Targeting glycolysis in endothelial cells to prevent intraplaque neovascularization and atherogenesis in mice

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Chapter 1

General introduction

Atherosclerosis

Atherosclerosis is a progressive inflammatory disease characterized by formation of lipid plaques inside the arterial vessel wall. Over time, plaque building can cause narrowing of the arterial lumen and lead to atherosclerotic vascular disease (i.e. coronary artery disease, peripheral artery disease).[1]

Although plaques often remain stable for years, they can also rapidly evolve to become unstable and rupture. This sudden event triggers intraluminal thrombus formation that can cause major adverse cardiovascular events such as acute coronary syndrome (ACS), myocardial infarction (MI) and stroke.[2 3] Elevated levels of low-density lipoprotein (LDL) in plasma is a major risk factor of atherosclerosis. LDL accumulates in the sub-endothelial space of the arterial wall and progressively undergoes oxidative modifications to form oxidized LDL (oxLDL).[4] This induces an inflammatory response characterized by overexpression of chemotactic proteins such as monocyte chemoattractant protein-1 (MCP-1), and adhesion molecules (vascular cell adhesion molecule-1 [VCAM-1], E-selectin and P-selectin) by endothelial cells (ECs).[5, 6] These adhesion molecules promote the infiltration of blood-carried monocytes into the inflamed arterial wall. After differentiation into macrophages, these cells engulf oxLDL and transform into foam cells which contribute to plaque development by secreting multiple mediators of the inflammatory process in the vessel wall.[7] The inflammatory response also promotes recruitment of circulating monocytes and T-cells that stimulate the migration of vascular smooth muscle cells (VSMCs) from the tunica media into the sub-endothelial space where they exhibit abnormally high proliferation and secrete extracellular matrix proteins that also contribute to atheroma growth.[5]

Advanced human plaques are characterized by a large necrotic core, many lipid-laden and activated macrophages, few VSMCs, intraplaque (IP) neovascularisation and haemorrhages, and a thin fibrous cap that separates the plaque from the blood stream.[8-10] Rupture of the fibrous cap of such high-risk vulnerable plaques leads to luminal thrombosis, arterial occlusion or embolism in distant vascular beds, resulting in MI, stroke or sudden death.[11] Figure 1 summarizes the components that can be usually found around the site of the atherosclerotic plaque.

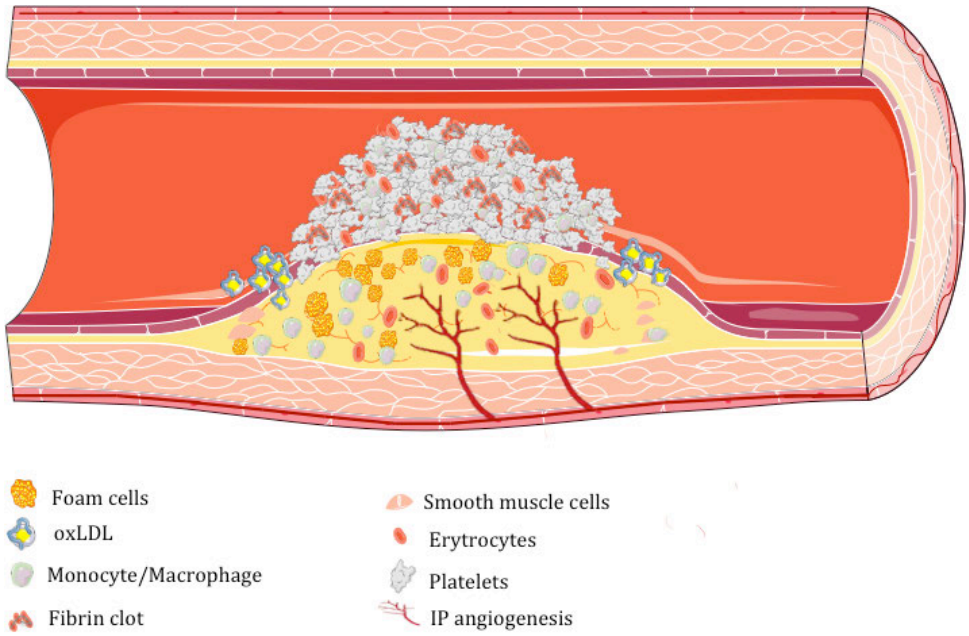

Figure 1. Schematic representation of the commonly observed components during various stages of plaque progression.

Endothelial cell dysfunction and subsequent accumulation of low-density lipoproteins (LDL) in the intima of the vessel wall are the early step of atherosclerotic plaque formation. The expression of adhesion molecules on the endothelium stimulates the recruitment of monocytes and T cells into the subendothelial space. After differentiation, macrophages turn into foam cells by “ingesting” oxidized LDL. The migration of vascular smooth muscle cells from the media into the plaque is promoted by growth factors and cytokines, derived from macrophages and T cells. Subsequent production of collagen results in the formation of a thick, protective fibrous cap. In response to hypoxia, immature intraplaque microvessels penetrate into the plaque. The atherosclerotic plaque becomes vulnerable because of a thinning fibrous cap and the formation of a large necrotic core. Plaque rupture exposes pro-coagulant material to the blood, thereby triggering thrombus formation.

Intraplaque neovascularization in advanced atherosclerosis

Intraplaque (IP) angiogenesis is a complex process that depends on the equilibrium between several pro- and anti-angiogenic molecules.[12] The presence of hypoxia in advanced atherosclerotic plaques correlates with IP angiogenesis in human carotid

arteries.[13-15] The response to low O_2 is mostly mediated by Hypoxia-inducible factors (HIFs), a family of transcription factors that control the expression of multiple pro-angiogenic mediators including vascular endothelial growth factor (VEGF), angiopoietin-1, angiopoietin-2, Tie2, platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), and monocyte chemoattractant protein–1 (MCP-1). Activation of HIF pathway induces localized angiogenesis by enhancing vascular permeability, endothelial cell proliferation, sprouting, migration, adhesion and tube formation.[16] From a molecular point of view, HIFs are heterodimeric proteins composed of one α and one β subunits. The α chain confers oxygen sensing to the complex and its expression is hypoxia-dependent. Each subunit has three isoforms expressed by three different genes, HIF-1α, 2α,3α and HIF-1β, HIF-2β HIF-3β HIF-1α and HIF-1β are widely expressed in normal tissues. The beta chain (three isoforms) is constitutively expressed and works as an aryl receptor nuclear translocator. Under normoxic conditions, the synthesized HIF-1α is rapidly degraded and the co-activators are blocked by oxygen-dependent enzymes; the prolyl-hydroxylases domain (PHD) enzymes. During hypoxia, PHD activity is reduced, allowing the dimerization of HIF-1α and HIF-1β. This active complex translocate to the nucleus where it binds to DNA and promotes transcription of downstream genes important for angiogenesis.[17, 18] Furthermore, it has been shown that HIF1α induces a switch from oxidative phosphorylation towards glycolysis which in turn regulates EC rearrangement during vessel sprouting.[19]

Besides hypoxia, inflammation is a strong inducer of angiogenesis as it promotes the synthesis of various angiogenic factors. During acute inflammation, several pro-angiogenic molecules can induce cell permeability, contributing to the infiltration of leukocytes in the inflammatory core and thereby provoking chronic inflammation.[20] Because the newly formed vessels growing into the plaque are immature, they are inherently leaky, permitting inflammatory cell infiltration and influx of blood constituents (including erythrocytes and blood platelets) into the plaque (Figure 2).[21] Moreover, IP neovessels can promote the entry of leukocytes into the plaque by upregulation of adhesion molecules such as ICAM-1 and VCAM-1.[22] The increased release of matrix metalloproteinases from activated macrophages and proteases secreted from mast cells cause further damage to the microvessels and facilitate IP haemorrhage.[23, 24]IP neovascularization also increases the risk of blood

haemorrhage inside the plaque which promotes plaque progression and instability.[25,26]

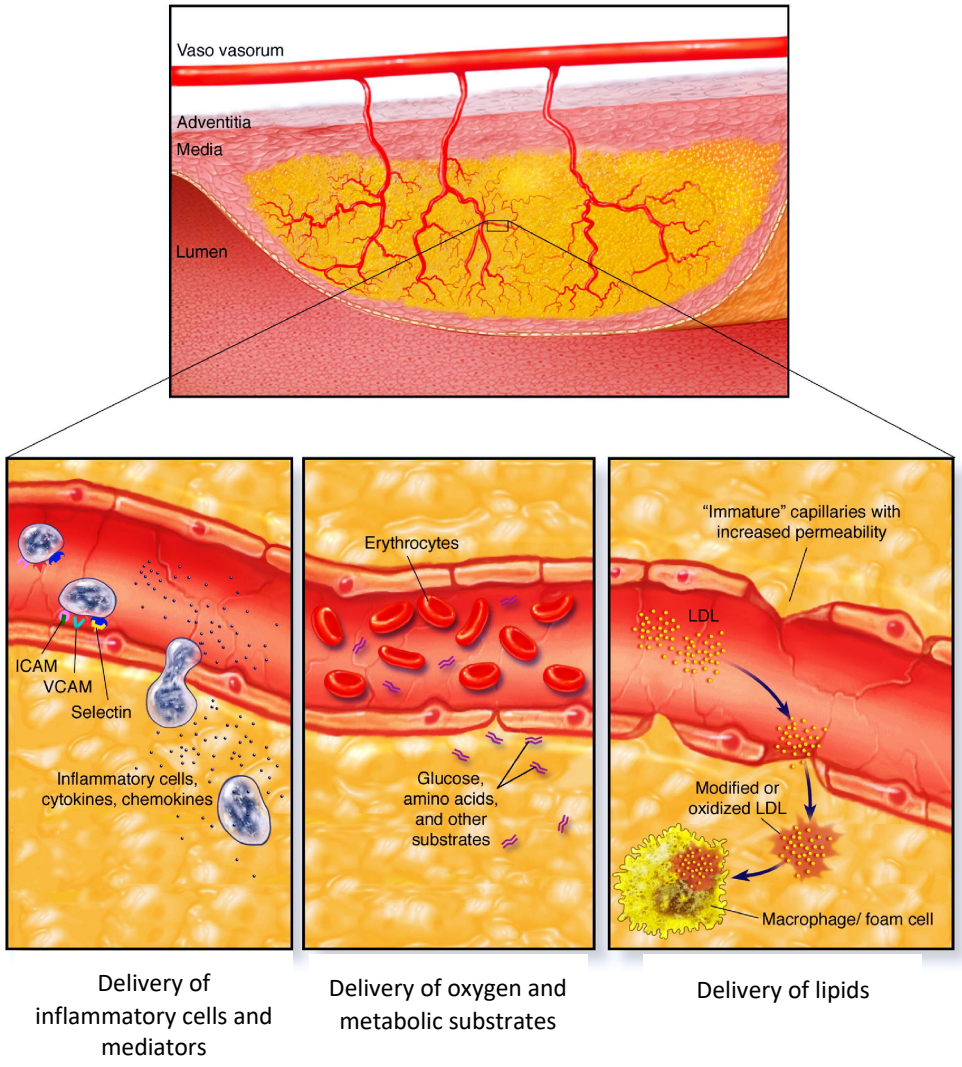

Figure 2. Contribution of intraplaque neovessels to plaque destabilization. (Adapted from Doyle et al. [27])

IP neovessels are often leaky and show high expression of vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1). These conditions further facilitate the entry of pro-inflammatory cells, erythrocytes and low-density lipoprotein (LDL) in the plaque microenvironment.

Intraplaque neovascularization: a novel therapeutic target in advanced atherosclerotic plaques

Thanks to cholesterol-lowering drugs (such as statins), the lifespan and wellbeing of patients has significantly improved. Recently, several new therapies have also emerged to treat high-risk patients (e.g. PCSK9 monoclonal antibodies to reduce the residual cholesterol risk).[28-31] However, a large group of patients does not fully benefit from current lipid-lowering strategies.[32] Indeed, despite major advances in cardio- and cerebrovascular research, plaque rupture remains the leading cause of acute events.[33, 34] Therefore, additional therapy that reduces atherosclerosis or prevents plaque rupture and its complications is needed.

Recent evidence suggests that IP angiogenesis promotes atherosclerosis progression and plaque destabilization.[35] Indeed, clinical data have found a strong association between IP angiogenesis and progressive unstable vascular disease.[8] Autopsy studies, for example, revealed a higher density of IP vasa vasorum microvessels in symptomatic (ruptured) plaques compared to asymptomatic ones.[24, 35] A comprehensive review of the published studies about the evidence and the impact of IP angiogenesis in human atherosclerosis progression can be found in **chapter 2** of this thesis**.**

Glycolysis inhibition as a novel approach to block IP angiogenesis and to promote plaque stability

Glycolysis is a metabolic process that converts glucose into two molecules of pyruvate and generates 2 molecules of ATP (adenosine triphosphate), 2 NADH (reduced nicotinamide adenine dinucleotide), and two water molecules (Figure 3).

Recent studies have demonstrated that proliferating ECs mainly rely on high glycolytic activity (>200-fold higher than glucose, fatty acid and glutamine oxidation), to generate up to 85% of the total cellular ATP content.[36] Although anti-VEGFA therapy has been widely used to inhibit neovascularization in oncology and eye

disease, modulation of EC metabolism has recently shown beneficial effects in cancer research, and this approach may represent an attractive new strategy to inhibit neovascularization in atherosclerosis as well.[37, 38] Further details about this approach are described in **chapter 2** of this thesis**.**

Vascular endothelial growth factor receptor-2 (VEGFR-2) signalling and expression of glucose transporter-1 (GLUT-1) as well as that of glycolytic enzymes, such as phosphofructo-2-kinase/fructose-2,6-bisphosphatases (PFKFB3) are increased in ECs under inflammatory conditions, (Figure 3).[39, 40] PFKFB3 is a key enzyme that modulates glycolytic flux and it has also been shown to promote EC migration. Deletion of this enzyme in ECs leads to defects in angiogenesis both *in vitro* and *in vivo.*[41]

Furthermore, it has been demonstrated that in ECs, normal atheroprotective blood flow increases the activity of the flow-responsive transcription factor Kruppel Like Factor 2 (KLF2) which, in addition to increasing VE-cadherin expression and barrier function, transcriptionally represses PFKFB3 expression and lowers glycolytic rates to sustain EC quiescence. Atheroprone turbulent flow lowers KLF2 activity and VE-cadherin levels, and upregulates PFKFB3 expression causing increased glycolysis and EC activation. Additionally, in atheroprone regions, low shear stress and oxLDL induce miR-92a expression to lower KLF2 levels.[39] Based on these findings, PFKFB3 could play an important role also on atherosclerotic plaques stabilization.

Previous studies in the oncological field presented a small molecule 3PO [3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one], which transiently inhibits glycolysis in proliferating ECs and impairs pathological angiogenesis in vivo without interfering with the metabolism of healthy cells.[42] 3PO targets the hyper-metabolism that is induced when ECs switch from quiescence to proliferation and migration.[43]

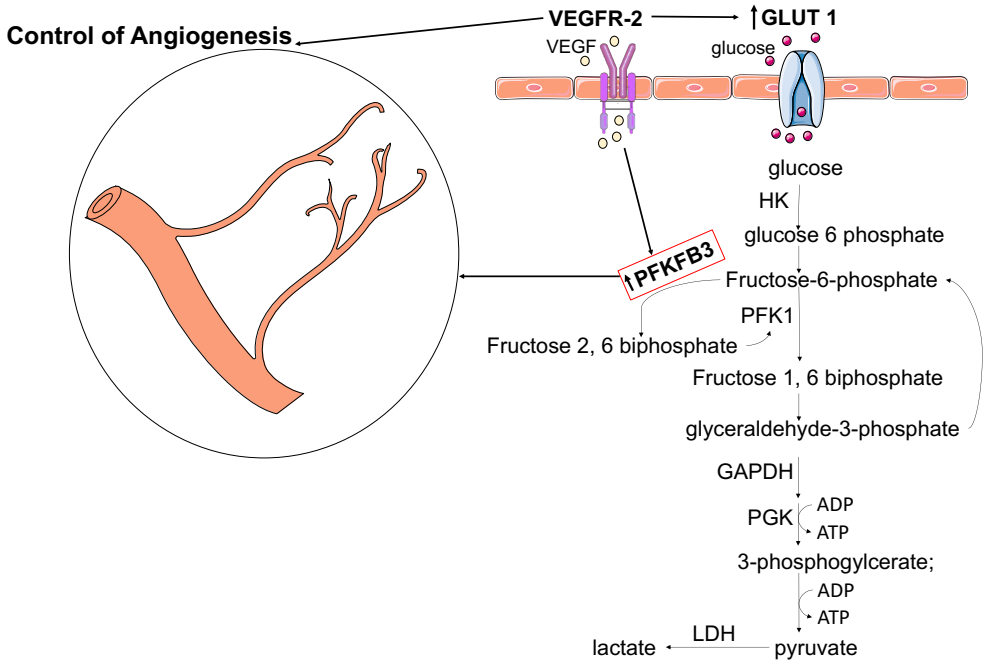

Figure 3. ***Glycolysis in ECs and its relation with angiogenesis***. Activated angiogenic endothelial cells rely mostly on glycolysis for ATP generation. Indeed, VEGFR-2 signalling (a major proangiogenic factor) increases the expression also of GLUT-1 as well as of other glycolytic enzymes, such as PFKFB3. HK (Hexokinase); PFK1 (Phosphofructokinase-1); GAPDH (Glyceraldehyde-3-Phosphate Dehydrogenase).

Animal models that develop vulnerable plaques with intraplaque angiogenesis

According to the American Heart Association (AHA), a 'vulnerable plaque' is defined as a lesion susceptible to complications, and identifies all thrombosis-prone plaques and those ones with a high probability of undergoing rapid progression. These plaques are often modestly stenotic lesions with specific morphological characteristics including a large core of lipid deposits, IP neovessels, necrotic cell debris and macrophages producing matrix-degrading enzymes such as metalloproteinases, with thinning of the fibrous cap.

These lesions as described above, are prone to rupture and may cause acute cardiovascular complications.[44]

In vivo studies with experimental animal models are essential to gain detailed insight into the molecular mechanisms of atherosclerotic plaque vulnerability and to develop better diagnostic and therapeutic tools for imaging IP neovessels. Over the years the most extensively characterized strains are apolipoprotein E-knockout ($ApoE^{-/-}$) mice and low-density lipoprotein receptor-knockout ($LDLr^{-/-}$) mice.[45] Although these models have permitted the study of atherogenic processes, to date they often do not develop vulnerable plaques with IP neovessels. This limitation can be overcome using the $ApoE^{-/-}Fbn1^{C1039G+/-}$ strain or other established mouse model of atherosclerosis (e.g.,$ApoE^{-/-}$, APOE*3-Leiden) that undergo vein grafting procedure. **Chapter 3** of this thesis presents all the current animal models of atherosclerosis including the animal models that have been used for the experimental work of this thesis, namely $ApoE^{-/-}Fbn1^{C1039G+/-}$ mice and regular $ApoE^{-/-}$ mice (with or without vein grafting).

Apolipoprotein E-deficient Fibrillin-1 mutant ($ApoE^{-/-}Fbn1^{C1039G+/-}$) mice

The extracellular matrix of atherosclerotic plaques is a complex network of predominantly elastin and collagen, which is essential to provide structural, adhesive and biochemical signalling support to the vessel wall. In elastic arteries, elastin is the most abundant protein. The elastic fibres comprise the elastin core, which is surrounded by a mantle of fibrillin-rich microfibrils.[46] The elastic-fibre-associated microfibrils have as the main structural component fibrillin-1, a large glycoprotein of about 350 kDa, whose major role is in binding and sequestering growth factors, such as transforming growth factor-β (TGF-β), as well as providing the scaffold for the deposition and the cross-linking of elastin.[47, 48]

Recently, our group reported the effect of an impaired elastin structure of the vessel wall on the progression of atherosclerosis by cross-breeding $ApoE^{-/-}$ mice with mice containing a heterozygous mutation ($C1039G^{+/-}$) in the fibrillin-1 (Fbn1) gene.[48] Mutations in the Fbn1 gene lead to the Marfan syndrome, a genetic disorder characterized by fragmentation of elastic fibres.[49] This results in increased arterial stiffening, elevated pulse pressure and progressive aortic dilatation.[48, 50, 51]

Moreover, the mutation leads to the development of highly unstable plaques in ApoE$^{-/-}$ mice, resulting in spontaneous plaque rupture with clinical end-points such as MI and sudden death.[48, 52] Importantly, these events do not – or only very occasionally – occur in ApoE$^{-/-}$ mice on a Western-type diet or in ApoE$^{-/-}$Fbn1$^{C1039G+/-}$ mice fed a normal diet.[52, 53] These findings underscore the importance of elastin fragmentation in combination with a Western-type diet as prerequisites for atherosclerotic plaque rupture in mice.

ApoE$^{-/-}$Fbn1$^{C1039G+/-}$ mice have significantly larger plaques with a highly unstable phenotype, characterized by a large necrotic core (occupying about 30% of total plaque area), and a strongly diminished collagen content. Accelerated atherogenesis in these mice is likely the result of enhanced vascular inflammation, leading to increased monocyte attraction, oxidation and accumulation of lipids.[54]

Extensive neovascularisation and IP haemorrhages consistently occur in the brachiocephalic and common carotid arteries of ApoE$^{-/-}$Fbn1$^{C1039G+/-}$ mice on Western-type diet. These features are rarely seen in murine atherosclerosis models but are known to highly affect plaque progression and vulnerability in humans.[35] In ApoE$^{-/-}$Fbn1$^{C1039G+/-}$ mice on a Western-type diet, IP neovessels, likely arising from adventitial vasa vasorum, clearly sprout out of the media.[55, 56] Neovessels are not only present at the base of the plaque but are also frequently observed in its centre, similar to the human pathology.[35, 56] Angiogenesis requires extracellular matrix degradation by proteases, including MMPs, to enable EC migration into the surrounding tissue.[57] In addition, degradation of the extracellular matrix induces the release of sequestered angiogenic factors such as vascular endothelial growth factor (VEGF) and TGF-β,[57, 58] also observed in ApoE$^{-/-}$Fbn1$^{C1039G+/-}$ mice on Western-type diet. The extent of neovascularisation in ApoE$^{-/-}$Fbn1$^{C1039G+/-}$ mice correlates with the degree of elastin fragmentation in the vessel wall. However, degradation of the extracellular matrix alone is not sufficient to induce neovascularisation in atherosclerotic plaques, because neovessels are not present in plaques of ApoE$^{-/-}$Fbn1$^{C1039G+/-}$ mice on a normal diet. Moreover, the presence of IP erythrocytes near neovessels at the base of the plaque points to intraplaque haemorrhages, substantiating ruptured neovessels as a source of IP bleeding.[35, 58, 59] Erythrocytes are important sources of free cholesterol, thereby increasing necrotic core size. Hence, neovascularisation, besides supplying plaques with leukocytes and

lipoproteins, can promote focal plaque expansion when neovessels rupture or become thrombotic.[35, 59, 60] These observations in ApoE$^{-/-}$Fbn1$^{C1039G+/-}$ mice are in line with current concepts of human vulnerable plaques. Therefore, ApoE$^{-/-}$Fbn1$^{C1039G+/-}$ mice on a Western-type diet offer the opportunity to investigate the role of key factors involved in plaque destabilisation, including IP neovascularisation, which will provide more insight into the mechanisms of plaque disruption and potential targets for therapeutic interventions.[52, 61-63]

Vein grafting in Apolipoprotein E deficient mice

Vein bypass grafting is a cardiovascular surgical practice for revascularization of occluded atherosclerotic arteries. The great saphenous veins are normally used as conduits for bypassing the occlusion.[64] Multiple lines of evidence show that 40% of the patients suffer from bypass failure within eight years and about 10% of all vein grafts fail due to acute thrombosis as a result of technical problems and de-endothelializations.[65-67] The high failure rate is due to the fact that veins grafted into an arterial environment undergo a complex vascular remodeling process resulting in intimal hyperplasia, which can lead to the rupture of the graft. These events are due to complex, dynamic interactions between various cell populations including ECs, mesenchymal smooth muscle-type cells, macrophages, activated thrombocytes and infiltrating inflammatory cells.[64, 66-68]

An important role in vein graft remodeling and subsequent rupture is played by ECs which have the ability to switch between a mature quiescent and an angiogenic state.[69] In recent years several mouse vein graft models have been described. These models vary in the location in which the graft is placed, primary in the carotid artery or the aorta. One of the most frequently used models for vein graft disease is the model described for the first time by the group of Xu [70] and currently used also by our group. In this model, the caval vein of a donor mouse is interpositioned in the carotid artery of a receiver mouse (Figure 4).

When this procedure is performed in apolipoprotein E-knockout mice (ApoE$^{-/-}$) or ApoE3*Leiden mice, a vein graft with high unstable lesions and IP angiogenesis is formed.[71] It has been demonstrated that these neovessels in vein graft lesions are dysfunctional, immature and they contribute to make the plaque bigger and unstable

by enhancing leukocyte recruitment and accumulation of cholesterol and platelets.[72, 73] Therefore vein grafting in ApoE$^{-/-}$ mice is an interesting and useful model to study potential therapeutic strategies in order to stabilize atherosclerotic lesions.

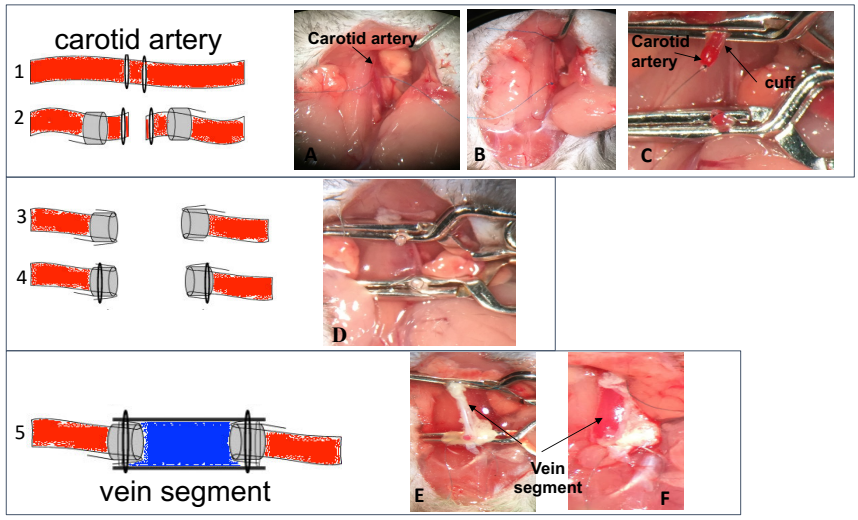

Figure 4. On the left side (1-5) : schematic overview of the different steps during vein graft surgery; on the right side (A-F): representative pictures of the procedures

1-2 Carotid artery becomes visible after it is dissected free from the surrounding fat and tissue. It is then ligated with two 8/0 silk sutures and cut in the middle (A-B). On both the proximal and distal artery end, a nylon cuff is sleeved which is fixated on the cuff handles with hemostatic clamps (C).

3-4 The carotid artery is reverted over the cuff and is tightened with an 8/0 suture (D).

5 The caval vein is positioned over both the cuffs and fixed with ligatures (E). Clamps are then removed (F).

In vivo imaging of atherosclerosis plaques

The assessment of plaque vulnerability with non-invasive imaging methods has become critically important for the diagnosis of rupture-prone plaques. Non-invasive computed tomography (CT) angiography and Doppler ultrasound are commonly used in the clinical practice to assign patient to medical or surgical intervention in presence of carotid atherosclerosis.[74] However, these methods mostly rely on

stenosis and often do not take into account other plaque parameters associated with high risk of rupture. For instance, it has been shown that significant atheroma burden with a high risk of subsequent cardiac events may arise even in absence of luminal stenosis due to outward artery remodeling.[75] Other imaging techniques, such as intravascular ultrasound (IVUS), optical coherence tomography (OCT) and near-infrared spectroscopy (NIRS), can provide a limited plaque characterization but, unfortunately, they are invasive and thus not ideal in early-diagnosis or follow-up.

To date Positron Emission Tomography (PET) is one imaging method that overcomes the previous limitation because it can detect and quantify the pathophysiological processes associated with atherogenesis and subsequent risk of plaque destabilization.[74, 76]

PET imaging as discussed in **chapter 7** is regularly used in the oncological field, and since 2002 has been used as research tool to measure pathophysiological processes in atherosclerosis.[77] [78] This imaging methodology shows higher sensitivity allowing better visualization of biological and biochemical processes involved in the development of atherosclerotic plaques. Although there is no optimal PET tracer for routine clinical imaging, numerous pathways and targets have been studied for PET imaging of atherosclerosis such as glycolysis, cell membrane metabolism (phosphatidylcholine synthesis), integrin αvβ3, low density lipoprotein (LDL) receptors (LDLr), natriuretic peptide clearance receptors (NPCRs), fatty acid synthesis, VCAM-1, macrophages, platelets, etc. Among the PET radiotracers that have been studied, only 18F-FDG has been approved for clinical use. However, carotid artery imaging using 18F-FDG PET is challenging due to the low spatial resolution of PET (≈ 4 mm in human PET and 1,2 mm in rodent PET), cardiac motion and myocardial spill over.[78] [79] To address the latter limitation, new imaging targets and radiotracers with lower unspecific myocardial uptake are currently under investigation. Non-invasive diagnosis and follow-up tools could also help in risk stratification and evaluation of the efficacy of anti-atherosclerotic therapies. **In chapter 7** the development of a new radiotracer that targets PFKFB3 enzyme in atherosclerosis is reported. This novel radiotracer (18F-radiolabeled PFKFB3-targeted ligand) has been tested in two mouse atherosclerosis models that develop rupture-prone plaques and has shown promising results.

The “low” spatial resolution of PET imaging often limits its use in preclinical studies where the characterization of atherosclerosis plaque progression and structure need to perform in small animals. To overcome this limitation, intravital microscopy (IVM) has been recently investigated. This method consists of high-resolution hardware able to study the microvasculature and explore cellular events in small sized animals using several light microscopy techniques such as widefield fluorescence, confocal, two-photon (2P).[80]

Ex-vivo imaging of atherosclerosis plaques

Although conventional histology is the gold standard for analysis of plaque morphology, it shows several limitations for assessing the three-dimensional (3D)-architecture of IP neovascularization. In every optical and microscopic technique, the capability of imaging deep into a biological sample is conditioned by the limited penetration depth of light within biological tissues that are optical media characterized by high turbidity.[81]

In **chapter 8**, a novel method of 3D reconstruction is described. This is based on immunolabeling-enabled 3D Imaging of Solvent Cleared Organs (iDISCO) and confocal microscopy. The technique is applied for the characterization of IP neovessels in $ApoE^{-/-}Fbn1^{C1039G+/-}$ an established atherosclerosis mouse model characterized by the presence of advanced plaques with evident IP neovascularization.

iDISCO is an optical clearing method that renders biological samples more transparent (‘cleared’) and allows such cleared segments to be visualized using confocal microscopy. [82] Application of this method to atherosclerotic arteries resulted in the visualization of the delicate IP neovascularization in carotid plaques. Furthermore, quantitative measurements of the neovessels entering the plaque can be obtained by using 3D analysis software.

This method may represent a useful tool for studies that aim to determine whether there is a causal relationship between the presence of intraplaque neovessel structures and atherogenesis or between angiogenic stimuli and plaque angiogenesis.

Aim of the thesis

Atherosclerosis is the main underlying condition that promotes the onset of cardiovascular disease, a leading cause in mortality in the western world.[32] Mounting evidence suggests that IP neovascularization can promote atherosclerosis progression and plaque destabilization in advanced stage. [24, 35]

The overall aim of this thesis is to investigate the biological role of IP neovascularization in atherosclerosis progression and plaque stability. In this context, ECs highly rely on glycolysis to sustain their activated pro-angiogenic behavior.[35, 69] Accordingly, this thesis also investigates whether **inhibition of EC glycolysis would be a novel target to prevent atherogenesis and/or atherosclerotic plaque destabilization.**

Thesis outline

Chapter 2 consists of a review in which we discuss the potential pharmacological strategies to inhibit IP angiogenesis. In particular, we focused our attention on inhibition of vascular endothelial growth factor signalling, inhibition of glycolytic flux, and inhibition of fatty acid oxidation. In **chapter 3** of this thesis, we review the animal models that have contributed to the understanding of atherosclerosis and its clinical consequences, and that have allowed significant improvement in atherosclerotic treatment.

Proliferating ECs in inflammatory conditions generate up to 85% of their ATP from glycolysis,[35, 69] thus targeting this pathway could be a promising approach. Indeed, IP neovascularization might represent a novel therapeutic treatment to promote plaque stabilization on top of the lipid-lowering therapies. Therefore, we investigate in **chapter 4** whether pharmacological inhibition of glycolytic flux by the small molecule 3PO [3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one] had a beneficial effect on plaque stability. The effect on general metabolism was first studied and then effects on IP angiogenesis, plaque composition and plaque formation in $ApoE^{-/-}$ $Fbn1^{C1039G+/-}$ mice were determined. The molecular pathway behind the in vivo effect was also studied. In **chapter 5,** we further investigate whether 3PO-mediated

glycolysis inhibition is directly related to its direct binding to PFKFB3 (6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase).

In **chapter 6,** we study the effect of specific endothelial PFKFB3 deletion in vein graft lesions of $ApoE^{-/-}$ mice. Firstly, the effect on general metabolism after PFKFB3 endothelial specific deletion was evaluated. Thereafter, IP angiogenesis and composition of vein graft lesions were analysed. Furthermore, in this chapter we evaluate in this chapter the effect of endothelial PFKFB3 deletion on native atherosclerosis.

Chapter 7 consists of a study performed in collaboration with the University of Aberdeen (UK) to investigate the uptake of a novel PFKFB3-targeted PET (Positron emission tomography) tracer ($[^{18}F]$ZCDD083) for atherosclerotic plaque imaging in $ApoE^{-/-}Fbn1^{C1039G+/-}$ mice and $ApoE^{-/-}$ mice.

Although conventional histology is the gold standard for analysis of plaque morphology, it shows several limitations for assessing the three-dimensional (3D)-architecture of IP neovascularization. Therefore, we describe in **chapter 8** a novel method based on iDISCO (immunolabeling-enabled 3D Imaging of Solvent Cleared Organs) and confocal microscopy, for a 3D reconstruction of IP angiogenesis in $ApoE^{-/-}Fbn1^{C1039G+/-}$ mice. Finally, **chapter 9** provides a summarizing discussion of the different chapters and future perspectives.

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