Intraplaque angiogenesis and therapeutic targeting of angiogenesis

Parma, L.

Chapter 1

General introduction

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Atherosclerosis

Cardiovascular diseases (CVDs) are the major cause of death globally, taking an estimated 17.9 million lives each year [1]. CVDs is a broad term that comprises pathological conditions affecting heart and blood vessels. The most common cause underlying cardiovascular diseases is atherosclerosis, a chronic disease marked by the formation of plaques in the intimal layer of the arteries.

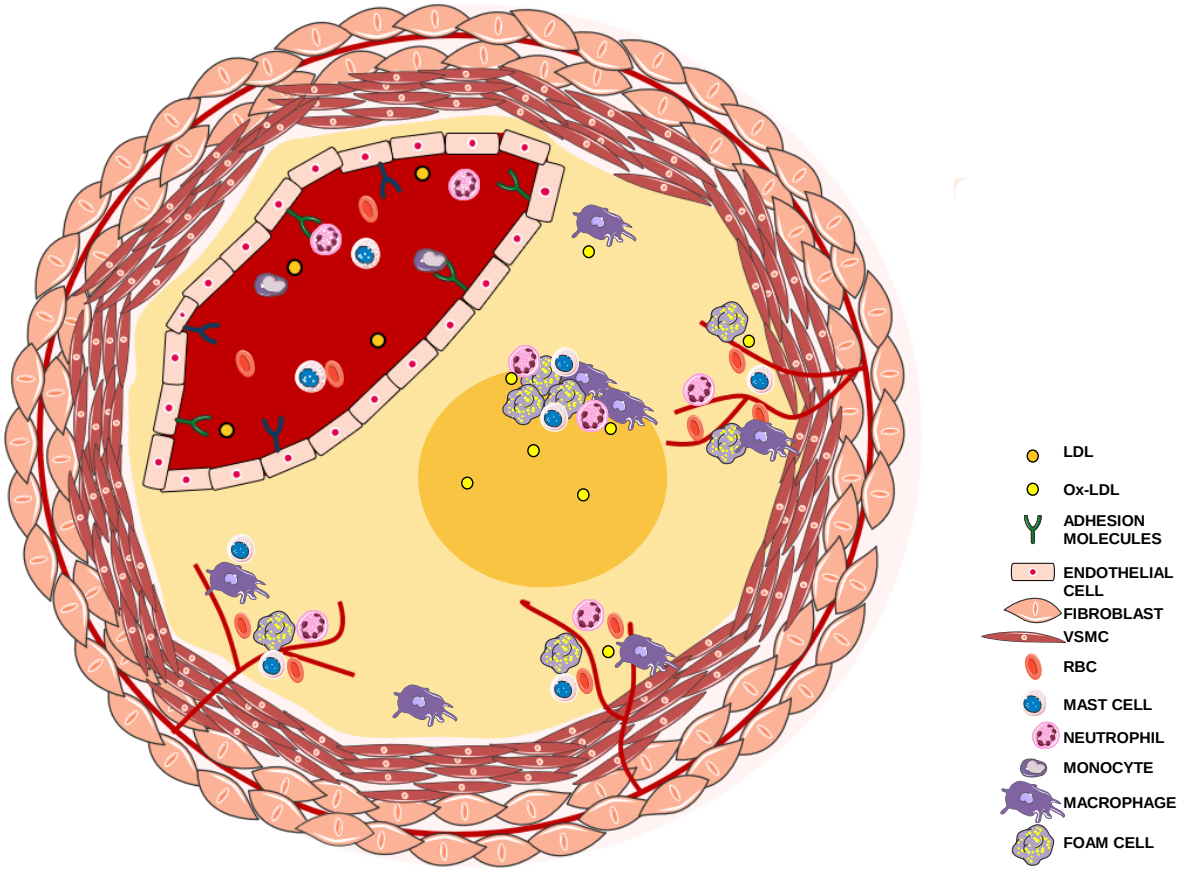

Figure 1. Unstable atherosclerotic plaque. Schematic representation of an advanced unstable atherosclerotic plaque. Features of plaque instability like intraplaque angiogenesis, haemorrhage, high inflammation and a large necrotic core are presents.

The earliest feature of atherosclerosis is characterized by oxidized low-density lipoproteins (oxLDL) accumulation in the aortic wall that provokes the expression of adhesion molecules on the dysfunctional endothelium and the production of inflammatory mediators resulting in the recruitment of monocytes [2,3]. Monocytes accumulate in the sub-intimal layer and differentiate into macrophages that engulf oxLDL and convert into lipid filled foam cells [4].

Engulfing of modified LDL by macrophages activates cytokine production that on its turn, promote the influx and activation of other inflammatory cells and their retention in the plaque [5]. Macrophages in atherosclerosis can be broadly divided in two groups. M1 pro-inflammatory macrophages play an important role in plaque progression by secreting pro-inflammatory cytokines and matrix metalloproteinases (MMPs) that drive the plaque toward an unstable phenotype [3,6]. In contrast, M2 anti-inflammatory macrophages promote tissue repair and hence favour plaque stability by reducing plaque size and cholesterol content [7,8].

As the plaque enlarges, the ensuing hypoxia and inflammation are thought to promote neovascularization [9]. These nascent immature blood vessels are leaky and permit extravasation of inflammatory cells and red blood cells into the plaque, a process called intraplaque haemorrhage, further contributing to necrotic core enlargement and plaque instability [9].

Intraplaque angiogenesis, intraplaque haemorrhage, high macrophage content and a large necrotic core are typical features of advanced unstable atherosclerotic plaques [10]. These plaques are more prone to rupture and the rupture of an unstable atherosclerotic plaque often results in thrombus formation and cessation of the blood flow, leading to the manifestation of severe clinical symptoms like myocardial infarction and stroke. The majority of acute cardiovascular events in patients is caused by occlusive thrombosis formed by rupture or erosion of an atherosclerotic plaque [11]. Despite improved insights into disease pathogenesis and therapeutic options, additional treatment strategies are required to block mechanisms involved in plaque destabilization [5].

Therefore, the aim of this thesis was to investigate the effects of new potential therapeutic strategies on the process of angiogenesis.

Angiogenesis

The formation of new blood vessels, angiogenesis, is a complex process that plays important roles in development, tissue and organ regeneration, as well as numerous pathological conditions [12,13]. The key players in this process are endothelial cells (ECs). Endothelial cells form the endothelium, a thin monolayer that lines the interior surface of a blood vessel.

During healthy adulthood most ECs are quiescent. During wound healing and in disorders fuelled by angiogenesis (for example cancer and atherosclerosis), quiescent ECs become proliferative and start rapidly to form new vessels [14]. The initial stimulus that triggers the process of angiogenesis is hypoxia [2,15]. Hypoxia is a state of lack of oxygen in which the tissue is in need for new vessels to restore its physiologic levels of oxygen. Hypoxia leads to the stabilization of the transcription factor Hypoxia-inducible factor 1-alpha (HIF1a), via preventing its degradation, and promotes its dimerization with the Hif1b subunit [16]. This complex activates the transcription of different genes, among which the pro-angiogenic factors like vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). These angiogenic growth factors work as stimuli that trigger the mobilization of endothelial cells and the formation of a new angiogenic sprout. Activated ECs release proteases that degrade the underlying basement membrane. Afterward, leading ECs start to migrate toward the angiogenic stimulus while the cells behind them proliferate and elongate the neovessel. At the end of the process a new mature vessel with a basement membrane and pericyte coverage is formed [14].

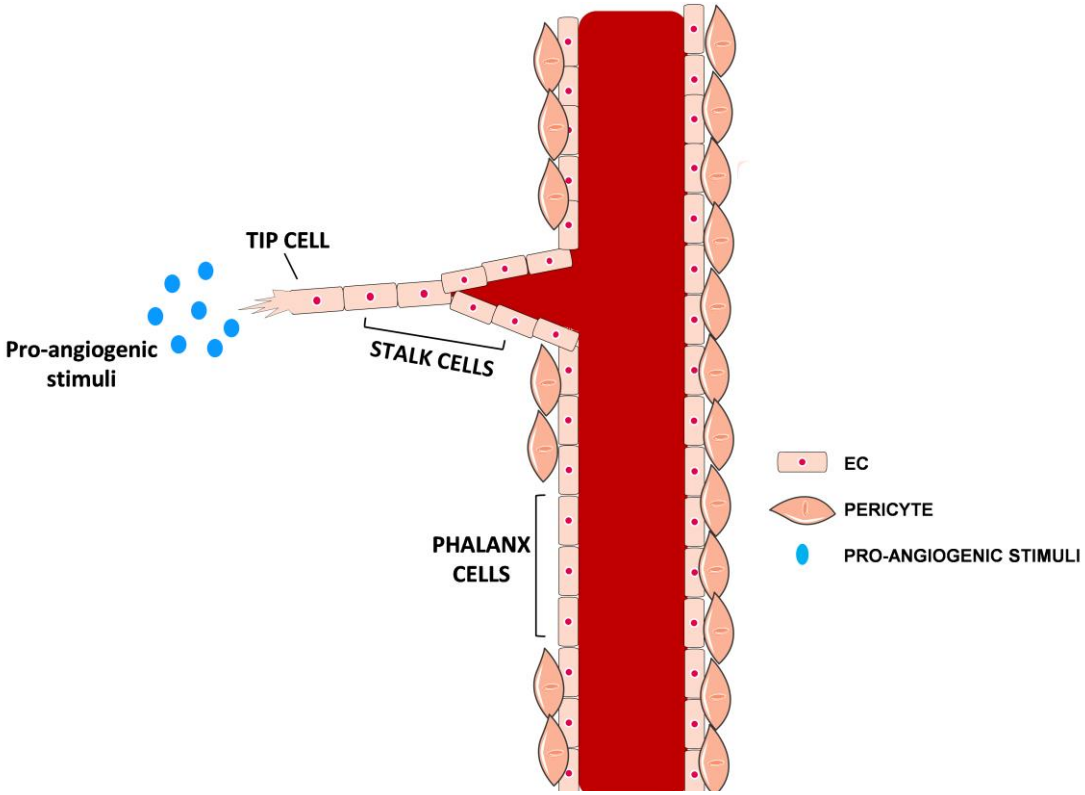

Figure 2. Process of angiogenesis. Schematic representation of the angiogenic process. Migrating tip cells migrate toward an angiogenic stimulus while stalk cells, through proliferation, elongate the nascent sprout. Phalanx cells line the lumen of quiescent vessels.

In a new vessel ECs with three distinct functions can be distinguished: phalanx, stalk and tip cells. Tip cells are motile and invasive and they quickly protrude filopodia that pull the cell forward. Tip cells lead the way to the nascent sprout sensing and migrating toward an angiogenic stimulus. Stalk cells are positioned behind the tip cells and elongate the stalk of the sprout. These cells proliferate, form junctions, lay down extracellular matrix and form a lumen [5]. However the EC phenotype is not static but dynamic. In fact, a stalk cell can become a tip cell, making sure that the fittest cell always leads the sprout [17]. The differentiation of tip versus stalk cell occurs via a Notch mediated lateral inhibition mechanism. Tip cells express high levels of VEGF receptor 2 (VEGFR2) in their filopodia following the VEGF pro-angiogenic signal. At the same time VEGF induces the expression of delta-like canonical Notch ligand 4 (Dll4). Dll4 activates Notch signalling in neighbouring cells and thereby suppresses VEGFR2 expression and tip cell behaviour and induces a stalk cell phenotype [18].

Once the new vessel has been formed, proliferating endothelial cells become quiescent phalanx cells. These cells form a cobblestone monolayer by sticking to each other through tight junctions, are covered by pericytes and are embedded in a thick basement membrane [19].

Phalanx, stalk and tip cells can also be characterized by their metabolism used mainly for redox homeostasis, biomass and energy production respectively [17]. Quiescent phalanx cells, exposed to high oxygen levels have to protect themselves from oxidative stress. To do so, phalanx cells have a 3-fold increase in their fatty acid oxidation (FAO), compared to proliferating cells, to ensure redox homeostasis via production of NADPH [20]. Proliferating stalk cells use the pentose phosphate pathway (PPP), the glutamine and serine pathways and FAO/TCA cycles for nucleotide and biomass production [21-23]. In contrast tip cells rely on PFKFB3-driven glycolysis to obtain fast energy to migrate [21].

Intraplaque angiogenesis in atherosclerosis

Intraplaque angiogenesis is the formation of neovessels inside an atherosclerotic plaque. During the process of plaque formation, large numbers of inflammatory cells enter the plaque. These cells are highly metabolic active and consume a great quantity of oxygen [24]. This process determines the status of intraplaque hypoxia, a status in which the oxygen demand is higher than the oxygen available. As explained above, hypoxia triggers the

recruitment of pro-angiogenic molecules that work as stimulus attracting endothelial cells coming from the vasa vasora. Most neovessels appear to originate from the adventitial vasa vasora, however in-growth of new vessels from the luminal endothelium has also been observed [25,26]. Endothelial cells migrate and proliferate from the existing vasculature of the vasa vasora toward the hypoxic area inside of the plaque following a gradient of pro-angiogenic cues and form new vessels.

An important feature of these vessels is that they are immature and therefore leaky [27]. They lack a complete pericyte coverage and moreover the tight junctions between endothelial cells are not completely formed leading to the extravasation of red blood cells and inflammatory cells from the neovessels to the inside the plaque, a process called intraplaque haemorrhage (IPH) [28]. Red blood cells in particular constitute the main cellular component of intraplaque haemorrhage [29]. Once red blood cells reach the highly oxidative intraplaque environment they lyse and their cellular membranes release un-esterified cholesterol [29]. At the same time extravasated inflammatory cells are rich sources of cytokines, growth factors, and proteases such as matrix metalloproteinases (MMPs), and can influence both plaque stability and plaque angiogenesis [2]. In addition, macrophages can secrete pro-angiogenic factors, like VEGF, and can therefore fuel intraplaque angiogenesis [30]. These processes contribute to increased plaque inflammation and instability, making the atherosclerotic plaque more prone to rupture.

One approach to stabilize atherosclerotic plaques and prevent their rupture is targeting intraplaque angiogenesis with the aim to reduce the number and/or increase the maturation of the neovessels.

Murine models of angiogenesis and atherosclerosis

Murine models are the preferred animal model to study atherosclerosis due to their rapid reproduction and ease of genetic manipulation. However naive atherosclerotic lesions in most of the traditionally used strains, ApoE KO, LDLR KO and ApoE3*Leiden, do not develop intraplaque angiogenesis most probably due to the small size of the lesions in which the hypoxia that occurs can be resolved by the regular levels of diffusion of oxygen via the lumen [5]. Currently two murine models have been developed with atherosclerotic lesions that show intraplaque angiogenesis. One model is based on vein graft surgery in ApoE3*Leiden mice on

high fat, high cholesterol diet. In this model, 28 days after surgery the lesions show typical characteristics of unstable human plaques, including intraplaque angiogenesis and haemorrhage, foam cells, calcification and cholesterol clefts [31]. The other is the ApoE KO Fbn1C1039G$^{+/-}$ mice model. A heterozygous mutation C1039G$^{+/-}$ in the Fbn1 gene results in the fragmentation of elastic fibers in the media of the vessel wall [32]. Those mice, if fed with a Western diet for 20 weeks, develop plaques that show intraplaque neovessels and intraplaque haemorrhage that can result in plaque rupture [33]. Both models are characterized by large atherosclerotic lesions and lack of elastic lamina together the cause of the displayed plaque angiogenesis. In this thesis we use one model of accelerated atherosclerosis, namely the vein graft model, that presents intraplaque angiogenesis. In this thesis two more animal models are used; to study intimal hyperplasia the femoral artery cuff model has been used and for studying in vivo angiogenesis, the murine Matrigel plug model was used.

Vein graft accelerated atherosclerosis in ApoE3*Leiden mice

Vein graft bypass surgery is mimicked in a mouse model of vein graft surgery. When this procedure is performed in mice that are prone to develop atherosclerosis, like the ApoE3*Leiden mice on a hypercholesterolemic diet, the lesion within the vein graft develop with an accelerated form of atherosclerosis [34,35]. During the surgical procedure the thoracic caval vein from a donor mouse is inter-positioned within the right carotid artery of a recipient mouse. Pulsatile flow through the venous conduit confirms a successful procedure. Donor mice are on chow diet until T=0, time point at which they are sacrificed. Recipient mice are on high cholesterol, high fat diet from three weeks prior the surgery until the day of sacrifice 28 days after surgery [35].

28 days after surgery the lesions formed highly resemble human native atherosclerotic lesions including intraplaque angiogenesis and haemorrhage, calcification, cholesterol clefts and the presence of foam cells [31].

Femoral artery cuff placement in C57BL/6 mice

A murine model to study intimal hyperplasia is the femoral artery cuff placement in C57BL/6 mice. In this model of intimal hyperplasia a non-constrictive cuff around the femoral artery of C57BL/6 mice is placed. Briefly, the left and right femoral arteries are isolated and a rigid, non-

constrictive polyethylene cuff is placed around the artery [36]. In response to this injury a smooth muscle cells (SMCs) rich neointima is formed within three weeks if this model is used under normocholesterolemic conditions. After 21 days, accelerated atherosclerotic lesions are formed in which is present a strong predominance of SMCs and no presence of intraplaque angiogenesis.

Matrigel plug model of in vivo angiogenesis in C57BL/6 mice

A widely used in vivo assay for the evaluation of pro- or anti-angiogenic factors is the in vivo angiogenesis plug assay, which makes use of the basement membrane Matrigel [12]. ECs migrate and proliferate in the Matrigel and here they form neovessels. During this procedure Matrigel extracellular matrix is injected into the subcutaneous space on the dorsal side of C57BL/6 mice on both flanks [37]. Matrigel is liquid at 4 °C and gels at higher temperatures and therefore it solidifies after injection forming a plug at body temperature. Over time, blood vessels will sprout into the plug and after 21 days new angiogenic sprouts are visible in the plugs [37].

Potential angiogenic targets for therapeutic interventions

In the past years, the number of compounds targeting different pathways to counteract angiogenic growth has greatly increased, mainly in the oncological field [38]. Due to their role in angiogenesis, growth factors and their receptors, as well as protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs), families of proteins that regulate the phosphorylation of these receptors, are main targets for angiogenic therapies. More recently, several studies showed that endothelial cell metabolism is a driver of angiogenesis and therefore the enzymes involved in different metabolic pathways, such as PFKFB3, represent new targets for anti-angiogenic therapies [17].

VEGF/VEGFR2 signalling

The VEGF family is composed of five structurally related factors: VEGF-A, VEGF-B, VEGF-C, VEGF-D and the placenta growth factor (PlGF) [39]. These ligands bind with different affinities to three VEGF receptor tyrosine kinases (VEGFR1-3) [39]. The receptors show an overlapping but distinct expression pattern with mainly VEGFR1 expressed in monocytes and macrophages, VEGFR2 in vascular endothelial cells, and VEGFR3 in lymphatic endothelial cells.

VEGFR1 signalling is involved in inhibition of angiogenesis, immune cell recruitment, fatty acid uptake, while VEGFR2 signalling activation promotes angiogenesis and VEGFR3 promotes lymph-angiogenesis [39,40]. VEGFR2 is part of the receptor tyrosine kinases family (RTKs). VEGF binding to VEGFR results in autophosphorylation of specific tyrosine residues in the cytoplasmic domain of VEGFR2 [41]. Phosphorylated VEGFR2 initiates downstream signalling pathways relevant to angiogenesis and produces several cellular responses in ECs including a strong mitogenic signal and survival signal [42]. The signalling network comprises the activation of signalling molecules and the cross talks involving these molecules modulates the process of angiogenesis [42].

Due to their important role in angiogenesis therapeutics targeting VEGF/VEGFR2 signalling were developed to block vessel growth in retinopathy and cancer [43]. In the context of atherosclerosis, it was shown that treatment with axitinib (inhibitor of VEGFR1, 2 and 3) attenuated plaque angiogenesis in $ApoE^{-/-}Fbn1C1039G^{+/-}$ mice [44]. Therefore, anti-VEGFR2 antibody treatment may have beneficial effects in stabilizing advanced atherosclerotic plaques, which is discussed in chapter 4.

bFGF/FGFR signalling

The basic fibroblast growth factor (bFGF), also called FGF2, is part of the fibroblast growth factors family that comprises 18 members that act as ligands and 4 members that represents the receptors (FGFR1-4) [45]. It is a very well-known regulator of angiogenesis and exerts is function by binding to one of the four FGFR. When promoting angiogenesis, bFGF binds primarily to FGFR1 on the surface of ECs [46,47]. bFGF has been shown to be involved in the pathogenesis of atherosclerosis [48] as well as in the regulation of processes that drive plaque instability [49,50].

Several studies showed that targeting FGF/FGFR axis could be beneficial for atherosclerosis. A study from Raj et al., showed that inhibiting FGFR signalling with the RTK inhibitor SU5402 in $ApoE^{-/-}$ mice fed a high-fat diet inhibited neointimal thickening by almost 85 % [51]. SSR128129E, a small-molecule multi-FGF receptor blocker was tested in a vein graft model in C57BL/6J mice and in $ApoE^{-/-}$ mice [52]. SSR128129E decreased neointima proliferation in both models [52]. However, it has been shown that a tight regulation of FGF signalling is necessary for vascular homeostasis and therefore the complete blockade of all the four

receptors could have adverse effects. To overcome this problem, we collaborated in the synthesis of the small molecule 3'-(propane-1,3-diyilbis(azanediyl)bis(oxomethylene)bis(1-(2,4-dichlorophenyl)-1,4-dihydro-thieno[3',2':4,5]cyclohepta[1,2-c]pyrazole-8-sulfonic acid), namely K5 that binds to bFGF and inhibits the bFGF/FGFR signalling [53]. The effects of K5 treatment on accelerated vein graft atherosclerosis in ApoE3*Leiden mice are described in chapter 5.

Pentose phosphate pathway

Besides the glycolysis, an alternative metabolic process for the breakdown of glucose is the pentose phosphate pathway (PPP) [54]. This pathway has two distinct phases: the oxidative phase and the non-oxidative phase. First, glucose-6-phosphate (G6P) is converted into ribose-5-phosphate (R5P) in the oxidative branch, generating NADPH [54]. The non-oxidative branch provides precursors for nucleotide synthesis and glycolytic metabolites by interconverting fructose-6-phosphate (F6P) and glyceraldehyde-3-phosphate (G3P) generating R5P [54].

A key enzyme involved in the pentose phosphate pathway is transketolase (TKT), which is a thiamine-dependent enzyme. This enzyme reversibly links the non-oxidative branch of the PPP to the glycolysis controlling nucleotide biosynthesis and energy production. Both ECs and macrophages rely on this metabolic pathway to carry out proliferation [55]. Intraplaque angiogenesis and inflammation are strongly connected in atherosclerosis. The interaction between these two processes forms a vicious cycle that modifies the microenvironment composition of the plaque and drives the plaque towards an unstable phenotype. It has been shown that PPP blockade in macrophages reduces cytokine secretion and inflammation [56]. The result of TKT blockade in macrophages and its effects on angiogenesis are described in chapter 6.

Protein tyrosine Phosphatases

The activation of receptor tyrosine kinases is controlled not only by the binding of specific ligands, but it is also regulated by protein tyrosine phosphatases (PTPs) [57]. PTPs are a family of endogenous modulators of RTKs-mediated signalling pathways that carry out the dephosphorylation of phospho-tyrosine residues [57]. Among RTKs regulated by PTPs there are some master regulators of angiogenesis like VEGFR, FGFR and PDGFR. PTPs acts by either

direct dephosphorylation of particular receptor tyrosine residues or of downstream signalling components [57].

Due to their broad function, the regulation or blockade of PTPs could be an approach to increase RTKs activation and subsequently angiogenesis. We investigated the effect of PTPs blockade using bis(maltolato)oxidovanadium (IV) (BMOV) on in vitro angiogenesis and VEGFR2 signalling in **chapter 7**.

Outline of the thesis

The aim of this thesis was to investigate the effects of new potential therapeutic strategies on angiogenesis. The first part of this thesis focusses on new possibilities to inhibit or decrease intraplaque angiogenesis in atherosclerosis. **Chapter 2** consist of a review on intraplaque angiogenesis and intraplaque haemorrhage in atherosclerosis. In this review we discuss the processes that drive intraplaque angiogenesis as well as new imaging techniques to detect intraplaque angiogenesis in patients. Moreover, we give an overview of angiogenesis targets and animal models used for the study of atherosclerosis.

In **Chapter 3** we study how restoring hypoxia via hyperoxygenation treatment using carbogen gas (95%O_2, 5%CO_2) in ApoE3*Leiden mice that underwent vein graft surgery, affects intraplaque angiogenesis and vascular remodelling.

In **Chapter 4** we describe the effect of VEGFR2 blockade using a monoclonal antibody, DC101, on accelerated atherosclerotic lesions in ApoE3*Leiden mice. We investigate the role of VEGFR2 blockade on vascular remodelling as well as on intraplaque angiogenesis and on the maturation of neovessels. Due to the great importance of growth factors receptors and their ligands in angiogenesis, in **Chapter 5** we examine the effect of bFGF blockade on the stability of advanced atherosclerotic plaques. We describe how a new small molecule, K5, by binding and inhibiting bFGF have effects on the remodelling of accelerated atherosclerotic vein graft lesions and their compositions, including intraplaque angiogenesis and haemorrhage. In **Chapter 6** we study how TKT inhibition affects the production of pro-inflammatory and pro-angiogenic cytokines in cultured human macrophages. Additionally, we show the functional consequences of macrophage TKT blockade on angiogenesis in vitro. The second part of this thesis focusses on a new strategy to increase in vitro angiogenesis. In **Chapter 7** we unravel the effect of BMOV mediated PTPs blockade on in vitro angiogenesis using human endothelial

cells. Moreover, we examine how this influences the activation of VEGFR2 and its downstream signalling.

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