial pressure[48, 49], augmented cell accumulation in the periphery[50] and local changes in matrix stiffness that regulate cellular functions such as adhesion and invasiveness[51]. VEGF-C mediated enhancement of lymphatic drainage and reduced intestinal inflammation in experimental chronic colitis[52]. Furthermore, human studies have shown increased levels of VEGF-C and LYVE-1 in postmortem atheroma samples compared with healthy controls which indicated that cytokine secretion activates the lymphatic system and this later causes increased lymphangiogenesis towards the inflamed atheroma plaque combined with increased lymphocyte/macrophage trafficking in atherosclerosis[53]. However, an increased absorption does not lead to automatic proper propulsion of the lymph through the collecting lymphatic vessels. Indeed, studies[10, 29, 54, 55] support the fact that more vessels do not necessarily lead to more functioning vessels overall.

Although treatments such as apolipoprotein A-I (apoA-I) rescue the lymphatic permeability associated with atherosclerosis by modulating platelet[29] or platelet extracellular vesicles adhesion to the lymphatic endothelium, unlike apoA-I treatment, early treatment with VEGF-C 152s had no significant effects on lymphatic collecting vessel permeability. This effect was further supported by analyses of lymphatic endothelial cell junctions where VE-cadherin levels did not differ between our different groups.

The VEGF-C/VEGFR3 axis was shown to promote mesenteric lymphatic vessel contractions in the rat under *ex vivo* conditions. Our results were observed *in vivo* and for the first time, we were able to follow changes in lymphatic vessel contraction frequency and amplitude even before the onset and progression of atherosclerosis. The intrinsic lymphatic vessel contraction frequency was significantly higher than control under all conditions. To assess the effects on muscle tonus, phenylephrine was injected in the footpad dermis of the mice. Lymphatic vessels possess no basal adrenergic tone[56]. Phenylephrine (0.1-1.0 μM) has already been shown to produce dose-dependent increases in frequency and decreases in diameter in the rat[16]. In the present study, results show that with the exception of the period of time where mice were fed on a HFD, the frequency of contraction was significantly higher than control in mice that had undergone early treatment with VEGF-C 152s. This suggests that PE

could not overcome the decrease in contractions that is well known to occur once mice are fed a chronic HFD[57]. Lymphatic endothelial cells serve as sensors of local lymph flow and transduce signals to lymphatic smooth muscle[58]. As smooth muscle cell coverage did not differ between conditions in our two different groups, we can exclude any alterations with respect to lymphatic vessel muscle coverage following VEGF-C 152s treatment. Furthermore, at the only time point (HFD) that we did not observe any differences in contraction pumping after addition of phenylephrine, other changes systemically due to the VEGF-C 152s treatment still counteracted the negative effects of the disease observed in control. Indeed, the intrinsic capacity of the lymphatic vessels resisted the negative effects of a HFD through systemic downregulation of TNF-α. The pro-inflammatory cytokine TNF-α inhibits lymphatic pumping via activation of the NF-κB-iNOS signaling pathway[59].

Although we did see some changes at the molecular level of different genes expression in lymphatic vessels, we stipulate that VEGF-C 152s seems to be important in a multi-faceted sequential way. From the very beginning of the atherosclerotic process, VEGF-C 152s increases VEGFR3 expression and maintains it throughout the atherosclerotic process, improves lymphatic contraction capacity, and reduces inflammatory cell accumulation. We propose that this is the instigating mechanism by which VEGF-C 152s modulates inflammation systemically and within plaque, both leading to a smaller and more stable plaque. Subsequently, VEGF-C 152s administration prevented downregulation of FOXC2 whose absence/reduction is well known to be implicated in lymphatic valve failure[38], thereby contributing to the protection of the associated lymphatic dysfunction while mice were switched to a HFD.

CONCLUSION

Altogether, our results highlight the multiple mechanisms underlying the proper role of lymphatic vessels throughout the process of atherosclerosis. These findings show that atherosclerosis can be modulated if early treatments that specifically target the lymphatic vasculature are administered. In this study, early treatments with VEGF-152s restrained the accumulation of plaque and contributed to its stabilisation thereafter. The responsible mechanisms interact at different stages of the disease and in different ways, all aiming to preserve the lymphatic network and allow for proper reverse cholesterol transport from plaque.

To our knowledge, we are the first to correlate *in vivo*, in a timely matter, that a rescue in lymphatic vessel contractions leads to beneficial effects with respect to plaque accumulation, its properties, and overall systemic inflammation that leads to many deleterious effects. Further investigations into different kinds of treatments targeting the lymphatic vessels, particularly at the level of the collecting vessels, crucially deserve further attention and might actually lead to not only an efficient atherosclerosis treatment, but above all, prevent the risk of developing an atherosclerotic lesion.

CONFLICT OF INTEREST

The authors have no conflicts of interest to disclose.

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AUTHOR CONTRIBUTIONS

AM and CM: Study conception and design; data acquisition, analysis and interpretation; manuscript writing and critical revision. AS: data acquisition support.

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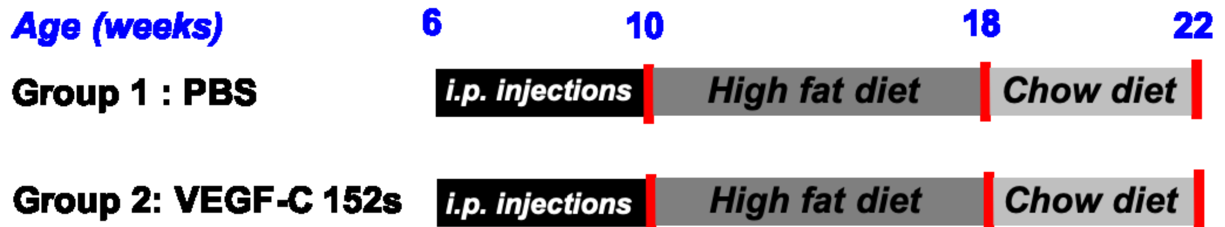
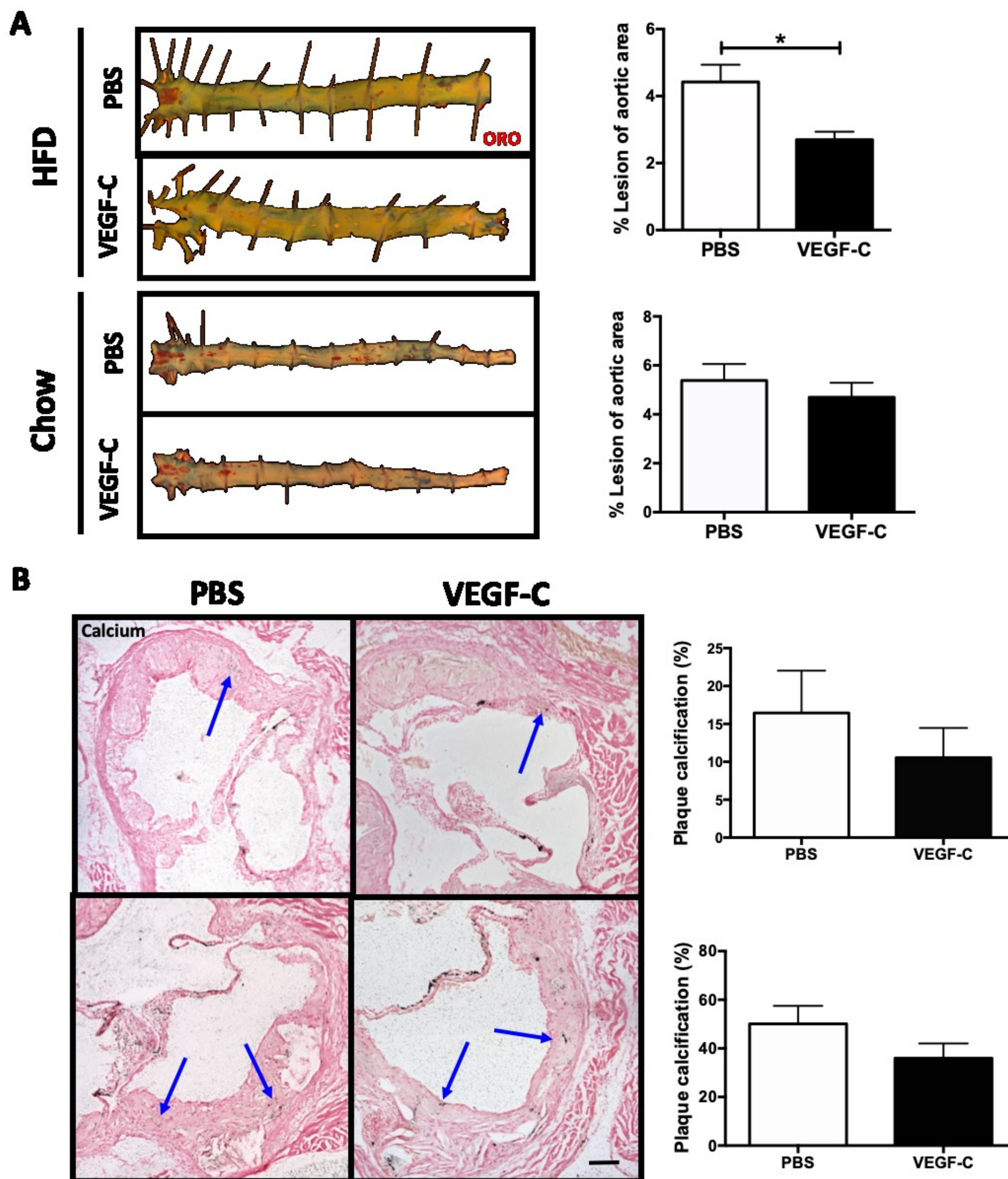
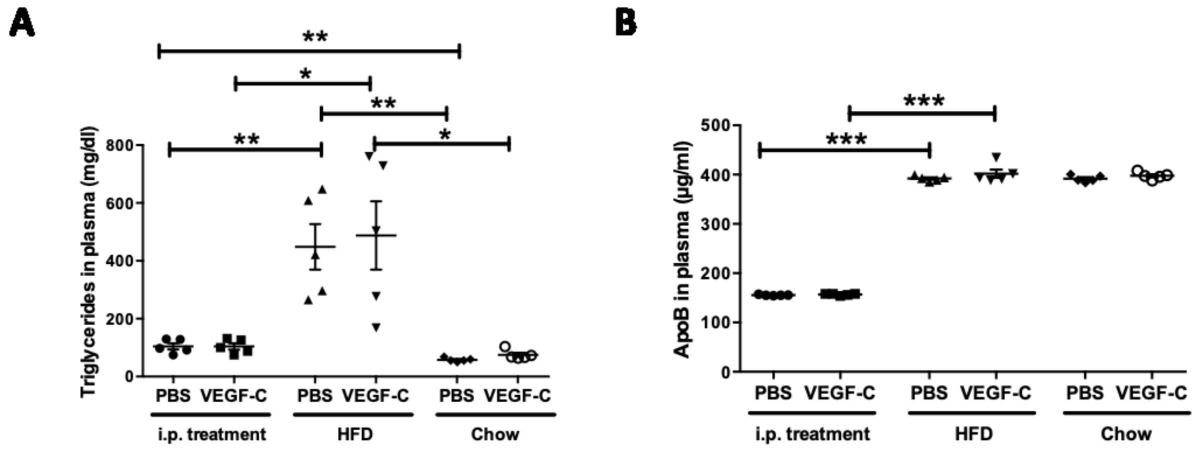
4.1.2 Supplementary data



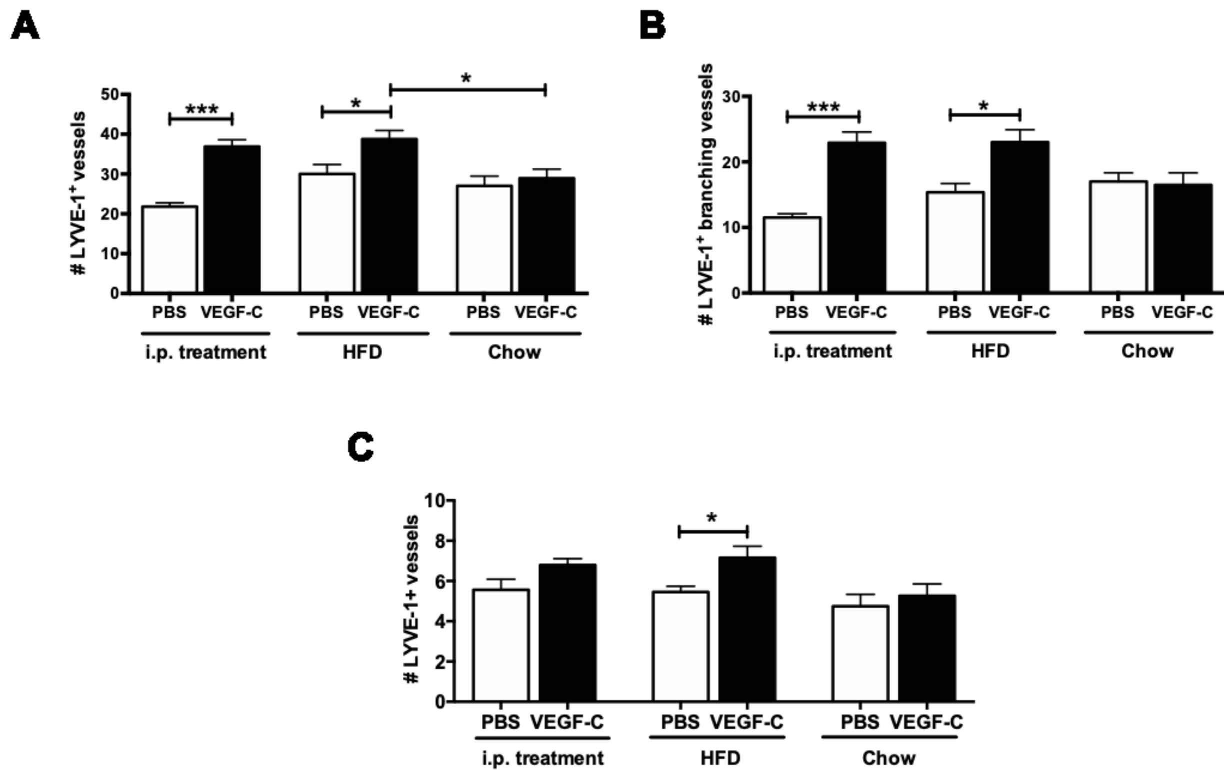
Supplementary Figure 1. Experimental setup. Before undergoing a pro-atherosclerotic regimen, *Ldlr*$^{-/-}$ mice at 6 weeks of age were injected i.p. with VEGF-C 152s (50ng/25g of body weight) or concentration matched control (PBS) for 4 weeks, fed on high fat diet (HFD) for an additional 8 weeks, and switched back on chow diet for 4 more weeks to stabilize plaque progression. A group of mice was sacrificed following each step (red bars).



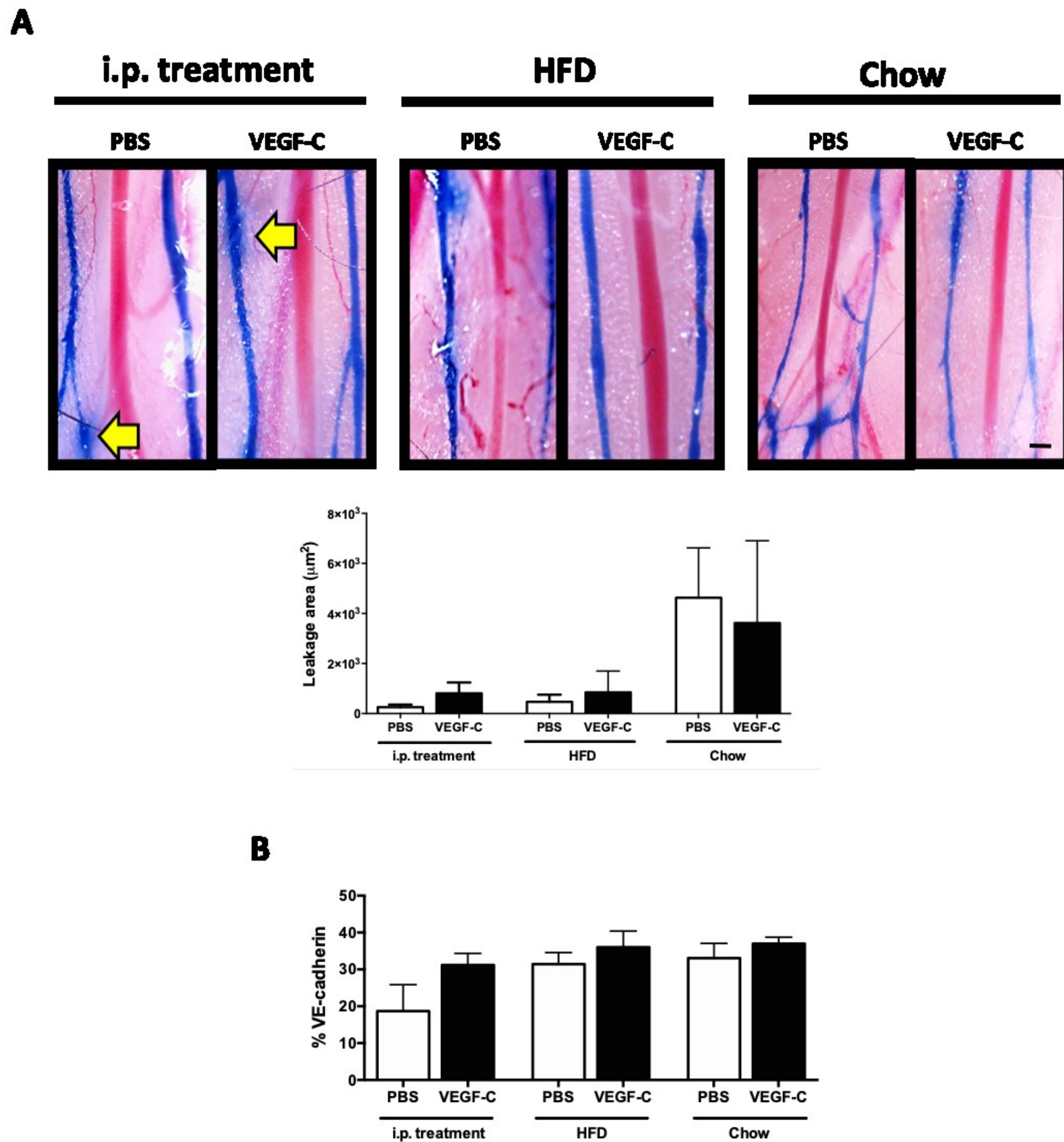
Supplementary Figure 2. Early treatment with VEGF-C 152s has partial effects on plaque progression in HFD- and chow-fed *Ldlr*$^{-/-}$ female mice. (A) Oil Red O (ORO) percentage within plaque was quantified in *en face* thoracic aortas. (B) The percentage of plaque calcification using Van Cossa staining (black regions, as indicated by the blue arrows) was quantified in 8-µm-thick aortic sinus sections. Experiments were performed with 8-10 mice per experimental group. Unpaired t-test. Scale bar=100µm. *$p<0.05$



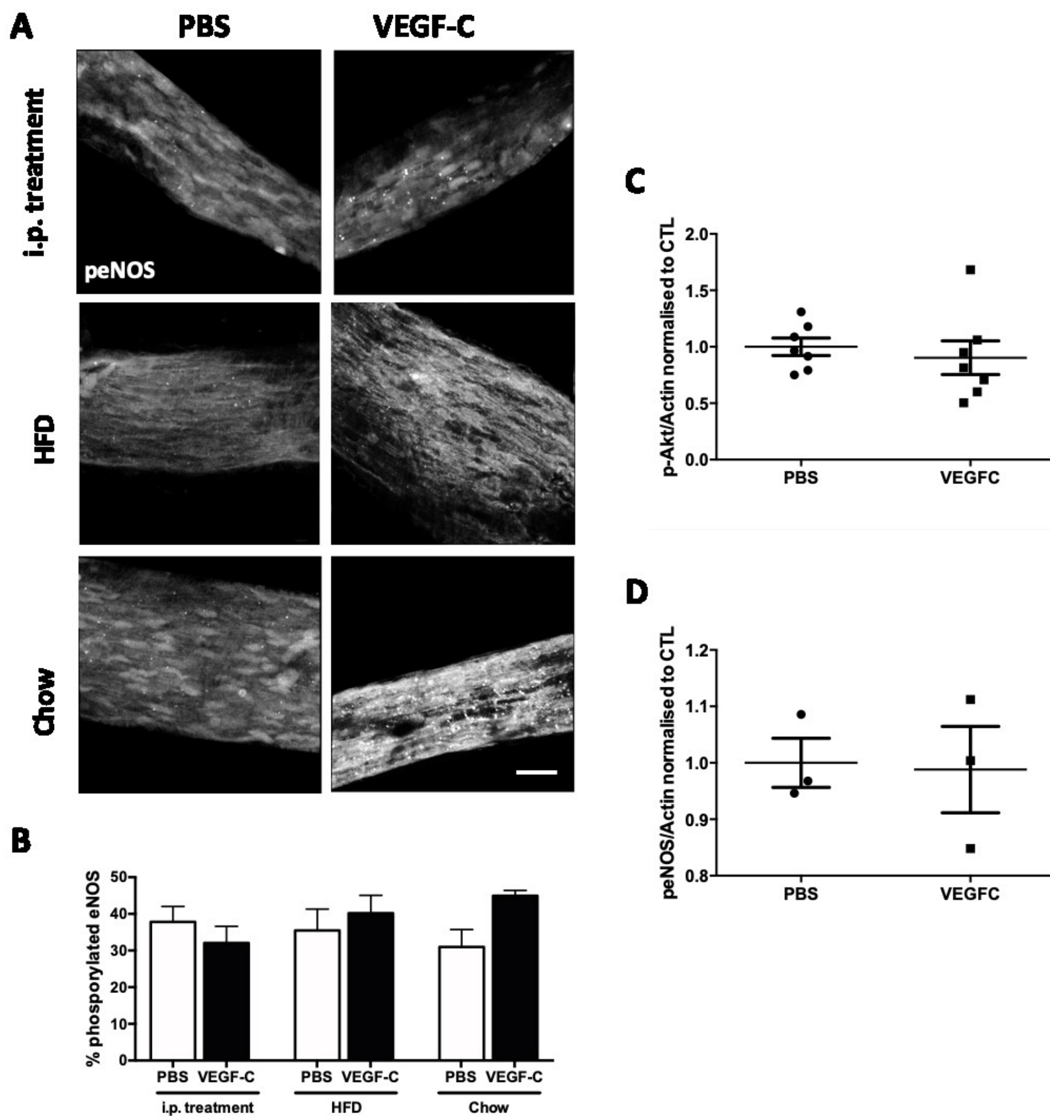
Supplementary Figure 3. Circulating levels of triglycerides and apoB are similar within all groups of mice. Both (A) triglyceride and (B) apoB circulating levels were measured in mouse plasma of PBS and VEGF-C 152s treated *Ldlr*$^{-/-}$ mice throughout the entire atherosclerotic process. Experiments were performed with 5 mice per experimental group and results show the average of duplicate measures. *$p<0.05$, **$p<0.01$ and ***$p<0.001$



Supplementary Figure 4. Early treatment with VEGF-C 152s promotes systemic lymphangiogenesis in *Ldlr*$^{-/-}$ mice. Immunofluorescence of initial lymphatic vessels (Lyve-1^{+}) in PBS (control) and VEGF-C 152s -treated *Ldlr*$^{-/-}$ mice. Quantification of the (A) number and (B) branching, ANOVA with Bonferroni post hoc test of Lyve-1^{+} vessels was assessed in the ear dermis. Experiments were performed using 5 to 9 mice per experimental group. (C) Quantification of initial lymphatic vessels (Lyve-1^{+}) in the aortic sinus. Only Lyve-1^{+} vessels of any circular shape were included and quantified. Experiments were performed using 8 to 10 mice per experimental group and 4 to 6 different sinuses per mouse were averaged for the final result. ANOVA with Bonferroni post hoc test. All image handling was performed using ImageJ software. *$p<0.05$ and ***$p<0.001$

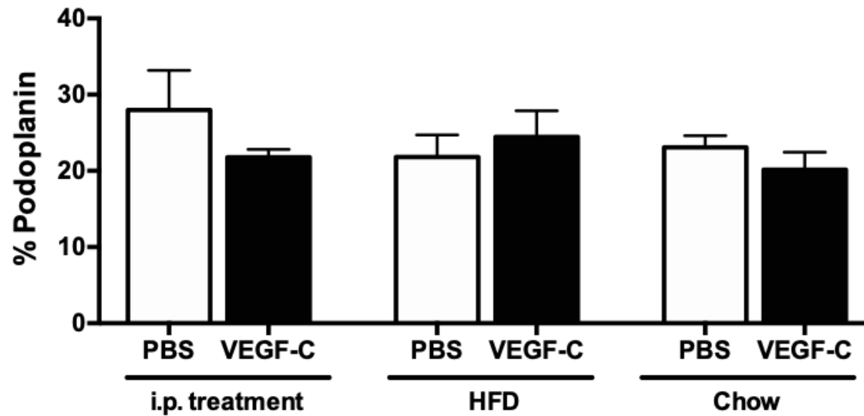


Supplementary Figure 5. VEGF-C 152s treatment modulates atherosclerosis development and progression independent of lymphatic collecting vessel permeability. (A) Lymphatic vascular integrity was assessed following Evans Blue (EB) dye intradermal injection in the footpad of PBS and VEGF-C 152s treated *Ldlr*$^{-/-}$ mice at our three different time points. Examples of EB dye leakage around the vessels (yellow arrows). The area was assessed and calculated using ImageJ software. ANOVA with Bonferroni post hoc test. Popliteal collecting lymphatic vessels were harvested and processed for immunofluorescence detection. (B) Quantification of the vascular endothelial cadherin (VE-cadherin) expression at the surface of the collecting vessels was reported. Experiments were performed with 5 mice per experimental group. Parametric t-test with Welch correction. Scale bar=100μm.

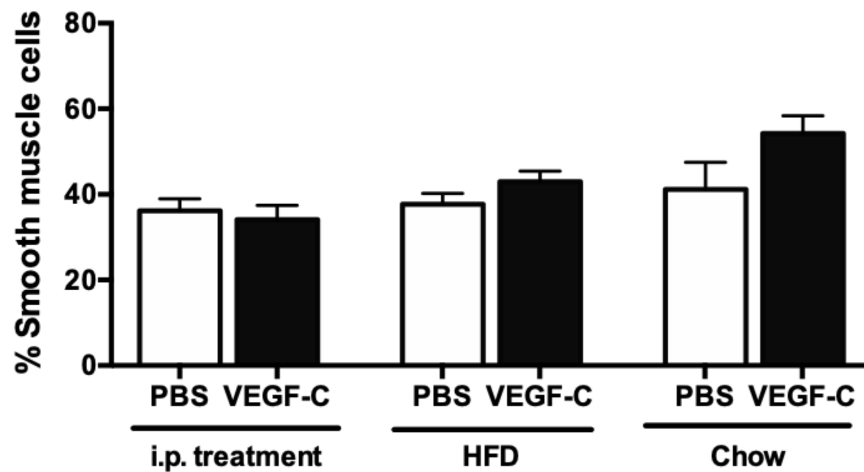


Supplementary Figure 6. Following early treatment with VEGF-C 152s, a low-fat diet preserves endothelial nitric oxide synthase (eNOS) regulation and endothelial function in Ldlr-/- mice at an advanced atherosclerotic stage. Popliteal collecting lymphatic vessels were harvested and processed for immunofluorescence detection. Representative images of (A) phosphorylated endothelial nitric oxide synthase (p-eNOS) expression and (B) quantification in collecting lymphatic vessels of PBS and VEGF-C 152s treated *Ldlr*$^{-/-}$ mice throughout the entire atherosclerotic process are illustrated. Experiments were performed with 5 mice per experimental group and three different regions of interest were analysed at random. Unpaired t-test. Western blot analysis was performed on human lymphatic endothelial cells treated with VEGF-C 152s for 24h and (C) phosphorylated-Akt and (D) phosphorylated-eNOS was quantified with respect to the beta-actin control. ANOVA with Bonferroni post hoc test. Scale bar=10µm. *$p<0.05$

A



B



Supplementary figure 7. Early treatment with VEGF-C 152s has no effect on the modulation of different lymphatic endothelial cell markers throughout the atherosclerotic process. Popliteal collecting lymphatic vessels were harvested and processed for immunofluorescence detection. Representative quantifications of (A) podoplanin and (B) lymphatic muscle cells (assessed by smooth muscle actin antibody) of PBS and VEGF-C 152s treated *Ldlr*$^{-/-}$ mice throughout the entire atherosclerotic process are illustrated. Experiments were performed with 5 mice per experimental group and three different regions of interest were analysed at random. The total fluorescence intensity was assessed as a percentage of total vessel area. ANOVA with Bonferroni post hoc test.

4.2 Presentation of the second article

Efflux of cholesterol from plaque depends on an efficient lymphatic transport, and in turn, functional lymphatics depend upon atherosclerotic disease severity. As atherosclerosis is a chronic inflammatory disease, treatments aiming not only to reduce circulating cholesterol, but also the inflammatory state, could be beneficial in rescuing hypercholesterolemia-induced lymphatic dysfunction.

Following preliminary studies, a contact-sensitization assay to assess dendritic cell transport demonstrated that cellular lymphatic transport in *apoA-I*$^{-/-}$ mice is greatly impaired compared to WT mice. This indicated that apoA-I might have a protecting role in lymphatic function, an effect that had not been studied *in vivo* to date.

An atherogenic diet leads to autoimmunity in *apoA-I*$^{-/-}$ mice, but lipid-free apoA-I therapy was shown to modulate regulatory T cell activity and decrease inflammation[732]. Most importantly, lipid-free apoA-I reduces atherosclerosis progression, without altering circulating cholesterol levels[733]. Furthermore, *in vitro*, in an inflammatory setting mediated by TNF-α, apoA-I administration was shown to increase lymphangiogenesis.

Since we also knew that apoA-I contributes to cholesterol efflux that is crucial for mRCT, among its diverse atheroprotective effects previously discussed, we now aimed to investigate whether and how lipid-free apoA-I would be beneficial in rescuing atherosclerosis associated lymphatic dysfunction.

<u>Our results suggest that treatment with low-dose lipid free apoA-I:</u>

- Preserves and restores collecting lymphatic vessel function through direct and indirect mechanisms that include platelet activity;
- Reduces plaque accumulation.

Participation of each author of the article:

AM: Project conceptualisation, troubleshooting, methods validation, experiments, results analysis and writing of the manuscript.

GJ: *In vitro* experiments.

FD: Experiment assistance and critical reading of the manuscript.

JCT: *Ldlr*$^{-/-}$ mice partial start-up funding.

YM: Platelet aggregation experiments.

MST: Resources (apoA-I) and scientific consultation.

CM: Project conceptualisation, resources, troubleshooting, methods validation, experiments, results analysis and writing of the manuscript.

4.2.1 Second article

ORIGINAL RESEARCH

Apolipoprotein A-I Modulates Atherosclerosis Through Lymphatic Vessel-Dependent Mechanisms in Mice

Andreea Milasan, MSc; Gabriel Jean, BSc; François Dallaire, MSc; Jean-Claude Tardif, MD; Yahye Merhi, PhD; Mary Sorci-Thomas, PhD; Catherine Martel, PhD

Background—Subcutaneously injected lipid-free apoA-I (apolipoprotein A-I) reduces accumulation of lipid and immune cells within the aortic root of hypercholesterolemic mice without increasing high-density lipoprotein–cholesterol concentrations. Lymphatic vessels are now recognized as prerequisite players in the modulation of cholesterol removal from the artery wall in experimental conditions of plaque regression, and particular attention has been brought to the role of the collecting lymphatic vessels in early atherosclerosis-related lymphatic dysfunction. In the present study, we address whether and how preservation of collecting lymphatic function contributes to the protective effect of apoA-I.

Methods and Results—Atherosclerotic *Ldlr*$^{-/-}$ mice treated with low-dose lipid-free apoA-I showed enhanced lymphatic transport and abrogated collecting lymphatic vessel permeability in atherosclerotic *Ldlr*$^{-/-}$ mice when compared with albumin-control mice. Treatment of human lymphatic endothelial cells with apoA-I increased the adhesion of human platelets on lymphatic endothelial cells, in a bridge-like manner, a mechanism that could strengthen endothelial cell–cell junctions and limit atherosclerosis-associated collecting lymphatic vessel dysfunction. Experiments performed with blood platelets isolated from apoA-I-treated *Ldlr*$^{-/-}$ mice revealed that apoA-I decreased ex vivo platelet aggregation. This suggests that in vivo apoA-I treatment limits platelet thrombotic potential in blood while maintaining the platelet activity needed to sustain adequate lymphatic function.

Conclusions—Altogether, we bring forward a new pleiotropic role for apoA-I in lymphatic function and unveil new potential therapeutic targets for the prevention and treatment of atherosclerosis. **(*J Am Heart Assoc*. 2017;6:e006892. DOI: 10.1161/JAHA.117.006892.)**

Key Words: apolipoprotein A-I • atherosclerosis • collecting lymphatic vessels • platelets

ApoA-I modulates atherosclerosis through lymphatic vessel-dependent mechanisms in mice

Milasan

Short Title: ApoA-I enhances lymphatic function

Andreea Milasan MSc[1,2], Gabriel Jean BSc[1,2], François Dallaire MSc[2], Jean-Claude Tardif MD[1,2], Yahye Merhi PhD[1,2], Mary Sorci-Thomas PhD[3] and Catherine Martel PhD[1,2]

[1] Department of Medicine, Faculty of Medicine, Université de Montréal, Montreal, Quebec, Canada

[2]Montreal Heart Institute, Montreal, Quebec, Canada

[3] Department of Medicine, Medical College of Wisconsin, Milwaukee, Wisconsin

Corresponding author:
Catherine Martel, PhD
Montreal Heart Institute Research Center
Department of Medicine, Faculty of Medicine
Université de Montréal
5000, Belanger street, Room S5100
Montreal, Quebec (Canada)
H1T 1C8
Telephone: (514) 376-3330 #2977
E-mail: catherine.martel@icm-mhi.org

Subject codes: Atherosclerosis, Platelets, Lipids and Cholesterol, Inflammation, Vascular biology

ABSTRACT

Background. Intradermally injected lipid-free apoA-I reduces accumulation of lipid and immune cells within the aortic root of hypercholesterolemic mice without increasing HDL-cholesterol concentrations. Lymphatic vessels are now recognized as prerequisite players in the modulation of cholesterol removal from the artery wall in experimental conditions of plaque regression, and a particular attention has been brought on the role of the collecting lymphatic vessels in early atherosclerosis-related lymphatic dysfunction. In the present study, we address whether and how preservation of collecting lymphatic function contributes to the protective effect of apoA-I.

Methods and results. Atherosclerotic *Ldlr*$^{-/-}$ mice treated with low-dose lipid-free apoA-I showed enhanced lymphatic transport and abrogated collecting lymphatic vessel permeability in atherosclerotic *Ldlr*$^{-/-}$ mice when compared with albumin-control mice. Treatment of human lymphatic endothelial cells (LECs) with apoA-I increased the adhesion of human platelets on LECs, in a bridge-like manner, a mechanism that could strengthen endothelial cell-cell junctions and limit atherosclerosis-associated collecting lymphatic vessel dysfunction. Experiments performed with blood platelets isolated from apoA-I-treated *Ldlr*$^{-/-}$ mice revealed that apoA-I decreased *ex vivo* platelet aggregation. This suggests that *in vivo* apoA-I treatment limits platelet thrombotic potential in blood while maintaining the platelet activity needed to sustain adequate lymphatic function.

Conclusion. Altogether, we bring forward a new pleiotropic role for apoA-I in lymphatic function and unveil new potential therapeutic targets for the prevention and treatment of atherosclerosis.

Keywords: ApoA-I**,** Collecting lymphatic vessels, Atherosclerosis, Platelets

Clinical Perspective

- We bring forward a new pleiotropic role for apoA-I in preserving and restoring lymphatic function by direct and indirect mechanisms that include platelet activity.

- Our work unveils new potential therapeutic targets for the prevention and treatment of atherosclerosis.

INTRODUCTION

The lymphatic system is an open, unidirectional system playing multiple roles in immunity, chylomicron transport and clearance of wastes from the periphery[1]. It is characterized by a network of vessels that carry a clear fluid called lymph. Sequentially, ultrafiltrates from peripheral tissues are absorbed by initial lymphatics (also called lymphatic capillaries), a highly permeable and specialized compartment constituted by discontinuous "button-like" junctions between endothelial cells[2]. These initial lymphatics are characterized by the absence of smooth muscle cells (SMCs) and the presence of lymphatic vessel endothelial hyaluronan receptor (LYVE-1) at the surface of lymphatic endothelial cells (LECs)[3]. Following its absorption, lymph moves on to converge into larger vessels called collecting lymphatic vessels (LVs) that are partially covered with SMCs, and characterized by the expression of podoplanin[4]. Collecting vessels are composed of contractile lymphangions that propel lymph in a unidirectional manner, with the help of intraluminal bi-leaflet valves, as well as SMCs. Eventually, lymph passes through the lymphovenous junction and reaches the blood circulation via the subclavian vein[5]. Junctional organization of LECs in initial lymphatics and collecting vessels is crucial to vessel integrity. The transcriptional factor Forkhead box protein C2 (FOXC2) has been identified to play a key role in cell-cell junctions and lymphatic valve integrity. Depletion of FOXC2 has led to major lymphatic dysfunction and lethality[6]. However, many other different transcription factors regulate lymphatic development. Interestingly, it has been shown that during embryogenesis, lymphatic system separation from blood vessels requires platelet activity[7]. Indeed, platelets regulate the blood/lymphatic vessel separation by inhibiting the proliferation, migration, and tube formation of LECs, upon the interaction of C-type lectin-like receptor 2 (CLEC-2) with podoplanin[8]. Platelet activity has also been shown to be required throughout life in order to maintain the lymphovenous junction integrity[5]. In addition to the hemostatic role of platelets in maintaining lymphatic[5] and blood vessels integrity[9], platelets have been shown to act through their secreted active relesates and platelet microparticles, also called extracellular vesicles (EVs), to promote inflammation[10-12] and conversely, to secure inflamed vessels[13, 14]. Platelet-derived EVs are abundant in blood circulation[15], and we have reported, for the first time, that platelet-derived EVs are present in mouse lymph[16].

The lymphatic network plays an important role in inflammatory and autoimmune diseases, cancer, lymphedema, graft rejection and wound healing[17-19]. The concept that lymphatic vessels could influence atherogenesis and lipoprotein transport has first been brought forward several decades ago[20-22]. However, we had to wait until recently to directly associate the lymphatic system to atherosclerosis[23-27], a disease driven by the accumulation of cholesterol in the artery wall, primarily by the low density lipoprotein (LDL), leading to increased plaque buildup[28]. It has been described that without a functional lymphatic network, cholesterol excreted from plaque macrophages cannot be properly conducted out of the artery wall, and thus cannot be evacuated[29]. The mechanisms responsible for the interplay between lymphatic function and the onset or progression of atherosclerosis remain under intensive investigation. In that perspective, our group has recently demonstrated that the lymphatic impairment observed during atherosclerosis first affects the collecting lymphatic vessels rather than the initial lymphatics, a defect that was detectable even before plaque formation[24].

Whereas the adventitial lymphatic vessels are now accepted as important modulators of cholesterol transport between the atherosclerotic lesion and the bloodstream, the apolipoprotein A-I (apoA-I) has been identified as a key regulator of cellular cholesterol efflux via the ABCA1 receptor present at the cell surface[30, 31]. ApoA-I is the main protein constituent of plasma high density lipoprotein (HDL) and participates to its highly heterogeneous properties[32, 33]. Whereas intradermal injections of low-dose lipid-free apoA-I treatment do not significantly increase plasma HDL-cholesterol (HDL-C) concentrations, lipid-free apoA-I reduces excess cellular cholesterol and reverses the autoimmune-like phenotype that develops in high cholesterol diet-fed *Ldlr*$^{-/-}$ *apoA-I*$^{-/-}$ double knockout mice[34]. Recently, the mechanistic basis explaining this protective effect of small and consistent amounts of apoA-I in reducing lipid and immune cell accumulation within the aortic root has emerged. HDL and apoA-I have been shown to modify atherogenic and antigen presentation properties by interfering with cell membrane lipid raft[35-37]. In the same vein, it has been demonstrated that apoA-I acts in hypercholesterolemic mice by systemically reducing excess cellular cholesterol accumulation in membrane lipid rafts[38]. The latter are tightly regulated microdomains contained in cell membranes and they form a platform responsible for organizing the signaling of receptors and proteins of various cell types. In a recent publication, *in vitro* treatment with apoA-I, on a tumor necrosis factor (TNFα) background, has been found to cause lymphangiogenesis. ApoA-I

treatment has been demonstrated to increase podoplanin mRNA level which could potentially help the CLEC-2/podoplanin interaction with platelets[39].

In the present study, we hypothesize that the protective effect of apoA-I is mediated at least in part by preserving collecting lymphatic vessel function by mechanisms that include modulating platelet adhesion on LECs. Our findings could bring forth a new pleiotropic role for apoA-I in lymphatic function and unveil new potential therapeutic targets for the prevention and treatment of atherosclerosis.

METHODS

Experimental setup. *Ldlr*$^{-/-}$ mice on a C57BL/6 background were purchased from Jackson Laboratories. Animals were housed in a pathogen-free environment under 12-hour light-dark cycles with free access to water. The experimental design of the *in vivo* section of our study is illustrated in Figure 1A. Female *Ldlr*$^{-/-}$ mice at 6 weeks of age were fed on high fat diet (HFD - adjusted calories diet, 0.2% total cholesterol and 42% from fat, Harlan 88137) for eight weeks, after which, they were separated into three age-matched groups for a period of six more weeks. To assess the direct effect of apoA-I on lymphatic function, one group was injected intradermally with lipid-free apoA-I (200 ug/ 25 g of body weight) 3 times a week for six weeks while still under a HFD. The control group received concentration-matched bovine serum albumin (BSA) alone. A third group was switched to standard chow diet, in order to lower plasma cholesterol and promote lesion regression[40]. At the end of the sixth week, lymphatic function assays were performed and blood and lymph were collected under anesthetic conditions. Following, mice were sacrificed by cardiac puncture following ketamine anesthesia (100 mg/ml ketamine administered at 0.10 ml/10g of body weight), or by carbon dioxide (CO_2), and were perfused with 15 ml phosphate buffered-saline (PBS). Finally, organs were collected. All experiments were performed in accordance with the Canadian Council on Animal Care guidelines and approved by the Montreal Heart Institute Animal Care Committee.

ApoA-I preparation. ApoA-I was purified from human plasma by sequential ultracentrifugation as previously described[34]. The apoA-I was lyophilized to dryness, dissolved in 6 M guanidine hydrochloride and then refolded by dialyzing exhaustively against

10 mM ammonium bicarbonate, pH 7.4. Mass spectrometry and 12% SDS PAGE were used to ensure that there were no contaminating proteins and that apoA-I methionines were not oxidized to the sulfoxide form, a common contaminant in apoA-I preparations. Protein concentration was determined using the Lowry assay[30].

ApoA-I kinetics. To ensure that the lipid-free apoA-I injected was absorbed by the initial lymphatics present in the back skin dermis and that apoA-I kinetics in lymph reflect that of blood, we used wild type (WT) mice on a C57BL/6 background purchased from the Jackson Laboratories (Figure S1). WT mice were anesthetized with isoflurane (4% for induction, 2-3% for maintenance). For lymph collection, the animal was anesthetized and positioned on its right side. A cannula was inserted into the thoracic lymph duct above the cisterna chyli between the transverse lumbar artery and the diaphragm as previously described[16]. Lymph was collected continuously for 45 minutes with a tube attached to a syringe coated with EDTA 0.1 M. Blood was collected by cardiac puncture with a syringe coated with EDTA 0.1 M followed by the animal sacrifice. Collected lymph was centrifuged at 1200 g for 10 minutes and blood was centrifuged at 2400 g for 10 minutes. To avoid thawing-related damage on lipoprotein conformation, sucrose (5%) was added before samples were stored at −80°C for further batch analysis. Human ApoA-I was measured by ELISA tests.

Lymphatic Functional Assessment. Lymphatic function was assessed by three complementary methods. First, the propensity of dendritic cells to migrate through the lymphatic vessels from the periphery to draining lymph nodes (LNs) was measured as described previously[41]. The animals were sacrificed 18 hours after the application of a solution that contains fluorescein isothiocyanate (FITC), dibutyl phthalate and acetone solution, in order to instigate an immune response and thus dendritic cell trafficking. Of importance, FITC was applied to a different location than the apoA-I injection site, as our interest was to assess the global lymphatic function. The corresponding skin-draining LNs were recovered and enzymatically digested in collagenase D for 25 minutes at 37°C. Cells were then passed through a 70 µm cell strainer, washed, counted, and stained for analysis by flow cytometry (BD Biosciences LSR II). Conjugated antibodies CD11b PerCp-Cy5.5 (BioLegend, CA101227-BL), CD11c PeCy7 (Tonbo biosciences, 60-0114), MHCII-VioletFluor 450 (Tonbo biosciences, 75-5321) and

CD45-APC (Tonbo biosciences, 20-0451) were used. The number of dendritic cells that uptake FITC and travelled to the corresponding skin draining lymph node was then counted based on the total LN cellularity (% of $FITC^+$ cells x # of cells/LN).

Second, lymphatic vessel permeability was assessed using Evans blue dye for tracing the path of lymph through popliteal LVs[24]. Mice were anesthetized with isoflurane and following Evans Blue intradermal injection in the footpad, popliteal collecting lymphatic vessels were visualized using a Stereo Discovery V8 (Zeiss). Both the effusion of Evans Blue around the vessel, as well as the area the leakage covers were analyzed using ImageJ software.

Third, lymphatic function was assessed by quantifying the dermal clearance of dextran by the initial lymphatics, as described previously[42]. Briefly, a total of 1 µl fluorescent (Cy5) dextran (70 kDa) at a concentration of 2 mg/ml in sterile PBS was injected intradermally in the ear pinnae of anesthetized mice. Due to its large size, the tracer is specifically uptaken by blind-ended lymphatic capillaries avoiding absorption by blood capillaries. Fluorescence decay was observed through the skin using a fluorescence stereomicroscope and images of the skin were acquired every minute for 30 minutes. The rate of clearance was determined by calculating the area under the curve (AUC) of fluorescence intensity at each time point and normalized to the initial value. The normalized rate of fluorescence decay was then calculated from the slope of AUC *vs.* time, which is considered proportional to the actual rate of dextran-Cy5 clearance.

Mouse platelet isolation. Mouse blood was withdrawn in 1 ml syringe containing 50 µL of diluted heparin (dilute stock heparin 1:10 to obtain 1000U/ml). Blood from 2-3 mice was pooled to obtain sufficient platelets. Blood was then diluted in Tyrode's buffer (1/2) containing 0.2 µg/ml of Prostaglandin E1 (PGE_1, Sigma) and centrifuged at 164g for 8 minutes. An additional centrifugation was made with diluted RBC in Tyrode's buffer containing 0.2 µg/ml of PGE_1 to obtain more platelets. 0.2 µg/ml PGE_1 was added to the pooled platelet-rich plasma (PRP) and centrifuged at 1000g for 5 minutes. The pellet was resuspended in modified Tyrode's buffer at a concentration of 250 x 10^6 platelets/ml.

Mouse platelet aggregation. Isolated platelets were submitted to a constant shear rate (1000 rpm) at 37 °C in a four-channel optical aggregometer (Chronolog Corp.). A volume of 250 µL of the washed platelet preparation was used per channel. Platelet aggregation was then induced with high concentration of thrombin (0.5 to 1 U/ml) in the first channel or podoplanin (5 µg/ml) in the second channel, and the percent aggregation was recorded when platelets reached a plateau.

Immunoblotting of mouse platelets. Following aggregations, platelets were lysed by adding 62.5 µL of 4X SDS in 250 µL of washed platelets, and heated at 95°C for 5 minutes. Samples were stored at -20°C until further analysis. Proteins from the three mouse groups' platelets were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by its transfer to nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk for 1 hour at room temperature. Antibodies against CLEC-2 (R&D Systems, AF1718) and pAkt (Cell Signalling, 9275) were incubated with the membranes overnight at 4°C, and an HRP-conjugated secondary antibody (Abcam, AB6741) was used for detection using the Western Lightning Ultra chemiluminescence kit (PerkinElmer).

Organ harvesting. LNs, ears, dermal back skin sections, aortas, hearts and popliteal collecting lymphatic vessels were harvested and either freshly processed for flow cytometry analysis and/or western blots, or fixed in 4% paraformaldehyde (PFA) and 10% formalin for future analysis.

Immunohistochemistry and immunofluorescence of the skin dermis. The back skin of the animals was shaved and harvested, fixed in 10% formalin, and embedded in paraffin. Eight-micrometer (µm) thick back-skin sections were stained with hematoxylin and eosin (H&E). Pictures were taken with an Olympus B45 microscope and visualized using ImagePro Plus 7.0 software. Another batch of 8 µm thick back-skin sections were incubated with anti-CD206 (Abcam, ab64693), -CD68 (Biolegend, 137001) and -LYVE-1 (Abcam, ab14917) antibodies. Secondary antibodies anti- Alexa-fluor 555 (Abcam, ab150074), -Cy3, and -Cy5 (Jackson ImmunoResearch, 712-165-150 and 711-606-152, respectively), as well as DAPI were then

added and images were acquired with an LSM 710 Confocal Microscope (Zeiss) equipped with a 63x/1.4 oil dic objective.

Immunofluorescence of the collecting lymphatic vessel. Popliteal collecting lymphatic vessels were identified following Evans blue dye intradermal injection as described above, and harvested. For analysis of vessel integrity, whole-mount immunofluorescence analysis following incubation with anti-SMA already coupled to FITC (Sigma) and -FOXC2 (R&D Systems, AF6989) antibodies was performed on isolated popliteal lymphatics of mice. Secondary antibody donkey anti-sheep 555 (Jackson ImmunoResearch, 713-165-147) and DAPI were then added. Images were acquired with an LSM 710 Confocal Microscope (Zeiss) equipped with a 63x/1.4 oil dic objective. All image handling was performed using ImageJ software.

Atherosclerotic lesion and initial lymphatic density quantification. The heart and aorta were removed and fixed in 4% PFA for 2 hours. The heart was transferred into PBS containing 30% sucrose (wt/vol) overnight at 4 °C before being immersed in optimal cutting temperature (OCT) compound and stored at −80 °C. Eight-micrometer-thick cryosections of the aortic sinus were prepared. Cross-sections of the aortic sinus were stained with anti-LYVE-1 (Abcam, ab14917) and anti-CD68 (Biolegend, 137001) antibodies, and then incubated with the appropriate secondary antibodies. As macrophages can also be positive for LYVE-1, adventitial initial lymphatics were identified as LYVE-1^{+}CD68^{-} cells forming vessel-like shapes. Whole-mount immunohistochemical analysis of the ear dermis to visualize lymphatic vessels was performed as described previously[43]. Ear dermis were stained for lymphatic capillaries (anti-LYVE-1, Abcam) at 4 °C, and then sections were incubated with Alexa Fluor 647-conjugated donkey anti-rabbit antibody and Cy3 donkey anti-rat (Jackson ImmunoResearch, 711-606-152 and 712-165-150, respectively). All imaging was performed on a Fluoview FV10i (Olympus). Vessel counts were performed by one observer. The relative quantification of the number of initial lymphatics (LYVE-1^{+} vessels), their diameter and the total surface area they occupy was determined by computer-assisted morphometric analysis. Aortas were cleaned by removing the surrounding fat and were then split along their outer curvature. Neutral lipid assessment in

atherosclerotic lesions in the aortic sinus and *en face* aorta were performed by Oil-red-O (ORO) staining (Sigma, O-0625).

Human platelet isolation. All experiments performed with human specimens were approved by our institutional review committee and every subject gave informed consent. Human blood from healthy donors was collected with a syringe containing Anticoagulant Citrate Dextrose Solution (ACD) and centrifuged at 200 g for 15 minutes. PGE_1 (1 µg/ml) was added to the PRP and centrifuged at 1000 g for 10 minutes. Platelets were washed with HBSS citrate buffer and the suspension was centrifuged at 800 g for 10 minutes. Lastly, platelets were resuspended in HBSS for a total concentration of 250 x 10^6 platelets/ml.

Cell culture. Primary human dermal lymphatic microvascular endothelial cells-adult (HMVEC-dLyAd) were cultured according to the manufacturer's protocol (Lonza) in EBM-2 medium containing the EGM-2 MV SingleQuots. Cells were seeded in one µ-Slide $VI^{0.4}$ Ibi-treated flow chamber (IBIDI) or in glass bottom culture dishes chamber (Mattek) for experiments that did not require a steady perfusion flow rate. In both these cases, HMVEC-dLyAd at 80% confluence were treated with apoA-I (0.6 mg/ml) for 24 hours.

Platelet perfusion under physiological lymph flow. Following treatment, for underflow, isolated human washed platelets were perfused over the HMVEC-dLyAd at a wall shear rate of 50 s^{-1} at 37°C for 8 minutes. PBS was then added to remove the non-adhered platelets. For static treatments, the HMVEC-dLy-Ad were treated with washed platelets for 1h30. Cells were then fixed with PFA 2% and immunofluorescence analysis was performed following incubation with anti-CD61 (Clone: VI-PL2, Biolegend).

Immunoblotting of human lymphatic endothelial cells. Proteins were extracted using radioimmunoprecipitation assay buffer (RIPA buffer) and the protein concentration was established using Bradford Protein Assay Kit (Bio Basic). Protein samples were diluted in 4X Laemmli buffer, then heated at 95°C for 5 minutes. Proteins were separated by electrophoresis on a 12% SDS-PAGE, then transferred on nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk in tris-buffered saline (TBST, 0.1% Tween 20) for 1 hour at

room temperature, then incubated with an anti-podoplanin (OriGene, DP3512S), an anti-VEGFR-3 (Abcam, AB27278), or an anti-beta-actin (Abcam, AB8227) overnight at 4°C. The membranes were washed with TBST and incubated with HRP-conjugated secondary antibodies (Abcam, AB6721 and AB6721) for one hour at room temperature. Western Lightning Ultra chemiluminescence kit (PerkinElmer) was used for detection. Each sample was normalized with its respective beta-actin expression.

PAF-AH activity. Plasma and lymph from the three mouse groups was used to assess total PAF-AH activity. We used the Cayman's PAF Acetylhydrolase Assay Kit (Cayman Chemical, 760901) and followed manufacturer instructions.

Statistics. Data are presented as mean and SEM. Statistical differences were assessed using a two-tailed parametric Student's t-test, ANOVA or non-parametric tests, with $p < 0.05$ reported as statistically significant, using Prism software version 6.0 c (GraphPad) or SPSS version 23.0 (SPSS Inc. Chicago, IL, USA).

Study approval. All animal studies were performed in accordance with the Canadian Council on Animal Care guidelines and approved by the Montreal Heart Institute Animal Care Committee. All experiments performed with human specimens were approved by our institutional review committee and every subject gave written informed consent prior to inclusion in the study.

RESULTS

ApoA-I treatment reduces aortic lipid without significantly affecting total cholesterol level in *Ldlr*$^{-/-}$ mice

As our goal is to assess the effect of apoA-I on the lymphatic vasculature *per se*, we performed intra-dermal injections to allow a more direct drug uptake by the dermal initial lymphatic vessels (experimental design illustrated in Figure 1A). After subtracting the background value, we first measured the time course appearance of human apoA-I in plasma and lymph following intradermal injection of lipid-free apoA-I in HFD-fed *Ldlr*$^{-/-}$ mice, and

confirmed that the injected apoA-I reach lymph efficiently. Approximately 1.5% and 3% of the injected human apoA-I appears and peaks by 3 and 8 hours post injection, in lymph and blood, respectively (Figure S1).

We next tested the effect of this route of injection on the extent of atherosclerosis in our three groups of mice. *En face* aorta ORO^+ staining is decreased following apoA-I treatment; Figure 1B shows representative images of the atherosclerotic lesions present in the thoracic aorta, as assessed after staining with ORO. The results of ORO quantification are expressed as percent of total surface (Figure 1C). *Ldlr*$^{-/-}$ mice that have been switched from a high fat diet (HFD) to a chow diet have significantly less plasma cholesterol than control- (BSA-) or apoA-I- treated mice on continuous HFD (Figure 1D). ApoA-I does not significantly decrease plasma (Figure 1D) or lymph (Figure 1E) cholesterol compared to control. Contrarily to mice that had been switched to a chow diet, apoA-I treatment, when injected intradermally, did not however, significantly reduce lesion size or ORO area, nor $CD68^+$ cells in the aortic sinus (Figure S2A, S2B and S2C, respectively) of *Ldlr*$^{-/-}$ mice fed for 8 weeks on HFD. Figures S2D and S2E show representative images of the aortic root stained with ORO and CD68 antibody, respectively.

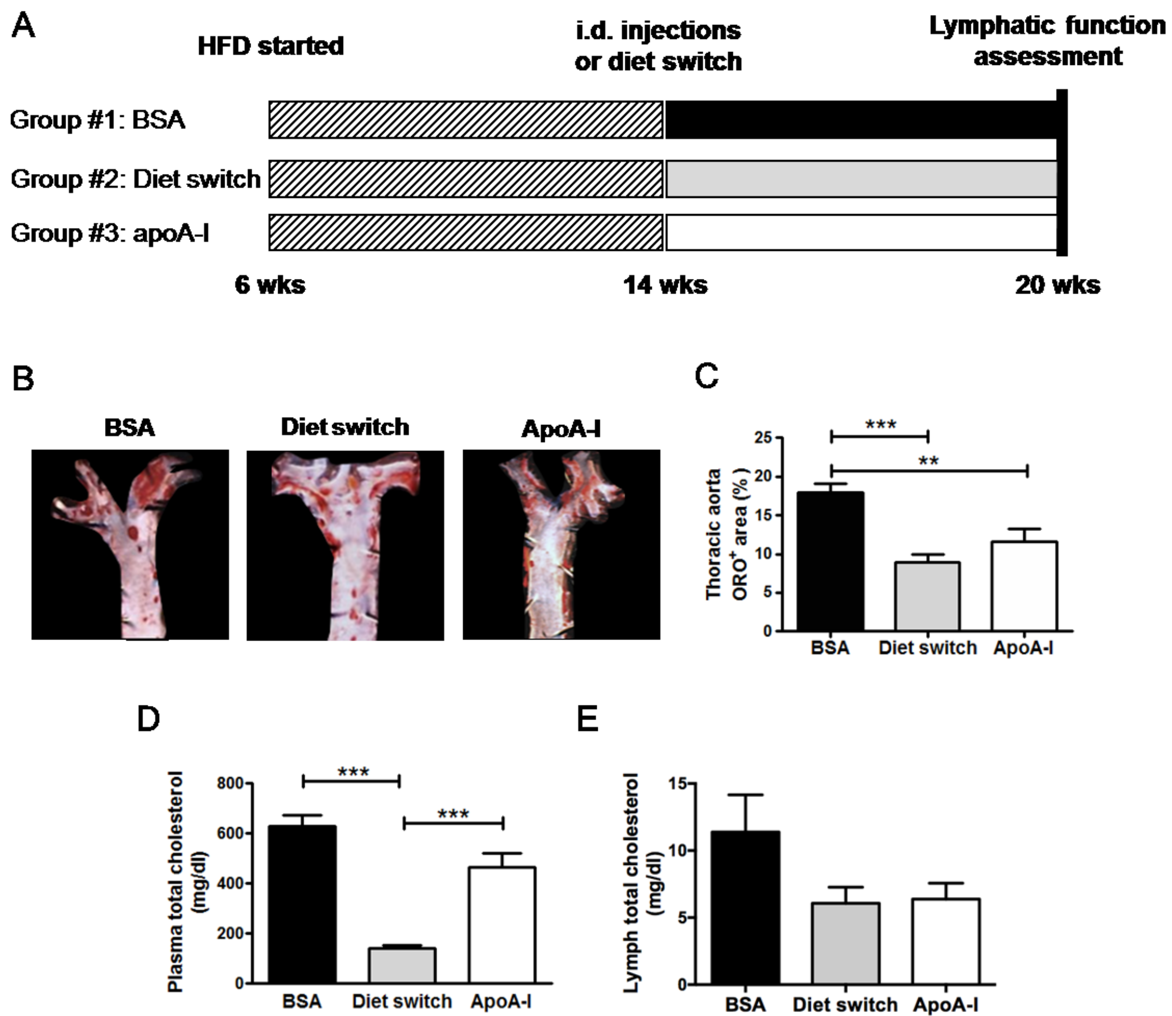


Figure 1. Study design and assessment of circulating total cholesterol and lipid deposition in *Ldlr*$^{-/-}$ mice. A, Pictogram of the experimental design used. At 6 weeks of age, female *Ldlr*$^{-/-}$ mice began a high fat diet (HFD, hatched bar). After 8 weeks, mice were divided into three groups. Two groups (groups #1 and #3) remained on the HFD during the treatment phase of the study and were divided as follow: group #1 – intradermal (i.d.) injections of 200 μg of bovine serum albumin (BSA), 3 times/week (black bar); group #3 - intradermal injections of 200 μg of lipid-free apolipoprotein A-I (apoA-I), 3 times/week (white bar bar). Group #2 was switched on a chow diet instead of receiving injections (light grey bar). After 20 weeks of age, the mice were evaluated and lymphatic function assessed. B, C, Neutral lipid- (Oil Red O) area was quantified in the three groups of mice (*en face*) using ImageJ software. Experiments were performed with 7-11 mice per experimental group. Total cholesterol was assessed in D, plasma and E, lymph of BSA, diet switch and apoA-I treated *Ldlr*$^{-/-}$ mice. Experiments were performed with 4 mice per experimental group. **p≤0.01 and ***p≤0.001, as determined by one-way ANOVA with Tukey's post-hoc test.

ApoA-I restores systemic cellular lymphatic transport in *Ldlr*$^{-/-}$ mice

To test whether apoA-I might exert its beneficial effects through its interaction with the lymphatic system, we first measured the transport of dendritic cells from the peripheral tissue (skin) to the corresponding draining lymph node through the lymphatic system in our different groups[41]. We show that apoA-I treatment rescues lymphatic function in atherosclerotic HFD-fed *Ldlr*$^{-/-}$ mice, with respect to the number of dendritic cells that have migrated from the skin to the corresponding draining lymph node (Figure 2A). In diet switch mice, this effect was not seen, despite a decrease in plasma cholesterol as previously illustrated (Figure 1D). In addition to improving dendritic cell transport from peripheral tissues, this low-dose apoA-I treatment was associated with fewer immune cells accumulation in the skin dermis of *Ldlr*$^{-/-}$ mice. CD206^{+} (Figure 2B) and CD68^{+} (Figure 2C) macrophage levels were decreased in apoA-I-treated mice. Whereas resident CD206^{+} macrophages are significantly less abundant in the diet switch group, CD68^{+} macrophages density was not significantly affected by this decrease in plasma cholesterol.

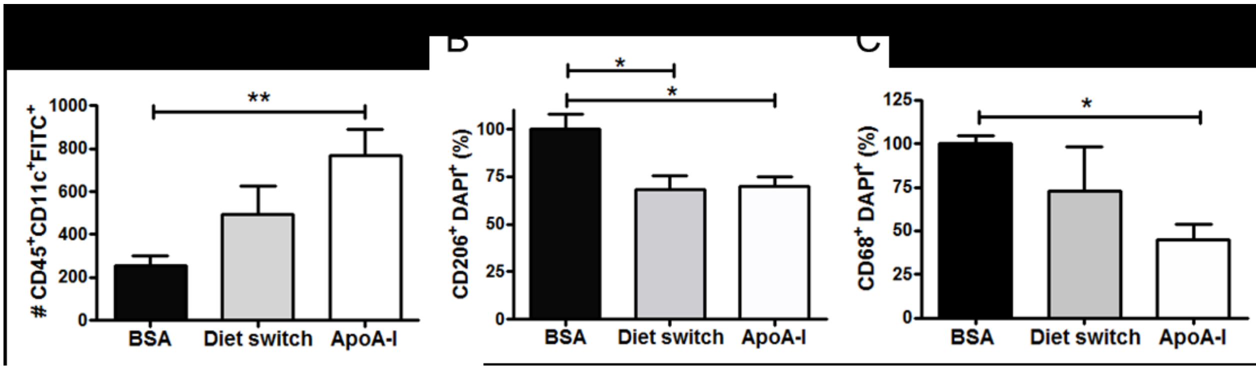


Figure 2. Effect of apoA-I on systemic cellular lymphatic transport in *Ldlr$^{-/-}$* mice. Using a contact sensitization assay, the assessment of dendritic cell migration through the lymphatic was assessed by flow cytometry. Skin draining lymph nodes were harvested after 18 hours and the number of dendritic cells that migrated from the skin to the lymph node was determined. We quantified the A, number of CD45$^+$CD11c$^+$FITC$^+$ cells in BSA, diet switch and apoA-I treated *Ldlr$^{-/-}$* mice. Experiments were performed using 5-8 mice per experimental group (mean ± SEM). Analysis was performed using a BD LSRII flow cytometer. The percentage of B, CD206$^+$DAPI$^+$ and C, CD68$^+$DAPI$^+$ macrophages were quantified in 10 µm-thick skin sections from the three groups of mice. Experiments were performed with 7-11 mice per experimental group and were quantified using ImageJ software. *p<0.05 and **p<0.01, as determined by one-way ANOVA with Tukey's post-hoc test.

ApoA-I does not alter obesity-related parameters

Obesity causes chronic systemic and local inflammation[44], and impairs lymphatic function[45]. We therefore sought to investigate whether apoA-I could have acted on lymphatic transport by first reducing obesity-related parameters. Our results revealed that subcutaneous adipose tissue (Figure 3A and 3B), visceral fat (Figure 3C) or total body weight variation (Figure 3D) in *Ldlr*$^{-/-}$ mice are not a priori affected following apoA-I treatment in our experimental design.

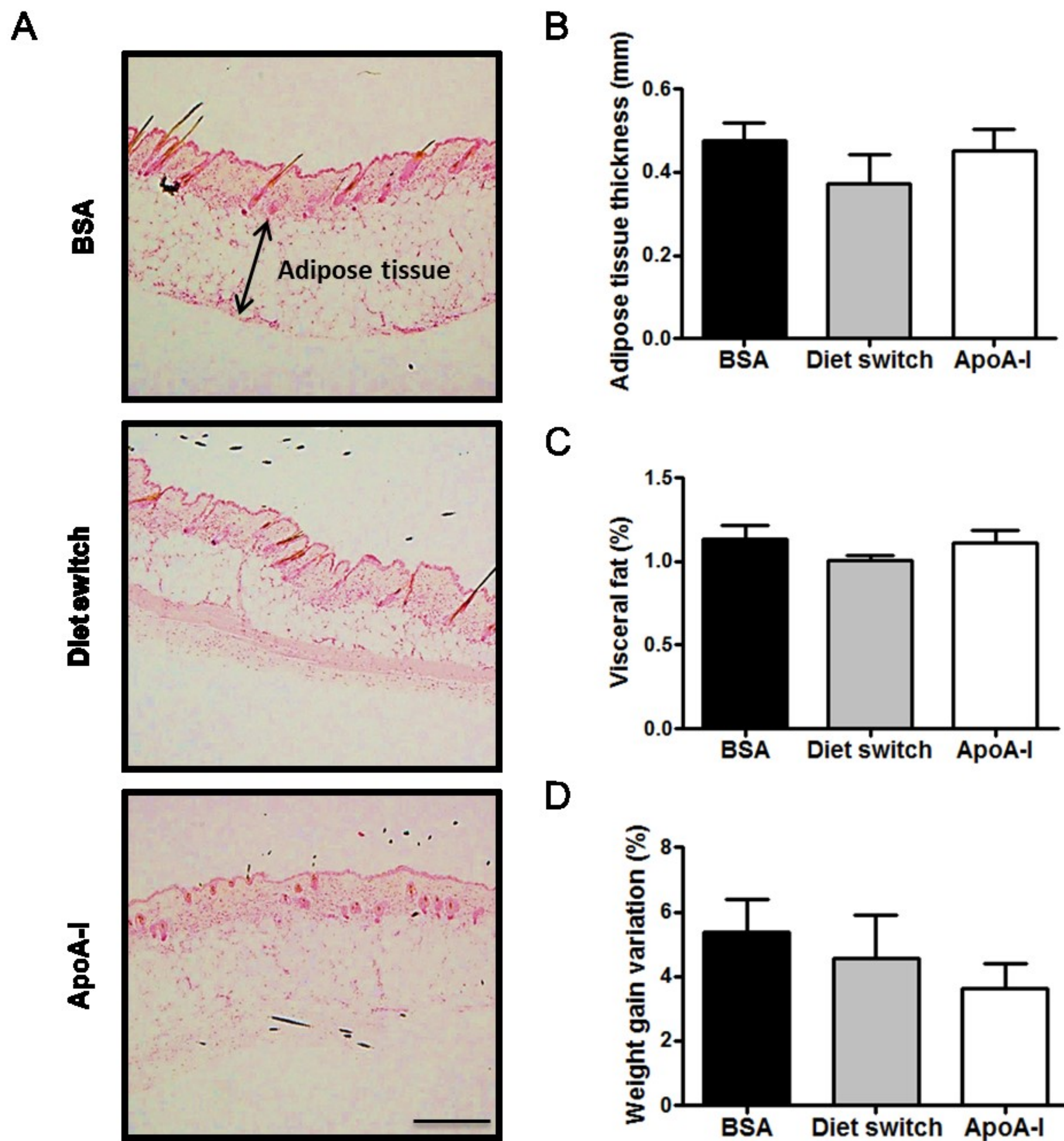


Figure 3. Obesity-related parameters in *Ldlr*$^{-/-}$ mice. A, Hematoxylin and eosin staining was performed on 8 µm- thick paraffin skin sections in BSA, diet switch and apoA-I treated *Ldlr*$^{-/-}$ mice. B, Quantification of adipose tissue thickness in back skin is illustrated. Experiments were performed with 5-7 replicates per experimental group (mean ± SEM). Pictures were taken with an Olympus B45 microscope and analyzed using ImagePro Plus 7.0 software. All image handling was performed using ImageJ software. C, Visceral fat was excised and weighed. Visceral fat percentage was calculated based on the visceral fat and the mouse total body weight. D, Weight gain variation percentage was calculated using the variation between the final and initial weight of the mouse. Experiments were performed with 10-12 replicates per experimental group (mean ± SEM). Scale bar = 500 µm.

ApoA-I enhances the prevalence of initial lymphatic vessel number without affecting their function

We have recently demonstrated that lymphatic dysfunction, at least in an early stage of the atherosclerotic disease, is mainly due to collecting lymphatic vessel impairment[24]. In our current experimental design, as the atherosclerotic plaque is already advanced, we do not exclude the fact that the defect in lymphatic transport could now reside in both the initial and the collecting lymphatic vessels. We therefore explored lymphatic vessel density in the skin dermis (back skin and ear dermis, Figure 4A and 4B, respectively) and in the adventitia of the aortic sinus (Figure 4C). Reversing the diet to a chow diet does not increase lymphangiogenesis (Figure 4D, 4E, 4F). Although no changes are observed in the back skin, apoA-I treatment is reflected by a small albeit significant increase of initial lymphatic vessels in the aortic sinus and in the ear dermis of *Ldlr*$^{-/-}$ mice. However, an increase in branching points or initial lymphatic hyperplasia could not be observed concomitantly (Figure 4G and 4H, respectively). To test whether this slight increase in LYVE-1^{+} vessels was sufficient to improve the uptake of interstitial molecules, we quantified the dermal clearance of a large molecule (dextran Cy5) by the initial lymphatics over a time course of 30 minutes and observed no changes in the dye uptake. (Figure 4I).

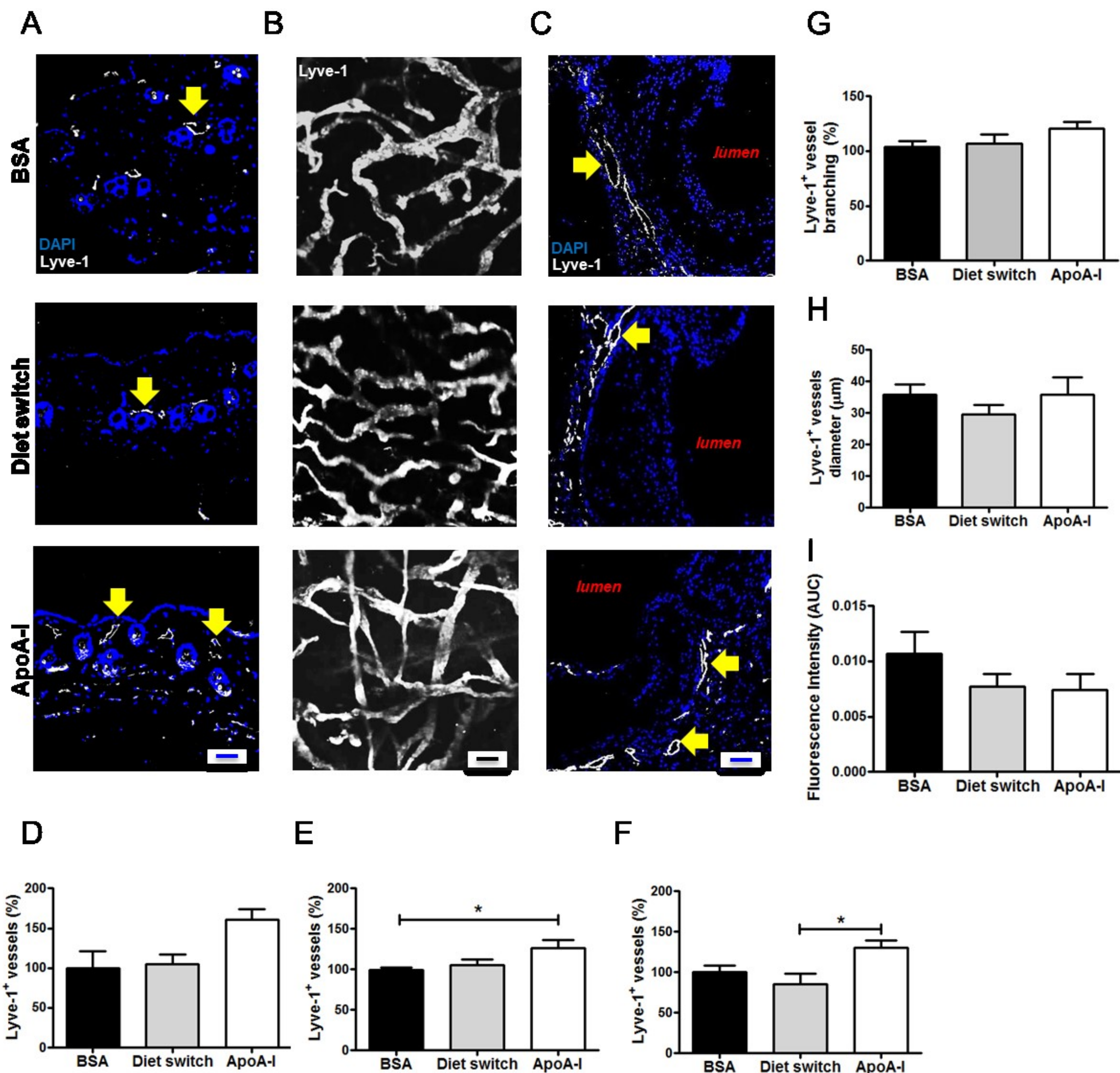


Figure 4. Assessment of initial lymphatic vessels morphology and function in *Ldlr*$^{-/-}$ mice. Initial lymphatic vessels (Lyve-1^{+}) were imaged by immunofluorescence and their number was quantified in A, D, the back skin, B, E, ear dermis and C,F, aortic sinus of BSA, diet switch and apoA-I treated *Ldlr*$^{-/-}$ mice. In back skins and aortic sinuses, only distinguishably round Lyve-1^{+} vessels were included, as indicated by the yellow arrows. Data are expressed as the percentage of BSA (control group) in each set of tissues. Experiments were performed with 7-11 replicates per experimental group (mean ± SEM). Quantification of the G, branching and H, diameter of Lyve-1^{+} vessels in the ear dermis. I, Lymphatic molecular transport was assessed by Cy5-labelled Dextran (70 kDa) injection in the ear dermis of BSA, diet switch and apoA-I treated *Ldlr*$^{-/-}$ mice. Experiments were performed with 5-11 replicates per experimental group (mean ± SEM). Pictures were taken using Fluoview FV10i (Olympus). All image handling was performed using ImageJ software. *$p \leq 0.05$ as determined by one-way ANOVA with Tukey's post-hoc test. Scale bars = 100 µm (B) and 50 µm (A, C).

ApoA-I reduces the atherosclerosis-associated increased collecting lymphatic vessel permeability

Whereas we observe an enhanced number of initial lymphatic vessels in the apoA-I-treated group, our results suggest that the interstitial fluid uptake is overall not seemingly improved. Thus, we hypothesize that it is only once reaching the collecting lymphatic vessel that lymph flow is compromised and that apoA-I would mediate its beneficial effect solely at that level during atherosclerosis progression. Therefore, we next wanted to investigate whether apoA-I adequately restores collecting lymphatic vessel function. We first determined the effect of apoA-I on collecting lymphatic vessel permeability by injecting Evans blue (EB) in the footpad skin of the mouse. Images of the lower limb were taken to visualize the popliteal lymphatic vessels. EB leakage surrounding the vessel was quantified (Figure 5A). Our results show that leakage is nearly inexistent in mice treated with apoA-I, indicative of proper lymphatic vessel integrity (Figure 5B, 5C). In the diet switch group, the area of leakage was also decreased, whereas the distance of leakage in punctual areas (perpendicular leakage distance) was as impaired as in the BSA group.

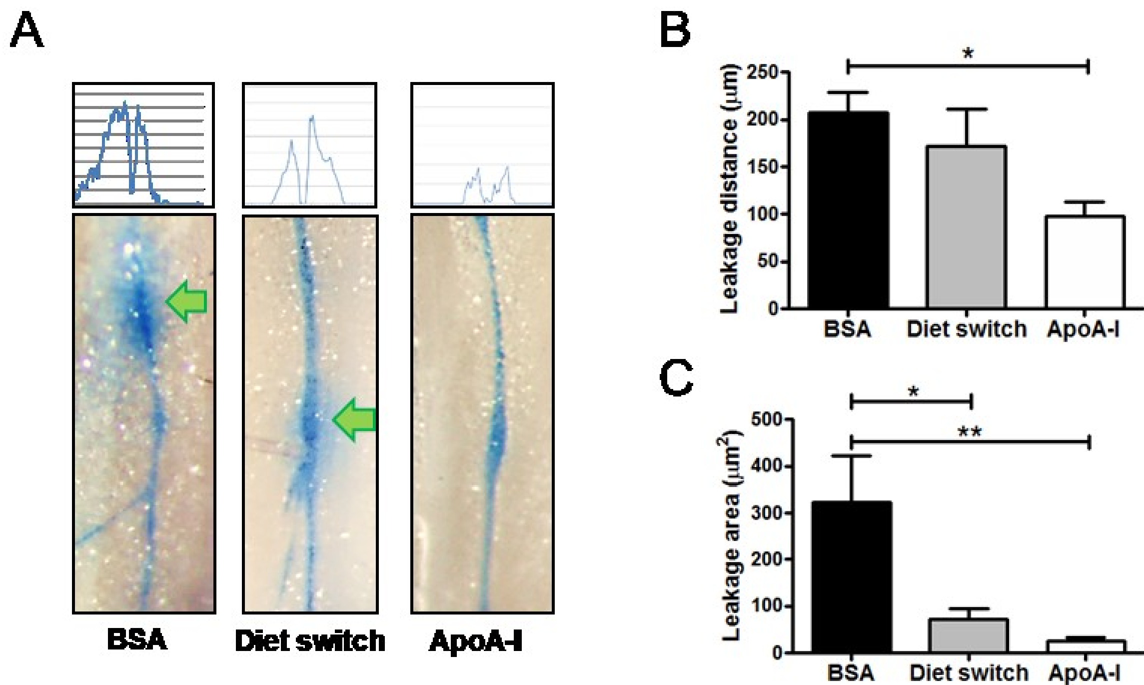


Figure 5. Assessment of collecting lymphatic vessel permeability in *Ldlr*$^{-/-}$ mice. A, Lymphatic vascular integrity was assessed following Evans Blue (EB) dye intradermal injection in the footpad of BSA, diet switch and apoA-I treated *Ldlr*$^{-/-}$ mice. After 30 minutes, lymphatic vessels were visualized using a Stereo Discovery V8. Pictures were taken by Canon Rebel XSI. BSA and diet switch mice display EB dye leakage around the vessels (green arrows). Histograms on top of each vessel illustrate the propagation of EB dye leakage. Both the B, leakage distance and C, its area were assessed and calculated using ImageJ software. Experiments were performed with 4 replicates per experimental group (mean ± SEM). **$p<0.01$ and *$p\leq0.05$, as determined by one-way ANOVA with Tukey's post-hoc test.

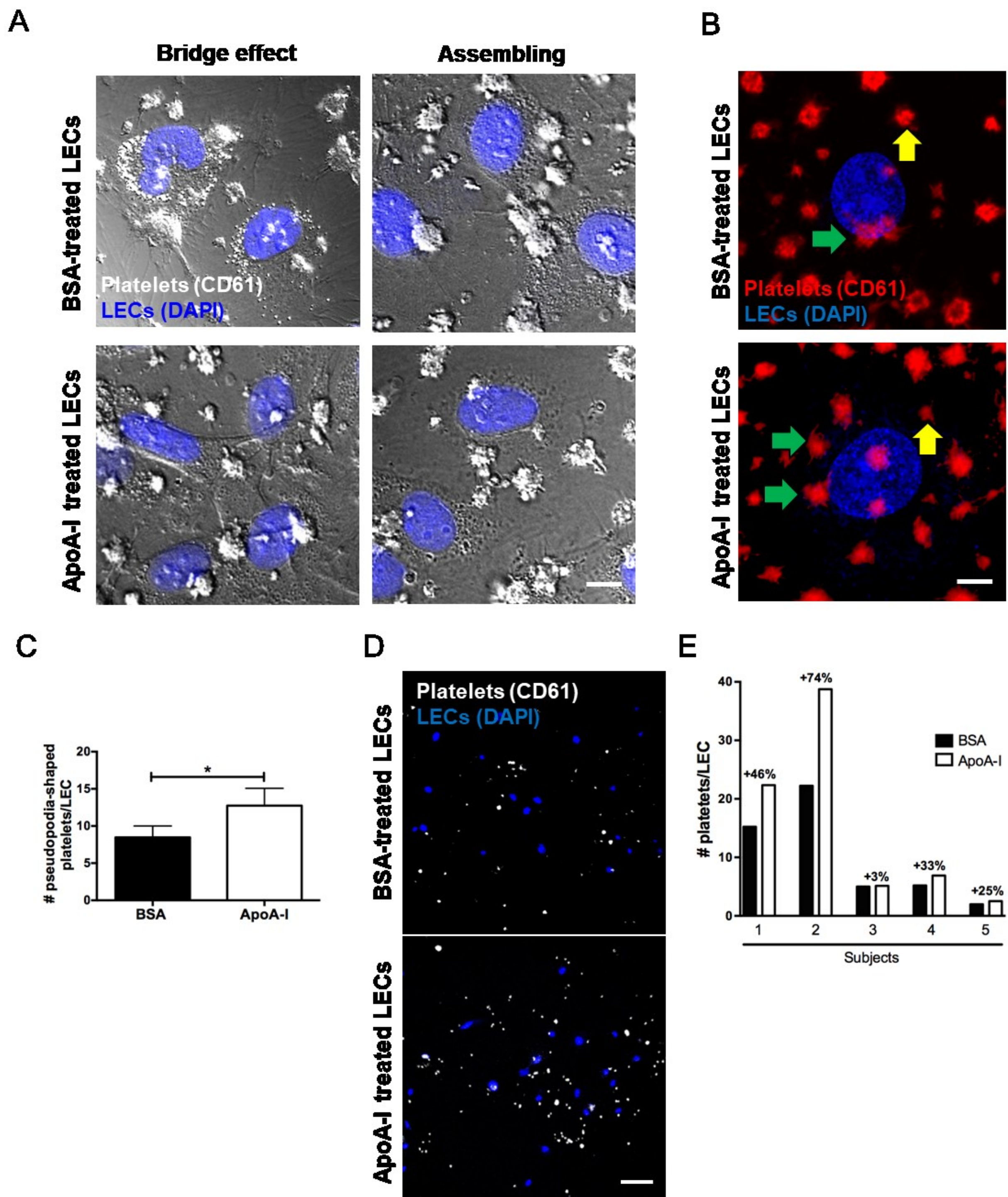
Several factors could account for the loss of lymphatic vessel permeability during atherosclerosis. Specific deletion of the forkhead transcription factor FOXC2 on LECs is associated with a loose and disorganized extracellular matrix, which is indicative of increased endothelial permeability[6]. To test whether apoA-I could regulate lymphatic permeability through FOXC2 expression, popliteal lymphatic vessels were harvested, and FOXC2 expression was assessed by immunofluorescence. Our images reveal that FOXC2 expression is similar in *Ldlr*$^{-/-}$ mice that underwent apoA-I treatment and control mice (Figure S3). However, feeding mice with chow diet appears to increase FOXC2 expression, suggesting that normalizing circulating cholesterol could enhance cell-cell junction stability and endothelial integrity. Our results indicate that apoA-I most likely uses another mechanism to improve lymphatic vessel permeability.

ApoA-I enhances human platelet adhesion to lymphatic endothelial cells in culture

Among the potential players involved in regulating vessel function and integrity, platelets are a target of interest in lymphatic physiology. Platelets are essential in proper lymphatic function: the interaction between CLEC-2 on platelets and podoplanin on LECs is crucial for the formation of the lymphovenous junction and for the lymphatic vessel integrity *per se*, during development and throughout life[5]. As platelets are gatekeepers for lymphatic endothelial cells, we thus sought to investigate the potential role of platelets in repairing the loss of lymphatic vessel permeability during atherosclerosis. First, we isolated platelets from human blood and assessed platelet adherence to primary HMVEC-dLyAd pretreated for 24 hours with either apoA-I or BSA (control). Our results show that, regardless of the treatment, platelets adhere to the lymphatic endothelium and exert a bridging effect through which as little as one platelet assembles together several LECs (Figure 6A, left panel). Furthermore, an assembling effect was observed, in which several platelets will aggregate together in an attempt to bring together several LECs (Figure 6A, right panel). Using the same experimental setting, quantification of pseudopodia-shaped platelet adhesion to the lymphatic endothelium allowed us to observe a significant increase in the number of strongly-adhered platelets[46] per LECs when the latter where pretreated with apoA-I (Figure 6B and 6C).

As podoplanin is more potent to bind to platelets under flow[47], we exposed apoA-I- or BSA-pretreated LECs to a constant venous shear for eight minutes using 6 channel μ-slides, as

depicted in Figure S4. Platelets isolated from five different healthy volunteers were perfused over HMVEC-dLy-Ad at a wall shear rate of 50 s^{-1} at 37°C for 8 minutes. Figure 6D illustrates that, under a physiological flow, the prevalence of $CD61^+$ platelet adherence per LEC ($DAPI^+$ cells) was increased when HMVEC-dLyAd were first treated with apoA-I. Platelet adhesion was improved in each individual donor, with an increase ranging from 3 to 74% in all our healthy volunteers, and an average increase in adherence of 36.2% ± 11.8% (mean ± SEM) was noted (Figure 6E). This large span between values is to be expected as human physiology is highly variable, but nonetheless, in all cases, we see an increase in platelet adherence to the lymphatic endothelium following apoA-I treatment ($p=0.043$ as determined by Wilcoxon Signed Rank Test).



(Figure legend on the following page)

Figure 6. Effect of lipid-free apoA-I treatment on platelet adhesion to lymphatic endothelial cells. A-C, Human platelets were isolated and incubated with a confluent monolayer of primary HMVEC-dLyAd for 1 hour at 37 °C. LECs and platelets were identified by immunofluorescence using DAPI and anti-CD61 antibodies, respectively. A, Representative images indicating that, under static conditions, apoA-I-treated LECs display a "bridge effect" mediated by platelets pseudopodia, thus assembling LECs together. B, Representative images and C, quantification of the number of adhered platelets interacting with HMVEC in BSA- (upper panel) and apoA-I- (lower panel) treated LECs. Results are the averages of five independent experiments. *$p<0.05$, as determined by one-tailed t-test. D-E, Human platelets were isolated and perfused over primary HMVEC-dLyAd seeded at maximum confluence in tissue culture treated flow chambers, at a wall shear rate of 50 s^{-1} at 37 °C for 8 minutes. D, LECs and platelets were identified using DAPI and anti-CD61 antibodies, respectively. E, The % augmentation in the number of adhered platelets following treatment is indicated above the bars. $p=0.043$, using a Wilcoxon Signed Rank test. Scale bars =10 µm (A, B) and 100 µm (E).

To test whether an increase in podoplanin expression on LECs could account for the beneficial effect of apoA-I, we performed immunoblotting of human LECs pretreated with apoA-I. Our results revealed no changes in podoplanin expression (Figure 7A). We therefore turned to another potential actor in lymphatic function regulation, namely the vascular growth factor receptor 3 (VEGFR-3). The detection of VEGFR-3 on HMVEC-dLyAd by western blots divulges that VEGFR-3 expression is increased when LECs are exposed to apoA-I (Figure 7B).

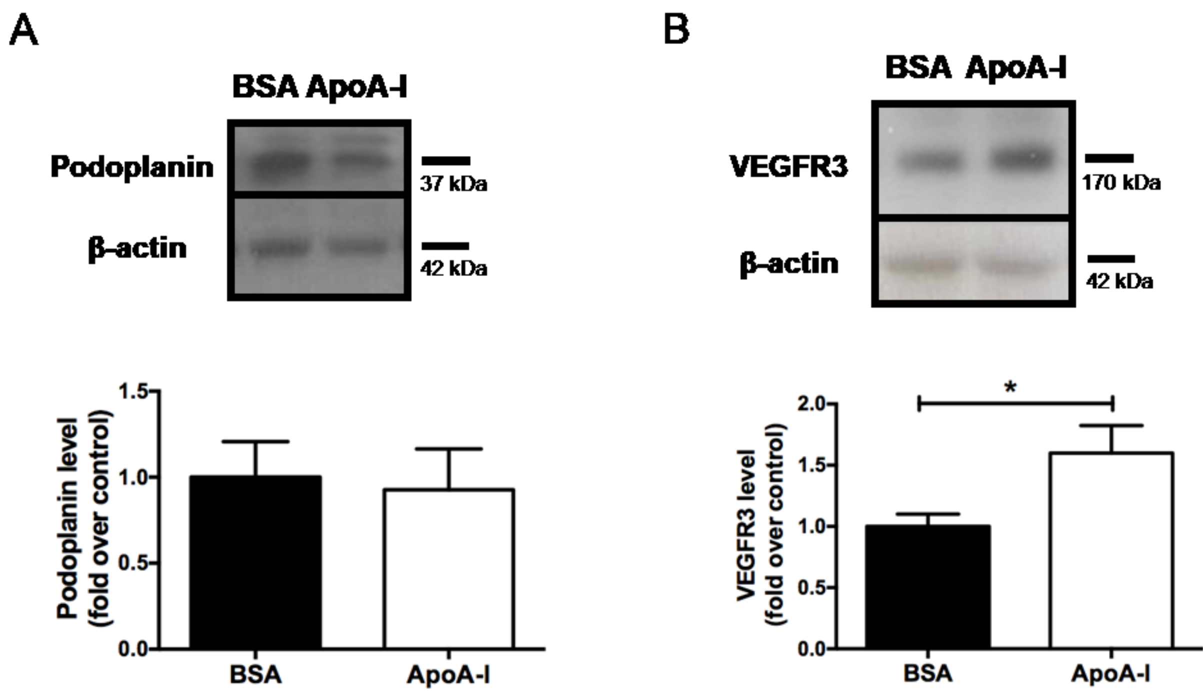


Figure 7. Effect of lipid-free apoA-I treatment on podoplanin and VEGFR3 expression. Primary HMVEC-dLyAd were seeded at maximum confluence and incubated for 20 hours with BSA or apoA-I. Cells were then analyzed by Western blotting with the use of A, podoplanin and B, VEGFR3. Podoplanin protein was observed at 37kDa and VEGFR3 was observed at 170kDa. Experiments were performed with 6 replicates per experimental group. *$p<0.05$, as determined by one-tailed t-test.

ApoA-I reduces *ex vivo* platelet aggregation

Activated platelets have long been known for their ability to promote thrombus formation and coagulation[48]. Increased platelet activation in blood is associated to the lethal outcomes of atherosclerosis[49]. Our results suggest that pretreating LEC with apoA-I prior to platelet infusion enhances platelet arrest at a physiological-like lymph flow on LECs *in vitro*, a phenomenon that would most likely be beneficial in preserving the lymphatic system integrity[5]. Therefore, we next evaluated whether apoA-I treatment in *Ldlr*$^{-/-}$ mice would solely enhance the platelet activity needed to maintain proper lymphatic function, while limiting the platelet activity that leads to blood thrombosis in circulation. We measured the effect of *in vivo* apoA-I treatment on platelet aggregation induced by platelet agonists *ex vivo*.

Close to two decades ago, Li et al. introduced the concept that apoA-I Milano inhibits arterial thrombus formation through inhibition of thrombin activity[50] and recent data have shown an inhibitory effect of isolated human apoA-I on human platelet activation and murine arterial/venous thrombosis[51]. Recently, it has been shown that, *in vivo*, apoA-I deficiency abrogates flow restriction-induced thrombosis in a mouse model of deep vein thrombosis (DVT), and intravenous human apoA-I infusion in WT mice decreases thrombi prevalence from 55% in vehicle-infused mice to 0%[52]. Figure 8A shows representative aggregation curves, obtained with a Chrono-log Optical aggregometer. Platelet aggregation was performed using either high concentrations of the classic agonist thrombin (0.5 to 1 U/ml, blue curves) or the CLEC-2 specific agonist podoplanin (5 µg/ml, black curves) on platelets isolated from BSA, diet switch or apoA-I treated *Ldlr*$^{-/-}$ mice. The histogram depicted in Figures 8B and 8C represents the mean data of percent platelet aggregation with thrombin and podoplanin, monitored under a constant shear (1000 rpm) at 37ºC. ApoA-I-treated *Ldlr*$^{-/-}$ mice displayed lower aggregation amplitude than that isolated from the BSA-treated or diet switch group once it reached a plateau after addition of thrombin (Figure 8B). At this arterial shear rate, there was a statistically insignificant trend of decreased aggregation of platelets isolated from apoA-I-treated mice with podoplanin (Figure 8C). At least in plasma, Figure 8C also revealed that podoplanin-induced platelet aggregation is decreased in apoA-I treated mice compared to BSA-control mice, as calculated by a two-tailed t-test.

To test whether apoA-I infusion could suppress flow restriction-induced aggregation due to enhanced baseline levels of CLEC-2 on platelets, we determined by immunoblotting the level

of CLEC-2 expression on resting isolated platelets. Neither the level of CLEC-2 (Figure 8D) or the phosphorylation of its downstream effector Akt (Figure 8E) were statistically affected by apoA-I or diet reversal. Platelet activating factor (PAF) is involved in platelet aggregation and is produced by a variety of cells, including platelets. Its production is controlled by the activity of PAF acetylhydrolases. Thus, as HDL and HDL- associated PAF acetylhydrolase (PAF-AH) has been shown to restore normal dendritic cell migration and priming[53], we sought to determine whether an increase in PAF-AH activity could have accounted for the beneficial effect on platelet activity, and consequently, on lymphatic vessel integrity. Figure S5 rather shows that PAF-AH activity, either in plasma or in lymph, is unchanged among the three different groups of *Ldlr*$^{-/-}$ mice, suggesting that apoA-I does not mediate its beneficial effect on lymphatic function via a PAF-AH-related mechanism.

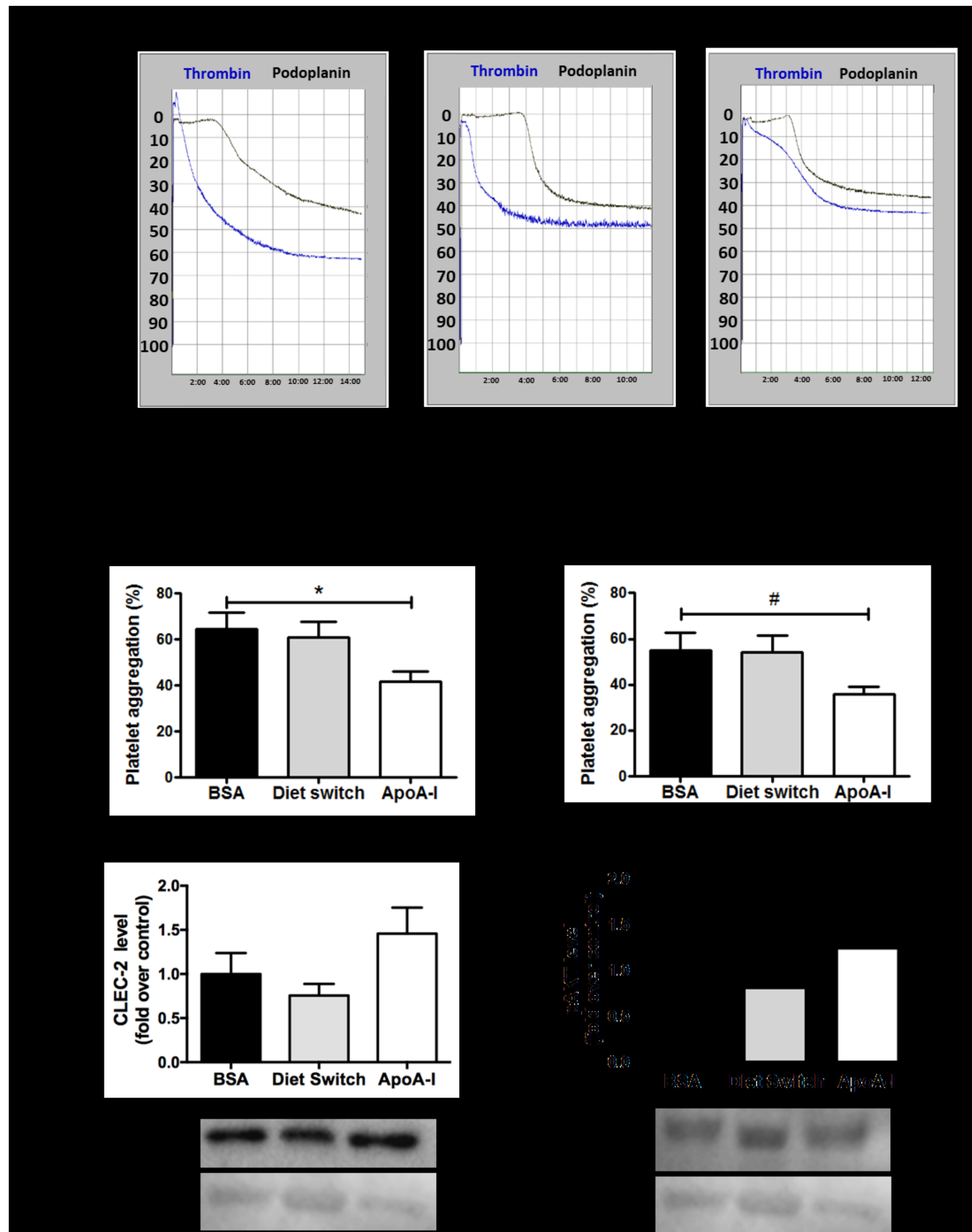


Figure 8. Effect of lipid-free apoA-I treatment on platelet activity. A, Representative aggregation curves, obtained with a Chrono-log Optical aggregometer. Platelet aggregation was performed using either thrombin (blue curve) or podoplanin (black curve) on platelets isolated from either BSA, diet switch and apoA-I treated *Ldlr*$^{-/-}$ mice. Histogram represents the mean data of percent platelet aggregation with B, thrombin (0.5 – 1 U/ml) and C, podoplanin (5µg/ml) monitored under a constant shear (1000 rpm) at 37ºC. *p<0.05 and $^{\#}$p<0.06, as determined by Kruskal-Wallis analysis with Dunn's multiple comparison post-hoc test. Extracts from resting platelets were analyzed by Western blotting with the use of D, CLEC-2 and E, Akt phosphorylation (pAkt). CLEC-2 protein was observed at 28 kDa and pAkt protein was observed at 60 kDa. Experiments were performed with 4 replicates per experimental group, each consisting of 2 mice per replicate.

DISCUSSION

Atherosclerosis is one of the principal causes of mortality worldwide, instigating most cardiovascular diseases (CAD). In recent years, a lot of emphasis has been put on increasing levels of blood HDL in clinical studies. However, from a global perspective, increasing HDL-C levels did not demonstrate any clinical benefits, nor improved macrophage reverse cholesterol transport (mRCT) or decreased CAD[54-56], leading scientists to redefine our understanding of the cholesterol excretion pathway. ApoA-I, the main protein constituent of plasma HDL, quickly became a target of interest. It has been shown to be a key player in mRCT regulation through its capacity to package large amounts of cholesterol following its interaction with ABCA1 on cell membranes[30, 57]. Interestingly, intradermally injected lipid-free apoA-I has been reported to reduce accumulation of lipid and immune cells within the aortic root of hypercholesterolemic mice without increasing HDL-cholesterol concentrations[34, 58]. Whereas apoA-I has been extensively studied in the past decade, the mechanisms by which it mediates its atheroprotective effect are still unclear. In the present study, we addressed whether and how preservation of collecting lymphatic function contributes to the protective effect of apoA-I.

Since several decades, the lymphatic system had been suspected as being a potential player in lipoprotein transport during atherosclerosis[21, 22, 59]. Few years ago, Martel et al. have quantitatively demonstrated, for the first time, that lymphatic vessels are important components involved in mRCT[26]. Therefore, the lymphatic system is now recognized as the missing link between the atherosclerotic plaque and the blood circulation. Improving lymphatic function to either prevent or abrogate atherosclerosis would be a potentially attractive therapeutic target. We therefore herein sought to connect apoA-I beneficial effects in atherosclerosis to lymphatic function, and we proposed that apoA-I might reduce the lymphatic dysfunction observed during atherosclerosis[26]. Our findings reveal that a continuous low-dose intradermal injection of diet-fed *Ldlr*$^{-/-}$ mice with lipid-free apoA-I reverses atherosclerosis-associated collecting lymphatic vessel dysfunction, without significantly affecting plasma nor lymph total cholesterol concentrations. The direct effect of apoA-I on LECs combined to its role on platelet activity highlight the versatility of this apolipoprotein in the modulation of lymphatic function.

Altogether, our work suggests that preservation of collecting lymphatic function contributes to the protective effect of apoA-I.

The method of injection chosen in our experimental design comes from the fact that lymphatic vessels are present and abundant in the skin dermis[60]. Therefore, to directly assess the effect of the drug on the lymphatic vasculature *per se*, we performed intra-dermal injections, rather than intraperitoneal injections as performed in previous work[38]. Following the injection, apoA-I is first retrieved in lymph before appearing in the blood circulation, thus acting upon the lymphatic vasculature as soon as it gets within the mouse. We observe longitudinal plaque regression in the thoracic aorta of *Ldlr*$^{-/-}$ mice following lipid-free apoA-I treatment, independently of cholesterol accumulation in lymph or plasma. As reported by Wilhelm et al., this lack of effect in plasma cholesterol could be explained by the potent effect of apoA-I on immune cell cholesterol balance instead of affecting whole body cholesterol balance[34]. ApoA-I have the ability to relieve excess cholesterol in lipid rafts/microdomains and thus to affect numerous types of signal transduction pathways that rely on these microdomains[31]. The same rationale could be applied to lymph: the positive effects of apoA-I on lymphatic and platelet function suggests that despite high plasma cholesterol, cells present in lymph have the ability to efflux their excess cholesterol via lipid-free apoA-I to the liver. Therefore, it is very likely that LEC and platelet signaling pathways depend on the amount of cholesterol in their microdomains, consequently modulating platelet aggregation and adhesion to the lymphatic endothelium. As we hypothesize that apoA-I plays a positive role in preserving lymphatic transport, the expected repercussion would be observable, primarily, at the level of the collecting lymphatic vessel, the entity that is first defective during atherosclerosis-associated lymphatic dysfunction[24]. By improving lymphatic vessel integrity, we view that apoA-I would improve lymph transport *per se*, thus promoting the movement of lymph content toward the blood circulation. Sequentially, cholesterol would be taken up by the initial lymphatics, and efficiently pumped out of the collecting lymphatic vessels to subsequently allow the exit of cholesterol from the lymphatic vessel, to eventually reach the liver to complete the RCT loop, rather than being stagnant in lymph.

Subcutaneous injections of apoA-I[34] had more potent effects than intradermal injections to reduce the inflammatory cell content in the aortic sinus. This is most likely due to a more direct access to the blood vasculature. However, we show that interstitial spaces near the skin

draining lymphatics had less macrophage accumulation in the apoA-I injected mice compared to the diet switch- or the BSA-injected control group. Following contact sensitization on the back skin dermis[41], we also show that dendritic cells are more potent to migrate to the corresponding draining lymph nodes when lipid-free apoA-I is injected, compared to the two control groups. Our results suggest that apoA-I, but not diet switch, can regulate immune cell content and improve lymphatic function prior to influencing obesity-related parameters such as subcutaneous fat accumulation.

Recent studies in animals provide evidence that affecting lymphatic drainage by promoting the growth of lymphatic vessels can modulate inflammation[61-64]. Lymphatic vessel hyperplasia has been associated to hypercholesterolemia[43], but we here show that apoA-I does not reduce vessel diameters. In addition, we report that the transport of larger molecules is not seemingly different between the groups, reflecting that the capacity of the peripheral ultrafiltrate to be uptaken is not modified, despite the increased number of lymphatic vessels observed solely in the ear dermis. Altogether, these observations point out that more numerous initial lymphatic vessels do not necessarily reflect better functioning lymphatic vessels in our atherosclerotic mouse model. We have recently reported that lymphatic dysfunction associated to the early stage of atherosclerosis appears to be first and foremost linked to a defect in the collecting lymphatic vessel *per se*[24]. Aging[65] and chronic high fat diet[66] have also both been associated to a defect in the collecting lymphatic vessels as well, stressing the importance of targeting this portion of the vessel in chronic inflammatory diseases. As the defect is first found in the collecting vessels, lymphangiogenesis by itself might not be sufficient to reduce plaque formation. Therefore, we next investigated collecting lymphatic vessel permeability in our models. We first showed that, following apoA-I treatment, *Ldlr*$^{-/-}$ mice have restored collecting lymphatic vessel integrity as shown by a decrease in EB leakage around the vessel, both with respect to the area and the perpendicular distance of leakage within the surrounding tissue. This is potentially reflected in previous studies where HDL was shown to increase endothelial barrier integrity, implicating sphingosine 1-phosphate (S1P) as a mediator[67]. In our case, we suspected apoA-I acting in a similar way on the lymphatic vessels. Surprisingly, although FOXC2 absence/reduction is well-known to be implicated in lymphatic valve failure[68], thereby potentially causing lymphedema and more importantly a dysfunction in the lymphatic collecting vessels, our results did not report a direct effect of apoA-I on FOXC2. The switch to

a chow diet from HFD however, significantly restored FOXC2 levels by immunofluorescence imaging, an avenue that deserves further attention. So far, variations in FOXC2 may have a minor role in body weight control and seem to be involved in the regulation of basal glucose turnover and plasma triglyceride levels, particularly in women[69]. For now, we believe that modulating FOXC2 might not be sufficient to rescue atherosclerosis-related lymphatic dysfunction and that apoA-I uses another mechanism to improve lymphatic vessel permeability.

Whereas an excess in platelet activation in blood circulation is often deleterious and associated to atherosclerosis clinical devastation outcomes, platelets are also known to support the semi-permeable function of the blood vessel endothelium[70]. In lymphatic physiology, they play a critical role in the development and maintenance of the lymphatic system. In addition to maintaining the integrity of high endothelial venules (HEVs) during lymphocyte trafficking to lymph nodes[71], it has recently been reported that platelet activation through its receptor CLEC-2 is essential for maintaining lymphatic vessel integrity[5]. In blood vessels, under homeostatic conditions, macrophages have been shown to interact with blood endothelial cells through their filopodia and exert a "bridge effect" between adjacent endothelial cells, thus strengthening the endothelial barrier in blood vessels [72]. Perivascular cells appear to be for blood endothelial cells what platelets would be for lymphatic endothelial cells, i.e. guardians of good vessel integrity. Here, we observe that platelets can indeed also carry a similar protective effect on lymphatic endothelial cells, and that apoA-I enhances the adherence of pseudopodia-shaped platelets. Platelets were even shown to cluster together in order to be able to reach and pull several LECs together, reminiscent of endothelium junctions formation. Despite the known literature concerning platelets as being preferred structural elements of blood and that they do not form a normal constituent of lymph from the thoracic duct[73], it does not eliminate the possibility that platelets might quickly adhere onto the collecting lymphatic vessel to exert their effects. The increased concentration of several released factors[70] and platelet-derived EVs in lymph that we observed in a previous publication[16] might in turn reflect platelet activation following their adhesion to the lymphatic endothelium. Recently, platelet rich plasma (PRP) with the platelet releasants was shown to affect lymphangiogenesis and limit lymphedema development, thereby showcasing a potential role in the regeneration of lymphatic vessels, as demonstrated by an increase in lymphatic cell proliferation. This leads us to suspect that not only do platelets exert an essential role in preventing blood from entering the lymphatic system

at the lymphovenous junction, but platelet fragments would similarly have a tendency to bind along the lymphatic vessels and ensure proper cell-to-cell junctions, thereby maintaining proper collecting lymphatic vessel integrity and potentially improve lymphatic vessel regeneration. Mechanisms of how platelets, or their counterparts (ie. platelet-derived EVs) may exert similar effects on collecting lymphatic vessels *per se*, remain to be elucidated.

Importantly, platelet assembly appeared to occur longitudinally on the endothelium itself, suggesting that lymph flow would not be impaired by excessive platelet aggregation. Navarro-Núñez et al. reported that LEC-induced aggregate formation is inhibited by αIIbβ3 inhibitors, but many individual platelets could still be seen attached to LECs[47]. They concluded that abrogation of platelet-platelet interactions by αIIbβ3 inhibitors increased overall surface coverage. In our model, we believe that the decrease in CLEC-2 pathway-independent *ex vivo* aggregation amplitude level observed in platelets isolated from apoA-I-treated mice could reflect the instigative mechanisms that would occur *in vivo*. ApoA-I would thus first limit the platelet aggregation frequently observed in atherosclerotic mice, and abrogate the formation of large aggregates that would, without treatment, shield the podoplanin surface downstream of flow. Afterward, platelets or platelet microparticles could adhere more efficiently to the lymphatic endothelium, thus promoting lymphatic endothelial cells integrity. The latter could be mediated directly by the physical properties of platelets and platelet microparticles binding to LECs, through the release of VEGF-C upon platelet activation[74], or via the transduction of intracellular signals mediated by noncoding microRNAs[75]. Among the signaling pathways that would be relevant figures VEGFR-3, one of the main receptors of VEGF-C[76].

The increase in VEGFR-3 levels that we observe in apoA-I treated cells reinforce the hypothesis that apoA-I might also act directly on LEC to disrupt lipid rafts and therefore modulate specific cell signaling pathways. As binding of VEGF-C to VEGFR-3 has been reported to alter the intrinsic and phasic pumping of collecting lymphatics in rat mesentery[77], our results reveal that apoA-I could promote lymphatic integrity through the upregulation of VEGFR-3 activity. Of interest, a recent manuscript reported that VEGFR-3 prevented excessive vascular permeability by limiting VEGFR-2 expression and VEGF/VEGFR-2 pathway activity in quiescent and angiogenic blood vascular endothelial cells[78]. Whether and how VEGFR-3 could modulate its own expression to subsequently regulate lymphatic contraction capacity remains to be tested.

CONCLUSION

The lymphatic system has emerged as a prerequisite for proper cholesterol excretion from plaque, and we conclude that low-dose lipid-free apoA-I treatment in atherosclerotic mice preserves and restores collecting lymphatic vessels function by direct and indirect mechanisms. This work suggests that lipid-free apoA-I mediates beneficial effects through the direct upregulation of the VEGFR-3 pathway. In addition, apoA-I might exert a protective effect on the lymphatic endothelium, as by limiting platelet aggregation, it would clear the way for platelet adhesion on LECs. Albeit the evidence of a morphological role for platelets in maintaining LECs integrity, this indirect mechanism could also subsequently involve the activation of signaling pathway to contribute to proper lymphatic function. Altogether, these studies bring forward a new pleiotropic role for apoA-I in lymphatic function and unveil new potential therapeutic targets for the prevention and treatment of atherosclerosis.

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DISCLOSURES

None.

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4.2.2 Supplementary data

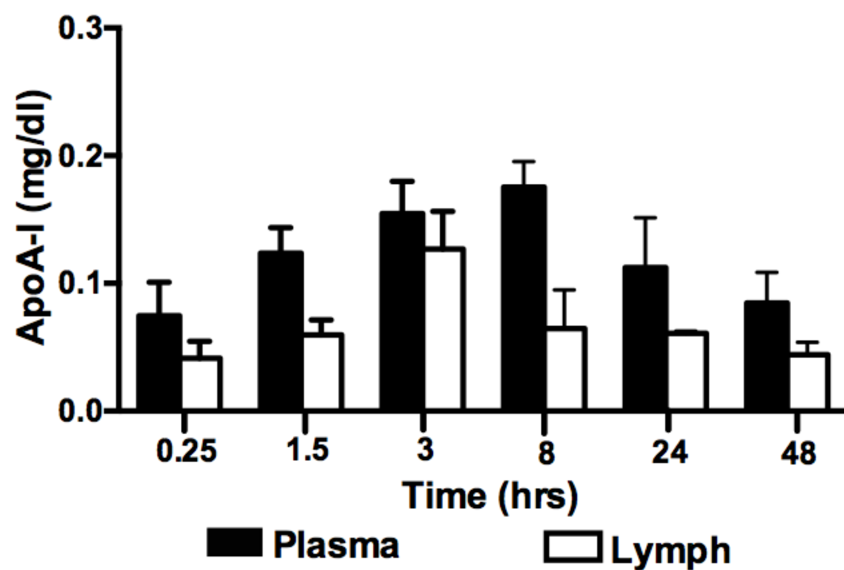


Figure S1. ApoA-I kinetics in wild type mice. Human lipid-free apoA-I was injected intradermally in the back skin of wild-type mice. Lymph and plasma were collected at different time points following injection. Human apoA-I levels were differentially detected in both lymph and plasma at each time point by ELISA and the background was subtracted. Experiments were performed with 4 mice per experimental group.

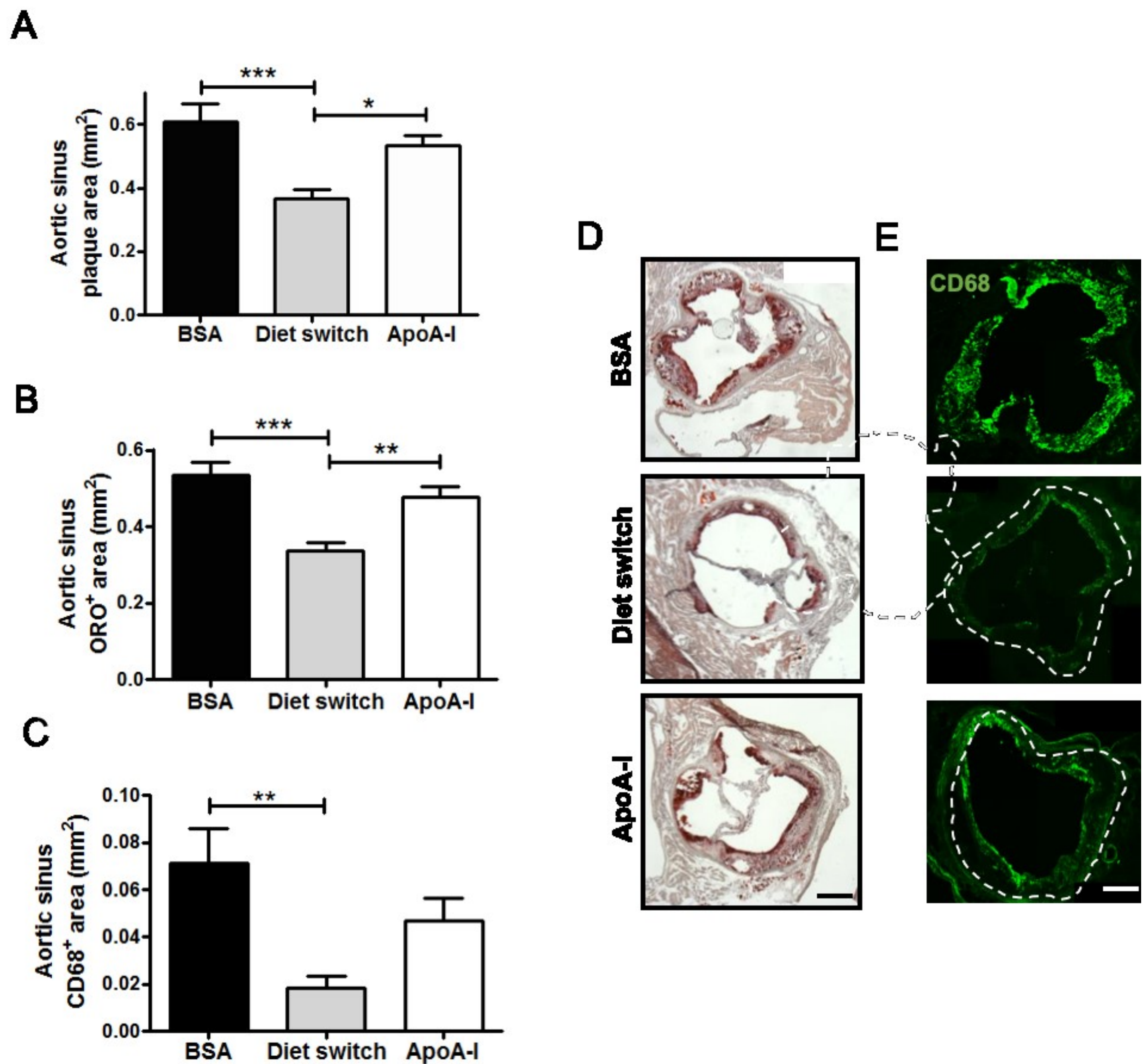


Figure S2. Assessment of atherosclerosis in *Ldlr*$^{-/-}$ mice. A, D, Total lesion, B, D, neutral lipid- (Oil Red O) and C, E, macrophage (CD68^{+})-positive areas were quantified in the three groups of mice in 8 µm-thick aortic sinus sections using ImageJ software. Experiments were performed with 7-11 mice per experimental group. *p≤0.05, **p≤0.01 and ***p≤0.001, as determined by one-way ANOVA with Tukey's post-hoc test. Scale bars = 500 µm.

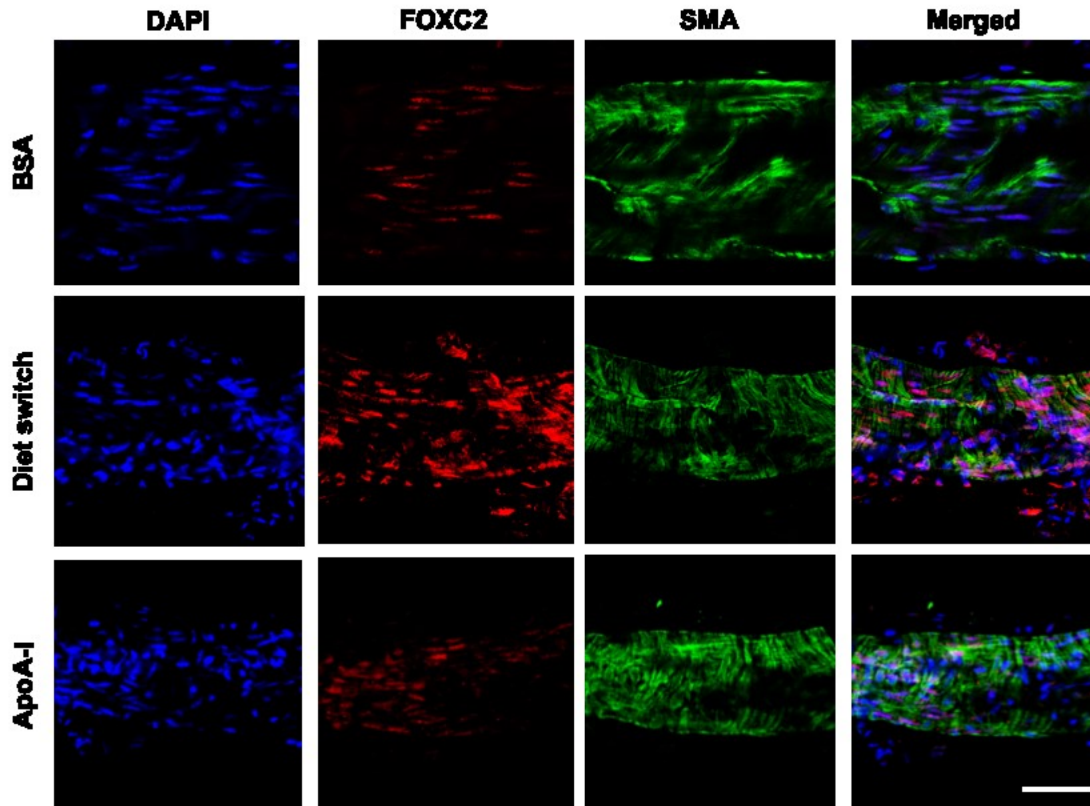


Figure S3. Effect of apoA-I treatment on FOXC2 expression on lymphatic endothelial cells. Representative images of FOXC2 and smooth muscle cells (smooth muscle actin, SMA) expression in collecting lymphatic vessels of BSA, diet switch and apoA-I treated *Ldlr*$^{-/-}$ mice. Popliteal collecting lymphatic vessels were harvested and processed for immunofluorescence detection. Longitudinally imaged single plan vessels were acquired with an LSM 710 Confocal Microscope (Zeiss) equipped with a 63X/1.4 oil dic objective. Experiments were performed with 4 replicates per experimental group. Scale bar = 50 µm.

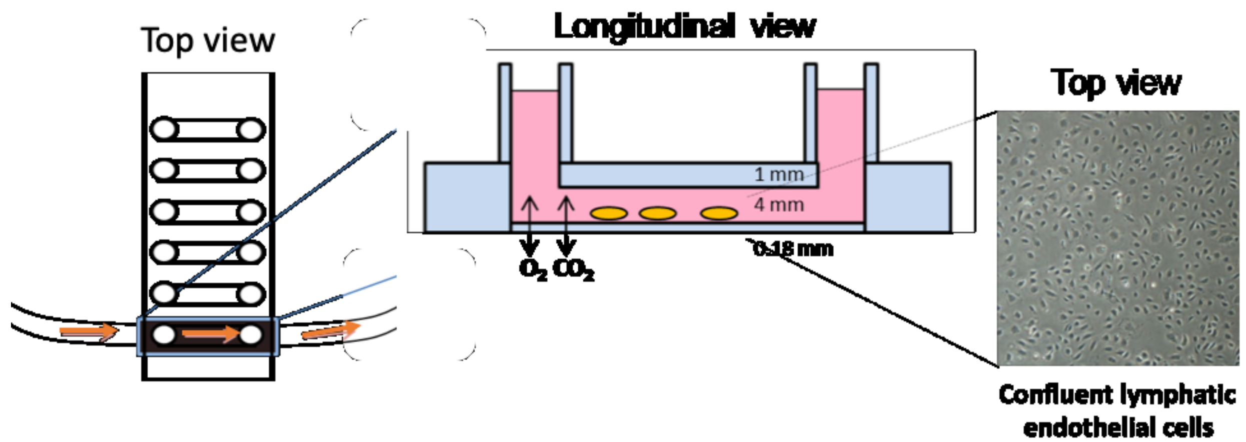
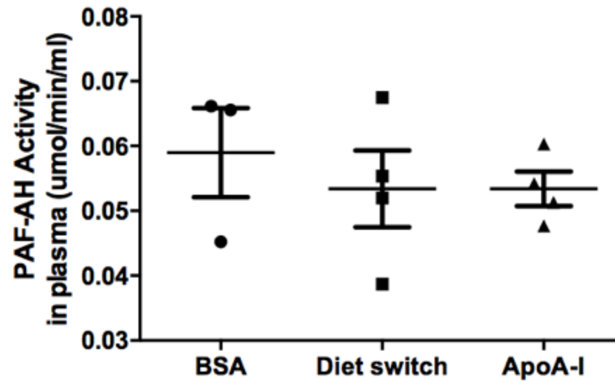


Figure S4. Analysis of platelet adherence on lymphatic endothelial cells under a physiological lymph flow. Human platelets were isolated and perfused over primary HMVEC-dLyAd seeded at 80% confluence in IbiTreated flow chambers at a wall shear rate of 50 s^{-1} at 37 °C for 8 minutes. Orange arrows indicate the direction of flow.



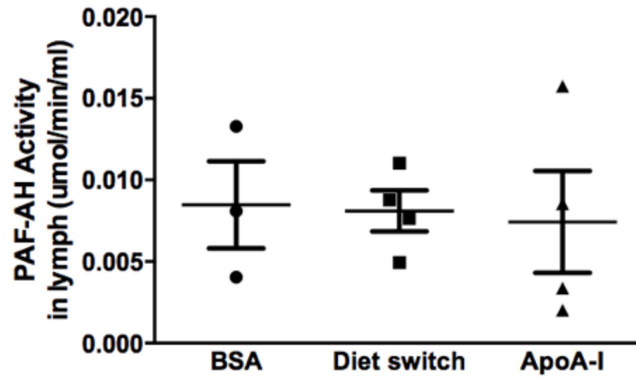


Figure S5. PAF-AH activity in plasma and lymph. Total PAF-AH activity was assessed in **A**, plasma and **B**, lymph of BSA, diet switch and apoA-I treated *Ldlr*$^{-/-}$ mice. Experiments were performed with 3-4 mice per experimental group.

4.3 Presentation of the third article

New probable players in atherosclerosis that came to our attention are proinflammatory cell fragments called extracellular vesicles that are found in atherosclerotic plaques and blood circulation. Although for a long time they were considered simple cellular debris, they are now suspected to be involved in many physiopathological processes such as thrombosis, autoimmune diseases and inflammation[307].

The composition of lymph is analogous to that of blood plasma as it also contains white blood cells and lipoproteins. Of interest, it is normally free of red blood cells, and poorer in nutrients. Furthermore, a broad array of cytokines, proteins and growth factors are contained within lymphatic fluid, which plays an important role in metabolism and cell proliferation[604]. Circulating blood contains extracellular vesicles that are derived from cells upon activation and/or apoptosis[332], but their presence had not been investigated in lymph. We suspect diverse EVs subsets to contribute to the maintenance or rather, the deterioration of the integrity of LECs and their function, especially in collecting LVs. Moreover, characterization of circulating EVs represents a valuable tool for diagnosing and monitoring of CVD, as well as lymphatic function, since circulating EVs offer noninvasive and ongoing access to circulating information.

<u>Our results demonstrate for the first time that:</u>

- Extracellular vesicles circulate in mouse lymph;
- The number of distinct subtypes of EVs differs in atherosclerosis.

Participation of each author of the article:

AM: Project conceptualisation, troubleshooting, methods validation, experiments, results analysis, writing and reading of the manuscript.

NT: Troubleshooting, methods validation, experiments, results analysis and reading of the manuscript.

ST: Electron microscopy experiment.

AB: Electron microscopy experiment and critical reading of the manuscript.

EB: Troubleshooting, methods validation, experiments, results analysis, writing and reading of the manuscript.

CM: Project conceptualisation, troubleshooting, methods validation, experiments, results analysis, writing and reading of the manuscript.

4.3.1 Third article

Journal of Extracellular Vesicles

co-action PUBLISHING

ORIGINAL RESEARCH ARTICLE

Extracellular vesicles are present in mouse lymph and their level differs in atherosclerosis

Andreea Milasan[1,2#], Nicolas Tessandier[3#], Sisareuth Tan[4], Alain Brisson[4], Eric Boilard[3*] and Catherine Martel[1,2*]

[1]Department of Medicine, Faculty of Medicine, Université de Montréal, Montreal, QC, Canada; [2]Montreal Heart Institute, Montreal, QC, Canada; [3]Centre de Recherche du Centre Hospitalier Universitaire de Québec, Department of Infectious Diseases and Immunity, Faculty of Medicine, Université Laval, Quebec City, QC, Canada; [4]UMR-CBMN, CNRS, University of Bordeaux, IPB, Pessac, France

Title: Extracellular vesicles are present in mouse lymph and their level differs in atherosclerosis

Running title: Extracellular vesicles in lymph

Andreea Milasan[1,2#], Nicolas Tessandier[3#], Sisareuth Tan[4], Alain Brisson[4], Eric Boilard[3*] and Catherine Martel[1,2*]

[1]Department of Medicine, Faculty of Medicine, Université de Montréal, Montreal, Quebec, Canada
[2]Montreal Heart Institute, Montreal, Quebec, Canada
[3]Centre de Recherche du Centre Hospitalier Universitaire de Québec, Faculty of Medicine, Department of Infectious Diseases and Immunity, Université Laval, Quebec City, Quebec, Canada
[4] UMR-CBMN, CNRS, University of Bordeaux, IPB, Pessac, F-33600, France

[#]These authors contributed equally to this work.

*Correspondence:
Catherine Martel, PhD
Montreal Heart Institute Research Center
Department of Medicine, Faculty of Medicine
Université de Montréal
5000, Belanger street, Room S3560
Montreal, Quebec (Canada)
H1T 1C8
Telephone: (514) 376-3330 #2977
E-mail: catherine.martel@icm-mhi.org

Éric Boilard, PhD
Centre de Recherche du Centre Hospitalier Universitaire de Québec
Department of Infectious Diseases and Immunity
Faculté de Médecine de l'Université Laval
2705, boulevard Laurier
Québec (Canada)
G1V 4G2
Telephone: (418) 525-4444 #46175
E-mail: Eric.Boilard@crchudequebec.ulaval.ca

Abstract

The lymphatic system works in close collaboration with the cardiovascular system to preserve fluid balance throughout the body and is essential for the trafficking of antigen-presenting cells and lymphocytes to lymphoid organs. Recent findings have associated lymphatic dysfunction to the pathogenesis of cardiovascular-related diseases such as atherosclerosis, inflammation and obesity. Whether and how lymphatic dysfunction is a cause or a consequence of these diseases is under intensive investigation. Extracellular vesicles (EV) are submicron vesicles released by diverse cell types upon activation or apoptosis and are considered important biomarkers for several inflammatory diseases. Thus, it is critical to characterize the presence of EVs in various biological tissues and fluids to thus delineate their origins and, subsequently, their functions. In the past few years, new techniques allowing the quantitative and qualitative analysis of EVs have emerged, thus facilitating the onset of studies bridging these vesicles to the lymphatic system. Using several state-of-the-art approaches, this article reports the presence of diverse EVs inclusively derived from red blood cells and platelets in lymph of healthy animals. Our results suggest that lymph from atherosclerotic mice display a higher concentration of EVs, bringing forward the concept that EVs contained in lymph could either be a biomarker for lymphatic dysfunction or conversely, for inflammatory disease progression.

Keywords: inflammation, biomarkers, lymphatic vessels, atherosclerosis, platelets

Introduction

The lymphatic system is the principal route of transport from tissues for antigen and immune cells [1]. It is necessary to maintain fluid balance, fight infection, and absorb dietary fat from the intestine to then transport it to the liver. Recently, dysfunction of this ubiquitous network has been linked to the pathogenesis of cardiovascular diseases, like aging [2], hypercholesterolemia [3-6] and atherosclerosis [7]. Our own work [7] and those of others [8, 9] sustain that absence or obstruction of lymphatic vessels may accelerate inflammation occurring mostly in the intimal layer of the artery. Whereas inflammatory cell accumulation in peripheral tissues has been suggested to be responsible for the subsequent lymphatic transport impairment in obesity [10], it is still not clear whether and how lymphatic dysfunction is a cause or a consequence of other pathologies such as atherosclerosis.

The lymphatic network consists of an open, unidirectional and low-pressure vascular system. Plasma ultrafiltrate in peripheral tissues is first absorbed through absorptive blind-ended initial lymphatic vessels. Lymph subsequently moves into lymphatic collecting vessels, the entities responsible of maintaining lymph flow through contraction of units called lymphangions. Bi-leaflet valves are present between the contractile units to prevent back flow [11] and the lymphatic endothelial cells (LEC) feature continuous "zipper-like" cell-cell junctions and a discontinuous SMC layer. Once they reach the lymph nodes (LNs), the afferent collecting vessels become the efferent collecting vessels, and are ultimately converging into the thoracic duct where lymph collected from all organs drains into the blood circulation at the subclavian veins through the lymphovenous (LV) junction [12]. Platelets have a unique role in lymphatic vessel development and maintenance, and protect both the LV junction and the thoracic duct from backward flow. Furthermore, this platelet-dependent process is required throughout life to prevent retrograde blood flow and to maintain lymphatic function [13].

The composition of lymph is analogous to that of plasma, as it contains white blood cells, notably lymphocytes, but is essentially absent of red blood cells (RBCs), poorer in nutrients, and richer in waste. A broad array of cytokines, proteins and growth factors are contained within lymphatic fluid, which play an important role in metabolism and cell proliferation [14]. Whereas studies have confirmed the presence of extracellular vesicles (EVs) of diverse origins in circulating blood [15], it is not clear yet whether these EVs can also be found in lymph *per se*.

EVs are derived from cells upon activation and/or apoptosis [15, 16], and are considered important biomarkers and key players in several inflammatory settings including atherosclerosis and rheumatic diseases [15, 17-21]. Physiologically, EVs also constitute a heterogeneous population, differing in cellular origin, number, size, antigenic composition and functional properties [22]. They comprise exosomes, vesicles stored in multivesicular bodies (50-150 nm), and microvesicles (100-1000 nm) produced by cytoplasmic-membrane budding and shedding. EVs generated from apoptotic cells are called apoptotic bodies (>1 μm). EVs are suggested to play key roles in intercellular communication by transporting messenger RNA, microRNA (miRNA) and proteins [23], and can interact with neighboring cells, causing structural and functional changes in tissues like the vascular wall, thereby affecting the endothelium [24] and myeloid cells such as macrophages [25] and neutrophils [26]. As one of the major roles of the lymphatic network is to orchestrate the clearance of cells and molecules from peripheral tissues, it is immunologically essential to maintain proper lymphatic vessel integrity throughout life. Recently, through *in vivo* near-infrared (NIR) imaging, exosomes have been shown to be rapidly transported within minutes from the periphery to the lymph node by lymphatics [27]. Using the most advanced complementary methods to date, we herein sought to qualitatively and quantitatively report the presence of EVs in healthy and atherosclerotic mouse lymph collected *in vivo*. Our results suggest that EVs derived inclusively, but not exclusively, from red blood cells and platelets are circulating in the lymph of healthy animals, and prevail in atherosclerosis.

Methods

Animals. Adult healthy eight-week old female wild-type (WT) C57BL/6 mice fed on regular chow diet and age- and sex-matched $Ldlr^{-/-}$ mice also on a C57BL/6 background were purchased from Jackson Laboratory. $Ldlr^{-/-}$ mice were fed with a high fat diet (HFD, Harlan Teklad 88137) for eight weeks to ensure the atherosclerotic phenotype. Animals were housed in a pathogen-free environment under a 12 hours light-dark cycles with free access to water and food. All experiments were performed in accordance with the Canadian Council on Animal Care guidelines and approved by the Montreal Heart Institute Animal Care Committee.

Thoracic Duct Cannulation. Mice were anesthetized with isoflurane (4% for induction, 2–3% for maintenance). The anesthetized animal was positioned on its right side and a cannula was

inserted into the thoracic lymph duct above the cisterna chyli between the transverse lumbar artery and the diaphragm. Lymph was collected continuously, on average for 45 minutes to one hour, with a tube attached to a syringe coated with EDTA 0.1M. Collected lymph was centrifuged at 1200g for 10 minutes at 4°C to remove most traces of whole cells, while being cautious not to create EVs due to high-speed centrifugation. Following, 5% sucrose was added to each sample to warrant a better preservation of the sample and then placed in a freezer at -80°C for further batch analysis. Albeit our technique ensures extreme care during collection, the absence of contaminating cells in lymph isolated from the thoracic duct was verified using a PC-based automated cell counter (Cellometer Auto X4, Nexcelom, equipped with a 470/535nm optic module) that allowed bright field and fluorescent cell counting using acridine orange (AO), a nuclear staining dye permeable that stains all nucleated cells to generate green fluorescence. Red blood cells were considered as AO^- cells larger than 5 µm.

Cryo-Transmission Electron Microscopy (EM) of lymph samples. Prior to EM analysis, lymph samples were labeled with 10 nm gold particles conjugated with Annexin-5 as follows. An 8 µL lymph aliquot was mixed with 1 µL annexin-5-conjugated gold particles at 10^{17} particles/L and 1 µL 20 mM Ca^{2+}. After 30 min incubation at ambient temperature, 4 µL aliquots of lymph samples were deposited on electron microscopy (EM) grids coated with a perforated carbon film. After draining the excess liquid with a filter paper, grids were quickly plunged into liquid ethane and transferred to cryo-boxes and stored under liquid nitrogen. For cryo-EM observation, grids were mounted onto a Gatan 626 cryoholder and transferred in a Tecnai F20 (FEI) microscope operated at 200 kV. Images were recorded with an USC1000-SSCCD camera.

Extracellular vesicle labeling. Lymph samples were rapidly thawed, which is considered optimal to maintain the EV integrity. Lymph (5 µl) containing D-Phenylalanyl-prolyl-arginyl Chloromethyl Ketone (PPACK, at 10 µM), an anticoagulant with no effect on Ca^{+2} dynamics, was diluted in a total volume of 100 µl filtered (0.2 µm) annexin V buffer 1X (BD Biosciences) containing V450 conjugated annexin V (BD Biosciences, 1.5 µL) following the manufacturer's instructions. PPACK was included as calcium addition was found to trigger formation of micro-aggregates in some biological fluids, which are confounders in flow cytometry analyses (EB, NT and AB, data not shown). Anti-CD41 antibodies conjugated with APC (Biolegend, 5 µg/ml)

and APC conjugated anti-Ter-119 (Biolegend, 2.5 μg/ml) were added, in two different labeling cocktails, to identify EVs from platelets and red blood cells, respectively. EVs were incubated in labeling cocktails for 30min at room temperature (RT), and the presence of esterase in EV was established by the addition of carboxyfluorescein succinimidyl ester (CFSE, eBioscience) at 5 μM for the last 15 min of incubation. Cocktails were diluted by the addition of 400 μl filtered (0.2μm) annexin V buffer 1X prior analyses by flow cytometry. As controls, antibodies conjugated with the same fluorochromes but directed against human antigens were used to position the negative flow cytometry gates, whereas 50 μM EDTA or detergent Triton-X100 (0.05%) were respectively added to cocktails in order to verify the specificity of annexin V staining and the membrane moiety of EVs. Only for the EDTA control, the whole labeling process has been done in PBS 1X instead of annexin V buffer 1X.

Flow cytometer sample analysis. To analyze EVs using flow cytometry, we used an improved high sensitivity flow cytometer (hs-FCM) Canto II special order product (SORP) equipped with a small particle option (BD Biosciences) [28]. The forward scatter (FSC) on this dedicated equipment is coupled to a photomultiplier tube (PMT) with a 488nm solid state, 100mW output blue laser (rather than the conventional 20 mW), and includes a 633 nm HeNe, 20 mW output red laser and a 405 nm solid state diode, 50 mW output violet laser. The hs-FCM is equipped with FSC-PMT and a Fourier optical transformation unit, reducing the background/noise and increasing the angle of diffusion, thus enhancing the detection of small diameter particles. The hs-FCM performance tracking was performed daily before all analyzes using the BD cytometer setup and tracking beads (BD Biosciences, San Jose, CA, USA). Size estimation was determined using silica beads of 100, 500 and 1000 nm (Kisker Biotech GmbH & Co. Steinfurt, Germany), whereas volume quantification was performed by adding a known number of 3 μm dim polystyrene microspheres to each sample (BD Biosciences).

Statistics. Data are expressed as the mean ± SEM. Statistical differences were assessed using a two-tailed non-parametric Student's *t* test, with $p < 0.05$ reported as statistically significant, using Prism software, version 6.0c (GraphPad). All experiments contained three or more replicate mice per experimental parameter.

Results and Discussion

Heterogeneous populations of EVs are present in lymph.

Recently, emerging small-particle option analysing systems have been optimized to fully characterize EVs, and access to these tools arouses the urge for a better understanding of the evident role of lymph as a route for EVs. Herein, we sought to assess their presence in circulating lymph using three different and complementary functional assays. First, to assess the purity of the lymph samples and the absence of possible contamination of blood that might occur during lymph collection (**Figure 1A**), the number of AO^- cells larger than 5 µm (*e.g.* red blood cells, leukocytes) was determined in lymph and compared to that of whole blood. We verified that, contrarily to blood, lymph only contained negligible trace of red blood cells (0.11% compared to whole blood), thus confirming the high purity of the lymph preparations used for this study (**Figure 1B**). In addition, by counting the number of nucleated (AO^+) cells in lymph before and after centrifugation, we demonstrate that centrifugation eliminated the vast majority of the cells in lymph samples (**Figure 1C**). These observations validate that our pre-analytical conditions were optimal for the assessment of EVs in lymph.

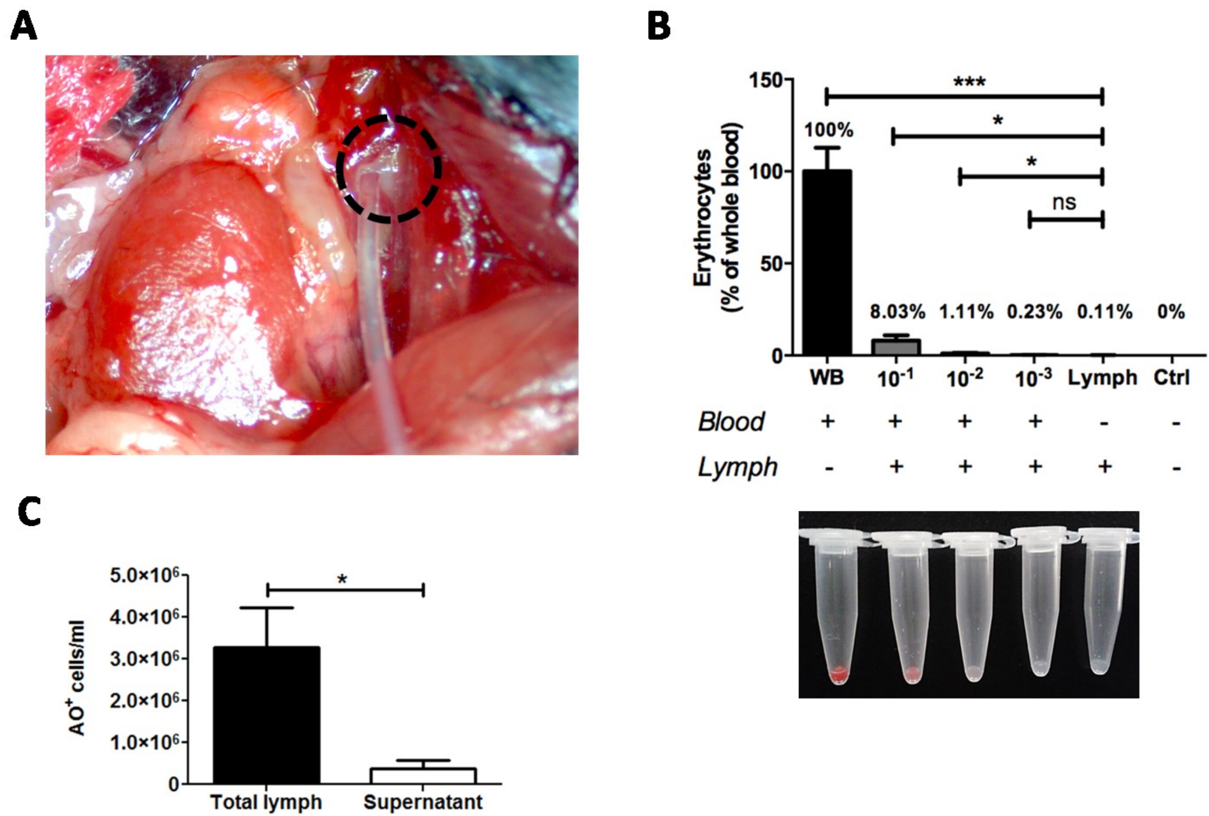


Figure 1. Assessment of lymph purity following collection and processing. (A) Lymph was collected from the thoracic duct (dotted circle) on EDTA (0.1M), and following sample collection, cell counting was performed with a Nexcelom AutoX4 cell counter before (total lymph) and after (supernatant) centrifugation. Cells were stained with Acridine Orange (AO), a nuclear staining (nucleic acid binding) fluorescent dye that stains all nucleated cells, in whole blood and total lymph. The number of AO^- cells larger than 5 μm in diameter (i.e. red blood cells) was assessed using brightfield and fluorescence imaging for AO. (B) Total lymph was purposely contaminated at different concentration (10^{-1}, 10^{-2}, 10^{-3}) with whole-blood (WB) and our sample of interest (Lymph) was then standardized to WB and compared to all concentrations and lymph supernatant (Control). (C) Total number of AO^+ cells was detected in total lymph and lymph supernatant using fluorescence imaging for AO. (n=4 per group; *$p<0.05$, ***$p<0.001$).

In a first technique using cryo-EM, we have detected spherical EVs with a diameter ranging from 50 nm to 800 nm (**Figure 2**). As compared to plasma samples [29], lymph samples seemed to contain a significantly higher amount of EVs. Hence, although cryo-EM is not a quantitative method, the fact that two or more EVs were frequently observed at close distance (**Figure 2D**) is in marked contrast with what is observed with pure plasma, which further excludes the impact of potential contamination of blood in lymph analyses. Of interest, most EVs were not labeled by annexin V conjugated with gold nanoparticles, indicating that most do not expose phosphatidylserine (PS) on their outer surface (**Figure 2A-C**). In addition to spherical EVs, some exhibiting multilayered structures (**Figure 2C**) were also observed. Furthermore, another class of objects was abundant in lymph samples. These objects present mostly a circular shape and a uniform greyness by cryo-EM. The absence of a lipid bilayer at their periphery enabled to distinguish them easily from EVs (**Figure 2B, F**), and will be referred to hereafter as lipoproteins. Surprisingly, lipoproteins were observed not only isolated in lymph medium (**Figure 2F**), but also encapsulated within EVs (**Figure 2C**). The size of lipoproteins varied from 100 nm to several hundred nm, yet smaller lipoproteins may well be present in lymph but are not resolved here. Thus, EVs of various dimensions are abundant in lymph, and a fraction of them expose PS.

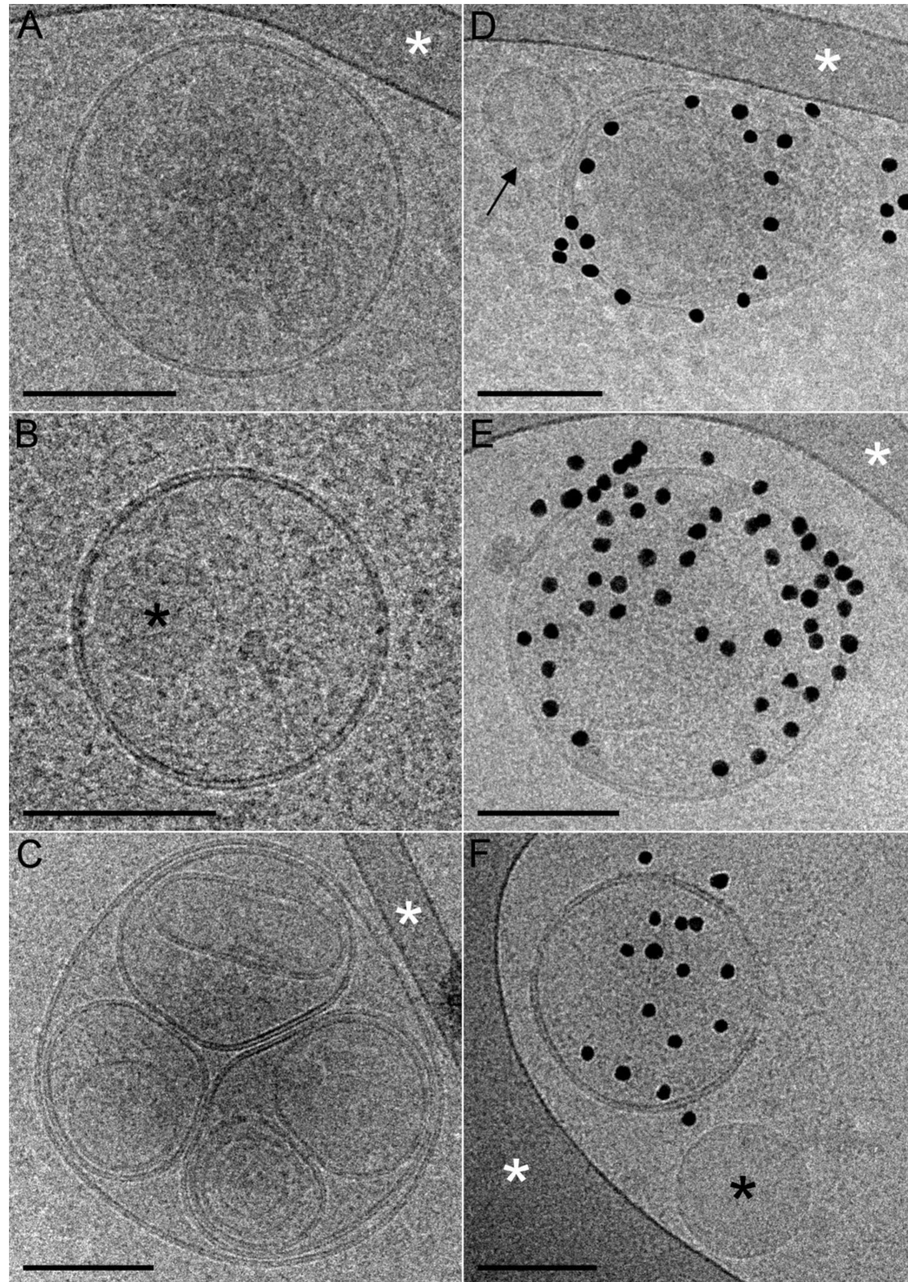


Figure 2. Representative cryo-EM images of lymph EVs. (A-C) EVs devoid of annexin V gold nanoparticles indicate the absence of phosphatidylserine (PS) on their outer membrane surface. (D-F) EVs are labeled by annexin V gold nanoparticles, with the exception of the small unlabelled EVs marked with a black arrow. (B, D) The black asterisks point to circular shapes devoid of a lipid bilayer, that could most likely be lipoproteins. (A, C-E) The white asterisks point to areas of the supporting perforated carbon net. EVs devoid of annexin V gold nanoparticles (G) and EVs labeled by annexin V gold nanoparticles in lymph of atherosclerotic LDLR$^{-/-}$ mice. Scale bars: 100 nm.

A second approach we used is high-sensitivity flow cytometry (hs-FCM) adapted with a small particle-option. Silica beads are more appropriate than polystyrene beads to obtain EV size estimation in flow cytometry because their refraction index is closer to the one of EVs [30]. We thus used silica beads of known dimensions and intact platelets to respectively determine the lower and upper limits of our EV gate (**Figure 3A-C**). To prudently assess EVs in complex fluids, we defined EVs as membrane cargo, containing active esterases ($CFSE^+$), and positive for at least one surface marker such as PS or protein receptor (*e.g*. CD41). Although we recognize that extracellular esterases, complexed with lipoproteins or lipoprotein-like particles, might also be labeled with CFSE, such combination of complementary markers greatly enhances the specificity of the approach. Having confirmed the specificity of our hs-FCM measurements using calcium chelation to prevent annexin V recognition of PS and of detergent to dissolve EVs (**Figure 3D-G**), we find profuse amounts of $CFSE^+$ particles in lymph, half of them exposing PS, suggesting that they were indeed EVs. While these observations contrast with those made using cryo-EM, which revealed the preponderance of EVs lacking surface PS, they also suggest that a proportion of PS^- EVs remains undetected by hs-FCM, consistent with the lower limit of the EV gate which we had intentionally set at approximately 100 nm accordingly to silica beads.

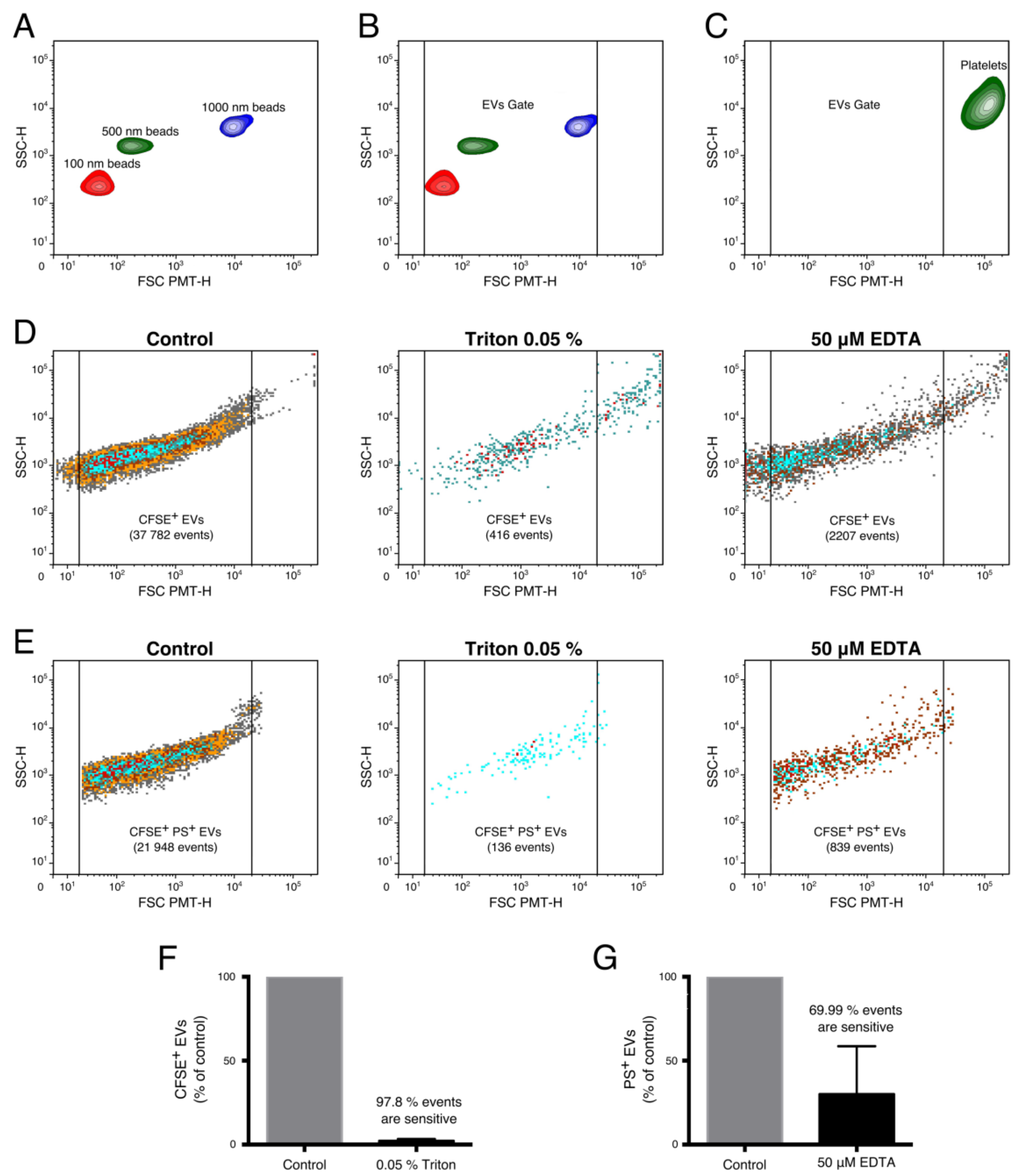


Figure 3. Detection of EVs in lymph using flow cytometry. (A-E) A Canto II flow cytometer modified with a "small particle" FSC PMT-H option was used to detect and quantify silica microspheres of 100 nm (red), 500 nm (green) and 1000 nm (violet) mean diameter and EVs. (A, B) An EV gate was designed for the detection of small particles from 100 nm to 1000 nm in diameter, based on the silica microspheres sizes (FSC PMT-H). (C) $CD41^+$ mouse platelets are excluded from the EV gate based on their size (FSC PMT-H). Representative flow cytometry dot plot of lymph (D) total $CFSE^+$ and (E) $CFSE^+PS^+$ EVs in control conditions, or treated with 0.05% Triton or 50 µM EDTA. Sensitivity to (F) Triton or (G) EDTA of EVs.

EV levels are modulated in lymph during atherosclerosis.

One of the main roles of the lymphatic system is to transport immune cells from peripheral tissues to lymphoid organs [1]. Inflammatory cell accumulation in peripheral tissues drives several chronic inflammatory diseases such as atherosclerosis [31] and obesity [32], in which lymphatic transport has been reported as being defective [7, 10]. A growing number of studies are reporting that EVs are active pro-inflammatory players also present in inflammatory sites, becoming important biomarkers in several pathological settings. Altogether, these recent studies converge toward a key role of the lymphatic network in the clearance and production of EVs, thus explaining why EVs are more numerous in chronic inflammatory diseases. However, to our knowledge, this hypothesis has never been directly addressed.

Lymph composition is different than that of plasma or serum, and its content also varies according to the pathological setting. Studies revealed that cholesterol-fed animals had higher phospholipid content, cholesterol to protein ratio and free cholesterol levels in their circulating lymph [33, 34]. In a first set of experiments, we sought to investigate whether the morphology and origin of EVs vary in WT versus atherosclerotic Ldlr$^{-/-}$ mice. Size estimation of nanoparticles was determined using hydrodynamics, which does not however distinguish EVs from aggregated proteins and lipoproteins. Although this approach might not be optimal for the examination of polydisperse EV populations, it successfully revealed that, compared to WT animals, the main population of nanoparticles in lymph in atherosclerotic mice displayed a modest increase in diameter (175±55 vs 133±41 nm). In addition, a second population of smaller nanoparticles (56±10 nm) could be observed in these animals (**Figure 4**). Whereas lymph composition appears altered in our atherosclerotic mouse model, further investigations are needed to determine whether this effect is due to hypercholesterolemia *per se*.

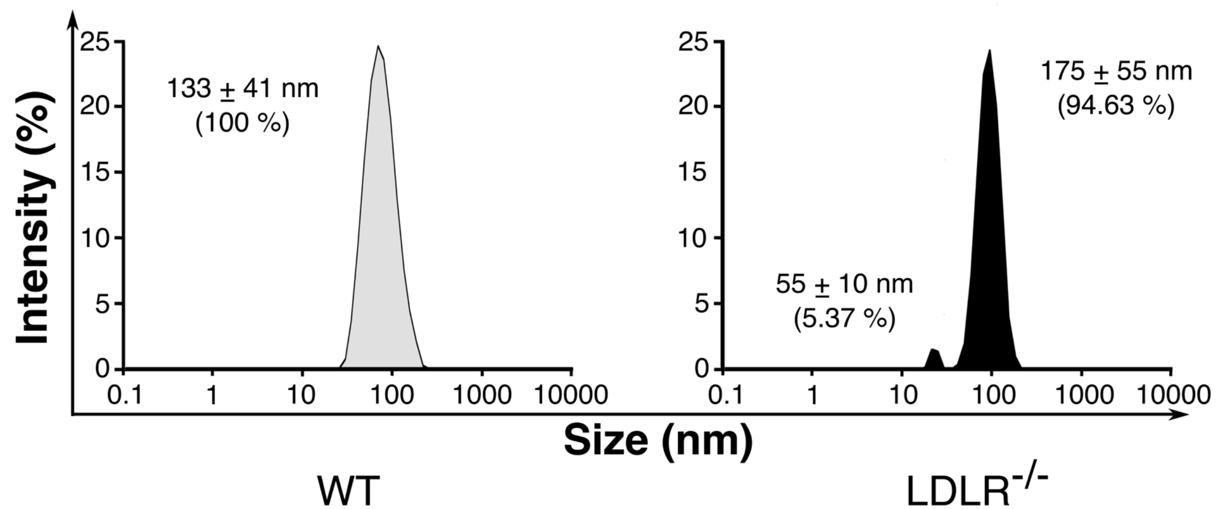


Figure 4. Dynamic light scattering size characterization of particles present in lymph of WT and LDLR$^{-/-}$ mice. Dimensions of the particles present in lymph from WT and LDLR$^{-/-}$ mice were determined with a Zetasizer nano S. Experiments were performed with 3 mice per experimental group.

The most studied and abundant EV populations recognized in blood are platelets and RBCs [29, 35]. In a normal physiological state, lymph is relatively free of RBCs. However, lymphatic transport impairment has been associated with improper lymphatic integrity, and linked to the presence of red blood cells in lymph due to a defective LV junction [13]. Platelets are key players in that process, mainly through their binding of the constitutively expressed on the surface of blood platelets C-type lectin-like receptor 2 (CLEC2) to LEC podoplanin, thus initiating a complex formed by fibrin-containing platelet thrombi that protects from backward flow. Therefore, we hypothesize that EVs derived from both platelets and RBCs could accumulate in lymph of mice displaying a defective lymphatic transport, as their lymphatic vessel integrity could be impaired.

To test our hypothesis, we used our adapted hs-FCM technique to measure platelet- and RBC-derived EVs. Overall, EV concentrations are increased in atherosclerosis (**Figure 5A**). Indistinguishably of the PS exposure, EVs from both platelet (CD41^{+}) and RBC (Ter119^{+}) origins are more abundant in lymph of atherosclerotic animals (**Figure 5C, E**), thus confirming our hypothesis. We suggest that the presence of RBC-derived EVs would most likely be due to

an inappropriate backward flow from the LV or to defective lymphatic endothelial cell-to-cell junction, thus reflecting an increased vascular permeability. Whether the large accumulation of platelet-derived EVs in atherosclerotic lymph is a cause or a consequence of lymphatic dysfunction remains to be tested. We bring forward new concepts that could explain the presence of platelet-derived EVs in lymph. Therefore, we hypothesize that the stagnation of platelet EVs in lymph could either reflect 1) the incapacity of the lymphatic vessels to propel lymph down the road, and thus to efficiently clear the interstitial space and 2) the incapacity of platelets to bind to LEC at the LV, which could lead to their shedding and backward flow in lymph. These hypotheses are currently under intensive investigation. Whereas PS^+ EVs from various origins were clearly increased, PS^- EVs were not significantly higher in sick animals (**Figure 5B, D, F**), reflecting that the impaired clearance of apoptotic cells in chronic inflammation and atherosclerosis is also observed in lymph.

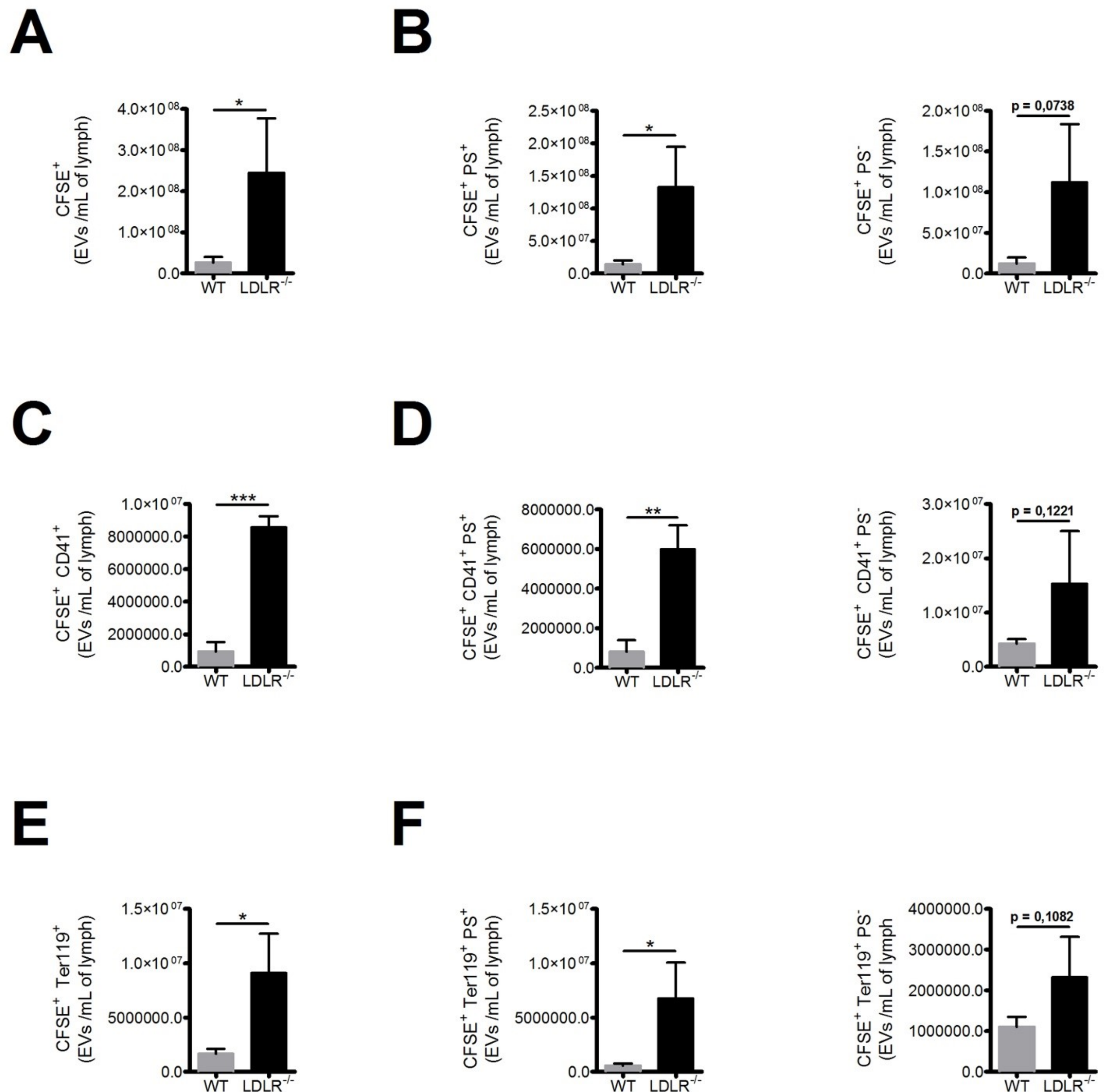


Figure 5. Characterization of EVs in lymph of atherosclerotic mice. Flow cytometry was used to identify (A) total CFSE$^+$ EVs based on their (B) PS expression. (C) CFSE$^+$CD41$^+$ and (E) CFSE$^+$ Ter119$^+$ EVs were analyzed based on their (D, F) PS expression. Experiments were performed with 3 mice per experimental group. (*p<0.05, **p<0.01, ***p<0.001)

Although the functional consequences of the presence of EVs in lymph remain to be established, this study reports that EVs, of diverse sources, are transported in lymph. EVs are involved in numerous physiological processes, and vesicles from both non-immune and immune cells have important roles in immune regulation. Whereas several stimuli, such as cytokines, lead to EVs release from macrophages and monocytes in the atherosclerotic lesion [36], EVs produced by blood endothelial cells (BEC) can also be found in blood [37]. As pro-inflammatory cytokines and nitric oxide can trigger BEC-derived EVs production [38], it would be of interest to test whether these players involved in lymphatic function could also promote LEC-derived EVs in atherosclerosis-related collecting lymphatic dysfunction [39]. Albeit the full assessment of the EV origin (*e.g.* immune cells) and subtypes (*i.e.* exosomes, microvesicles or apoptotic cells) using complementary biochemical approaches [40] would have been of interest, the volume of lymph collected was not suitable to perform these additional experiments and other studies will without any doubt characterize them in a near future. Presently, we speculate that lymph contains both exosomes and microvesicles, and that the $CD41^+$ and $Ter1119^+$ EVs, with their size and markers, correspond mainly to microvesicles derived from platelets (or megakaryocytes) and RBCs, respectively. We observed that a major proportion of EVs did not harbour surface PS. Considering that PS is implicated in the rapid clearance of EVs [22], PS^- EVs may predominantly accumulate in the lymphatic system where they might interact with other cells and lymphoid organs. Whereas the routes and mechanisms of EVs uptake have been described extensively in the past few years [41], our results complement these findings and bring forth a possible direct role of the lymphatic network in the transport, but also the production of EVs. Our results suggest that EVs derived from red blood cells and platelets, inclusively but not exclusively, circulate in lymph of healthy animals, and are morphologically different and abundant in atherosclerosis. Whether increased concentrations of EVs in lymph in atherosclerosis and other chronic inflammatory diseases reflect a cause or a consequence of lymphatic dysfunction remains under intensive investigation.

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Conflicts of Interest

The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

Author Contributions

Andreea Milasan, Nicolas Tessandier, Éric Boilard and Catherine Martel designed and carried out experiments, analyzed data, and wrote the manuscript. Sisareuth Tan and Alain Brisson contributed to design of experiments, revised the manuscript and analyzed data.

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DISCUSSION

5 Fundamentals of the thesis

Dysfunction in lymphatic transport is at the core of numerous pathologies that affect millions of people worldwide[734]. It now comes as no surprise that this impairment also affects atherosclerosis. With the recent emerging evidence that the lymphatic vessels play an important role in mRCT, accounting for more than 50% of cholesterol delivery from cholesterol-loaded macrophages into the plasma compartment[735], we herein sought to better characterize the lymphatic dysfunction associated with atherosclerosis by studying closely the physiological and temporal origin of the malfunction. During my master's degree, we studied from the initiation of atherosclerosis, to the progression of the advanced atherosclerotic lesion, the physiology of the two main components that form the lymphatic vessels, the initial and collecting LVs. Our results suggested that the lymphatic dysfunction is present before the onset of atherosclerosis, and we started to suspect that this dysfunction is primarily associated with a defect in the collecting lymphatic vessels, thereby limiting lymph transport from peripheral tissues to the blood.

Several factors can modulate the lymphatic contraction that leads to collecting LV inefficiency. As previously described, the lymphatic collectors are modulated by extrinsic factors such as the lymphatic muscle cells surrounding it. An increase in fat around the vessels suggests a decrease in the contracting muscle surrounding the vessels, which may further diminish their contractile ability. Furthermore, enlarged adipocytes recruit macrophages and promote inflammation[736], generally resulting in tissue swelling, lymphatic vessel leakage, and decreased lymphatic transport of fluids and dendritic cells. Clinically, spontaneous development of lymphedema in obese patients has been reported[737].

To get a better grasp on how lymphatic vessels may be affected during atherosclerosis, we acquired data using several methods to look not only at the contraction capacity of the lymphatic, but also their permeability, and endothelial junction integrity as well. The origin of the lymphatic dysfunction is now clear in both pre-atherosclerotic, as well as atherosclerotic mice, as we excluded the possibility that the lymphatic capillaries are a cause of lymphatic impairment. Although initial lymphatics can be modulated by growth factors like VEGF-C, leading to an increase in number and branching points, we do not believe their function was affected, as demonstrated in our diverse mouse models during my master's degree and

throughout this thesis. However, lymphangiogenesis is a side effect that worries the scientific community with respect to lymphatic growth factor therapy and should not be taken lightly. In our study however, twelve weeks following the interruption of the treatment, when mice were no longer on the pro-atherogenic regimen, VEGF-C 152s lost its lymphangiogenic potential, despite the sustained lymphatic function improvement observed at this stage. As such, lymphangiogenesis does not seem to significantly contribute to atherosclerosis progression and further improving the VEGF-C 152s treatment to have no effect on lymphangiogenesis, and solely act on the mechanism that induces lymphatic vessel contraction, is an avenue that warrants attention. We believe it is safe to conclude that the initial defect associated with atherosclerosis onset occurs in the collecting, rather than the initial LVs, reinforcing that more vessels does not always necessarily mean more functional vessels.

That is why, when it comes to the role of lymphatics in mRCT, and ultimately atherosclerosis, it is no surprise that further understanding the physiological characteristics of LV pumping and general dynamics is required. Important to keep in mind, is that age is a major factor influencing the body, and so, changes in lymphatics throughout age should also be taken into consideration. A decrease in contractility and pumping efficiency, as well as in ECM and contractile proteins, are physiological losses that appear with age. Furthermore, impaired pathogen clearances and impaired permeability leading to leakiness in aged collectors, were also seen[698]. Additionally, we also believe that with time and disease progression, as collecting LVs age and a variety of stressors further impede on their mechanisms of action and integrity, the impairment will also translate to the initial LVs and impair absorption.

Although only recently thoroughly investigated, atherosclerosis is characterized by excessive inflammation that is increasingly associated with the presence of EVs released by diverse cell types upon activation or apoptosis. In the last decade, the identification of paths involved in the atherosclerosis-related inflammatory process have emerged. While we identified EVs in lymph, we suspect that their presence is not only due to their transport from the peripheral tissue to the collecting LVs, but also due to lymphatic vessel degradation. Since EVs are now known to be key players in several physiological functions and even emerging as biological entities with high biomarker potential[738], we believe that their presence could not only reflect LV function, but in turn, modulate it as well. We suspected that EVs would initially be properly absorbed by the initial lymphatic vessels but would gradually accumulate in lymph in the

absence of an effective lymphatic propulsion mechanism. The accumulation of EVs in the advanced atherosclerotic lesion is thought to be due to poorer LV clearance due to lymphatic dysfunction observed under the same conditions[324]. Their impaired contractility and/or hyperpermeability are potential causal elements, as observed in studies 1 and 2. Since the endothelial permeability is increased in inflammatory situations, we believe that blood backflow occurs in lymph at the level of the subclavian vein. Preliminary studies from the lab (Gabriel Jean, MSc thesis) indicate that RBC-derived EVs are associated with an increase in oxidative stress that leads to increased LEC permeability *in vitro*. Alternatively, considering the pre-established role of platelets in maintaining lymphatic integrity via interactions between the CLEC-2 receptor and PDPN[860], and their recently demonstrated adhesion to LECs potentially involved in the restoration of lymphatic function during atherosclerosis, we suspect that the increased presence of pdEVs in lymph during atherosclerosis is involved in the restoration of lymphatic integrity. This new concept has opened the opportunity to test diverse hypotheses with respect to their effect on lymphatic function, and as well, EVs present in lymph have the potential to eventually become biomarkers of lymphatic dysfunction during the development of atherosclerosis, as well as therapeutic vehicles.

The novel discoveries within this thesis have allowed the lab to pursue the fundamental path investigating the mechanisms underlying collecting LV integrity and contraction capacity in atherosclerosis. The published studies prompted alternative therapeutic perspectives for the treatment and even prevention of atherosclerosis.

6 The blood and the lymphatic vasculature: interconnected

First and foremost, since the blood and the lymphatic systems exert very similar structural and regulatory characteristics, it is important to note that some bias may be introduced when studying the effects of each system separately, which is why results need to be carefully interpreted and thoroughly investigated before assumptions are to be made. More specifically, the use of different pharmacological agents, inhibitors and other components may affect one system as much as the other. While the studies herein required the circulatory network in its entirety, we took some precautions to make sure that specific targeting of the lymphatic vessels is achieved to the best of our technical abilities.

Treatment delivery is important to take into account. Intradermal injections were administered in our studies when we needed to ensure that the treatment reached the lymphatic vasculature, despite also being recovered in the blood circulation. By developing our skills in lymph collection, we were able to always compare its contents to those in blood, further illustrating differences between the two parallel, but complementary systems. In study #2 the apoA-I kinetics clearly demonstrated its increasing presence in lymph as time progressed. While the effects of apoA-I and HDL were well known, as previously mentioned, we are confident that although some of the beneficial effects observed in the *Ldlr*$^{-/-}$ mice could be mediated by the blood circulation, as for example HDL is shown to decrease inflammation systemically, which relieves the pathological state of the animal as a whole and could contribute to disease attenuation, the effects observed both *in vivo* on the collecting lymphatic vessel permeability, as well as *in vitro*, the latter which will be further elaborated upon below, support the added benefit of improving the lymphatic function with apoA-I treatments.

Alternatively, intraperitoneal injections were used to administer the VEGF-C 152s treatment systemically and allow for even further discussions. Although the growth factor was specifically designed to target the lymphatic circulation, we did not assess any of its potential effects that we could have observed on the blood circulation systemically, or near the plaque, following its administration. While VEGF-C has been shown to promote angiogenesis, as well as lymphangiogenesis when over-expressed[739], plaque angiogenesis is known to contribute to plaque progression, destabilisation and thromboembolic effects[740]. However, even if our study did not focus on analyzing any of the effects that VEGF-C 152s might have on blood

vessels, if we focus on the angiogenesis, even if it did increase it, that would mean that it could have potentially worsened the phenotype, when compared to control.

Nonetheless, although treatments possibly act on both the blood and the lymphatic systems, the positive effects we see on the lymphatic side represent undeniable complementary aid in disease prevention and progression. It is important however, that in future studies, a more thorough analysis be implemented to exclude benefits originating on the blood side and pinpoint specifically the positive contributions of the lymphatic network. Assessing systemic and plaque angiogenesis, in parallel to lymphangiogenesis, and ensuring the treatment is present in both blood and lymph following administration, should be a standard comparison in future studies.

An additional remark common to most of our studies, is the fact that the treatment could directly modulate cholesterol levels, independent of its supposed effects on the lymphatic vasculature. To clarify this possibility, in study #1 we assessed apoB levels to give a more accurate predictive estimate of atherogenic risk than do total cholesterol or LDL cholesterol levels[741]. A more complete assessment, such as a low apoA-I/apoB ratio, would indicate susceptibility to CVD[742] and should be kept in mind for future studies. As both groups were faced with the same risk of developing atherosclerosis, our study further reinforced the importance of functional lymphatics before lesion onset, independent of any direct cholesterol effects.

Since we cannot fully inhibit the functions of the blood vasculature to solely observe the effects of the lymphatic one, as long as experiments are carried out in a comparative manner and individual contributions clearly identified, in future years a lot more interesting insights will emerge and be increasingly defined. With respect to mRCT, specific identification at which steps the lymphatic vessel improvements occur is needed, but in parallel, cholesterol efflux from plaque is not to be neglected as it represents an important rate-limiting step in the whole process. Further limitations encountered, potential experiments to be included and alternative pathways of action will be discussed in the following sections.

7 Potential therapies for plaque prevention

Diagnosis and treatment of atherosclerosis should be targeted at various stages of the disease, from the early stages of vascular injury to the stabilisation of the vulnerable plaque. Although we have not yet identified the precise downstream mechanism through which VEGF-C 152s acts to promote all the beneficial effects observed in our study, its multifactorial actions paint a broad picture where different players are at interplay and are needed from the very early stages of atherosclerosis development. Potential mechanisms of how each may affect lymphatic function will be described herein.

7.1 VEGF-C/VEGFR-3 axis

A polymorphism in the gene encoding *Vegf-c* led to lower levels of circulating VEGF-C and was associated with CAD. Moreover, in the Framingham Heart Study, different metabolic parameters and dyslipidemia negatively correlated with VEGF-C levels in plasma[743]. As previously mentioned, mutation of the *Vegfr-3* gene in humans is a cause of primary lymphedema[744]. Despite this, it was not yet known if the role of these genes in heart disease relates with lymphatic biology in atherosclerosis. While VEGF-C may have multiple roles in disease, it is worth wondering if VEGF-C increases as a mechanism to combat disease progression, in this case acting to improve lymphatic transport of cholesterol from plaque, similar to how CXCL5 or innate IgM against oxLDL rise with disease activity and combat disease progression[743].

In *apoE*$^{-/-}$ mice VEGF-C was elevated in the atherosclerotic aortic walls, but despite this increase, adventitial lymphatics were shown to regress during the course of atherosclerosis progression. Moreover, elevated soluble VEGFR2 found in the aortas of these mice binds to and diminishes the activity of VEGF-C, thus impairing lymphatic drainage and aggravates atherosclerosis formation. As such, VEGFR2 in atherosclerosis could potentially be a new target for the release of additional endogenous VEGF-C, aiding in the prevention of lymphatic regression[745]. Treatment with VEGF-C 152s, like in our case, seems to also be a good alternative. By targeting the lymphatic vessels directly, especially early on before lesion onset, may offer these vessels a protective advantage against the negative inflammatory effects observed throughout the atherosclerotic process. In study #1, while circulating VEGF-C levels

were not measured, modulation of the VEGFR-3 receptor was observed following VEGF-C 152s administration. This makes us believe that the elevated levels of circulating VEGF-C, mutant and innate combined, increased this receptor's expression to protect lymphatic vessel integrity from the very early beginnings of atherosclerosis onset. Importantly, we cannot assess whether the VEGFR-3 levels were rescued to normal levels, since in our study we did not compare the *Ldlr*$^{-/-}$ mice to WT mice to see if levels are similar or significantly lower. In study #1 VEGFR-3 expression was measured by IF, whereas in study #2 we looked at VEGFR-3 levels *in vitro* by WB. This could be of importance to assess in future studies. Interestingly, despite VEGFR-3 levels also increasing in control mice once the disease progressed, this effect seems to occur too late to make a significant difference on the lymphatic function.

VEGF-C binding was shown to lead to VEGFR-3 autophosphorylation[746] and transduces signals that promote LEC survival, proliferation, and migration[744, 747]. VEGF-C/VEGFR-3 binding on LECs also induces activation of PI3K/Akt and leads to the phosphorylation of P70S6K, PLCγ1, Erk1/2 and eNOS[748], thus regulating the pumping activity of collecting lymphatic vessels[722]. While effects on these downstream effectors need to be further investigated, we were faced with a significant limitation at the time. Since collecting LVs are very small, the quantity of proteins needed to assess these by WB is insufficient. However, more recently we have developed skills to also isolate the flank collecting LVs in parallel to the popliteal vessels, so WB analyses may be carried out in the future. While the flank vessels are similar to the popliteal vessels, because they have significant muscle coverage and demonstrate strong contraction abilities[749], it is important to keep in mind when pooling LVs from different regions of the body that they may not always exert similar traits and mechanisms of action. Alternatively, using *in vitro* cells treated with VEGF-C 152s, we observed that Akt and eNOS phosphorylation did not differ between groups. However, we cannot conclude entirely that no effects were observed since the actual collecting LVs were not analyzed. Since then, quantitative polymerase chain reaction (qPCR) methods have been optimized in the lab and for future studies, analyzing these downstream effectors in collecting LVs isolated from treated mice should be possible.

An abundant presence of VEGFR-3 on LECs and their responsiveness to VEGF-C surely has an important impact on different signaling pathways and cellular structures of the lymphatic endothelium. Older microarray studies demonstrated that in response to VEGF-C stimulation, a

large number of differently expressed genes were transcription factors and cell cycle related, as well as angiogenesis. Some genes were also involved in tumorigenesis and tumor invasion, and the transport of proteins, solutes, and lipids. A number of genes related to lipid metabolism, as well as neurogenesis and neurodegeneration, were also responsive, further showcasing the broad spectrum of importance of the VEGF-C/VEGFR-3 axis[750]. However, VEGF-C binds to both VEGFR-3 and VEGFR-2 to exert broad effects. Two popularly used mutants of VEGF-C, VEGF-C 156s and VEGF-C 152s, specifically bind VEGFR-3. The VEGF-C 156s human mutant has a replacement of Cysteine with Serine to inhibit the homodimerization required for VEGFR-2 binding. Treatment of a mix of blood and LECs with VEGF-C 156s led to PI3K dependent phosphorylation of Akt and Protein kinase C dependent activation of mitogen-activated protein kinase (MAPK)[744]. Another option is its analog, VEGF-C 152s rat recombinant protein which similarly mutated specifically binds to VEGFR-3. Recently, VEGFR-3 activation on LECs through VEGF-C 156s led to downregulation of several genes that drive inflammation, thus suggesting that VEGFR-3 stimulation has direct anti-inflammatory effects systemically [751].

Building on these results, in *Ldlr*$^{-/-}$ mice pretreated with VEGF-C 152s we observed a decrease in the proinflammatory cytokine TNF-α when mice were fed a HFD. Hence, as inflammation can severely impair collecting lymphatic vessel contractions, the superior pumping capacity in these mice, when compared to control, may have been in part due to this diminished inflammatory background. A deeper understanding and analysis of VEGFR-3 modulation will strengthen our knowledge of how and which inflammatory genes it modulates and will provide new opportunities for intervention with selective molecules that promote specifically chosen pathway activities.

7.2 Lymphatic and muscle cell innervation

The presence on LVs of sympathetic and parasympathetic innervation has been declared but remains greatly underexplored. A significant decrease in nerve fibers was observed in aged collectors and is associated with the alteration of lymph flow[752].

Although observations of lymphatic innervation and the mechanisms regulating this process are notably under-studied, sympathetic adrenergic nerve fibers seem to be the dominant

neural innervation of the lymphatic vasculature. α-adrenergic stimulation was shown to increase contractile tone, amplitude and frequency[753], while these effects are countered by β-adrenergic receptor activation[754]. Bovine mesenteric LVs have nerves in their walls that can increase in frequency of spontaneous lymphatic contractions following stimulation but can be blocked by α-adrenergic antagonists. Similarly, α-adrenergic blockers decreased lymphatic flow in anesthetized sheep[753]. While a more diffuse presence of nerve fibers was observed in mesenteric and femoral LVs, compared to cervical ones, in lymphatic capillaries, few nerve fibers positive for neuropeptides and neurotransmitters were detected. The human thoracic duct also has both sympathetic and parasympathetic innervation. As expected, an important reduction of all specific nerve fibers analyzed was detected in LVs from elderly subjects[752].

EphrinB (EFNB2) is important for the endocytosis of VEGFR2 and VEGFR-3 in endothelial cells following ligand stimulation, which allows for downstream signaling activity. Selective deletion of EFNB2 in mice results in a failure of VEGFR-3 internalization following VEGF-C stimulation and thereby, reduced activation of Rac1, Akt and Erk1/2. The loss of function effects of EFNB2 in mice led to reduced lymphatic sprouting and defective development of both blood and the lymphatic vasculature[513]. Phenylephrine is a sympathomimetic amine, part of the phenylethanolamines class and causes vasoconstriction of the vessels[755]. While PE can act on both SMCs and the endothelium, recently, it increased expression of EFNB2 in human umbilical vein endothelial cells, and when the ERK inhibitor U0126 was added to the treatments, the increase was abolished or greatly diminished[756]. Interestingly, in human umbilical artery endothelial cells, exposure to PE similarly augmented the expression of EFNB2, but only when treated with VEGF at the same time. Although studies to date demonstrate that α-adrenergic activation promotes marker expression in human endothelium, this interaction needs to be further investigated in LECs and special attention should be warranted to the integrity modulation of LVs by PE through EFNB2 in an ERK dependent manner[756]. As observed in our studies, following PE, mice having been pretreated with VEGF-C 152s had significantly higher contractions than control. It was impressive to see the difference in contraction frequency in mice whose lymphatic function was assessed immediately following the one month of VEGF-C 152s treatment. Since PE was injected in the footpad of the mouse and had a five-minute window to act upon the LVs, it will be particularly

interesting to see if it caused VEGFR-3 internalization that led to downstream signaling to support the contractions.

The stimulation of LMC α_1-adrenoceptors in the mesenteric artery induces contractions that can be suppressed by the endothelium through a release of NO and efflux of K^+ from both LECs and LMCs. However, the release of NO and endothelium-derived hyperpolarizing factor (EDHF) can be induced indirectly by agents which act only on SMCs. Hence, activation of the endothelium is not due solely to endothelium-specific vasodilators or to changes in endothelial cell shear stress[757]. In studies of bovine lymphatic smooth muscle, norepinephrine (NE), a hemodynamically similar vasopressor to PE, increased the duration of the action potential and led to an increased force of contraction. This suggests that NE improves lymphatic pumping due to its positive inotropic effect and this effect is suspected to be due to the suppression of the outward K^+ current[758].

LMCs can be targeted by a wide variety of stimuli that modulate their contractile properties and activity, so further studies are greatly needed in the field. It is particularly imperative to identify the precise mechanisms through which treatments, such as VEGF-C 152s can modulate collecting LV contraction capacity and take into account the susceptibility of different factors to act through both endothelial and muscle cell mechanisms of action.

7.3 Lymphatic markers modulation

Throughout our different studies, we observed important lymphatic markers modulation following different treatments and dietary conditions. While scarce literature exists regarding this particular phenomenon, we believe it plays a crucial role in atherosclerosis development and progression, which warrants it further attention.

FOXC2. One of the most interesting discoveries we have made to date is the modulation of FOXC2 in LECs. Although we have not had the opportunity to thoroughly investigate its expression in valves particularly, its presence all along the collecting LVs was assessed and downregulated while on a HFD. Supporting our observations, individuals with FH combined with hyperlipidemia express lower levels of *Prox1* and *Foxc2* genes in adipose tissue[759]. FOXC2 was also shown to be involved in the control of the types and distribution of adipose tissue, adding to it an important role in lipid metabolism[760]. Furthermore, decreased levels of FOXC2 in *Foxc2*$^{+/-}$ mice exacerbated the severity and vulnerability to experimental colitis, as it

dilated LVs and functionally impaired mesenteric LVs[761]. Studies now link FOXC2 signaling to the maturation of collecting LVs, valve development and vascular mural cell recruitment, emphasizing its important role in both lymphatic development and maturation[557, 567]. Interestingly, HFD-induced obesity elevates expression of FOXC2, which in turn activates the Akt/mTORC1 and ERK/mTORC1 signaling pathways, thus enhancing proliferation of preadipocytes and inhibits their aberrant apoptosis[762]. As such, it seems that under dietary stress, the transcription of *Foxc2* is focused in adipose tissue and is downregulated in other tissues, such as the LVs. Significantly, both VEGF-mediated signaling pathways, PI3K and the ERK/MAPK pathways, modulate the transcriptional activity of *Foxc2* [763] thus explaining why the VEGF-C 152s treatment in study #1 might have prevented the FOXC2 downregulation that we previously observed during the two months of HFD in study #2. However, it is always important to take into consideration the pleiotropic effects of specific lymphatic markers modulation as we need to maintain a balance between atherosclerosis prevention or regression, and cancer. For example, certain studies demonstrated that the downregulation of FOXC2 in colorectal cancer cells promotes cancer cell death. Based on these results, the study hypothesizes that the ablation of FOXC2 expression could lead human cancer cells to become sensitive to chemotherapy[564]. A better understanding of whether FOXC2 modulation is organ-specific under certain pathological conditions would be interesting to assess. In cancer patients that suffer from atherosclerosis, treatments like VEGF-C would not be ideal since it would potentially have dual negative effects through lymphangiogenesis that helps the cancer spread, as well as maintenance of high FOXC2 levels that would need to be diminished to promote cancer cell apoptosis. The complexity of FOXC2 make it an interesting therapeutic potential and it would be of particular interest to study how exactly VEGFR-3 specific molecules like VEGF-C 152s could be modulated to specifically target FOXC2 presence on LVs.

LMC. As previously discussed, whether muscle cells are irregularly recruited to the collecting LVs, or atypically located on initial LVs, it contributes to vessel failure and leads to lymphedema[764]. However, the mechanisms controlling LMC recruitment and its role in vessel maturation are still scarce. Particularly of interest is the discovery of the implication of PDGF subunit B in the regulation of LMC recruitment to the collecting LVs. Deletion of *Pdgf subunit B* in LECs prevented muscle cell recruitment which caused dilation of the vessels and impaired their pulsatile contraction. However, PDGF subunit B overexpression in LECs did not

also recruit LMCs to the initial LVs, which was explained by the fact that ECM is required for PDGF subunit B binding and its presence is limited around those vessels[764]. This indicates a particular important role of the ECM in proper LMC deposition. Additionally, it also further implicates platelet activation and their role in maintaining proper LV integrity and function, thus confirming our suspicion of platelet involvement in lymphatic function in study #2. PDGF subunit B is actually synthesized, stored in the α granules of platelets, and released by platelets upon activation[765]. As such, it would be particularly interesting to assess if among the increased circulating pdEVs that we observed in atherosclerotic mice in study #3, some of those EVs contain some or abundant levels of PDGF subunit B .

Podoplanin. PDPN is upregulated in a variety of cell types during inflammation and it plays important roles by interacting with other proteins located in the same cell or in neighbouring cells. Downstream effects of its binding to respective ligands leads to modulation of pathways that regulate proliferation, contractility, migration, as well as remodeling of the ECM[766]. The PDPN/CLEC-2 axis activates pathways in platelets that release granules and leads to platelet aggregation that seals the separation zone of lymph sacs and the cardinal vein. This interaction allows for the prevention of backflow at the lymphovenous junction and recently, platelet CLEC-2 and podoplanin were also shown to form fibrin-containing thrombi near valves in LVs. Therefore, the PDPN/CLEC-2 axis acts as a safeguard for the lymphatic network not only *in utero* but also throughout life[577]. We took particular interest in studying this interaction as venous thrombosis occurs during inflammation, and platelet depletion protects against deep vein thrombosis in mice[767]. While PDPN upregulation in the wall of the inferior vena cava exacerbates deep vein thrombosis, upregulation of podoplanin and cluster formation in the lymphatic apical side leads to impairment of LEC proliferation, thereby maintaining LVs integrity[768, 769].

Recently, a novel role for the PDPN/CLEC-2 axis was reported in increasing megakaryopoiesis and platelet production in the bone marrow[770]. Furthermore, both TNF-α and LPS upregulated PDPN expression in infiltrating peritoneal macrophages, but not the resident ones[771]. Additionally, only PDPN$^+$ macrophages are able to bind and activate platelets via CLEC-2, which further demonstrates the importance of this axis in inflammatory processes such as atherosclerosis. In line with this, postmortem histological analysis of abdominal aortas showed an increased PDPN expression in SMCs and macrophages reflective

of advanced atherosclerotic lesions and necrosis[772]. Therefore, podoplanin may exert differential and opposing roles while present in both the blood and lymphatic systems, and as such, a more thorough understanding of its effects needs to be pursued.

8 Plaque regression and/or stabilization

While treatment with VEGF-C 152s provided us with potential for plaque prevention, we also observed unexpecting results when following the switch to chow diet, mice plaque did not regress, but rather stabilized compared to control. Although the study demonstrated efficacious modulation of the lymphatic pump throughout the entirety of the atherosclerotic progression, we hypothesize that sustained treatment with VEGF-C 152s beyond the four weeks of initial treatment may have further delayed plaque onset and progression, or at the very least significantly reduced it. As well, the chow diet was administered for only one month, but for a longer period of time of at least two months we may have observed a more pronounced plaque regression, especially with respect to plaque size.

A regressing plaque is characterized by reduced macrophage content, increased fibrosis, less apoptosis and smaller necrotic cores than when plaque development is at its apogee, thereby leading to a more stable plaque that is less susceptible to rupture and thrombus formation[773]. Reversal or regression of the atherosclerotic plaque was thought to be improbable until not too long ago, since many characteristics of advanced atherosclerotic plaques seem permanent and terminal, like necrotic core formation, calcification and fibrosis. On the basis of studies that extend back to the early 1900s, with the help of genetically modified mice, now known possible mechanisms responsible for lesion diminishment include decreased retention of apoB within the arterial wall, efflux of cholesterol and other toxic lipids from plaques, emigration of foam cells out of the arterial wall, and influx of phagocytes to remove the necrotic debris of the plaque[774].

An aortic transplant from *apoE*$^{-/-}$ mice into *apoE*$^{-/-}$ recipient mice that contained the human apoA-I transgene and were fed a chow diet, resulted in rapid lesion regression[775]. In *apoE*$^{-/-}$ mice with established atherosclerosis fed a HDF, injections of recombinant apoA-I Milano resulted in lesions characteristic of atherosclerosis regression[776]. Similarly, in HFD fed *Ldlr*$^{-/-}$ mice, following a switch to a chow diet combined with an antagonist of miR-33, a miRNA that downregulates genes in HDL generation, resulted in improved HDL function and induced lesion regression[777]. In our study #2, we support the anti-atherogenic role of apoA-I with results that show an effective plaque regression following treatment of *Ldlr*$^{-/-}$ mice with respect to plaque size, as well as macrophage content.

An emerging concept has demonstrated that growth and maintenance of advanced plaques is not dominated by the recruitment of monocytes, but rather by the propagation of local macrophages within the plaque towards the luminal periphery[778]. As such, therapies that halt macrophage proliferation within plaque are important regulators of the transition between an advanced atherosclerotic plaque progression and regression. Targeted inhibition of macrophage proliferation in HFD-fed *apoE*$^{-/-}$ mice with simvastatin nanoparticles led to reduced plaque inflammation and increased markers of plaque stabilization, despite unchanged plaque size[779]. However, all three mechanisms of macrophage depletion, which are increased macrophage egress, reduced monocyte recruitment and attenuated macrophage proliferation, may act conjointly to resolve the atherosclerotic lesion[773]. Interestingly, other studies reported that regardless of plaque state, whether progressive or regressive, macrophages localize upon entry into the plaque and cannot escape[780].

In our studies we assessed the presence of both CD68 proinflammatory macrophages, as well as resident CD206 macrophages within plaque. Both following apoA-I and VEGF-C 152s treatment, we saw a decrease in CD68 macrophage presence indicative at the very least, of a more stable plaque, since its size did not change following the switch to a chow diet. However, we cannot conclude at the present time through which mechanism macrophage content was modulated since no functional tests were performed. Following VEGF-C 152s treatment, we can suspect that there was less macrophage recruitment as MCP-1 mainly recruits monocytes to sites of inflammation, and its presence was decreased in plasma. However, the other mechanisms cannot be excluded. Furthermore, a thorough analysis of macrophage location characterization within plaque would be particularly interesting in future studies. Firstly, the distance between macrophage location within plaque, to the adventitia of the vessel, would be reflective of how deep they're located and how easily they are to be reached and contribute to cholesterol efflux and clearance. Markers such as Ki67 should also be co-stained with macrophage-specific markers to reflect their proliferation[781]. Cell tracking studies would also be interesting to not only confirm monocyte recruitment, but also to assess their differentiation within the plaque[782].

While macrophages play diverse roles within plaque, they can also serve as a source of VEGF-C and promote growth or hyperplasia of LVs by inducing sprouting of pre-existent lymphatics[783]. In inflamed tissues, VEGF-C released by CD11b^{+} macrophages increased

immune cell transport to draining LNs, thus reducing inflammation[784]. While we did not assess specifically $CD11b^+$ macrophages in our studies, this could be one mechanism in both studies #1 and #2 where there was a reduced number of proinflammatory macrophages in the atherosclerotic lesion. Alternatively, macrophages may transdifferentiate into LECs, by first forming cellular aggregates and then fuse with sprouting lymphatic vessels[783]. Additionally, LYVE-1^+ macrophages situated around the blood vessel protect against arterial stiffness by controlling the expression of collagen by SMCs through MMP9 regulation[785]. Supporting data showed that LYVE-1^+ macrophages were downregulated in the adventitia of $apoE^{-/-}$ mice with atherosclerosis[745]. Consequently, a measure of LYVE-1^+ macrophages present in the adventitia should be included in future studies from the lab and correlated to plaque size.

In conclusion, the results from our studies, taken together with emerging data, emphasize the complexity of the atherosclerotic plaque, and while some therapies may not decrease its size, it is not to mean that they are not efficient, especially systemically. Furthermore, elucidating the specific mechanisms through which macrophages are affecting lesion gravity are currently of upmost importance. Specific subtypes of macrophages seem to have dual roles and act positively upon both the blood and the lymphatic vasculatures, thereby rendering them critical throughout the whole development of the atherosclerotic disease.

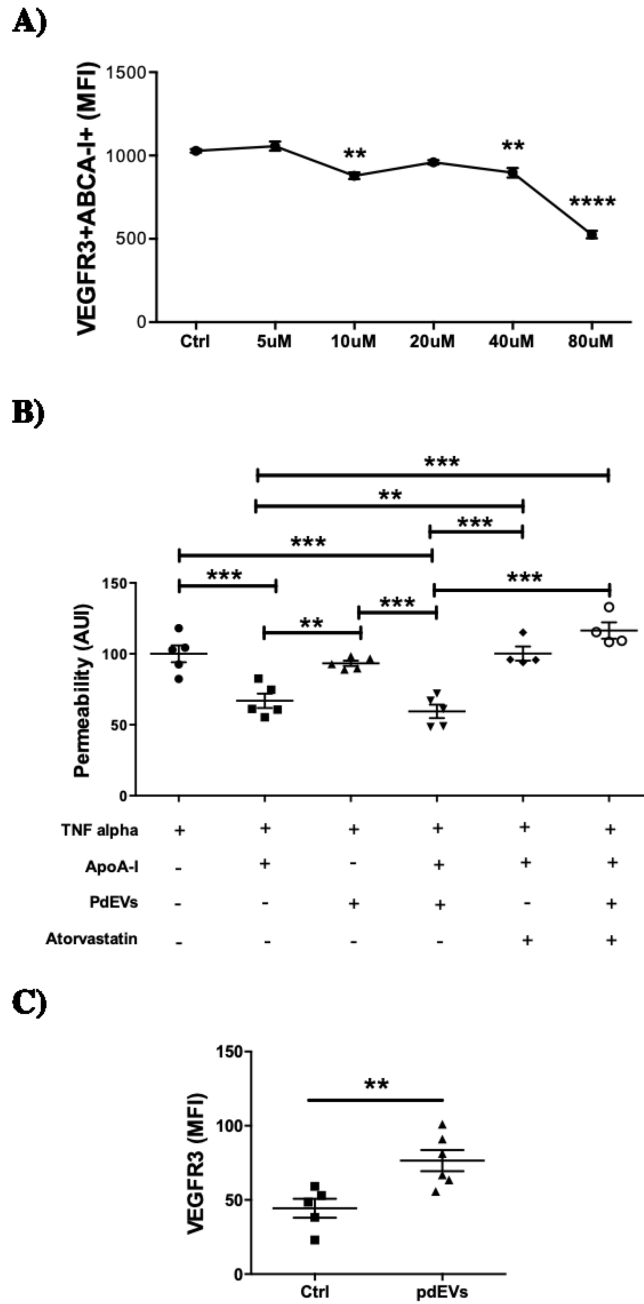
9 ApoA-I and lymphatic integrity

Thus far, we have shown that apoA-I modulates lymphatic function through mechanisms that may rely on platelets and pdEVs. Platelets were shown to adhere to the lymphatic endothelium that was pretreated with apoA-I *in vitro*, resembling a preventive patching between the LEC junctions that could explain the *in vivo* decrease of collecting LVs permeability in mice administered the apoA-I treatment. The precise mechanisms through which apoA-I modulates LV integrity, whether directly, by affecting the LEC junctions, or indirectly, by promoting pdEVs adherence and/or internalization to the lymphatic endothelium, will be thoroughly studied in the lab.

Transwell studies have permitted us to assess *in vitro* the permeability of a cultured monolayer of human dermal microvascular lymphatic endothelial cells (HMVEC-dLyAd). Transwell inserts are used by adding endothelial cell growth basal medium to the multiple plate wells, followed by adding the transwell inserts. Lastly, cells were seeded in the inside compartment and incubated at 37°C for 72h. Subsequently, different treatements were added for 24h each, starting with TNF-α (10ng/ml) accompanied by an ABCA-I inhibitor[786], Atorvastatin (40 μM) or its appropriate vehicle control, apoA-I (0.6mg/ml) and lastly, pdEVs (1 million per ml) isolated from healthy volunteers. Following, ovalbumin 488 (OVA488, 1:40) was added to the inserts for 20 minutes at room temperature and the liquid that was picked up from the plate wells was assessed by a fluorescence plate reader.

To date, our data demonstrate that *in vitro*, following apoA-I treatment, leakage as assessed by ovalbumin 488 is significantly decreased compared to control, thus reinforcing the reduction in Evans Blue leakage surrounding the collecting LVs previously observed in study #2. We believe the rescue is dependent on an ABCA1 mechanism, as downregulation of this receptor with Atorvastatin (Figure 16A) reversed the rescue in LEC permeability observed with the apoA-I treatment (Figure16B). However, no effects on permeability were observed when cells were also incubated with pdEVs. Nonetheless, pdEVs did increase VEGFR-3 expression in cultured LECs on a TNF-α background, as assessed by flow cytometry (Figure 16C). This similar effect was previously first observed in study #2 by platelets, as assessed by WB.

Figure 16. Lipid-free apoA-I treatment *in vitro* modulates LEC permeability.



(A) Atorvastatin-mediated downregulation of the ABCA1 receptor at the surface of LECs was assessed at specific concentrations and compared to control (Ctrl). (B) Transwell experiments demonstrate a role for apoA-I to decrease *in vitro* LEC hyperpermeability on a TNF-α background, an effect that's inhibited following 24h incubation with Atorvastatin. No effect on LEC permeability was observed following 24h incubation with pdEVs. C) PdEVs incubated for 24h with LECs increased VEGFR-3 expression, as assessed by flow cytometry. $^{**}p<0.01$, $^{***}p<0.001$ and $^{****}p<0.0001$

ApoA-I seems to have a direct role on LECs to preserve their integrity and potential mechanisms of actions are discussed below. Our results with Atorvastatin indicate that ABCA1 may be implicated, but as assessed by our dose-response curve, future studies need to be pursued to better understand how Atorvastatin acts upon LECs at different concentrations. While we based our experiments on the highest concentration of Atorvastatin (40 μM) previously used in the literature to inhibit ABCA1 in macrophages[786], we were intrigued to see that doubling the concentration nearly doubled the inhibition in LECs and would be interesting to see the adverse effects of such a strong inhibition on lymphatic function. Furthermore, while pdEVs did not seem to have a direct role on LEC permeability within the 24h, they were shown to increase VEGFR-3 at the surface, an effect that may prove beneficial more long term and would be worth investigating *in vivo*. Possible mechanisms of action of pdEVs will also follow in the next sections.

9.1 ApoA-I and intercellular junctions

Atherosclerosis is a chronic inflammatory disease that is characterized by a disturbed flow that increases cell proliferation and apoptosis of blood endothelial cells, which leads to increased vascular permeability and exacerbation of the disease[787]. On the other hand, LECs generally adapt to changes in shear stress by strengthening their cellular junctions[788]. Hence, we suspect that a disturbed lymph flow is the cause for the increased permeability observed in the atherosclerotic *Ldlr*$^{-/-}$ mice. As such, apoA-I would be able to rescue this leakage by modulating LEC junctions, or shear-stress induced transcription factors, like FOXC2 or PROX-1. Indeed, HDL has been shown to modulate connexin43 gap junction channels[789]. Additionally, genome-wide analysis associated low HDL levels and a specific region on chromosome 16q24.1, believed to be FOXC2[790], suggesting that HDL-based therapies could be associated to FOXC2 modulation and affect collecting LV function. While FOXC2 expression is similar in *Ldlr*$^{-/-}$ mice that underwent apoA-I treatment and control mice, a more thorough assessment of these LEC integrity markers needs to be investigated using additional techniques such as transmission electron microscopy and qPCR. Additionally, effects of the HFD administered to the mice may have been sufficiently strong to render apoA-I efficacy inadequate. It would be interesting to test, similar to the VEGF-C 152s treatment, apoA-I

treatment before a HFD and onset of atherosclerosis and see if administered pre-emptively, this may rescue FOXC2 downregulation once mice are switched to the HFD.

Immunofluorescence staining of human dermal LECs showcased the presence of different types of junctions, continuous or discontinuous, and demonstrated that zona occludens-1(tight junction marker) and VE-cadherin are co-localized[791]. In the lab to date, we have not had the chance to assess colocalization of these junctions in LECs. It would be important to assess if apoA-I treatment directly strengthens zona occludens-1 and VE-cadherin co-localization in cultured LECs, and/or increases their presence, following inflammatory-induced de-localization and downregulation of their individual expression[792, 793]. Furthermore, phosphorylation of the adhesion molecule VE-cadherin at tyrosine residues modulates the opening of endothelial junctions during inflammatory reactions[794]. Assessing phosphorylated VE-cadherin by WB would further evaluate lymphatic junction integrity following apoA-I treatment.

In light of all these possibilities and potential mechanisms of action, we cannot exclude that apoA-I may be able to directly modulate cell junctions in LECs, potentially through an ABCA1 dependent mechanism, thereby rescuing any negative junction modulation associated with atherosclerosis.

9.2 ApoA-I and lipid raft modulation

An alternative concept through which apoA-I may be modulating LV integrity is by affecting LEC lipid rafts. The latter are cholesterol-rich microdomains exhibiting functional properties, such as cell signalling, that depend largely upon their lipid composition. Since cholesterol plays a key role in assisting lipid raft formation, modulating cholesterol in lipid rafts provides a promising anti-atherogenic strategy. Importantly, activation of proteins and subsequent downstream signalling pathways behave differently when they are located in lipid rafts compared to when they are in nonraft membranes[795].

Recently, ABCG1, the principal transporter involved in HDL-mediated cellular cholesterol efflux, was shown to play an important role in T cell homeostasis, via lipid rafts[796]. The latter is increased when ABCG1 is absent on T cells and this leads to their differentiation into Tregs via a downregulation of the mTOR pathway which triggers the phosphorylation and activation of signal transducer and activator of transcription 5[797].

Similarly, the ABCA1 transporter requires a signalling process that activates mTOR, and inhibition of its upstream effector Akt has been shown to derange lipid rafts[798]. Of particular interest is that mTOR inhibition by rapamycin inhibits LEC tube formation by downregulating VEGFR-3 expression[799]. As previously described, the VEGF-C/VEGFR-3 axis is crucial to lymphatic vessel function, particularly collecting LVs pumping. Therefore, we suspect that mTOR modulation could also be involved in the maintenance of collecting LV integrity in our apoA-I-treated mice. By modulating lipid rafts, apoA-I may enhance the signalling pathway of VEGFR-3, which rescues LEC integrity in conditions of impaired physiological flow where shear stress-induced genes such as *Foxc2* are downregulated. Upregulation of VEGFR-3 has already been confirmed in our studies when cells were treated with pdEVs, and since these entities were shown to transport lipoproteins, this lipid-raft dependant mechanism of action could definitely be one possible explanation for our results.

In addition to the rescue in hyperpermeability associated with the apoA-I treatment *in vivo* and *in vitro*, if the VEGFR-3 expression is increased and functionally more active, it would be worth assessing if this leads to better lymphatic vessel contractions as well.

9.3 ApoA-I and cytokine modulation

Studies show that TNF-α alters the normal distribution of cell junctions and affects the endothelial barrier of cultured LECs. An patchy distribution of VE-cadherin at the cell periphery reflects the lymphatic hyperpermeability[800]. Recently, it was shown that apoA-I restores the neovascularization capacity of the lymphatic system during TNF-α-mediated inflammation[801]. It would therefore be interesting to see if apoA-I mediates lymphatic vessel permeability in part by decreasing circulating inflammatory cytokines such as TNF-α which play a crucial role in lymphatic vessel impairment. If differences are observed, it would also be interesting to see if lipid-free apoA-I injected in TNF-α-transgenic mice would rescue the lymphatic contractions similar to the TNF-α inhibitor[802]. Furthermore, it would be important to assess circulating TNF-α in the lymph of *Ldlr*$^{-/-}$ mice on a HFD, which we have not had a chance to do in the published studies herein. As lymph quantity per mouse is very limiting (100uL of lymph are needed per mouse to perform a CBA test), in future studies a significantly higher number of mice will need to be planned for this experiment. Nonetheless, investigating

this possibility is worthwhile, as therapies such as VEGF-C 152s did downregulate plasma cytokines such as TNF-α, which is believed to have contributed to the increase in contraction capacity of the collecting LVs, as observed. Our *in vitro* experiments were performed on a TNF-α background to mimic the inflammatory environment found in atherosclerotic mice. *In vivo*, our mice had up to 15 pg/ml of circulating TNF-α in plasma, and several studies employ concentrations between 10pg/ml to 10ng/ml *in vitro*[803]. Since previous data on LECs were performed with 10ng/ml[721, 801], and this value is also used as the physiologic concentration of human recombinant TNF-α[804], we deemed fit to align our results with these studies.

9.4 ApoA-I and EVs

Preincubation of lipid-free apoA-I on LECs appears to improve platelet adhesion to the lymphatic endothelium under physiological flow. These results highlight the potential role of apoA-I in the stabilisation of the lymphovenous junction, thus most likely preventing backflow. Furthermore, impaired interactions between LECs and platelets lead to a reduced lymph flow that impairs mature collecting lymphatic vessel remodelling and decreases the expression of shear stress upregulated lymphatic valve genes[560, 805].

Lymph, physiologically, does not contain a large number of platelets. However, upon cell activation and death, EVs are released. While our initial studies were published using platelets on the lymphatic endothelium, reflective of the lymphovenous junction integrity, future studies in the lab will be pursued using pdEVs to assess their diverse effects on LECs.

9.4.1 EVs adherence/internalization on the lymphatic endothelium

Currently in the field, it is not well defined whether EVs uptake is a cell type specific process. While a variety of studies show that EVs can be uptaken by practically any cell type, others suggest that the uptake is highly specific and may only take place if a cell and the EV possess the proper ligand-receptor interaction. Nonetheless, there is consensus that multiple mechanisms are responsible for the communication between EVs and targeted cells, and that more than one strategy can be used by different cell types. For example, inhibition of a certain integrin required for internalization impaired EVs uptake in DCs and macrophages, but not in microglia[349].

Adherence of pdEVs to the lymphatic endothelium may be achieved through different mechanisms. The principal one we suspected was the CLEC2/PDPN interaction. While preliminary results did not increase expression of neither CLEC-2 on the surface of platelets or PDPN on the surface of LECs, we halted this possibility. However, platelets could be treated with apoA-I to see if it induces the production of more CLEC-2^{+} pdEVs. As previously mentioned, lipid rafts have been observed to be necessary for the requirement of PS^{+} EVs, so they may modulate a lot more markers on their surface that we are not currently aware of. Assessments of their cargo components would also be interesting. Moreover, *in vitro*, following pdEVs adherence to the endothelium, a transwell test should be performed to assess lymphatic endothelium permeability. If no differences are observed, the binding strength between CLEC-2 and PDPN could be modulated instead and should be studied more thoroughly under conditions of flow.

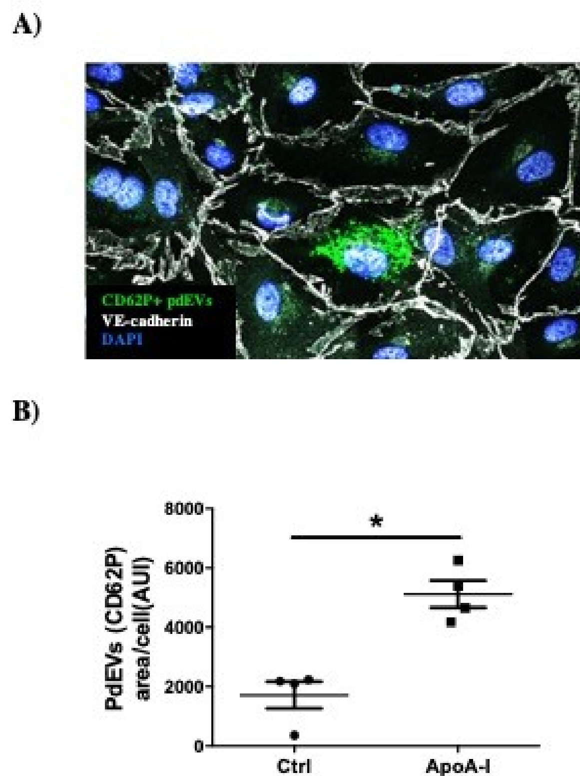
Alternative ways of pdEVs adherence should also be investigated. Since CD31 is expressed by the LECs at the junctions between the cells, it is possible that it may also be involved in the adhesion of pdEVs to HMVECs *in vitro*. CD31 is involved in homophilic interactions with other cells, which allows adhesion and transendothelial migration of monocytes and lymphocytes expressing CD31[806]. Since pdEVs also express CD31[401], this hypothesis is very likely. It has actually been reported that CD31 is involved in adhesion and platelet aggregation on an injured blood endothelium[807]. Furthermore, in a sepsis mouse model, blocking PS on platelets or $\alpha v\beta 3$ integrin, which promotes cell adhesion on ECs, impaired platelet clearance that led to increased circulating platelet counts in circulation[808]. It is therefore very likely that the binding of PS and/or CD31 to integrins and CD31 respectively, on the surface of HMVECs is partly responsible for the adhesion of pdEVs *in vitro*.

While platelets were seen to only adhere to the endothelium, pdEVs are mainly internalized, and at least partially, through the mechanisms mentioned in the introductory part of this thesis. The labeling of LECs with a membrane marker should also be carried out in order to ensure the exact location of the EVs in LECs by IF. Once internalized EVs may be targeted to the lysosome where they will be degraded, but alternatively they could also be recycled and may associate with proteins and signalling factors found within the cell, thus leading to additional effects once re-released by the acceptor cells[809]. Alternatively, pdEVs may also be

stained with dyes such as CFSE or CellRed and their internalization monitored by flow cytometry.

To date, during preliminary troubleshooting, we observed that internalization as assessed by IF occurs rather quickly, with most pdEVs being observed in the LEC within an hour (Figure 17A). Further data showed that similar to their larger counterparts, there was a marked difference between adherence/internalization of pdEVs to the lymphatic endothelium pre-treated with apoA-I for 24h *in vitro*, compared to control (Figure 17B). Assessing EVs contents and seeing precisely what they are bringing into the cell, such as any mRNA or growth factors that would enhance lymphatic function, should also be assessed by WB.

Figure 17. PdEVs adhesion/internalization in cultured lymphatic endothelial cells.



A) Immunofluorescence of cultured LECs stained with VE-cadherin and DAPI, following 1h incubation at 37°C with pdEVs stained with CD62P. Image was acquired with an LSM 710 Confocal Microscope (Zeiss) equipped with a 63X/1.4 oil dic objective. **B)** 24h preincubation of lipid-free apoA-I with LECs at 37°C increases were assessed IF. Experiments were performed with 5 mice per experimental group and three different regions of interest were analyzed at random. *p<0.05

9.4.2 EVs contents

An increasing amount of evidence highlights the beneficial influence of certain EVs of diverse cellular sources in cardiovascular biology. EVs are now considered of crucial importance in the modulation of vascular inflammation and atherosclerosis. However, major limitations include their detailed characterization and isolation procedures, which should be payed particular attention to, as mentioned in the introduction.

In recent years, different EV releasees were shown to be important for lymphatic function. Now that we confirmed EVs to be present in lymph, their direct interaction with the lymphatic endothelium warrants much needed attention both with the perspective of intercellular communication, but also as potential targeted therapy.

While EVs contain a multitude of diversified biological content that they transfer into target cells, miRNAs are among the most extensively studied. Overexpression of miR-31 isolated from human blood was shown to repress FOXC2 and other LEC-associated genes. Moreover, both miR-31 and miR-181a derived from human colorectal cancer cells can target PROX1 and repress LEC-specific genes, such as VEGFR-3[810]. As such, it would be important to see which, if any, types of EVs that possess these miRs and are released from different types of cells may modulate lymphatic function by affecting their integrity, especially before and during atherosclerosis progression. MiR-9 targeted NF-κB, downstream of TNF-α signaling, and regulated TNF-α-mediated inflammatory mechanisms. Additionally, miR-9, induced in rat mesenteric lymphatics in response to TNF-α, increases VEGFR-3 expression, indicating a possible role in lymphangiogenesis, but more importantly, represents its role as a potential rescuer of collecting LV function[811]. While they did not assess for this possibility, the miR-9 present within the LECs may have originated from internalized EVs cargo, that are increased in circulation under inflammatory conditions, as we reported in study #3. Important as well, miR-126 is abundantly found in platelets and is a known contributor to vascular integrity, as well as modulating the VEGF response[812]. Hence, while specific expression of these miRNAs, and many others alike, remains to be confirmed in pdEVs, their presence warrants attention and will likely be investigated in future studies from the lab.

Although pdEVs have been found to participate in a variety of biological and pathological processes via intracellular communication, they were particularly helpful in

promoting angiogenesis to restore endothelial integrity after vascular injury[813]. In fact, pdEVs are filled with a variety of growth factors, associated with the Akt and Erk pathways[814], which are not only central to angiogenesis and neurogenesis, but more importantly in our context, these pathways are important for LEC function as well[815]. Promising avenues lie ahead as mesenchymal stem cell derived EVs were shown to contain some VEGF-C[816], that could also be useful as a treatment delivered by EVs, and aid with contractions and plaque modulation. ApoA-I may actually be responsible for the presence of different growth factors from platelets, particularly VEGF-C. The latter is known to be released from platelets upon activation[817] and in our study we saw that apoA-I modulates lymphangiogenesis and increases VEGFR-3 expression on LECs[818]. This may explain the rescue in lymphatic hyperpermeability in the *Ldlr*$^{-/-}$ mice and remains to be investigated if it could also increase their contraction capacity *in vivo*.

Additionally, as assessed by cryo-EM, we also observed the presence of lipoproteins within EVs further enticing the possibility of administrating apoA-I treatments to LECs directly. A more recent study actually quantified thoroughly plasma EVs proteome identifying 1187 different proteins. Amongst them, we have confirmation of apoA-I, -II, -IV, apoB, LDLR, TGFβ, fibroblast growth factor, angiotensin, and transforming growth factor, just to name a few of the more relevant ones for this context[819]. Additionally, Takov *et al*. also identified a multitude of angiogenic factors, such as VEGF-C among many other proteins and cytokines, with ET-1 being the most abundant[820]. It would be of upmost importance to do a similar screening of the proteome of lymph EVs and see how contents differ. *Mycobacterium tuberculosis* was actually shown to release EVs that contain a variety of lipoproteins as well as lipoglycans that block the phagosome, inhibit antigen presentation and modulate cytokine production[821]. Even neurons and astrocytes release EVs that contain growth factors and promote paracrine responses[336].

Due to their ability to carry such a diverse cargo, EVs can also be used as a therapeutic tool for the treatment of cardiovascular disease. Importantly, circulating EVs can be chemically modified to optimize specific delivery of bioactive molecules to a certain cell type[822]. EVs can be fused to synthetic liposomes resulting in decreased immunogenicity and longer half-life in circulation[823]. Fleury *et al*. engineered T cell-derived EVs enriched with Sonic hedgehog, a member of the Hedgehog signaling pathway involved with cell division, tissue development,

and morphogenesis[824]. Administration restored endothelial function by stimulating angiogenesis and increasing NO production in mouse ischemia/reperfusion models[825]. In another study, exosomes released by $CD34^+$ hematopoietic stem cells overexpressing the Sonic hedgehog transgene were shown to exhibit increased healing effects on cardiomyocytes and overall heart function, as they possess anti-apoptotic properties through induction expression of anti-oxidant enzymes in ECs[826].

EVs derived from cardiac progenitor cells, particularly exosomes, represent another promising avenue as drug delivery vehicles as they have been shown to stimulate migration of endothelial cells[827] and to protect the ischemic myocardium from reperfusion injury[828]. To date, small exosomes containing a lipid bilayer have proven beneficial in settings of acute MI, as embryonic stem cell derived EVs improved cardiac function in the infarcted area[829]. *In vivo* administration of EVs derived from EVs from mesenchymal stromal cells to mice increased both blood reperfusion and angiogenesis by acting on VEGF receptors, thus improving hindlimb ischemia recovery[830]. As EVs can be applied to a large amount of different pathologies, another possibility is to target EVs from DCs and load them with small siRNA that specifically silence expression of β-secretase 1, for example, a therapeutic target in Alzheimer's disease[831]. Following injection, specific delivery was achieved and down-regulation of β-secretase 1 in the mouse brain[347].

EVs are actually currently considered as useful therapeutic agents and used in a variety of pathologies as drug delivery vectors, immunomodulatory or regenerative therapies, and antitumor and pathogen vaccines. With several clinical trials completed and some ongoing, EVs therapy is generally accepted safe and relatively well tolerated[832]. A detailed identification and characterization of EVs shed by different components of the cardiovascular system in normal and pathological conditions will aid in the identification of new signaling pathways and open promising doors for cardiovascular therapy among many others.

9.4.3 EVs cellular origin

One of our recent studies suggests that EVs derived from diverse cell subtypes circulate in the lymph of healthy animals and some are increased in atherosclerosis[303]. An alternative hypothesis is that apoA-I modulates EV levels and origin in atherosclerosis. Mouse plasma and lymph need to be collected and EVs should be assessed by flow cytometry using markers for

platelets (CLEC-2, CD61 or CD62P), lymphatic endothelial cells (podoplanin and VEGFR-3), red blood cells (Ter119) and EV-specific markers (CD81, CD63, flotilin, CD9, PS, AnnexinV). Several results are likely to emerge. ApoA-I may decrease circulating pdEVs in lymph indicative of more adhesion and/or internalization within lymphatic vessels. To test this assumption, plasma and lymph from apoA-I mice should be collected and using the markers above, different populations are to be identified. It is imperative to use as many markers as technically possible concomitantly to ensure specific origins of the EVs. Depending on the origins that will be identified and whether they are present in plasma or lymph, multiple hypotheses can be expected. An increase in LEC derived EVs in lymph could indicate LV dysfunction, as the integrity may be compromised when compared to control and leads to apoptosis and disturbance of collecting LVs cells. We would expect apoA-I to limit LEC derived EVs levels, as its effects were shown to act through rescuing of the LV integrity and permeability. Furthermore, less RBC-derived EVs in lymph would reflect an adequate blood-lymphatic separation at the level of the lymphovenous junction. Alternatively, if more are present in lymph, as observed in study #3, in atherosclerotic mice, this could indicate that there is impairment in proper lymphatic/blood delimitation causing backflow. By reducing inflammation, potentially by decreasing TNF-α, ApoA-I may also modulate proinflammatory T-cell and BEC derived EVs in plasma, which could translate to a decrease in T cell effector cells that aggravate the plaque, as well as reduced endothelial dysfunction. A multitude of effects are to be expected, placing EVs at the forefront of novel treatments and also their potential use as biomarkers in atherosclerosis.

10 Rethinking atherosclerosis therapies

Since we demonstrated that the lymphatic dysfunction associated with atherosclerosis commences early, even before plaque onset, in mice and likely in humans as well, we need to rethink the timing of primary prevention of atherosclerosis-related diseases. Certain treatments will not only need to be administered earlier, but certain changes in currently prescribed therapies, such as statins which represent the primary therapy to reduce atherosclerosis and anti-platelet therapies used to reduce thrombus formation[833], may be needed as well.

10.1 Antiplatelets

Platelets play a key role in the initiation of hemostatic mechanisms during vascular injury and antiplatelets are often prescribed as a means of primary prevention for patients at risk of CVD. However, considering our recently published data supporting the importance of platelets in the maintenance of LV integrity, physicians should carefully weigh the potential benefits of cardiovascular risk reduction with the likelihood of harm, related to hemorrhagic complications but also to lymphatic function. While the precise mechanisms need further investigation, a thorough clinical research would help optimize therapy duration and intensity, which could then be personalized for each patient's needs[291]. Furthermore, a controlled trial to investigate the antiplatelet therapy effect on extracellular vesicles (AFFECT EV) in acute MI is underway to better understand whether lower pdEVs concentrations are associated with improved clinical outcomes in patients treated with P2Y12 antagonists, ticagrelor and the less potent clopidogrel. Healthy aged-matched controls were also enrolled to be able to assess physiological levels of pdEVs and their procoagulant activity, as demonstrated by the levels of PS, CD62P and fibrinogen. They expect to see a lower level of procoagulant pdEVs in patients administered the more potent platelet inhibitor, since it reduces platelet activation more rigorously[834].

Adherent platelets at the lymphovenous junction and lymphatic valves are likely capable of driving secondary activation after PDPN stimulation, so while this concept remains to be elucidated, it is clinically significant because such response is the target of commonly used antiplatelet drugs such as aspirin and adenosine 5′-diphosphate receptor antagonists. Thereby, this potentially leads to disruption of the lymphovenous junction in pathological conditions with

elevated venous pressure, which ultimately causes reduced lymph flow and impaired lymphatic valve function[587].

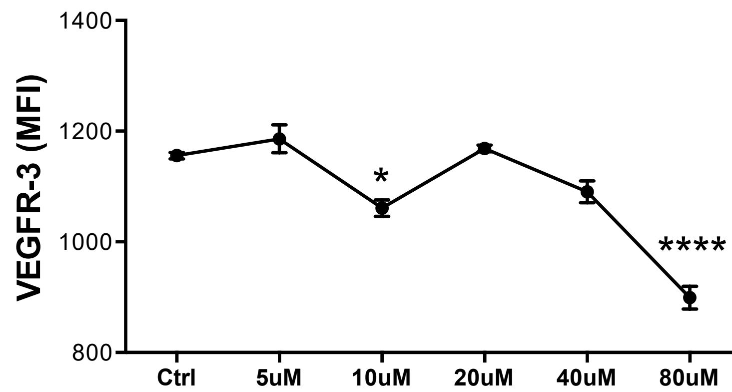
Interestingly, in lymph, coagulation occurs much slower than in blood, despite existing levels of fibrinogen and vWF. Contrarily to blood, lymph contains increased levels of heparin and increased fibrinolysis[835]. Future studies in disease settings will be needed to reveal the precise effect of antiplatelet therapies on lymphovenous hemostasis and lymphatic function, especially in the early stages of atherosclerosis development.

10.2 Statins

Besides the lowering of LDL, statins have pleiotropic effects that extend to improved endothelial function, reduced thrombus formation and reduction of inflammation[836]. Many studies continue to demonstrate the efficacy of statins in patients with CAD.

Recently, statins were even shown to suppress lymphangiogenesis and lymphedema development. Atorvastatin reduced pathological lymphangiogenesis by inhibiting VEGF-C expression and secretion by lesional $CD11b^+$ macrophages[837]. While in patients with pathological lymphangiogenesis that promotes tissue inflammation in some disease states such as inflammatory bowel disease (IBD)[838], and cancer propagation, this may seem like a potentially new and effective therapy, in our studies atorvastatin decreased VEGFR-3 levels on cultured LECs (Figure 18). It seems that both a low dose of atorvastatin, as well as a much higher dose has detrimental effects on LEC VEGFR3 expression. Worrisome is that for example, the American Heart Association Guidelines recommends either moderate intensity (atorvastatin 10-20 mg) or high intensity (atorvastatin 40-80 mg) therapy[839], which similar to the concentrations used in mice, may in fact have deleterious effects on LECs. Although functional tests remain to be pursued to enlighten this data, taking into account everything we know to date, we believe that statin therapy might not be as efficient as previously thought in patients at risk of CAD, especially in the early stages before atherosclerosis development. If this turns out to be the case, as statins are prescribed especially to patients with FH, this means that they are put at risk of impaired lymphatic function from the very early beginnings and as seen in our *Ldlr*$^{-/-}$ mice, VEGFR-3 expression is required especially before the lesion onset to delay plaque. A thorough reorganization and rethinking of atherosclerosis therapies is necessary now more than ever.

Figure 18. Atorvastatin decreases VEGFR-3 expression at the surface of LECs in a dose-dependent manner.



Expression of VEGFR-3 was assessed by flow cytometry in cultured LECs following 24h incubation with different doses of Atorvastatin. Each dose was compared to control (Ctrl). *$p<0.05$ and ****$p<0.0001$

11 Translational perspective

Many of the patients presenting with the first episode of CVD do not have a traditional risk factor profile and CRP levels that correlate minimally with the individual components of the Framingham Risk Score[836]. CRP is believed to play a grand role in all the phases of atherosclerosis and acts as an important marker of inflammation that has been widely used to predict future cardiovascular events[840]. Therefore, application of the traditional primary prevention strategies may not apply to some patients. The possibility to better control lymphatic function early enough to be able to delay atherosclerosis onset and progression is recent. Translational projects are underway in the Martel laboratory at the Montreal Heart Institute that will allow for identification of early lymphatic dysfunction in different patients, as well as alternative circulating entities such as EVs, ultimately connecting these to an abnormal lymphatic function that modulates atherosclerosis progression in humans.

11.1 Lymphatic dysfunction as a biomarker of cardiovascular disease

A method to monitor lymphatic function could greatly aid in the development of new therapies, predict the patient's susceptibility to develop atherosclerosis, and properly evaluate in a timely manner the patient's condition and response to therapy and treatment. As such, we have developed a clinical trial where systemic lymphatic function can be assessed in the skin of human subjects by a newly optimized non-invasive imaging technique, *in vivo* near infrared fluorescence (NIRF), where Indocyanine Green (ICG) is injected as a lymph contrast agent to dynamically follow lymphatic trafficking in subjects. Currently, ICG is the only fluorophore approved clinically for use in humans[841]. NIRF allows for visualization of the initial, precollecting and collecting lymphatics, as well as the draining LNs of the limb. While camera designs vary in sensitivity, non-invasive NIRF imaging can detect lymphatic vessels located as deep as 3 to 4 cm beneath the skin surface[842]. NIRF lymphatic images can be collected rapidly with millisecond acquisitions which allows for detailed study of the function of collecting LVs and the quantitative assessment of the contraction frequency and velocity. To date, NIRF has been used to image LVs in patients with metastatic cancer[843, 844] and to show that manual

lymphatic drainage therapy and pneumatic compression therapy modulate lymphatic pumping[845-847]. Furthermore, studies in animal models under a panoply of conditions have shown that NIRF results that are consistent with invasive, intravital fluorescence measurements using FITC-dextran[842]. Contrary to other imaging techniques, like lymphoscintigraphy, NIRF is superiorly accurate and has allowed for earlier detection of lymphoedema in the upper limbs[848]. Magnetic resonance lymphography is based on variations in photon relaxation in different tissues and works adequately to image lymphatic function, but its picture resolution and sensitivity are relatively poor when compared to NIRF. Alternative techniques like positron emission tomography and contrast-enhanced ultrasound are more useful for larger abnormalities as observed in metastatic cancers in the LNs, as they lack precision and, in some instances, may lead to false positives[849]. Although lymphatic function is assessed systemically, the information collected is still the most reflective of the actual function of LVs in the heart. The technique needs to be non-invasive and even the most recent imaging techniques cannot reach deep enough to directly measure coronary lymphatics. Nonetheless, as long as this limitation is kept in mind, the limbs reflect momentarily the closest assessment of lymphatic function that we are able to achieve clinically and the results collected will surely provide new insights into how the lymphatic network is specifically affected in different patients throughout the entire atherosclerosis development.

The studies published to date in the lab have offered a solid foundation to pursue translational studies. We have established that lymphatic dysfunction appears even before the onset of the atherosclerotic lesion and is precociously associated with a defect in the propelling capacity of the collecting LVs. This may potentially be associated to a defect in genes associated with FH, such as *Ldlr* which is present on LVs, but in a cholesterol-independent manner. From here on, we need to focus on enhancing lymphatic transport prior to lesion onset which could limit atherosclerosis progression and favour atherosclerotic plaque stability and even regression.

To do so, the lymphatic function in patients with or at risk of CAD can be evaluated and correlated with atherosclerotic plaque progression, lymphatic function, and EVs characterization. Furthermore, a direct method of obtaining the functional capacity of the endothelium could be to measure the LEC response to direct stimulation such as the release of NO to mediate lymphatic relaxation. Infusions of L-NAME in patients, could rescue lymphatic pumping if too much NO is present. Certain studies have already seen promising results

following administration of L-NAME in pathologies such as cardiogenic shock and hypotension[850, 851].

This project will be a great asset in understanding the specific mechanisms that contribute to lymphatic function modulation in patients with atherosclerosis and identifying a lymphatic dysfunction early enough as to be able to treat it.

11.1.1 Lymphatic imaging differences in mice and humans

As thoroughly discussed, the lymphatic system has diverse functions and responds dynamically especially under inflammatory conditions and altered hemodynamics. Visualization of these complex processes in human patients and animal models of disease is key to better understand how the LVs are affected and in turn, how they modulate disease progression[842]. Longitudinal, non-invasive, and repeated imaging is required to broaden our understanding of atherosclerosis progression and regression both in a basic science and clinical context[842]. To date, several imaging tools have been used to assess lymphatic function, such as NIRF imaging, fluorescence microlymphangiography, magnetic resonance imaging lymphangiography and in some limited cases X-ray computed tomography, in both mice and humans [852-858]. These techniques each offer some specifics depending on what the objective of the study is. In our case, to assess popliteal collecting LV function we used fluorescently based imaging but with commercially available fluorochromes such as OVA488 that preferentially gets uptaken by the LVs instead of travelling directly into the bloodstream, if injected intradermally.

Mice are an experimental tool of choice for most inflammatory diseases and have helped yield tremendous insights into the workings of the human. Nonetheless, differences need to be acknowledged especially when we base our interpretation of results on *in vivo* imaging. Since each method differs in the way the subject is positioned, the invasiveness of the experimental setup, and the volume of contrast agent injected, it is important to take these parameters into consideration as they can significantly affect the functional measurements of lymphatic vessels.

Lymphatic function can be assessed in different manners. One way in the human is by measuring the time between the intradermal injection of ICG in the dermis of the back of the hand and the time it took for it to travel a certain measured distance[859]. This is the method that is employed in the clinical trial at the Montreal Heart Institute aforementioned. In mice, it

is by monitoring the depot clearance of intradermally injected OVA488 in the footpad of the mouse that reaches the popliteal LN[860]. While in humans the technique is non-invasive since no skin needs to be removed for the visualization of the vessels, in mice, skin removal is often employed to allow for proper imaging of the vessels. As per our observations, contrarily to humans, where imaging begins before the injection so as to measure the velocity of the dye through the LVs as it reaches a designated region of interest, in mice, the foot of the mouse needs to be moved a few times to allow for the dye to appear in the vessels.

A recent study investigated how lymphatic function can be influenced by different factors in mice. They concluded that fluorescence microscopy performed on mice in a prone position reflected a statistically lower contraction frequency compared with mice sitting upright. In our case, to keep the mice at a physiological temperature for the duration of the experiments, we deemed best to place the mouse in a prone position on a heat pad that kept the mouse body temperature as close to normal as possible. Furthermore, the lowest frequencies were observed after 20μL injections, but despite the frequency increasing at 4μL, a higher volumetric flow was observed following the 20μL injection and so, to them, contraction frequency alone is not sufficient to understand lymphatic transport[861]. Nonetheless, in our study, we believe that we acquired data that demonstrated significant differences between the different groups of mice when placed and analysed under the same conditions. In our experimental design, mice were injected with 10μL of OVA488, and this amount created a visible bulb in the area of the footpad, that we deemed reasonable. Injecting a higher amount of volume caused too much strain on the skin, as the mice we worked with were relatively small. As such, we did not want to sever any initial LVs and further damage the surrounding environment in any way. Conversely, injecting 5μL of OVA488 was an insufficient amount of dye to keep the LVs visible throughout the entire acquisition time of 30 minutes in total. Finally, with the help of NIRF imaging Bouta *et al.* demonstrated that skin removal to better visualize the lymphatic vessels also reduced contraction frequency[861]. This latter affirmation we cannot confirm nor reject, but we can agree that removing the skin causes tremendous physical stress on the foot of the mouse and we are not surprised that it would have some influence on the physiological pumping of the LVs. To palliate as much as possible to this effect, saline was administered at regular intervals, so the tissue does not dry during handling and image acquisition.

Ex vivo studies are also a very interesting alternative to studying the effects of different agents on lymphatic vessel frequency and amplitude. The pressure-myograph is a widely employed technique that requires impressive dexterity, especially when studying LVs which are very small, with a diameter ranging between 30μm to 100μm from our observations. However, many successful experiments have been performed to date in different laboratories[862-866]. Important information can be extracted from such experiments, but they are sometimes prone to critique, as some deem the environment in which the vessel is placed insufficient and non-reflecting of actual physiological conditions within the body[867]. Furthermore, tremendous care needs to be taken when isolating the vessels and mounting, as the slightest technical alteration during handling can affect the muscle cells surrounding the collecting LV, or the LECs themselves, which could lead to biased observational results.

In summary, the clinical data, in addition to preliminary studies performed in mice, need to be interpreted very carefully and further troubleshooting may be needed to ensure the most reliable translation of results, especially when testing specific treatments identified in the basic science context.

11.1.2 Taking into account sex differences

Diverse notions from our lab regarding the lymphatic vasculature and its role in CVD, were based on experiments carried out on female mice. However, there are actually several differences when it comes to arterial disease between men and women, including, but not limited to the prevalence, the clinical manifestation of the disease, treatment efficacy, and prognosis[868]. Sex is considered a biological variable, and several studies have demonstrated differential effects in both human and animal models of atherosclerosis, with respect to the mouse model studied, its genetic background, the time point analysed, the type of diet issued and the location of lesion analysis[869-871].

Microcirculatory differences between the sexes are prominent, reflecting higher levels of vasoconstrictors like ET-1, higher oncotic pressure that prevents fluid from leaving the capillaries, lower platelet counts in blood and a higher release of TNF-α in circulation, when comparing men to women[872]. Additionally, females demonstrated a stronger cell-mediated immune response compared to males[873]. As such, since both the blood and lymphatic circulation are relatively similar, some level of sexual dimorphism can be expected and should

be taken into account when studying inflammatory diseases such as atherosclerosis. It would be particularly interesting to recreate our experimental designs and observe if the treatments are as efficient in *Ldlr*$^{-/-}$ male mice. For example, female mice are known to have delayed atherosclerosis onset, despite having more pronounced lesions once the disease is established [868]. Would VEGF-C 152s therapy still delay plaque onset in male mice? Would the treatment significantly reduce further lesion size? In lymph collected from male mice, are pdEVs as prominent and would they offer the same protective effect with respect to lymphatic vessel integrity? If males have more circulating TNF-α, would the apoA-I and VEGF-C 152s treatments still be as efficient? These questions merit further investigation.

One important distinction between sexes is the hormonal differences, especially estrogen which has been reported to offer vascular protection and regulates the expression of several genes, including *Ldlr* and *eNOS*[874, 875]. Moreover, estrogen was demonstrated to reduce lymphedema[876], which may explain why postmenopausal women have a decrease in lymphatic pumping when compared to age-matched men[699]. However, on a diabetic type 2 background, while the lymphatic vessels are significantly enlarged, with a severe permeability defect, this phenotype occurred independent of sex[877]. In our hands, no differences between sexes with respect to lymphatic pumping or permeability have yet been observed in patients at different stages of the atherosclerotic disease, but the study is ongoing, and the statistical power needs to be increased.

With all these established differences between sexes, on patients and animal models, it is now expected that all animal studies that investigate atherosclerosis as an end point be performed in both male and female mice, with the data separated by sex[878]. A global overview analyzing a multitude of aspects needs to be acquired from both sexes before a final therapeutic initiative gets approved for a clinical trial.

11.2 Other inflammatory diseases

Advancements in the understanding of lymphatic system physiology and integrity is relevant in many different disease states, particularly those with a major inflammatory component.

While systemic alterations in lymphatic function are observed in CAD, changes in the intestinal lymphatic network are also found in humans[879], such as observed in Crohn's disease, which is a type of autoimmune IBD, that leads to significant adipose tissue deposition and impaired lymphatic contractile activity[880]. In order to relieve these effects and compensate for the protein loss and significant lymphedema, lymphangiogenesis has often occurred in the gut[881]. It has also been shown that IBD promotes cytokine dysregulation, including lymphatic-active cytokines[882]. In mice, the tissue damage sustained following infection is poorly healed and this causes hyperpermeable LVs in the mesentery which impair antigen and immune cell transport to the appropriate draining LN, further impeding on sustained proper immunity[883]. Furthermore, using three dimensional analyses, the path towards the draining LN in the collecting LVs was shown to be disrupted by the formation of tertiary lymphoid structures composed of B cells, thereby affecting lymph flow and its composition[167]. Taken together, these studies indicate that lymphatic function in immune cells and lipid transport is much more active than previously thought and is more complex than just passive draining of fluid from tissues and between cells.

Although the vascular system has long been acknowledged as an active player in IBD, it is now easier to assess and compare differences in the flow of fats, immune cells, and other molecules in both blood and lymph. Generally, IBD studies are performed in several mouse models like *Il10*$^{-/-}$. In these mice, blocking VEGFR-3 resulted in hyperplasic LVs in the colon, as well as increased edema and leukocyte infiltration[219]. Other research showed reduced lymphatic vessel density and reduced lymphatic clearance of Evans blue[884]. Moreover, genetic studies and diverse mouse models have stressed the role of genetic predispositions, as well as exposition to microbes and harsh environmental factors which can further cause complications[885]. Similar with how we prevented plaque onset in study #1, pre-emptive treatment with targeted VEGF-C 152s may prevent IBD onset by reducing inflammation severity and improving drainage through the lymphatics[886]. While we did see changes in macrophage number present in the atherosclerotic lesions in both studies #1 and #2, antigen clearance from the inflamed colon was accelerated by systemic delivery of VEGF-C[887] and further supports our results. Contrarily to our results, in IBD a lower density of lymphatic vessels has been linked to an increased risk of Chron's disease recurrence[888]. Hence, although modulation of lymphangiogenesis is regarded as an attractive therapeutic strategy, administering

non-targeted VEGF-C treatments was shown to increase angiogenesis and inflammation and thus worsen atherosclerosis in IBD patients[889].

IBD, like CAD, is a multifactorial disorder and current treatments include the use of anti-inflammatory agents such as TNF-α. One of the first ones available is infliximab, currently used to treat Chron's disease. Not only was this treatment able to significantly reduce circulating levels of TNF-α, but also CRP[890]. As previously mentioned, TNF-α plays a variety of roles in pathological states, and TNF-α therapy should definitely be considered for future experiments that assess lymphatic function in atherosclerosis. By blocking its release, we can not only identify its precise roles in CAD at all stages of disease development, but we can also use it as control to eliminate any effects that it may be responsible for, thus allowing us to discover other pathways of disease action. As previously detailed in section 9.3, this particular cytokine offers great potential insights for our understanding of lymphatic vessel impairment in inflammatory conditions.

Interestingly, the use of anti-TNF medication did not show reduced risk of CAD in IBD patients but did effectively lower it in patients with rheumatoid arthritis[891]. The latter is an autoimmune inflammatory disease driven by immune complexes that affect the joints due to overwhelming inflammation, leading to lymphatic vessel structural damage, loss of contraction, and reduction in lymphatic clearance[802]. A commonly used mouse model of rheumatoid arthritis is the TNF-α transgenic mice that spontaneously develops chronic joint inflammation that can be restored by anti-TNF-α therapy through restoration of lymphatic vessel contractions and promotion of inflammatory cell egress[802]. Lymphatic endothelial cells efferent to inflamed joints produce iNOS and inhibit lymphatic vessel contraction and drainage[892]. Additionally, adenoviral delivery of VEGF-C in the joints of TNF-α transgenic mice decreased inflammation, and significantly improved leg motility and clearance of ICG out of the footpads[886]. This demonstrates that similar to the lymphatic vessels that drain the atherosclerotic plaque, modulating collecting LV function increases drainage and relieves some of the burden associated with these similar pathologies. Nonetheless, it is important to consider that cytokines and growth factors often have pleiotropic effects, making it difficult to distinguish between direct and indirect mechanisms. It therefore becomes crucial to identify specific pathways of action depending on each disease state.

The lymphatic network is now under much deserved research focus as it plays a central role in homeostasis and is involved in many diseases, such as IBD and arthritis, but also Alzheimer's disease, hypertension, chronic bronchitis and cancer, which have not been thoroughly explored herein.

CONCLUSION

Based on our data and work from other groups, there is accumulating evidence that enhancing lymphatic transport can limit atherosclerosis progression and favour plaque regression/stabilization. A great deal of research has identified lymphatic markers and growth factors that allowed for exploration of the roles of lymphatic vessels in health and disease. As such, pro-lymphangiogenic therapies that use VEGF-C to counteract fluid retention disorders such as lymphedema and to enhance the local healing process have emerged. While most studies are investigating the lymphangiogenic effects of VEGF-C, especially studies related to cancer and lymphedema, we herein aimed to better understand its ability to modulate the contraction capacity of the collecting lymphatic vessels. Our previous studies identified a breach in the integrity of these entities in *Ldlr*$^{-/-}$ mice, with no effects on the absorptive capacity of the initial LVs. Thus, with data supporting their role as main effectors in the early stages of atherosclerosis development, an in-depth characterization of their contractile capacity throughout the atherosclerosis development was necessary. We showed that when injected with VEGF-C 152s, before the administration of a pro-atherogenic regimen, *Ldlr*$^{-/-}$ mice were protected from excessive plaque formation and long-term, had a more stable plaque. The sustained contraction capacity of the collecting LVs and the enhanced expression of VEGFR-3 and FOXC2 observed in the VEGF-C-152s treated mice contributed to the clearance of harmful components contained in peripheral tissues such as the atherosclerotic lesion. While advancements in lymphatic physiology are on the rise, with numerous excellent experiments assessing lymphatic function in depth both *in vivo* and *ex vivo*, we are the first to correlate *in vivo*, in a timely matter, that a rescue in LV contractions leads to beneficial effects with respect to plaque accumulation, its properties, and the overall systemic inflammation that leads to many deleterious effects. Although in the clinic most patients show up with an already established atherosclerotic lesion, study #1 offers novel insights regarding how specific treatments can modulate lymphatic function and in turn may prevent atherosclerosis lesion onset and progression in susceptible patients.

Generally, mechanisms that increase HDL capacity for cholesterol efflux have been the most extensively characterized. Specifically, as apoA-I injections decreased plaque accumulation without directly affecting circulating cholesterol levels, a thorough description of alternative mechanisms that were shown to implicate the lymphatic vasculature, are needed to correctly assess its anti-atherogenic properties in health and disease. In study #2, we treated

atherosclerotic *Ldlr*$^{-/-}$ mice with lipid-free apoA-I and assessed its effect on lymphatic function and morphology, to ultimately observe its effects on plaque regression. ApoA-I efficiently increased the number of initial lymphatics when compared to control or mice switched from a HFD to a chow diet to reverse the hypercholesterolemic phenotype. This systemically increased LYVE-1^{+} vessel density, despite a modest total cholesterol decrease in plasma, once again indicated that similar to the VEGF-C 152s treatment, apoA-I also acts on lymphatics in a cholesterol-independent manner. Contrarily to VEGF-C 152s, apoA-I treatment abrogated the lymphatic leakage observed in atherosclerotic mice, reflecting its beneficial effect on the collecting LV integrity. The precise mechanisms underlying this beneficial effect of apoA-I on collecting lymphatic vessel permeability and possibly contractility remain to be investigated. We strongly believe that apoA-I treatment may directly regulate lymphatic endothelium dynamics and cell-cell junction stability in the collecting lymphatic vessels, both directly and with help from platelets.

While few platelets are present in lymph, we turned our attention to attractive new players that are instead released from platelets, namely platelet derived EVs. The latter are more potent mediators and are responsible for a panoply of effects, but their presence had not yet been identified in the lymph. As such, we are the first to ever demonstrate the presence of EVs of heterogeneous origin in the lymph of mice, using three different techniques. This will now allow for a better understanding of their specific involvement in the lymphatic dysfunction associated with atherosclerosis. Populations of EVs derived from RBCs and platelets were similar in composition to those found in blood, but in addition, their concentration was even greater in *Ldlr*$^{-/-}$ mice fed on a HFD. EVs derived from other cell types were also detected, although their origins and specific functions remain to be more thoroughly investigated. Of interest, preliminary results suggest that the effect of EVs on lymphatic endothelial cell integrity depends on their cellular origins so we gather that specific subsets of EVs would prevent atherosclerosis-related lymphatic dysfunction, potentially in synergy with other treatments such as lipid-free apoA-I, or by carrying lymphatic growth factors such as VEGF-C in their cargo, whereas the accumulation of other subsets in lymph would be harmful to proper lymphatic function. Understanding the molecular and cellular properties of EVs is of upmost importance and relevance for all disease states, especially atherosclerosis.

The studies undergone during my master and doctoral thesis offer tremendous hope for the development of targeted treatment to collecting lymphatic vessels that in turn may be administered to patients with the use of biological therapeutic vehicles such as EVs. Future studies assessing lymph flow would be necessary to specifically assess lymphatic drainage. However, it is important to keep in mind that cholesterol efflux remains the rate-limiting step in mRCT, so a combination of therapies is most likely to help prevent and regress the atherosclerotic plaque. As the Montreal Heart Institute has a unique infrastructure in Canada to study atherosclerosis in clinical settings, the lab is eager to build on the published data from this thesis and pursue translational projects to assess the correlation between lymphatic function and atherosclerosis in patients. With the collaboration of local and international experts, the aim is to identify strategies that may enhance lymphatic function and lead to the prevention and regression of atherosclerosis.

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APPENDIX: List of publications

1. **Milasan A**, Smaani A and Martel C. Early rescue of lymphatic function limits atherosclerosis progression in Ldlr$^{-/-}$ mice. Atherosclerosis, 2019. 283: p. 106-119.

2. Pasquin S., Laplante V, Kouadri S, **Milasan A**, et al., Cardiotrophin-like Cytokine Increases Macrophage-Foam Cell Transition. J Immunol, 2018. 201(8): p. 2462-2471.

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