Targeting intraplaque angiogenesis : imaging and therapeutic interventions

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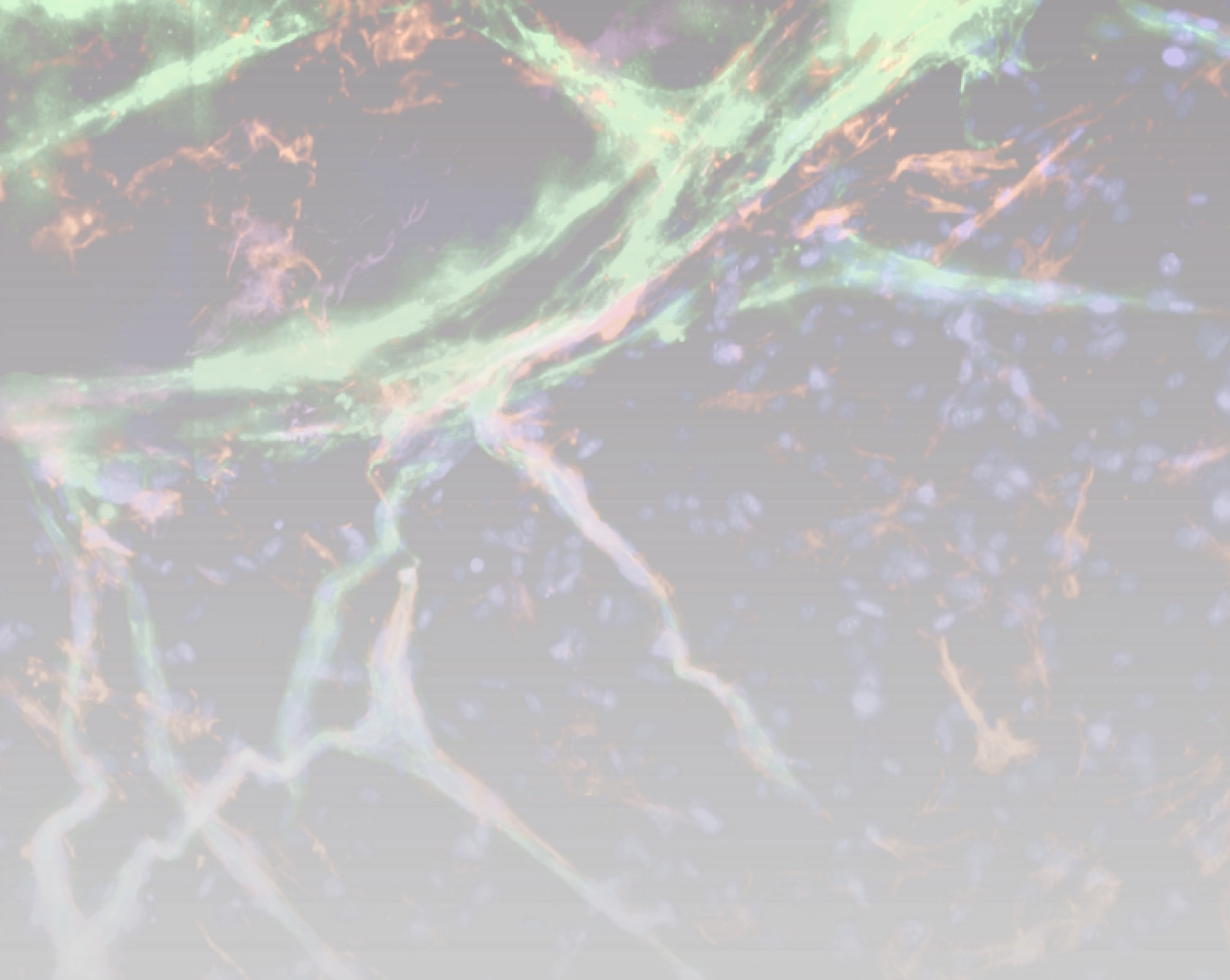

Chapter 1.

General Introduction and Outline of the Thesis

ATHEROSCLEROSIS

Atherosclerosis, which is chronic inflammatory lipid-drive disease, consists of the build-up of a plaque (starting in the inner layer) of medium and large arteries that compromises blood flow. The formation of these lesions is a slow multifactorial process and in general remains asymptomatic for decades.[1] Narrowing of the arterial lumen, which initially can be compensated by small wall enlargements with no change in the lumen caliber, becomes more severe after decades of living.[2] When symptoms arise, they usually relate to chronic ischemia, by critical narrowing of the lumen (>75%), or, more often, to acute ischemia in the form of plaque rupture or erosion and subsequent thrombosis.

Clinical manifestations, depend on the organ and/or artery affected.[3] When an atherosclerotic plaque progresses in the coronary arteries affecting heart's own circulation, it can cause chronic ischemia, such as stable angina pectoris, but also acute events such as myocardial infarction.[4] Plaque progression, in the carotid artery can lead to acute ischemic stroke, and in the peripheral arteries, it[5] can jeopardize limb viability.[6]

Cardiovascular diseases (CVD) include all heart and blood vessel diseases and despite the improved treatment, acute arterial ischemia is the major cause of mortality worldwide.[7] In most CVD, the main underlying pathology is atherosclerosis.[4] Therefore, it is important to understand the processes involved and identify new therapeutic targets to prevent the initiation, growth and rupture of an atherosclerotic plaque.

PLAQUE FORMATION

Dysfunctional changes in the endothelium induced by disturbed shear stress and the cumulative exposure to oxLDL over the years leads to endothelial cell (EC) activation.[2, 3] Activated ECs upregulate the expression of several adhesion molecules, such as ICAM-1, VCAM-1 and selectins, promoting leukocytes arrestment and adherence to the endothelium. These leukocytes subsequently migrate through the EC junctions by combined actions of PECAM-1 and chemokines produced in the intima.[7, 8] These infiltrated inflammatory leukocytes increase in number by local proliferation.[9, 10]

OxLDL, which can no longer be recognized by LDL receptors (LDLRs)[11], is taken up by macrophage scavenger receptors, thereby inducing foam cell differentiation, a hallmark in early atherosclerotic lesions.[12] Moreover, oxLDL particles also induce inflammation by furnish neo-epitopes that stimulate humoral and adaptive immunity.[13, 14] For instance, phosphorylcholine (PC) epitopes, which mediate the binding of oxLDL to scavenger receptors[15], are recognized as damage associated molecular patterns (DAMPs)[16], trigger complex immunoinflammatory responses, induce toxic oxidative stress, apoptosis, EC activation[17] and dysfunction[18].

Elevated LDL cholesterol levels can be reduced by statins and PCSK9 inhibitors. Both effectively control LDL cholesterol and reduce major adverse cardiovascular events (by ≈50%).[1] However, other risk factors, such as hypertension and insulin resistance, can also promote inflammation and/or aggravate atherosclerosis. Angiotensin II is implicated in hypertension pathogenesis, and activate nuclear factor-κB (NF-κB) pathway.[19] Insulin resistance, the underlying cause of type 2 diabetes mellitus, is associated with elevated levels of C reactive protein, plasminogen activator inhibitor-1, and fibrinogen.[20] These extravascular sites of

inflammation can affect distant artery walls, by releasing soluble inflammatory mediators such as cytokines.[21]

Atherosclerosis is also early accompanied by the migration of VSMCs from the media to the intima.[22, 23] Intimal VSMCs are considered to be the most important source of extracellular matrix (ECM) components, such as collagen, elastin, proteoglycans and glycosaminoglycans. [22, 23] These proteins can entrap lipoproteins within the intima,[24] undergo modifications, and thereby trigger inflammation. Until recently, most researchers considered monocyte-derived macrophages the only precursors of foam cells. However, oxLDL by binding to intimal proteoglycans, forms aggregates that are cleared by the LDLR-related receptors in VSMCs. VSMCs, like macrophages, become engorged with lipids, contribute to lesion growth.[25]

PLAQUE GROWTH AND PLAQUE INSTABILITY

An environment rich in growth factors and cytokines can trigger arterial wall remodeling. Proteases, such as MMPs, plasmin, cathepsin can degrade the ECM and stimulate the migration of VSMCs.[26-29] As VSMCs migrate from the media to the intima, they can lose their contractility, differentiating into a proliferative and secretory phenotype that can contribute to vascular wall thickening which on its turn can result in gradual loss of luminal patency.[30] ECM degradation products can act as endogenous ligands for toll-like receptors (TLRs), thereby driving inflammatory responses, such as the invasion of T cells, NK cells, and mast cells.[31] Inflammation and excessive protease activity can promote cellular apoptosis. Macrophages and VSMC, undergo in particular due to oxLDL uptake, programmed cell death being the basis of the necrotic core formation of the advance plaques.[32,

[33] However, advanced atherosclerotic plaques do not necessarily lead to clinical events. Plaques, with limited lipid accumulation and thicker fibrous caps can remain stable for years. but they may also in time, or eventually, become unstable.

Unstable plaques are characterized by necrotic core expansion, increased cholesterol content, calcifications, and ECM remodeling. ECM remodeling and VSMCs apoptosis decrease collagen synthesis, affecting VSMCs ability to maintain the skeleton of the fibrous cap.[34, 35]

Concomitantly, severe stenosis compromises oxygen diffusion (from vasa vasorum (VV) and lumen) and active inflammatory cells increase oxygen consumption, aggravating hypoxia.[36-38] A hypoxic large necrotic core drives strong changes in the plaque, such as intraplaque angiogenesis (IPA) and successively intraplaque hemorrhage (IPH), both frequently observed features of unstable plaques. Plaque rupture exposes thrombogenic material in the plaque core the ultimate and most dreaded complication of atherosclerosis.

INTRAPLAQUE ANGIOGENESIS AND INTRAPLAQUE HEMORRHAGE

Although angiogenesis arises as a natural physiological response to the increased oxygen demand in the necrotic core, IPA is a major plaque instability factor due to the active and immature nature of the neovessels.

During hypoxia, hypoxia-inducible transcription factors (HIF)-1α, HIF-2α and HIF-1β heterodimerize, triggering the expression of genes containing hypoxia-responsive elements, such as the vascular endothelial growth factor (VEGF). Activation of the VEGF receptor 2 (VEGFR2) in a quiescent vascular network, induces microvessel disturbance and EC sprouting.[39, 40] Through a VEGF gradient,

ECs proliferate and migrate towards the intima, **as extensively discussed in chapter 2.**

Also, during hypoxia, ANGPT2 is released from storage granules called Weibel–Palade bodies, antagonizing ANGPT1-Tie2 downstream signaling in ECs and in pericytes.[41, 42] ANGPT1 and ANGPT2, are highly homologous but exert different effects on the Tie2 receptor.[41, 43] ANGPT1, continuously secreted by mural cells, is a canonical agonist of Tie2 receptor, promoting vessel maturation by stimulating pericytes recruitment[44, 45] and junctional accumulation.[46, 47] ANGPT2 destabilizes the vasculature to potentiate the angiogenesis triggered by VEGFA[48], inducing junction internalization between ECs and pericytes.[49, 50] Microvessel growth is tightly controlled under physiological conditions, however, when the balance between pro- and anti-angiogenic molecules balances toward angiogenic inducers, neovessels maturation is strongly compromised.[51-53]

Neovessel maturation, which comprehends the structural integrity and composition of the endothelium, basement membrane and pericyte coverage, is a determining factor in the regulation of vascular permeability. Ruptured atherosclerotic plaques often present extended angiogenic microvasculature with poor pericyte coverage and lack of several junctions with a low ANGPT1/ANGPT2 ratio.[54]

Moreover, immature microvessels haven been co-localized with the presence of extravasated erythrocytes, constituting the main source of IPH.[55, 56] Erythrocytes trapped in the plaque tend to lyse delivering a potent combination hemoglobin (Hb), iron, phospholipids membranes and free cholesterol.[57]

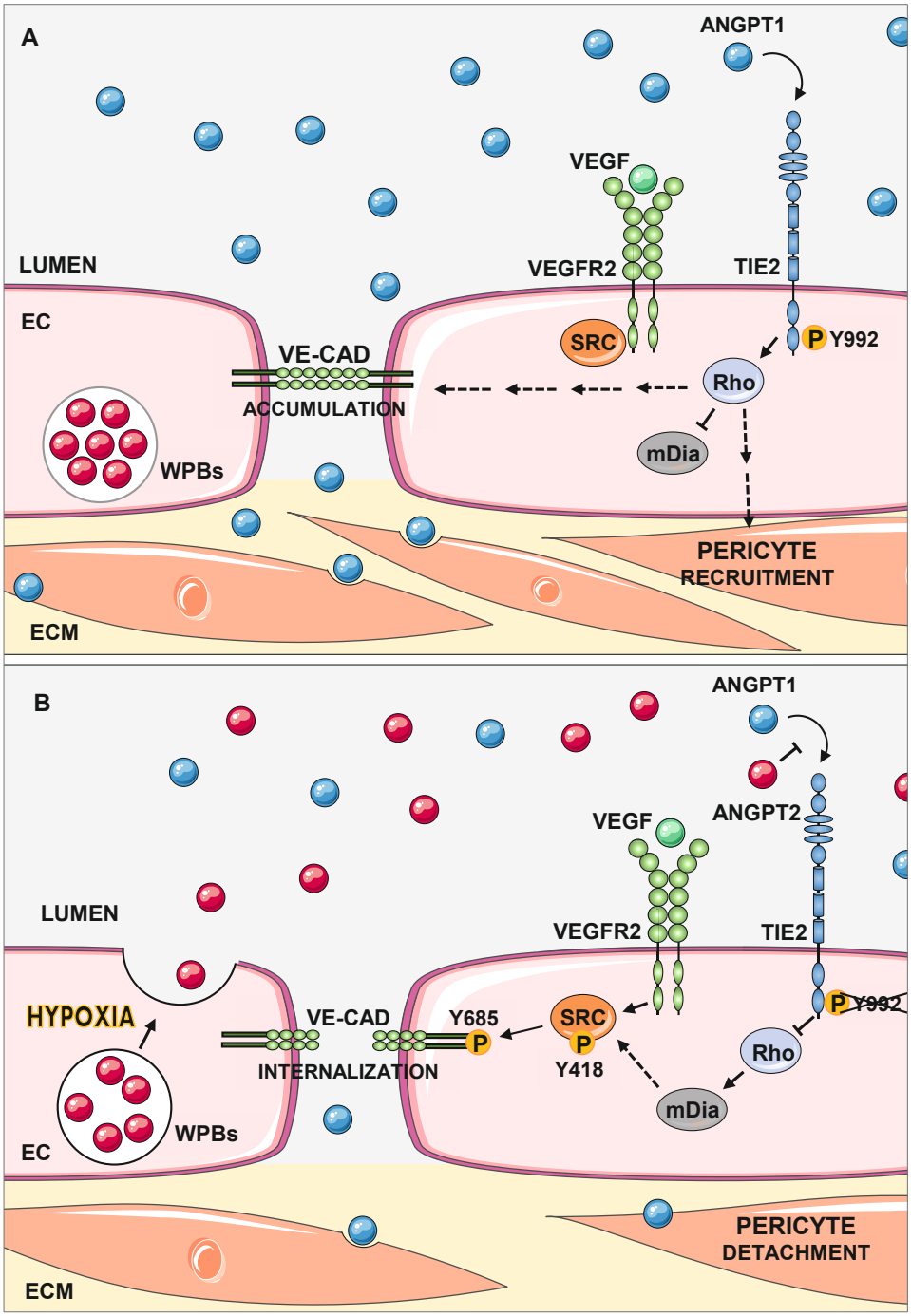

Figure 1. Graphical representation of the Angiopoietin-Tie2 axis. (a) Expression of the receptor Tie2 is heavily enriched in the endothelium. Tie2 is activated by ANGPT1 which

is secreted by platelets and peri-endothelial cells. Activation of Tie2 receptor, culminates in recruitment of pericytes and junctional accumulation between ECs. **(b)** ANGPT2 is almost exclusively expressed by EC and thereby acts as dynamic autocrine modulator of ANGPT1/Tie2 signaling. In the context of hypoxia (or even inflammation), ANGPT2 stored in Weibel–Palade bodies (WPBs) is released and antagonizes ANGPT1, inducing vascular leakage by VE-Cadherin (VE-CAD) junctional internalization.

Hb binds to haptoglobin (Hp) creating hemoglobin:haptoglobin (Hb:Hp) complexes. Intake of hemoglobin by the hemoglobin-haptoglobin receptor CD163 leads to a distinct alternative non-foam cell anti-inflammatory macrophage phenotype, previously considered atheroprotective.[58, 59]

Guo, Akahori, et al. showed that CD163+ macrophages (M($CD163^+$) are abundantly present in human atherosclerotic plaques, mainly near the neovascularized, VEGF-positive hemorrhagic areas.[58] Genetic analysis of cardiovascular disease patient cohorts revealed an association between an polymorphism that increases CD163 expression and plaque rupture and plaque angiogenesis (independently of traditional cardiovascular risk factors). Furthermore, these authors also showed that Hb:Hp uptake by macrophages increases iron export. M($CD163^+$) intracellular iron deprivation inhibits PHD2 activity, inducing of HIF1α activation. Consequently, $CD163^+$ macrophages secrete VEGF-A, promoting angiogenesis, vascular permeability and VCAM-1 endothelial expression that enhances inflammatory response.[60]

Additionally, iron is also strong lipid oxidizer, and extravasated erythrocytes increase plaque lipid content by accumulation of free cholesterol and phospholipidic membranes.[57, 61] In combination with the impaired mechanisms regulating the clearance of debris and apoptotic cell in advance plaques, IPH drives necrotic core expansion by feeding a vicious cycle of IPA and inflammation.[62, 63.]

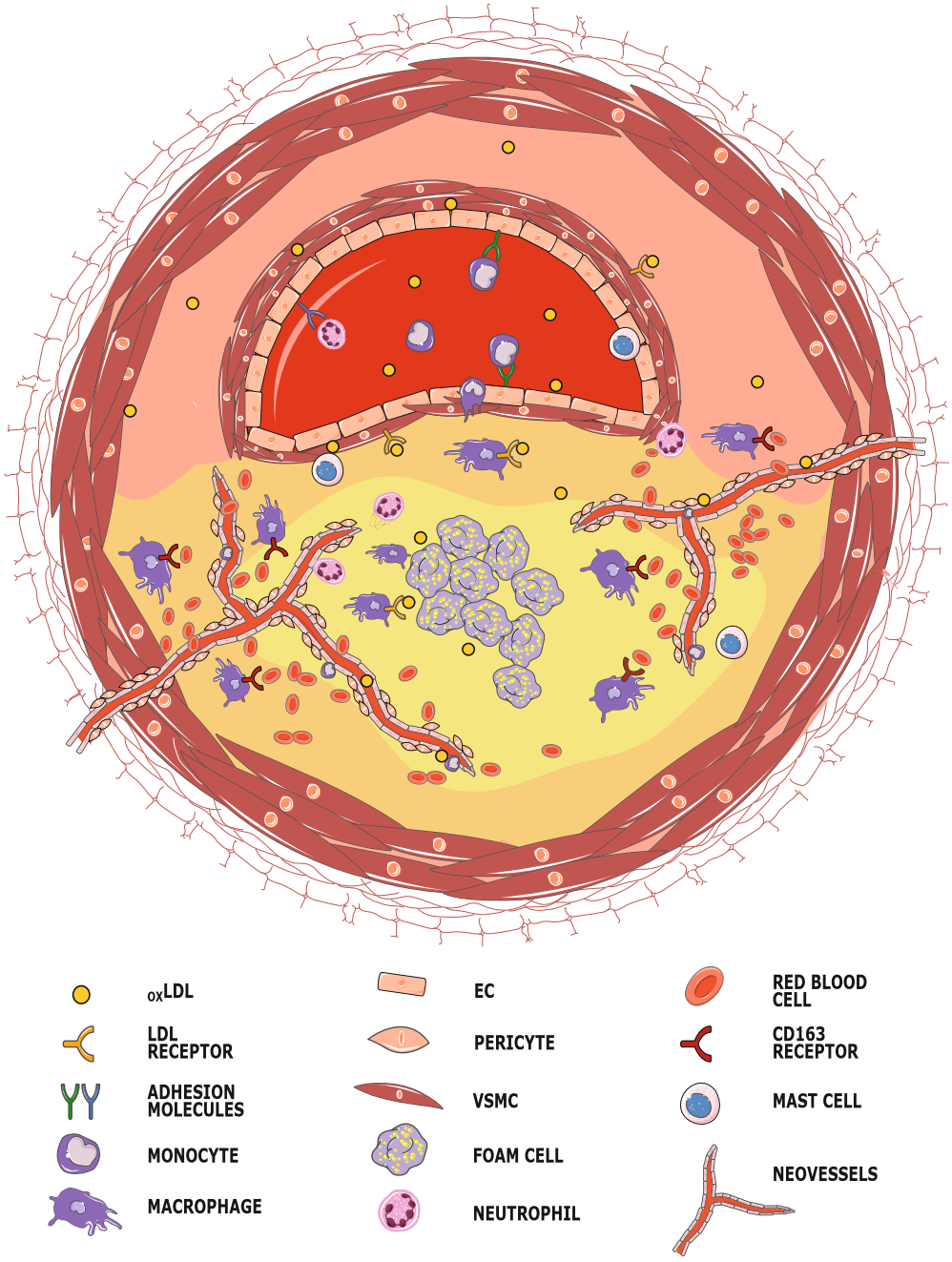

Figure 2. Plaque angiogenesis. Once in the plaque macrophages engulf oxLDL and become lipid filled foam cells. Their accumulation activates cytokine production that promotes the influx of neutrophils, mast cells and monocytes. The high oxygen consumption of these inflammatory cells leads to hypoxia. HIF-1α and VEGF, together with an unbalanced

presence of the destabilizing factor Ang2 bound to its receptor Tie2, trigger the formation of leaky neovessels. (Magnification) Intraplaque hemorrhage, with extravasation of red blood cells and inflammatory cells, is due to immature neovessels, lacking proper enveloping pericytes and poor tight junctions between endothelial cells. Macrophages phagocytize extravasated red blood cells.

VEIN GRAF FAILURE

Vein graft (VG) surgery is part of the standard revascularization strategies for patients with coronary and peripheral artery diseases and more than two million surgeries are performed worldwide annually.[64, 65] VG surgery can markedly improve survival and symptoms in selected patients, however within in one month after surgery, 10% of the VGs fail due to acute thrombosis. After one year, 15% of the VG occlude due to pathological intimal thickening. By 10 years after surgery, only 60% of VG are still patent, pointing out that VG failure (VGF) is a serious clinical problem.[66-68] VGF results from complex pathophysiological processes that lead to a partial or complete occlusion of the graft. The progression of VGF over time involves vascular wall remodeling and inflammation as central processes throughout all distinct phases, **as extensively discuss in the chapter 5.**

Vessel narrowing, unstable atherosclerotic lesions and subsequent plaque rupture are the main causes of late VGF.[67, 69] Although the start of the lesion deviates as well as some morphological differences, there is a marked resemblance between vein graft accelerated atherosclerosis and native atherosclerotic lesions. Therefore, VG surgery in a preclinical model mouse model has been used to study instability features of advance atherosclerosis lesion, such as intraplaque angiogenesis and intraplaque hemorrhage, as will be discussed in the next section.

MODELS FOR ADVANCED ATHEROSCLEROSIS

The use of animal models for accelerated atherosclerosis is essential to understand the pathophysiological mechanisms behind plaque rupture since it is a systemic process with multiple organs and tissues involved; and is of importance to study the potential of (new) treatments for plaque instability.

Mice have a different lipid profile compared to humans (most of the cholesterol is transported in HDL instead of the atherogenic LDL and VLDL), making them relatively resistant to atherosclerosis. However, due to their fast reproduction, easy genetic manipulation, and introduction of modifying diets (such as hypercholesterolemic and high-fat) and additional atherosclerosis risk factors (such as diabetes), mice became prevalent in experimental atherosclerosis.[70, 71]

APOE AND LDLR KO MICE

ApoE KO mice and LDLR KO mice, the most common used models for atherosclerosis,[72] result from the genetic deletion of LDLR (that withdraws LDL from the blood) and ApoE (a ligand for the LDLR on LDL and VLDL).[70] Such deficiencies result in increased cholesterol plasma levels, driving the formation of spontaneous atherosclerosis in regions of low and/or oscillatory shear stress.[73] Furthermore, hypercholesteremic and high-fat diets further increase cholesterol plasma levels, resulting in a faster and more advanced atherogenic phenotype.[74] However, LDLR and ApoE are crucial proteins in LDL clearance, and due to their absence, established human lipid-lowering drugs such as statins, fail to lower cholesterol plasma levels in ApoE KO mice.

APOE3*LEIDEN MICE

ApoE3*Leiden mice are a transgenic strain generated using a genomic 27-kilobase DNA construct (containing the ApoE gene, ApoC1 gene and all regulatory elements) found in a large Dutch family with a genetic form of hyperlipidemia.[75, 76] Due to the defective binding of the ApoE3*Leiden protein to the LDLR, mice have an impaired clearance of chylomicron and VLDL remnant lipoproteins.[75, 76] As a consequence, ApoE3*Leiden mice are able to develop diet-dependent hyperlipidemia and are highly susceptible to diet-induced atherosclerosis. Contrary to ApoE and LDLR-KO mice, in which atherosclerosis is not diet dependent, ApoE3*Leiden mice allows titration of cholesterol plasma levels with different cholesterol content diets, **as discussed in chapter 4**.

Although ApoE3*Leiden mice are less prone to spontaneous atherosclerosis than ApoE KO, when fed with hypercholesterolemic diets, ApoE3*Leiden atherosclerotic lesion are similar to ApoE KO. Additionally, the ApoE3*Leiden lipoprotein profile resembles the human situation more closely.[77]

Moreover, they have the ability to synthesize functional mouse ApoE, which is useful to study atherogenesis (as they offer a possibility to evaluate increased lipid levels effects without disturbing macrophage functions[78]) but also LDL-lowering drugs (and pleiotropic) effects, **as discussed in chapter 4.** Also, ApoE*3 Leiden mice express ApoE in the liver and in macrophages, keeping them able to respond the statins (as discussed below in more detail), whereas in ApoE KO mice this does not occur. Therefore, the hypercholesterolemic ApoE3*Leiden is considered one of the atherosclerosis models closest to humans.[79, 80]

Hypercholesterolemic APOE3*LEIDEN Vein Graft Model to study Intraplaque Angiogenesis and Intraplaque Hemorrhage

Because similarities were found between lesions in VG and atherosclerosis, a murine model of VG graft disease has been established. One of the most frequently used models for vein graft disease is the model described by Xu et al.[80]

In this model, the caval vein of a donor mouse is interpositioned in the carotid artery of a receiver mouse. The carotid artery is dissected from its surrounding from the bifurcation at the distal end toward the proximal end. On both the proximal and distal artery end a nylon cuff is sleeved and fixated on the cuff-handles with hemostatic clamps. After dissecting the ligature from the distal artery end, the artery is everted over the cuff and this procedure is repeated on the proximal artery end. The caval vein is fixed over both the cuffs with ligatures. Pulsatile flow through the venous conduit confirms a successful procedure. Within 28 days after the surgery, a vein graft develops from a few cell layers at the start of the engraftment up to a massive thickened vessel wall. After surgery, the endothelial cell lining of the graft is severely damage due to handling and dilatation.

These VG procedure has been used in both, ApoE KO and ApoE3*Leiden mice, and their VG lesions presented typical histological features of late-stage atherosclerosis.[81-83] Hypercholesterolemic ApoE3*Leiden VG lesions, in particular, show severe VG thickening (up to 50 times the original), microcalcifications, cholesterol clefts, lipid-loaded foam cell accumulation dissections and erosion areas, 28 days after surgery.[79, 81, 82, 84] Remarkably, these lesion present vasa vasorum derived neovascularization stimulated by hypoxia[84], with the unique presence leaky intimal neovessels and intraplaque hemorrhage.[62] The majority of

plaque neovessels in VG have heterogeneous basement membrane and poor pericyte-coverage related to an ANGPT1/ANGPT2 unbalance.[62, 84] Moreover, CD163 macrophages can be abundantly found throughout the vein graft lesion but mostly in close proximity of neovessels.[62]

The fact that hypercholesterolemic ApoE3*Leiden VG lesions show a complete range of plaque destabilization features occurring within four weeks, emphasizes its advantages to study rupture-prone atherosclerotic plaques.

IMAGING OF INTRAPLAQUE ANGIOGENESIS

Imaging of vulnerable plaque features can represent a step forward in the detection of high-risk atherosclerotic patients. Significant investments have been made in the cardiovascular imaging field, and the most prominent human imaging technologies, such as PET, MRI and CT have shown to be crucial for detection of plaque angiogenesis, (**as discussed in Chapter 2**).[34] However, these clinical imaging techniques do not have sufficient resolution to image the detailed microvessel network in small-sized preclinical animal models, providing low benefit when it comes to the development of target-tracers and target-therapeutic approaches. Therefore, high-resolution hardware has emerged to visualize the microvasculature and explore cellular events in small size animals, *in vivo.*[60]

Intravital microscopy (IVM), in particular, due to its high resolution provides unparalleled insights into dynamic molecular aspects of angiogenesis.[85, 86] IVM was first described in the 19th century by Wagner, who applied brightfield microscopy to visualize leukocyte trafficking in translucent tissues of a frog.[87] Under visible light, intravascular leukocytes appeared colorless, and only cells

slowed by adhesive processes could be distinguished from rapidly flowing cells in the background.[88]

Currently, it can be performed using several light microscopy techniques including widefield fluorescence, confocal, two-photon (2P), and others. The main considerations rely on the depth and/or detail needed to image the area of interest. If the area of interest is more than 50–100 μm depth, which is the case of plaque neovessels, two-photon (2P)-IVM is required.[89] Equipped with fluorescence-tracers and pulsed infrared two-photon laser, 2P-IVM allows visualization and quantification of microvessel permeability in real time.

2P-IVM differs from traditional fluorescence microscopy, in which the excitation wavelength is shorter than the emission wavelength, as the wavelengths of the two exciting photons are longer than the wavelength of the resulting emitted light.[90] 2P-IVM typically uses infrared light to minimize scattering. Together these effects increase the light penetration depth, with a good signal/background ratio until 500 μm deep.[90] Moreover, 2P-IVM pulsed approach decreases phototoxicity, allowing recurrent imaging over prolonged periods.[89] Remarkably, the development of imaging windows, allows long-term 2P-IVM to track single EC proliferation and migration in the same area in living animals, with need for recurrent surgeries.[91]

The usefulness of these techniques, however, can be adversely affected by limited possibilities of a unified standard of quantification software. Most manufacture software is aimed for general applications, lacking specific software tools for quantification of more detailed aims.

Nevertheless, 2P-IVM can be an interesting tool to study intraplaque leaky neovessels in murine advance atherosclerotic lesions, **as discussed in chapter**

3. Moreover, these techniques can potentially improve in preclinical assessment of antiangiogenic therapies.

OUTLINE OF THE THESIS

Despite the available treatment options and sophisticated imaging technologies for monitoring lesion development, the morbidity and mortality from acute cardiovascular events remain unacceptably high.

While cholesterol-lowering, anti-inflammatory and anti-platelet therapies benefits can increase survival as a primary or secondary prevention, they are not sufficient for plaque rupture prevention. Moreover, the most advance imaging technologies to detect high-risk atherosclerotic patients fail to visualize and explore cellular events in small preclinical models. Therefore, there is a clear need for the development of new therapies and the application of high-resolution imaging modalities.

In the current thesis, we evaluated new possibilities to inhibit and image intraplaque angiogenesis, as highlighted by the following scope:

In **Chapter 2**, we review the new insights of atherosclerotic neovascularization, including the effects of leaky neovessels on intraplaque hemorrhage, both in experimental animal models and humans. Moreover, we give an overview of therapeutic interventions targeting angiogenesis and *in vivo* imaging modalities used to study atherosclerosis.

In **Chapter 3,** we use 2P-IVM to visualize and study the architecture of adventitial and intimal plaque neovessels in murine advanced atherosclerotic vein graft lesions. Moreover, we report a 2P-IVM method to assess passive diffusion by

quantification of labeled-dextrans extravasation in healthy microvessels as well as plaque microvessels in real time.

In **Chapter 4,** we evaluate both, the lipid lowering-dependent and independent effects of atorvastatin on vein graft atherosclerosis, including intraplaque angiogenesis and intraplaque hemorrhage. We also present evidence of the pathophysiological and molecular mechanism of atorvastatin-mediated inhibition on neovascularization.

In **Chapter 5**, we review the pathophysiological mechanisms underlying the development of vein graft failure, emphasizing the role of immune response and associated factors related to VG remodeling and failure. Moreover, we discuss potential therapeutic options that can improve patency based on data from both preclinical studies and the latest clinical trials.

In **Chapter 6**, we describe the effect of a newly constructed humanized monoclonal antibody against phosphorylcholine PC (PCmAB) on accelerated atherosclerotic lesions in ApoE3*Leiden mice. PC is one of the main oxLDL epitopes and plays a central role in atherosclerosis, triggering complex immunoinflammatory responses, EC activation and dysfunction. We investigate the role of PCmAB on vascular remodeling, plaque stability, inflammation as well as on intraplaque angiogenesis and intraplaque hemorrhage. Additionally, we investigated the isolated effect of PCmAB in in vitro angiogenesis assays and $CD163^+$ macrophage cultures.

In **Chapter 7**, a summarizing discussion of the different chapters and future perspectives, concludes this thesis.

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