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Intraplaque neovascularization as a novel therapeutic target  
in advanced atherosclerosis

## **Abstract**

**Introduction:** Atherosclerosis is a lipid-driven inflammatory process with a tremendously high mortality due to acute cardiac events. There is an emerging need for new therapies to stabilize atherosclerotic lesions. Growing evidence suggests that intraplaque (IP) neovascularisation and IP haemorrhages are important contributors to plaque instability.

**Areas covered:** Neovascularization is a complex process that involves different growth factors and inflammatory mediators of which their individual significance in atherosclerosis remains poorly understood. This review discusses different aspects of IP neovascularization in atherosclerosis including the potential treatment opportunities to stabilize advanced plaques. Furthermore, we highlight the development of accurate and feasible *in vivo* imaging modalities for IP neovascularization to prevent acute events.

**Expert opinion:** Although lack of a valuable animal model of IP neovascularization impeded the investigation of a causal and straightforward link between neovascularization and atherosclerosis, recent evidence shows that vein grafts in ApoE\*3 Leiden mice as well as plaques in ApoE<sup>-/-</sup> Fbn1<sup>C1039G+/-</sup> mice are useful models for intraplaque neovessel research. Even though interference with vascular endothelial growth factor (VEGF) signalling has been widely investigated, new therapeutic opportunities have emerged. Cell metabolism, in particular glycolysis and fatty acid oxidation, appears to perform a crucial role in the development of IP neovessels and thereby serves as a promising target.

**Keywords:** Atherosclerosis, animal model, cell metabolism, imaging, neoangiogenesis, neovascularization, VEGF

## 1. Introduction

Atherosclerosis is a lipid-driven inflammatory disease that occurs in the intima of the arterial vessel wall and typically leads to the formation of atherosclerotic plaques [1]. Subendothelial retention of low density lipoprotein (LDL) and the subsequent modification into oxidized (ox)LDL is the initial start of this inflammatory process [2]. OxLDL activates endothelial cells (ECs) to enhance the expression of different cell adhesion molecules such as vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1), which recruit monocytes and lymphocytes into the early atherosclerotic lesion [3–5]. Monocytes differentiate into macrophages and ingest large amounts of modified lipoproteins, which results in lipid accumulation and foam cell formation [6]. Lymphocytes are important mediators in the early stages of lesion formation, but they also affect key functions later on in the atherosclerotic process by secreting cytokines and by serving as memory cells to specific antigens [7–9]. Importantly, focal storage of lipids in the intima of the vessel wall is considered safe and may not directly yield clinical effects [10]. However, activated macrophages stimulate the release of various pro-inflammatory cytokines and matrix-degrading molecules such as matrix metalloproteases (MMPs). In response to pro-angiogenic signals, disruption of medial elastic laminae gives the vasa vasorum (i.e. microvasculature in the adventitia of large arteries) the opportunity to sprout into the plaque, thereby leading to a network of growing microvessels. Intraplaque (IP) neovascularization represents a new entry for even more oxLDL and inflammatory mediators [11]. This process of plaque destabilization results in the formation of thin cap fibroatheroma (TCFA), which is the precursor lesion of a ruptured plaque. TCFA comprises typical morphological features such as a large, lipid-rich necrotic core and a highly inflamed mass with IP neovessels surrounded by a thin fibrous cap [12]. Rupture or erosion of plaques followed by luminal thrombosis is a major cause of clinical complications such as myocardial infarction, stroke and even sudden

death [13,14]. The morbidity and mortality from acute cardiac events remains high because of the tremendous complexity of lesion progression towards an unstable plaque. Therapies treating luminal stenosis or thrombosis include expensive and quite often surgical interventions. Moreover, primary/secondary prevention of acute events by drug treatment such as cholesterol-lowering drugs may deliver a remarkable benefit in survival but still does not suffice to prevent acute complications in all treated patients. Therefore, the need for innovative strategies to stabilize atherosclerotic plaques is high and cannot be underestimated. The identification of patients susceptible to clinical manifestations caused by atherosclerotic plaque rupture is based on established risk factors and conventional imaging modalities such as coronary angiography, intravascular ultrasound (IVUS) and optical coherence tomography (OCT). These imaging techniques represent the 'gold standard' to determine the degree of luminal stenosis and thus plaques at high-risk of rupture. However, a large body of evidence shifted the determination of a vulnerable plaque towards the cellular composition rather than the flow-limiting features of a lesion. Indeed, the composition of an atherosclerotic lesion has already been very valuable in the prediction of future cardiovascular events [15]. The present review will focus on neovascularization in the atherosclerotic plaque and its impact on plaque stability, as well as on the possibilities for novel treatment options.

## **2. Intraplaque neovascularization as a trigger of plaque rupture**

### **2.1 Intraplaque neovascularization in human lesions**

Neovascularization comprises the formation of new blood vessels from existing blood vessels to nourish every part of our body, a process also known as angiogenesis and widely investigated in cancer research [16]. Even though large and midsized arteries have their own supply of oxygen and nutrients via the vasa vasorum, it does not meet up with the increasing

demand of the growing plaque. In order to develop novel therapeutic approaches against atherogenesis and plaque destabilization, IP neovascularization has become an emerging concept in cardiovascular research. Importantly, microvessels located in the intima of the vessel wall are quite rare and only seen in pathological conditions such as atherosclerosis. Koester and colleagues [17] were the first to present a link between IP neovascularization and atherosclerosis, followed by the work of Winternitz et al. [18], but an explanatory mechanism for this association was lacking until 1938 when Patterson et al. [19] reported that capillary rupture concomitant with leakage of erythrocytes and platelets into the plaque (IP haemorrhage) is causative for plaque progression, plaque rupture and coronary thrombosis. Since the early 1980s, research focused more and more on scrutinizing the importance of IP neovascularization in the complexity of plaque progression and rupture [11,20–22]. Hitherto, observational studies confirmed that human stenotic lesions are characterized by an extensive network of IP neovessels, which are associated with areas of high inflammatory infiltration, a large necrotic core and substantial lesion size. Moreover, IP haemorrhages appear to be important stimuli of plaque progression, instability and rupture [11,23,24]. It should be noted, however, that even without IP haemorrhage IP neovascularization is related to plaque vulnerability, as it is a source of lipids. In addition, microvessels in the plaque expose increased expression of adhesion molecules (e.g. ICAM, VCAM, E-selectin, CD40), thereby promoting the migration of inflammatory cells in the plaque. IP haemorrhage without IP neovascularization does not occur.

To our current knowledge, neovascularization is caused by the increasing demand for oxygen and nutrients by different cell types in fast proliferating tissues such as a growing atherosclerotic lesion. In metabolically active tissues, such as a highly inflamed plaque, oxygen has a limited diffusion distance of  $<250\mu\text{m}$  from the nearest capillary towards different cell types. The media of the vascular wall consists of a specific amount of lamellar

units depending on the type of blood vessel. In physiological conditions, a threshold of 29 lamellar units restricts the diffusion distance of oxygen [25,26]. Hence, development of a network of microvessels into the plaque is stimulated to provide the necessary oxygen and nutrients. Thomlinson and Gray [27] postulated that hypoxia is the driving force for tumor neovascularization as the limited diffusion distance of oxygen cannot meet the higher demand for fast expanding tissues. Also in atherosclerosis, once vessel wall thickness exceeds a critical dimension due to the accumulation of lipids and inflammatory cells, neovascularization is initiated from the vasa vasorum into the plaque. Microvessels originating from the luminal side of the plaque are rarely observed but do appear from time to time. Interestingly, plaque hypoxia is mainly upregulated by the presence of inflammatory mediators and their increasing demand for oxygen in the plaque, while a decreasing supply of oxygen due to lipid accumulation is of minor importance [28]. This explains why plaques of similar intimal thickness can differ in IP microvessel density. Besides inducing hypoxia, inflammation is an autonomous catalyst of the process by producing various angiogenic factors [16,29,30]. In response to acute inflammation, several pro-angiogenic molecules serve as inducers of cell permeability, contributing to the infiltration of leucocytes in the inflammatory core and thus provoking chronic inflammation [31]. Moreover, microvessels in the plaque overexpress adhesion molecules to promote the migration of inflammatory mediators in the plaque as mentioned above. Finally, it is noteworthy that a persistent inflammatory stimulus takes part in permanently shifting the EC phenotype towards a migratory state [32].

In general, three clear steps are essential for efficient microvessel development (figure 1). First, ECs undergo an angiogenic switch from a quiescent, non-proliferative state, to an active and proliferative state. In physiological conditions, quiescent ECs of the vasa vasorum are separated from ambient mural pericytes by a basement membrane. In response to pro-

angiogenic (e.g. VEGF, angiopoietin [Ang] 2) signals, pericytes are stimulated to detach from the basement membrane, which allows ECs to disrupt their cellular junctions such as VE-cadherins and claudins [33]. Second, ECs will differentiate into ‘tip’ (navigating) or ‘stalk’ (proliferating) cells. Tip cells will work their way through the hypoxic region, whereas stalk cells have to proliferate and embody the developing vessel [34]. VEGF signalling, which is an important factor for migration and proliferation of ECs, can be downregulated in cells with activated Notch signalling by lowering the levels of VEGF receptor (VEGFR). Accordingly, Notch signalling can control the sprouting pattern of blood vessels during angiogenesis. VEGF and Notch signalling pathways cooperate in tight coordination to specify and balance the tip and stalk cell phenotype between the ECs that constitute the sprouts during the angiogenic process. To this end, Notch signalling acts in a negative feedback loop with VEGF signalling during angiogenesis. The initial angiogenic response is induced by VEGF gradients established in the hypoxic plaques. Under VEGF stimulation, DLL4 (Delta like ligand 4) expression is upregulated in the tip cells. In turn, DLL4 ligand activates Notch signalling in the stalk, consequently suppressing the tip cell phenotype [35]. Finally, microvessels should regain functionality and blood flow. Expression of pro-maturation signals induces nascent ECs to recover their quiescent state and protective packaging with mural cells. However, maturation is disturbed so that newly formed vessels do not display the qualities of healthy mature vessels. Indeed, they have a leaky appearance with insufficient endothelial lining. The incomplete and disturbed maturation of microvessels is due to an imbalance in pro-angiogenic factors (e.g. VEGF, ang 2) and pro-maturation factors (e.g. platelet-derived growth factor [PDGF], ang 1). In symptomatic atherosclerosis, IP microvessels in coronary and carotid arteries appear thin-walled with ECs showing abnormal features such as basement membrane detachment and open (leaky) EC junctions [20,22]. Thus, neovascularization not only serves to provide the necessary nutrients and to remove metabolic remnants, the increased

permeability of newly-formed microvessels also creates a new gateway for lipoproteins and other blood components into the plaque, further promoting lesion growth. This statement is supported by previous findings showing that unstable and ruptured plaques contain a higher density of microvessels and IP haemorrhages than stable and non-ruptured plaques [23,36,37]. Critical in the progression of IP haemorrhage towards plaque rupture is the enlargement of the necrotic core (by both plasma and membrane-derived cholesterol from erythrocytes and platelets) and the increased influx of macrophages into the plaque, both provoking plaque destabilization [11]. Moreover, leakage of erythrocytes and platelets into the plaque promote lesion progression by becoming rapidly phagocytized by macrophages. In literature, discrepancies exist concerning the significance of erythrocyte and platelet phagocytosis in plaque destabilization. On the one hand, several studies claim that these actions lead to subsequent activation of macrophages (characterized by the expression of the inducible nitric oxide synthase [iNOS]), iron deposition and more pronounced foam cell formation. Indeed, several studies of human plaques indicate a distinct association between iNOS-expressing macrophages and the presence of microvessels in the lesion [11,21,38]. However, other studies indicate that a novel atheroprotective macrophage subpopulation is generated upon IP haemorrhage [37,39]. This subset of macrophages clears hemoglobin more quickly and shows increased expression of ferroportin (a transmembrane protein that transports iron outside of the cell) so that intracellular iron and oxidant stress is reduced. Reduced ROS drives transcription of ABC transporters, thereby inhibiting foam cell formation (Finn et al. 2012).

## 2.2 Molecular mediators implicated in the development of intraplaque microvessels

Neovascularization is initiated during hypoxia or inflammation in the plaque via upregulation of hypoxia inducible factor (HIF)-1 $\alpha$ . HIF is a heterodimeric transcription factor that consists

of a constitutively expressed  $\alpha$ -subunit and  $\beta$ -subunit that is regulated by oxygen status [40,41]. In oxygen-deprived situations, HIF-1 $\alpha$  is responsible for maintaining O<sub>2</sub> homeostasis and initiating neovascularization to restore blood flow by activating the transcription of multiple genes [42,43]. Hypoxia responsive elements that contain HIF-1 binding sites are found in the proximity of the promoter region of genes such as Ang 2, iNOS and members of the VEGF family, all involved in neovascularization [44–46]. Remarkably, the expression of hypoxia-inducible proteins, such as HIF-1 $\alpha$  and placenta growth factor (Plgf), correlates with symptomatic plaques and with a higher incidence of plaque haemorrhage. Moreover, these growth factors are spatially and quantitatively linked to IP neovascularization in human lesions [23,36].

### 2.2.1 VEGF in neovascularization

A key player in IP neovascularization is the VEGF family, consisting of 5 close-related members (VEGF-A, B, C, D and PlGF) of which VEGF-A has been thoroughly investigated over the last decade. In normal physiological conditions, low levels of VEGF-A are released in an autocrine manner by a broad range of cells including ECs, SMCs and macrophages to sustain vascular homeostasis. However, high levels of VEGF-A induce sprouting of microvessels with VEGFR-2 (also known as KDR/Flk1) as its main receptor in controlling the development of the IP angiogenic network [47]. Activation of VEGFR-2 enables several downstream pathways, all of them resulting in the activation of different EC functions such as survival, permeability, migration and proliferation (figure 2). Interestingly, VEGF-A is abundantly present within advanced human coronary and carotid atherosclerotic plaques [36,48]. In normal coronary arteries, VEGF-expression is noticeable, whereas in lipid-rich coronary lesions lucid expression of VEGF-A is observed. Moreover, stenotic coronary

plaques display very intense positivity for VEGF-A, particularly in ECs and macrophages surrounding microvessels [48]. VEGF-A upregulation results in an increased permeability of the EC layer, which is one of the reasons why microvessels are not able to mature properly. VEGF-A induces cell permeability by binding VEGFR2, thereby promoting endocytosis of VE-cadherin, a crucial adhesion molecule that maintains endothelial barrier function [49]. Proliferation and migration results in the recruitment of progenitor cells and the formation of the growing body of neovessels. On the other hand, the VEGF homologue Plgf primarily regulates inflammation in neovascularization via the recruitment and adhesion of monocytes. A study performed in ApoE<sup>-/-</sup> mice showed that neointimal macrophages are associated with an increased expression of Plgfr [50]. In the early stages of identifying this new growth factor, it was already observed that Plgf potentiates the activity of VEGF [51]. Patients with symptomatic carotid atherosclerosis show higher levels of Plgf in their lesions than asymptomatic patients, with more inflammation and also microvessels present in these advanced lesions [52]. However, whereas VEGF has an important function during embryonic development, Plgf has not, which constrains its role solely to pathological conditions by binding VEGFR-1 (or Flt-1) [47]. The other vascular growth factors B, C and D have not been explored to the same extent as VEGF-A. However, it appears that they all manifest their individual function in IP neovascularization, frequently reinforcing the other growth factors [53,54]. VEGF-B is primarily present in pathological neovascularization [53], whereas VEGF-C and -D are valuable in both neovascularization and lymphangiogenesis [54,55].

### 2.2.2 Fibroblast growth factor

Fibroblast growth factor (FGF) 1 (acidic FGF) and FGF 2 (basic FGF) as well as their key receptors (FGFR 1-4) are involved in various biological activities including morphogenesis,

cell proliferation and migration. Because the FGF-family is widely studied in ischemic revascularization therapy, potential aggravation of atherosclerosis has to be considered, even though studies about FGFs in atherosclerosis are scarce and contradictory findings have previously been published. For example, one study indicates that FGFs are expressed in both early and advanced human plaques [60], but that only FGF2 holds a key role in IP neovascularization. Another study claims that FGF 1 is of importance in plaques showing abundant IP neovascularization, because expression of FGF 2 is lacking [57]. Expression of FGFRs varies subtle along the different stages of the growing lesion, supporting the idea that the receptor function and impact of FGF differ according to lesion progression [56].

### 2.2.3 Angiopoietins and platelet-derived growth factor

Angiopoietins (Ang) 1 and 2 are growth factors particularly engaged in vessel stabilization and destabilization [58]. Whereas the VEGF system is engaged in several steps of the angiogenic process, Ang 1 and 2 both have a key role in the final maturation phase of neovascularization with opposite functions (figure 2). Ang 2, induced by HIF-1 $\alpha$  along with VEGF-A, destabilizes the interactions between mural cells and ECs, counteracting Ang 1 that acts as a stabilizing factor aspiring for sufficient vessel sustainability [58]. It has been hypothesized that the stimulation of vessel maturation prevents newly formed IP vessels from becoming leaky, thereby preventing the creation of a novel entrance for inflammatory mediators and lipids. Preclinical studies have provided evidence that neovascularization is indeed a complex cascade of different growth factors [58–60]. Ang 1 and PDGF seem to be an indispensable team in stimulating the maturation of newly-formed microvessels, whereas VEGF-A and Ang 2 collaborate in the first step of blood vessel destabilization (i.e. detachment of mural cells from ECs) [60]. PDGF is associated with pericyte recruitment and

turns nascent vessels into sustainable vessels. In particular PDGF-BB and its receptor PDGF receptor (PDGFR)- $\beta$  have a critical role in vessel permeability (figure 2), fragility and impaired perfusion as observed in embryos lacking PDGF-B and PDGFR- $\beta$  [61]. The combination of both VEGF-A and Ang 1 improved the total hypoxic area by strengthening ECs structurally and functionally [62,63], clearly stating a significant interaction in vessel formation and maturation.

#### 2.2.4 Inflammatory mediators in intraplaque neovascularization

Besides hypoxia-induced transcription and translation of angiogenic proteins, inflammatory mediators accelerate neovascularization as well [16,30,64]. In the last few years, evidence emerged that neovascularization and immunity have a reciprocal association. A vicious cycle starting with more inflammatory cells in the plaque that need more oxygen and nutrients will stimulate neovascularization, which in turn creates an entrance for new inflammatory mediators to enter the plaque and thus reinforces the cycle at the beginning. In human plaques, areas with a spacious network of neovessels are associated with an increased expression of angiogenic chemokines (IL-1 $\beta$ ) and chemokine receptors that stimulate recruitment of multiple inflammatory mediators. Moreover, enhanced expression of interleukin (IL)-8, transforming growth factor (TGF)- $\beta$  and hepatocyte growth factor (HGF) is observed in human atheroma tissue as compared to normal vessels and appears to exert a regulating function in initiating and sustaining IP neovascularization [65–67].

#### 2.3 Anti-angiogenic factors

Neovascularization occurs under physiological and pathological conditions, and is controlled at a transcriptional level. Besides pro-angiogenic genes (vide supra), anti-angiogenic genes

are preserved in the genome as well. The regulation of anti-angiogenic proteins such as angiostatin, endostatin, endostar and platelet factor (PF) 4 has been explored in tumor research but is still merely unexplored [68–71]. For the majority of endogenous neovascularization inhibitors, the effects are strictly limited to ECs. Recently, a novel endothelial-derived neovascularization inhibitor was identified, namely vasohibin [72]. This protein is a VEGF-inducible neovascularization inhibitor that is expressed in human carotid atherosclerotic plaques and its expression correlates with the degree of neovascularization and inflammatory load [73]. Nevertheless, despite the presence of endogenous angiogenic inhibitors in atherosclerotic plaques, pathological neovascularization persists in the lesion. Administration of exogenous neovascularization inhibitors already delivered a promising outcome, resulting in the prevention or decrease of lesion formation [71,74].

### **3. Imaging modalities for intraplaque neovascularization**

Accurate and feasible imaging modalities for the composition of a plaque including the presence of IP neovascularization may imply a big step forward in the prevention of acute events such as myocardial infarction or stroke, particularly because many patients that endured a cardiac event have no prior symptoms. Up to this day, histopathological analysis has been the only method capable of discernibly visualizing newly-formed vessels in unstable lesions with the drawback of requiring tissue samples [75]. Immunohistochemical staining of ECs with CD31/CD34 or von Willebrand factor is commonly used in order to characterize and quantify the neovascular network in human lesions (figure 3). Anti-TER-119 staining is associated with glycophorin-A on erythrocytes and demonstrates on-going flow in neovessels and IP haemorrhages. In addition, staining of the membrane proteoglycan neural/glial antigen 2 (NG2) is characteristic of pericytes and thus marks the maturation status of IP neovessels.

However, in a preventive manner, histology does not offer any leverage because a surgical intervention is necessary.

Therefore, several non-invasive imaging methods are currently being optimized in experimental models and clinical settings. However, given that only a few imaging methods are able to give a detailed representation of plaque composition, non-invasive imaging of IP neovascularization remains challenging. Positron emission tomography/computed tomography (PET/CT), single-photon emission computed tomography (SPECT) and contrast-enhanced ultrasound (CEUS) are molecular imaging techniques that visualize cells in their own microenvironment without invasively manipulating tissues or taking biopsies. First, PET/CT imaging provides a resolution up to 5mm in clinical settings for early and late detection of lesion formation with already various tracers thoroughly investigated. Possible targets for PET/CT that are implicated in IP neovascularization are glycolysis, fatty acid synthesis (FAS) and  $\alpha_v\beta_3$  integrins [76,79]. Because IP neovascularization consists of fast proliferating and metabolically highly active ECs, more energy is consumed (such as glucose and fatty acids) through the upregulation of glycolysis and FAS, both discovered in oncological research [79,80]. Fluorodeoxyglucose (FDG, labelled with  $^{18}\text{F}$ ) and acetate (labelled with  $^{11}\text{C}$ ) are glucose and acetyl-coenzyme-A analogues, respectively. Emission of  $^{18}\text{F}$ -FDG or  $^{11}\text{C}$ -acetate signals reflects the tracer uptake by metabolically active cells. Up to date,  $^{18}\text{F}$ -FDG is one of the most-frequently used PET tracers for *in vivo* imaging of atherosclerosis, but is unsuitable in coronary atherosclerosis as background myocardial uptake is generally higher than overall plaque incorporation [81]. Interestingly,  $\alpha_v\beta_3$  integrins are cell surface glycoproteins that are highly expressed on angiogenic ECs and macrophages in atherosclerotic lesions [76].  $^{18}\text{F}$ -galacto-RGD is a peptide tracer that binds with high affinity to cell surface  $\alpha_v\beta_3$  integrins. Both PET/CT and SPECT/CT have demonstrated distinct

visualization of these integrins in atherosclerotic lesions, and thus revealed to be convenient methods for distinct visualization of IP microvessels [78].

Secondly, CEUS, based on the combination of conventional medical sonography and contrast ultrasound imaging, offers the leverage of high spatial and temporal resolution. In this particular case, antibody-targeted gas-filled microbubbles (to for example  $\alpha_v\beta_3$  integrins) represent a useful tool in visualizing IP neovascularization because of their ability to specifically target vasa vasorum and IP microvessels as an intravascular tracer. Moreover, untargeted gas-filled microbubbles are able to cross the smallest human vessels as a contrast agent without affecting the microenvironment [82]. Microbubbles give a nonlinear response to the ultrasound signal. This allows for distinguishing between the microbubbles response and linear tissue response. Carotid intraplaque neovascularization quantification software (CINQS) was recently designed to quantify IP neovascularization in CEUS images [83]. Both animal and human experimental studies have provided evidence that CEUS is a feasible technique with optimal properties to predict instable plaques at risk [84–86]. With this technique, contrast-agent enhancement in carotid arteries is correlated with neovessel density obtained through histology [85,87].

Magnetic resonance imaging (MRI) is a valuable technique to detect IP haemorrhages, though it is clinically more relevant to detect IP microvessels because they can be detected before IP haemorrhages occur. Patients will benefit much more from an imaging modality that allows early detection of IP neovascularization. Thus, PET/CT, SPECT/CT and CEUS imaging in IP neovascularization yield the most promising qualities, albeit more research is needed to elaborate these techniques in cardiovascular imaging.

#### **4. Animal models of atherosclerosis and plaque rupture**

Current knowledge on the significance of neovascularization in atherosclerosis is mainly based on studies with human atheroma tissue. In spite of obvious scientific proof (see section 2.1.), a causal and straightforward relation between plaque rupture and IP neovascularization has never been confirmed due to the lack of a relevant animal model of atherosclerosis with human-like characteristics such as IP neovascularization. Most commonly used in the investigation of IP microvessels are genetically modified mice, though animals such as rabbits [85,88], pigs [71] and rats [89] are also used. Pigs and rats do not display IP microvessels, which makes them unsuitable for exploring IP neovascularization in plaque development. Rabbit atherosclerotic plaques induced by a combination of 0.2 % cholesterol diet and double balloon endothelial denudation contain microvessels [90]. However, mice are preferred because they are easy to handle. Moreover, some genetically modified mice (e.g. apolipoprotein E [ApoE<sup>-/-</sup>]-deficient mice with a heterozygous mutation in the fibrillin (Fbn)1 gene, vide infra) show spontaneous IP neovascularization even without surgical interventions.

For a long time, ApoE<sup>-/-</sup> mice were considered the most human-resembling animal model to study plaque development. A second animal model widely used in atherosclerosis research is the LDLR knockout (LDLR<sup>-/-</sup>) mouse [91]. ApoE<sup>-/-</sup> and LDLR<sup>-/-</sup> mice that are fed a high fat diet develop lipid-rich plaques with human-like components such as foam cells, a necrotic core and a fibrous cap rich in SMCs [92]. However, plaque rupture or coronary thrombosis almost never occurs in these models, which makes them unsuitable to investigate unstable plaques. A major cause of plaque rupture in human atherosclerosis is the development of IP microvessels with subsequent IP haemorrhage, which are also lacking in advanced lesions of these animals. Multiple studies investigating plaque development and IP neovascularization were performed in ApoE<sup>-/-</sup> and LDLR<sup>-/-</sup> mice based on the angiogenic activity occurring in the adventitial layer of the vessel wall [74,93]. The impact of a treatment is most often established

through the analysis of plaque development and composition, which are indirect parameters of IP neovascularization. These observations do not suffice to unravel the exact role of IP neovascularization since much more parameters are implicated in lesion formation.

Besides hyperlipidaemia-induced atherosclerosis, mouse models of arterial injury, vein graft or vessel transplantation have been explored to establish a substitute model for the human progress of atherosclerotic pathology. In 2002, vein grafts in ApoE\*3 Leiden mice [94] were among the first lesions in animals with features resembling those in human plaques such as intimal dissection, intramural thrombosis and IP neovascularization (figure 4A) [95]. Given that this model is based on vein graft surgery, it requires a complex intervention to induce microvessel formation via neovascularization. Immunohistochemical staining of the basement membrane and pericytes surrounding these microvessels pointed out that there was an intact basement membrane, but efficient pericyte covering was lacking. Diffuse erythrocytes were observed in the vicinity of the newly-formed microvessels, suggesting that they are leaky and immature, similar with microvessels in human plaques [95]. More recently, the first animal model with spontaneous plaque rupture was developed, namely the ApoE<sup>-/-</sup> Fbn1<sup>C1039G<sup>+/-</sup></sup> mouse [96]. A heterozygous mutation C1039G<sup>+/-</sup> in the Fbn1 gene results in the fragmentation of elastic fibres in the media of the vessel wall. Fragmented elastic fibres combined with a western-type diet in ApoE<sup>-/-</sup> mice leads to enhanced plaque formation with features typical of human unstable lesions such as IP neovascularization and haemorrhages (figure 4B), and triggers sporadic rupture and myocardial infarction without any surgical interventions [97]. It seems that permeabilization of the extracellular matrix (due to the disruption of elastic fibres) as well as IP hypoxia (due to the highly inflamed status of the plaques) is required to enable migrating ECs of the vasa vasorum to penetrate into the plaque [97]. Overall, this unique mouse model gives the opportunity to further identify key players in

atherosclerosis and provides the possibility of investigating novel targets such as IP neovascularization, as well as therapeutic interventions.

## **5. Expert opinion**

To our current knowledge, a critical balance between pro-angiogenic and pro-maturation factors is indispensable for the integral development of mature microvessels in plaques. However, the question remains whether the inhibition of IP neovascularization will result in stable lesions. Hitherto, a conclusive answer is lacking even though preclinical studies are testing potential angiogenic inhibitors. It should be noted that the lack of an appropriate animal model gave rise to discrepancies in the outcome of several studies, making it impossible to make scientifically-based conclusions [93,98–102]. In the last decade, there has been a substantial increase in compounds targeting VEGF or its downstream pathways to counteract angiogenic growth. Bevacizumab, a monoclonal antibody against VEGF-A, inhibited IP neovascularization with smaller atherosclerotic lesions as a result [100]. This finding nourished the presumption that targeting IP neovascularization will result in more stable lesions and opened a new field of interest in scrutinizing the development of atherosclerosis. However, because VEGF-A takes part in normal physiological processes, a balance in VEGF-A levels is necessary to maintain its function in vasodilatation, wound healing and thrombosis. Indeed, the homeostatic function of VEGF-A seems disturbed during anti-VEGF treatment in cancer, as (sometimes severe) cardiovascular side effects occur such as hypertension and thrombo-embolic events [103]. Therefore, we feel that antibody therapies targeting VEGF-A are not an ideal approach to stabilize atherosclerotic plaques.

Besides antibodies against VEGF-A [104], antibodies blocking VEGFR2 (DC101) revealed smaller lesions and less IP haemorrhages in ApoE\*3 Leiden mice [105]. VEGFR2 blockade

had no effect on IP neovessel density even though there were less extravasated erythrocytes, indicating that the newly-formed microvessels had fully matured [105]. Furthermore, tyrosine kinase inhibitors are an important subgroup of new anti-cancer compounds specifically targeting VEGFR-induced neovascularization [106]. Tyrosine kinases are signalling proteins that act by promoting proliferation, survival and migration of ECs, which makes them very attractive targets in blocking angiogenic growth. We assume that compounds targeting VEGFR1-3 (e.g. axitinib) may provide an interesting approach to investigate the potential of VEGF signalling in the stabilization of atherosclerotic plaques.

While VEGF and its downstream pathways have been widely investigated to regulate and to inhibit neovascularization, recent studies in the field of oncology present evidence from a different point of view. Indeed, modulation of cell metabolism (glycolysis or fatty acid oxidation) has already shown beneficial effects in cancer research, and we speculate that this approach could be of value in atherosclerosis as well. Oxygen has always been considered as the driving force of neovascularization. However, it has been demonstrated that proliferating ECs generate up to 85% of their ATP from glycolysis [80]. This finding makes this metabolic pathway an interesting alternative target for the inhibition of neovascularization. The enzyme 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3) exerts a critical role in glycolytic flux during neovascularization. It activates the conversion from fructose-6-phosphate (F6P) to fructose-2,6-bisphosphate (F2,6P2), which is an allosteric activator of 6-phosphofructo-1-kinase (PFK-1), a rate-limiting enzyme in glycolysis [80,107]. Inhibition of PFKFB3 results in a decreased glycolytic flux and reduces angiogenic growth, at least in tumors [107]. More importantly, partial and transient inhibition of the enzyme suffices to inhibit pathological neovascularization in a significant way [108]. 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO), a small-molecule inhibitor of PFKFB3, was the first compound that made it possible to target the metabolism of fast proliferating cells by

inhibiting neovascularization. More recently, another approach was assessed by focusing on the fatty acid oxidation pathway [79]. Inhibition of carnitine palmitoyltransferase 1A (CPT1A), a rate-limiting enzyme in fatty acid oxidation, affects proliferation during vessel sprouting by blocking *de novo* nucleotide synthesis [79,80] without causing energy stress or a redox imbalance, which is actually the case with the transient inhibition of glycolysis [109]. We hypothesize that both pathways will provide a new angle in anti-angiogenic research in atherosclerosis, with a lot of potential to be investigated in preclinical studies.

Statins reduce cholesterol levels via inhibition of hydroxymethylglutaryl co-enzyme A (HMG-CoA) reductase, the rate-limiting enzyme in cholesterol biosynthesis, and are widely used in primary and secondary prevention of cardiovascular disease. Growing evidence indicates that statins trigger pro-angiogenic effects at low (nanomolar) concentrations and anti-angiogenic effects at higher (micromolar) concentrations [110]. They display these pleiotropic functions beyond lipid lowering, which provide additional benefit in the reduction of atherosclerosis. HMG-CoA reductase is an enzyme that regulates the synthesis of mevalonic acid, a precursor of cholesterol as well as geranyl geranylpyrophosphate (GGP). This last intermediate seems to play an important role in the anti-angiogenic properties of statins as the supplementation of GGP reverses the angiostatic effects [110]. Indeed, ApoE<sup>-/-</sup> Fbn1<sup>C1039G<sup>+/-</sup></sup> mice treated with atorvastatin show much less IP neovascularization as well as a reduction in cardiovascular morbidity and mortality without obvious changes in plasma cholesterol [111]. Accordingly, we presume that patients suffering from atherosclerosis can benefit from the anti-angiogenic properties of statins, even without elevated cholesterol levels.

At present, vulnerable plaque imaging remains largely investigational with long-term, clinical end point trials needed before widespread adoption of such an approach is warranted. It is possible to use plaque echogenicity to assess tissue composition with echolucent lesions demonstrating features of vulnerability such as IP haemorrhage. MRI can distinguish stable

fibroatheromas from those with thin or ruptured caps within the human carotid artery but it is technically challenging to image coronary plaque by MRI. One particularly important attribute of MRI is the ability to detect IP haemorrhage [112]. Interestingly, a possible target for PET/CT is glycolysis, which is implicated in IP neovascularization. However, to our opinion it is too early to recommend one particular imaging technique to visualize IP neovascularization and haemorrhage.

Finally, we consider vein grafts in ApoE\*3 Leiden mice as well as the spontaneous development of unstable, rupture-prone plaques in ApoE<sup>-/-</sup> Fbn1<sup>C1039G<sup>+/-</sup></sup> mice very useful animal models for experimental research. Vein grafts in hypercholesterolemic ApoE\*3 Leiden mice develop advanced lesions just four weeks after surgical manipulation, which enables rapid screening of several compounds that might counteract IP neovascularization and lesion development [95]. However, development of an IP network of microvessels occurs in a venous vessel wall, whereas in humans atherosclerosis develops in large to midsized arteries. Immediate translation of results to human patients is therefore not possible. The ApoE<sup>-/-</sup> Fbn1<sup>C1039G<sup>+/-</sup></sup> mouse, on the other hand, develops unstable plaques in its arteries without the need of a surgical intervention, but requires up to 25 weeks Western-type diet to allow formation of unstable lesions with features such as abundant IP neovascularization, plaque rupture and myocardial infarction [97]. Accordingly, we feel that this model is not the first choice for large drug screening, but rather complementary with the vein graft model in scrutinizing the role of a pre-screened compound because of the human-like characteristics of lesion development. Overall, we conclude that plaque neovascularization represents an interesting therapeutic target to acquire stable lesions. In particular, therapeutic manipulation of microvascular cell metabolism might offer promising opportunities to inhibit neovascularization in atherosclerosis and to prevent formation of unstable plaques.

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## Figure legends

### Figure 1. **Schematic overview of microvessel sprouting in atherosclerotic plaques.** (1)

Upregulation of pro-angiogenic factors stimulates pericytes to detach from the basement membrane and endothelial cells (ECs) to unfasten their cellular junctions. (2) ECs experience an angiogenic switch, inducing two different phenotypes, namely tip cells and stalk cells. Tip cells work their way into the hypoxic zone by sprouting from the vasa vasorum through the media into the plaque. They carry actin-rich filopodia at their edges mediating the migration of ECs. Stalk cells follow the lead of tip cells and proliferate to extend the developing microvessel. (3) Because maturation of microvessels is disturbed, ECs lack a dense barrier. This enables lipids, inflammatory mediators and blood cells to intrude the plaque, a process known as intraplaque haemorrhage promoting destabilization of the advanced lesion.

### Figure 2. **Growth factors in intraplaque neovascularization.** Hypoxia emerges in the

atherosclerotic lesion due to the incessant accumulation of lipids and inflammatory mediators. In hypoxic conditions, hypoxia-inducible factor 1 alpha (HIF-1 $\alpha$ ) signalling mediates the upregulation of several genes to preserve the continuity in vessel formation. HIF-1 $\alpha$  induces upregulation of vascular endothelial growth factor (VEGF)-A, placenta growth factor (PlGF), fibroblast growth factor (FGF) and angiopoietin 2 (Ang2), all included in the stimulation of neovascularization in the plaque. Via their specific receptors, a cascade of signalling pathways is accelerated, resulting in the differentiation, proliferation, migration and survival of ECs and their progenitor cells. In order to obtain stable and fully matured microvessels, mural cells have to surround the developing structure, stimulated by Ang1 and platelet-derived growth factor BB (PDGF-BB). However, the balance between pro-angiogenic and pro-maturation factors is disturbed, resulting in an insufficient amount of stimuli to grow into

functional blood vessels. Leaky vessels enable more lipids and inflammatory mediators to enter the plaque and stimulate plaque progression.

**Figure 3. Intraplaque neovascularization in human atherosclerotic plaques.** (A) Example of an advanced human atherosclerotic plaque in a carotid endarterectomy specimen showing a large lipid-rich necrotic core with extensive intraplaque microvessels. The plaque is immunohistochemically stained for endothelial cells using anti-vWF antibodies. (B) High-power micrograph of the boxed area in panel A, illustrating microvessels (arrowheads) surrounding and infiltrating the necrotic core. Scale bar = 50µm. M: media; NC: necrotic core.

**Figure 4. Intraplaque neovascularization in vein grafts of ApoE\*3 Leiden mice and in plaques of ApoE<sup>-/-</sup> Fbn1<sup>C1039G<sup>+/-</sup></sup> mice.** (A) Example of microvessels (arrows) in CD31 stained vein grafts of ApoE\*3 Leiden mice. Scale bar = 100µm. (B) ApoE<sup>-/-</sup> Fbn1<sup>C1039G<sup>+/-</sup></sup> mice on a western-type diet (WD) develop intraplaque neovascularization in the common carotid artery, brachiocephalic artery and aortic arch. Example of multiple microvessels (arrows) in an haematoxylin-eosin stained section of the brachiocephalic artery of ApoE<sup>-/-</sup> Fbn1<sup>C1039G<sup>+/-</sup></sup> mice after 25 weeks of WD. Scale bar = 50 µm. M: Media.