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## Targeting intraplaque angiogenesis : imaging and therapeutic interventions

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# Chapter 2.

Plaque Angiogenesis and Intraplaque Hemorrhage in Atherosclerosis.

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## **ABSTRACT**

Acute cardiovascular events, due to rupture or erosion of an atherosclerotic plaque, represent the major cause of morbidity and mortality in patients. Growing evidence suggests that plaque neovascularization is an important contributor to plaque growth and instability. The vessels' immaturity, with profound structural and functional abnormalities, leads to recurrent intraplaque hemorrhage.

This review discusses new insights of atherosclerotic neovascularization, including the effects of leaky neovessels on intraplaque hemorrhage, both in experimental models and humans. Furthermore, modalities for in vivo imaging and therapeutic interventions to target plaque angiogenesis will be discussed.

## INTRODUCTION

The majority of acute cardiovascular events in patients is caused by occlusive thrombosis formed by rupture or erosion of an atherosclerotic plaque.<sup>1</sup> Despite improved insight into disease pathogenesis and therapeutic options, additional treatment strategies are required to block mechanisms involved in plaque destabilization.

Advanced atherosclerotic lesions are characterized by large necrotic cores with thin fibrous caps, cholesterol deposits, inflammatory cells and calcifications.<sup>2</sup> Recent insights in the pathophysiology of atherosclerotic lesions have shed new light on the formation of unstable lesions. For instance, it has been shown that, of the total number of foam cells a significant portion is derived from smooth muscle cells (SMCs) rather than from macrophages<sup>3</sup>. Also the role of calcification is more clarified, it has been shown that extended calcification can stabilize atherosclerotic plaques<sup>4</sup>, whereas spotty micro-calcifications contribute to plaque destabilization.<sup>5-7</sup> Furthermore, it is becoming more and more clear that plaque angiogenesis and intraplaque hemorrhage (IPH) are important contributors to unstable lesions.<sup>8, 9</sup> Plaque angiogenesis is a physiological response to the increased oxygen demand in the plaque but can have adverse effects by facilitating IPH and influx of inflammatory mediator.<sup>10</sup>

This review is focused on plaque angiogenesis, the relation with inflammatory mediators, and the subsequent effects of IPH on plaque instability, both in experimental models and in humans. Moreover, options to target plaque angiogenesis for imaging and therapeutic purposes will be discussed.

## ANGIOGENESIS-DRIVING PROCESSES

### Hypoxia

The molecular mechanism regulating angiogenesis in atherosclerosis involves signaling pathways that are mainly driven by the lack of oxygen.<sup>11</sup> Hypoxia occurs when oxygen supply is decreased and or oxygen demand is increased. The ability to sense and respond to changes in O<sub>2</sub> concentration is a fundamental feature of all nucleated cells. Cell survival in a hypoxic environment leads to a general shut-down of energy-consuming transcription and translation, with one major exception — the hypoxia-inducible factor (HIF) pathway.<sup>12, 13</sup>

Hypoxia promotes monocyte/ macrophage survival and oxLDL uptake by macrophages.<sup>14</sup> It also enhances the expression of matrix metalloproteases by a variety of cells in the plaque contributing to the instability of the plaque.<sup>15</sup> Furthermore, due to the hypoxic state of macrophages ATP depletion occurs, causing cell death and expansion of the necrotic core leading to a feedback cycle between plaque expansion and hypoxia. Fong et al. has shown that exposure to hypoxia accelerates the plaque growth of ApoE KO mice fed with a high cholesterol diet.<sup>16</sup> In contrast, when atherosclerosis prone mice were exposed to carbogen (95% O<sub>2</sub>, 5 % CO<sub>2</sub>) oxygenation, plaque growth was inhibited.<sup>12</sup> Sluimer et al. showed extensive hypoxia in the center of advanced human carotid atherosclerotic plaques.<sup>17</sup> Pimonidazole, a hypoxia marker, was co-localized with CD68 positive macrophages, HIF1- $\alpha$  and VEGF expression, suggesting the involvement of the HIF pathway in the regulation of human plaque angiogenesis and lesion progression.<sup>17</sup>

HIF is a heterodimeric protein composed of  $\alpha$  and  $\beta$  subunits. The  $\alpha$  chain confers oxygen regulation on the complex and its expression is hypoxia-dependent and has three isoforms, HIF-1 $\alpha$ , 2 $\alpha$  and 3 $\alpha$  of which only HIF-1 $\alpha$  and HIF-1 $\beta$  are widely expressed in normal tissues.<sup>18-20</sup> The beta chain (three isoforms) is constitutively expressed and works as an aryl receptor nuclear translocator. Under normoxic conditions, the synthesized HIF-1 $\alpha$  is rapidly degraded and the co-activators are blocked by oxygen-dependent enzymes, the prolyl-hydroxylases domain (PHD) enzymes<sup>21, 22</sup>. During hypoxia, PHD activity is reduced, allowing the dimerization of HIF-1 $\alpha$  and HIF-1 $\beta$ .<sup>23, 24</sup> This active complex binds to DNA starting the transcription of downstream genes involved in angiogenesis and inflammation.<sup>25</sup>

HIF-1 $\alpha$  mediates inflammation by promoting pro-inflammatory cytokines expression (stromal cell-derived factor 1, VEGF-A) and consequently inflammatory cell recruitment.<sup>26, 27</sup> Arrup et al. have shown that HIF-1 $\alpha$  expression also modulates the macrophage glycolytic pathway, by increasing glucose uptake and glucose transporter 1 mRNA expression, enhancing oxygen consumption. In addition, HIF-1 $\alpha$  reduces the mRNA expression of the major cholesterol transporters<sup>28</sup> and activates the lectin-like OxLDL receptor-1 scavenger receptors that mediates oxLDL uptake in macrophages<sup>29</sup>, leading to the expansion of the foam cell population. Interestingly, hypoxia can also reduce macrophage migration, by mediating the expression of retention molecules that stimulate their accumulation and prevent egression from the plaque.<sup>30</sup> Thus, hypoxia induced overexpression of HIF-1 $\alpha$  not only regulates plaque angiogenesis, but also has additional effects stimulating the growth of atherosclerotic plaques.

Moreover, an extensive crosstalk between HIF and nuclear factor- $\kappa$ B (NF- $\kappa$ B), two important molecular players in atherosclerosis, has been reported. They have common activating stimuli and share regulators and targets.<sup>31, 32</sup> Only the canonical NF- $\kappa$ B pathway is sensitive to hypoxia, while both the inhibitor of NF- $\kappa$ B subunit alpha (IKK $\alpha$ ) and the inhibitor of NF- $\kappa$ B subunit beta (IKK $\beta$ ) can be hydroxylated by PHD.<sup>33, 34</sup> Marsch et al. shows that PHD1 knockout mice display a protective cardiovascular metabolic phenotype with lower plasma cholesterol levels and glucose tolerance improvement.<sup>35</sup> Furthermore, NF- $\kappa$ B has been shown to play a role in basal and stimulated HIF-1 $\alpha$  mRNA expression. The p50 and p65 NF- $\kappa$ B subunits can bind to the HIF-1 $\alpha$  promoter in response to hypoxia and when these subunits are overexpressed, an increase in HIF-1 $\alpha$  mRNA levels and promoter activity is observed.<sup>36</sup> The full mechanism between HIF and NF- $\kappa$ B in hypoxia is not yet completely understood but these features highlight the complex and interrelated hypoxia and inflammatory signaling cascades in atherosclerosis.

### **Endothelial Cell Sprouting**

Angiogenic sprouting involves the invasion of avascular areas by proliferating and migrating ECs. The mechanism of angiogenesis has been deeply studied and the process of neovessel formation has been described in detail.<sup>37-40</sup> In a nascent sprout, three phenotypically distinct EC types can be recognized: tip, stalk and phalanx cells. Tip cells are motile and invasive, protrude filopodia and lead the way to the nascent sprout since they are located at the forefront of the vessel branches, sensing and responding to guidance cues in the microenvironment, while migrating toward an angiogenic stimulus. Stalk cells trail behind the tip cells and elongate the stalk of the sprout. These cells



proliferate, form junctions, lay down extracellular matrix and form a lumen. Phalanx cells are the most quiescent ECs, lining vessels once the new vessel branches have been consolidated.<sup>41</sup>

The differentiation of tip versus stalk cell occurs via a Notch mediated lateral inhibition mechanism. VEGF-A and VEGF receptor 2 (VEGFR-2) signaling induces tip cells formation and delta-like canonical Notch ligand 4 (Dll4) upregulation. Expression of Dll4 in tip cells activates Notch in adjacent ECs, thereby decreasing the expression of VEGFR-2 and inducing stalk cell differentiation.<sup>42</sup> Interestingly, it was shown that Notch signaling promotes the progression of atherosclerosis in vivo.<sup>42</sup> Blockade of Dll4–Notch signaling by anti-Dll4 antibody administration, suppresses atheroma progression in the aorta of LDLR KO mice that were fed high-cholesterol/high-fat diet for 24 weeks. Blockade of this angiogenesis related pathway leads to a reduction in the accumulation of macrophages in the aorta of the mice treated with neutralizing anti-Dll4 antibody<sup>42</sup>, showing the tight interaction of these processes.

VEGF-A, VEGF-C and their receptors VEGFR-2 and VEGFR-3 participate in the detachment process of ECs from the ECM and guide the behavioral switching of the ECs. To ensure optimal fitness of the tip cell leading the sprout, the EC with the highest responsiveness to VEGF will occupy the tip position. Chronic inflammatory cell infiltration in the atherosclerotic plaque activates ECs and enhances the expression of different cell adhesion molecules like vascular cell adhesion protein 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1), which recruit monocytes and lymphocytes.<sup>43</sup>

A stabilized and mature vascular plexus includes adoption of a quiescent endothelial phalanx phenotype, branch regression, basement membrane

deposition and coverage with pericytes that will stabilize the endothelial tubes and help regulating the capillary diameter and vessel permeability. Fusion of sprouting neovessels, which is necessary to form vascular networks, is controlled by bridging-macrophages.<sup>44</sup> Macrophages accumulate at sites of vessel anastomosis and interact with filopodia of neighboring tip cells during fusion. It was shown in zebrafish embryos that macrophages can act as cellular chaperones for endothelial cell fusion by bridging tip cells from different vessel segments.<sup>45</sup>

Although it is widely accepted that angiogenesis is mainly regulated by hypoxia, other factors like hemodynamic forces may also regulate angiogenesis.

### **Hemodynamic forces**

Blood flow plays crucial roles in angiogenesis by generating frictional force that develops between flowing blood and the vascular endothelium.<sup>46</sup> ECs covering the inner surface of blood vessels are constantly exposed to different types of shear stress. Shear stress is pulsatile in normal physiology, but can be oscillatory in pathologies such as atherosclerosis, affecting endothelial function and morphology. The EC response to shear stress is closely linked to the regulation of vascular tone, blood coagulation and fibrinolysis, angiogenesis, and vascular remodeling, and it plays an important role in maintaining the homeostasis of the circulatory system.<sup>47</sup> Shear stress induces collateral artery growth as well as capillary growth and it was shown that endoglin played a crucial role in this process.<sup>48</sup> Furthermore, it is known that shear stress modulates the expression of thrombospondin 1 and its receptor CD36 during angiogenesis in vivo.<sup>49</sup> Impairment of the EC response to shear stress leads to

the development of vascular diseases such as hypertension, thrombosis, aneurysms, and atherosclerosis.<sup>47</sup> The mechanisms and sensors by which ECs initially recognize shear stress have yet to be confirmed, but the sensors most likely involved in angiogenesis are Piezo1<sup>50</sup>, calcium<sup>51</sup> and primary cilia<sup>52</sup>.

## **ANGIOGENESIS IN THE ATHEROSCLEROTIC PLAQUE**

Pathological angiogenesis of the vessel wall is a consistent feature of atherosclerotic plaque development and progression of the disease<sup>53</sup> however the source of plaque neovessels is not fully established. The general idea is that endothelial cells (ECs) grow from the existing adventitial vasa vasorum triggered by a gradient of VEGF, into the plaque.<sup>54</sup>

Adventitial angiogenesis is thought to be the main source of neovessels. In addition, it has been suggested that angiogenesis may also occur from the luminal side. However, clear evidence for the extent of this phenomenon is lacking.

Due to its important role in atherosclerotic plaques, vasa vasorum has been studied as a therapeutic target. Langheinrich et al. reported a significant decrease of lesion size in ApoE KO LDL KO mice treated with 3-Deazaadenosine, an anti-inflammatory and anti-proliferative drug. This was accompanied by a significant decrease of vasa vasorum neovascularization, although no effects on intraplaque angiogenesis were reported.<sup>55</sup>

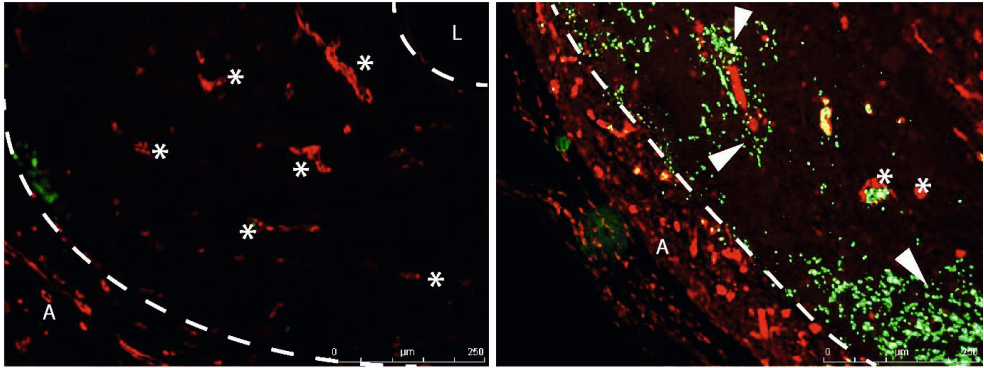
Descriptive and cross-sectional studies in humans suggest a clear association between the neovessel density and atherosclerotic progression and vulnerability.<sup>11</sup> A large longitudinal atherosclerosis plaque biobank study

(AtheroExpress) demonstrated that plaque neovascularization but also IPH significantly relate to adverse cardiovascular outcome during clinical follow-up.<sup>54</sup>

### **Intraplaque hemorrhage**

Neovessels in vulnerable plaques are immature, irregular and fragile due to the compromised structural integrity.<sup>56</sup> In fact, they are characterized by discontinuous basement membrane and a low number of tight junctions between ECs.<sup>57</sup> Moreover these premature vessels are relatively poor in pericytes coverage and are therefore immature and highly susceptible to leakage of circulating cells<sup>58</sup>, leading to intraplaque hemorrhage (IPH).

In the oncological field, newly formed vessels have been reported to have the same features. Tumor vessels are heterogeneous<sup>59</sup>, and many are hyperpermeable<sup>60</sup>. In their walls, there are inter endothelial openings and trans endothelial channels, resulting in a wide range of pore sizes.<sup>61</sup> The hyperpermeability of tumor vessels allows plasma to flow to the interstitial space.<sup>61</sup> Moreover tumor vessels are organized in a chaotic fashion and do not follow the hierarchical branching pattern of normal vascular networks.<sup>62</sup> Of interest is that compared to normal individuals, patients with acute coronary syndrome have red blood cells with higher amounts of cholesterol in the membranes. Consequently the leakage of these red blood cells may lead to an increased cholesterol deposition, atheroma growth and decreasing plaque stability. Thus, the cholesterol content in the red blood cell membrane could be a marker for the growth and vulnerability of the atherosclerotic plaque.<sup>63, 64</sup> This is further illustrated in Figure 1.



**Figure. 1. Vein graft lesions in hypercholesterolemic ApoE3\*Leiden mice 28 days after surgery.** (a) Vein graft lesions show extensive neovessels (\*). (b) Red blood cells dispersed in the extracellular matrix outside the neovessels demonstrate intraplaque hemorrhage (arrow head) in a vein graft lesion. CD31 positive endothelial cells (red), TER119 positive red blood cells (green) (A) adventitia, (L) lumen.

The key players in vessel maturation are VEGF its receptors and the members of the angiopoietin system. VEGF and its main receptors VEGFR-1 and VEGFR-2 drive EC proliferation and tube formation as well as the attachment and detachment of pericytes during the maturation of neovessels.<sup>37</sup> Angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2) are ligands of the endothelial receptor Tie-2, and both have a major role in the final maturation phase of neovascularization with opposite functions. HIF-1 $\alpha$  and VEGF-A, induce Ang-2 to destabilize the interactions between pericytes and ECs, and thus allows vessels to grow. Ang-1 together with platelet derived growth factor (PDGF) acts as a major stabilizing factor that increases the stability of the junctions between the EC, thus promoting vessel maturity and stability and reducing leakiness.<sup>65</sup> The balance between Ang-1 and Ang-2 expression was explored in human plaques. A positive correlation was observed between Ang-2 expression and microvascular density within the plaque, as well as with the ratio Ang-2/Ang-1.<sup>66</sup> Also, PDGF-BB and its receptor (PDGFR)- $\beta$  are known to be important in vessel permeability,

fragility, and impaired perfusion<sup>43</sup>, because of their pivotal role in the establishment of functional blood vessels by recruiting and stabilizing perivascular cells. These findings suggest that the neovessels are subjected to regular leakage associated with extravasation of red blood cells, leucocytes and plasma lipids to the neighborhood.

### **Red blood cells**

Extravasated red blood cells (RBCs) constitute the main cellular component of IPH, which with their hemoglobin content and cell membrane components, enriched in non-esterified cholesterol, participate in both the cholesterol accumulation and the oxidative process.<sup>67</sup> Once trapped in the highly oxidative environment of the atherosclerotic plaques, RBCs tend to lyse quickly.<sup>68</sup> The cytoplasm of RBCs is rich in hemoglobin, which can attract multiple monocytes and neutrophils to the plaque.<sup>58</sup>

Cholesterol retention in the atherosclerotic plaque leads to cholesterol crystal formation. This can originate directly from free cholesterol or from cholesterol esters endocytosed by foam cells. RBCs membrane have a high cholesterol content with a percentage of lipids up to 40% of the total weight of the cells.<sup>69</sup> It has been suggested that RBC membranes are very important contributors to lipid deposition and lipid core expansion upon IPH. This is further illustrated by the presence of iron and glycophorin A, a characteristic protein of the RBC membrane, which co-localizes with cholesterol crystals within the plaques, suggesting that cholesterol crystals could originate from erythrocytes phagocytized by macrophages.

## Inflammation

Inflammation is a key factor in all stages of atherosclerosis progression. In the initial phase of atherosclerosis, oxidized low-density lipoproteins (oxLDL) accumulation in the aortic wall triggers the expression of adhesion molecules that facilitate the migration of monocytes into the aortic wall.<sup>10</sup> Monocytes differentiate into macrophages that engulf oxLDL and convert into lipid filled foam cells. Accumulation of modified LDL by macrophages activates cytokine production that promote the influx and activation of other inflammatory cells and their retention in the plaque.

Most inflammatory cells in the plaque, and especially macrophages, are metabolic very active cells that exhibit high oxygen consumption which leads to oxygen deprivation in the plaque.<sup>70</sup> In addition, monocytes/macrophages release pro-angiogenic factors such as VEGF and by interacting with vascular smooth muscle cells (VSMC), macrophages induce unbalanced synthesis of the extracellular matrix (ECM) leading to secretion of VEGF by VSMC.<sup>71</sup> Furthermore, in endarterectomy samples obtained during surgery an accumulation of mast cells was observed in neovessel-rich areas of atherosclerotic plaques.<sup>72</sup> Whereas, in animal experiments it was demonstrated that mast cells situated near the newly formed vessels contained fibroblast growth factor (FGF), a potent pro-angiogenic factor.<sup>73</sup> In line with these reports, it was stated that vasa vasorum vessel density in atherosclerotic lesions of ApoE KO mice highly correlates with the occurrence of inflammatory cells foci.<sup>71, 74-76</sup>

In advanced lesions, neovessels leakage constitutes the main entrance for inflammatory cells. The influx of RBCs facilitate the extravasation of circulating inflammatory cells. The influx of RBCs facilitate the extravasation of circulating

inflammatory cells. RBCs can change the forces on an interacting cell, by giving the ability to interact with the endothelium at higher shear stress, increasing the contact frequency and duration with the endothelium.<sup>77</sup> RBCs increase the numbers of rolling and adhering monocytes by increasing the normal force and/or the frequency of collision of monocytes interacting with the endothelium. The increase in cell capture requires the physical presence of RBCs, indicating that RBC-induced mechanical forces may facilitate leukocyte-endothelial cell interactions in vivo.<sup>77</sup> This invasion leads to reactive, inflammatory and apoptotic environment where the instability of the plaque is profoundly affected. Not only monocytes are increased, also neutrophils and mast cells were increased, that can release their granular content rich in serine proteases and matrix metalloproteases.<sup>67</sup> These proteases digest components of elastic fibers (elastin) and of the basement membrane (collagen, laminin and fibronectin). This high proteolytic activity can ultimately lead to fibrous cap thinning and plaque erosion.<sup>78, 79</sup>

Furthermore, the influx and lysis of RBCs drives a higher request of macrophage activity in order to phagocytose the RBC remainders. In combination with the hampered efferocytosis response in the atherosclerotic lesions causing an impaired clearance of these apoptotic cells by lesional macrophages this may explain why these macrophage accumulate in the atherosclerotic necrotic core, and may potentiate vascular inflammation.<sup>80, 81</sup> Also, their ability to efferocytose, phagocytosis of dying/dead cells, is defective. This malfunctioning increases the inflammation state and reduces cholesterol efflux contributing to necrotic core expansion and ultimately, the increase of risk of plaque rupture.<sup>82</sup>



## IMAGING OF ANGIOGENESIS

The detection of patients with atherosclerotic plaques at risk is a major challenge for the cardiovascular research field. It has inspired the development of invasive and non-invasive imaging technologies to visualize the atheroma in detail. The significant investments in these imaging technologies are not only justified by the need to early diagnose patients with atherosclerosis but also by the development of drug programs.<sup>83</sup>

The most prominent imaging technologies are already in used in clinical studies and their value to identify crucial characteristics of vulnerable plaques is undeniable.<sup>84</sup> Plaque angiogenesis is one of these features and its detection in vivo can represent a step forward in diagnosis and follow up of atherosclerosis.

The most advanced technique in humans to visualize angiogenesis is positron emission tomography, PET.<sup>85-87</sup> This high sensitive tool uses 18F-fluorodeoxyglucose (FDG), a glucose analogue tracer. After intravenous injection, 18F-FDG is taken up by cells that metabolize glucose, where it becomes trapped after phosphorylation. Due to the high glycolytic rate of endothelial cells, plaque neovascularization can be monitored by 18F-FDG uptake.<sup>84</sup> However, PET images do not give structural information. This has to be assessed using PET with combined techniques such as computed tomography (CT), magnetic resonance imaging (MRI).<sup>88</sup> Another disadvantage is the low resolution, as a result of this imaging of angiogenesis in small size animal models is still a challenge.

Near-infrared fluorescence (NIRF) appears to be a highly versatile platform for in vivo molecular imaging due to their picomolar sensitivity and microscopic

resolution.<sup>89</sup> Matter et al. developed a sensor for NIRF that targets the extra-domain B of fibronectin, inserted into fibronectin during angiogenesis. In this study, blood vessels were visualized with a good target-to-background ratio.<sup>90</sup> NIRF can also be used for the identification of specific plaque features, such as MMP activity<sup>91</sup> or flow patterns.<sup>92, 93</sup> NIRF however does not provide any structural information on the plaque. This limitation can, in part, be overcome by multimodal imaging such as NIRF–optical coherence tomography (OCT) and others.<sup>94, 95</sup>

OCT is an imaging technique also based on infrared light, which can be used to study atherosclerotic plaques with extreme spatial accuracy. OCT imaging presents a strong correlation to histology and specificity to distinguish plaque phenotypes. OCT has been used to identify patients with risk of plaque rupture by measuring calcified nodules, fibrous cap thickness, lipid pool extension and also neovascularization.<sup>96</sup> However, contrary to PET and NIRF, OCT does not allow specific molecular targeting.

No perfect technique is yet available but the combination of multimodal technologies seems to be a promising opportunity for imaging.<sup>94</sup> Furthermore, the strong correlation between angiogenesis and plaque progression suggests a useful application of imaging technologies as a therapeutic approach for patients with atherosclerosis.

## ANGIOGENESIS TARGETS

### VEGF, Ang2 and Endostatin

In the last decade, there has been a substantial increase in compounds targeting different pathways to counteract angiogenic growth, mainly investigated in the oncological field. Interestingly, lately more emphasis is put on stabilizing neovessels rather than blockade of angiogenesis due to unwanted side effects.<sup>97, 98</sup> Several approaches have been investigated in order to block angiogenesis in the atherosclerotic plaques, such as the use of anti-angiogenic agents and blocking pro-angiogenic factors.

Endostar is a novel modified recombinant human endostatin<sup>99</sup> a broad-spectrum angiogenesis inhibitor that interferes with the pro-angiogenic action of growth factors such as basic fibroblast growth factor (bFGF/FGF-2) and VEGF. A study in ApoE KO mice study showed that prolonged treatment with endostatin reduced plaque growth.<sup>100</sup> More recently, Endostar has been tested in a swine model.<sup>101</sup> The combination of hypercholesterolemic diet with balloon injury resulted in early atherosclerotic lesions. The use of Endostar in this model attenuates vasa vasorum neovascularization, vessel wall inflammation and the progression of atherosclerosis.

A different therapeutic approach, besides the use of anti-angiogenic agents, could be the blockage of pro-angiogenic factors. Bevacizumab, a fully humanized anti-VEGF antibody is a well-known inhibitor of angiogenesis and is widely used in clinical oncology. Although Bevacizumab does not recognize murine VEGF<sup>102</sup>, it showed profound effects in a murine model by causing disruption of the endothelium and consequently accelerated atherosclerosis in ApoE KO mice.<sup>103</sup>

In a New Zealand rabbits model, Bevacizumab-eluting stent implantation in iliac arteries inhibits neovascularization without affecting re-endothelialization. Local gene delivery of VEGFR-1 in the iliac artery of a rabbit in which an atherosclerotic plaque was induced by high-lipid diet in combination with balloon catheter injury, reduced lesion formation. This occurred most likely via an inhibitory effect on atherosclerotic plaque angiogenesis, which hints at the clinical utility of sFlt-1 in atherosclerosis therapy.<sup>104</sup>

Apart from VEGF, another angiogenic target under study is Ang-2. Blockade of Ang-2 on experimental atherosclerosis in LDLR KO ApoB100/100 mice on high cholesterol diet was shown to result in delayed fatty streak formation and decreased plasma triglyceride levels. However, Ang-2 deletion did not prevent plaque progression or changes in plaque stability and did not affect adventitial neovessel density.<sup>105</sup>

So far, the right anti-angiogenic target in atherosclerosis is yet to be found, but the potential of anti-angiogenic approaches in the tumor field, suggest that anti-angiogenic treatments in atherosclerosis will be defined in the near future. Of note should be that targeting the vasa vasorum neovascularization is a different approach than blocking the intraplaque angiogenesis, despite the fact that the intraplaque angiogenic capillaries have their origin in the vasa vasorum.

## **EC Metabolism**

In the field of (tumor) angiogenesis, it is well recognized that endothelial cell metabolism changes during hypoxia, switching to glycolysis-dependent ATP production <sup>106</sup>. In atherosclerosis, plaque progression is associated with macro and micro endothelial dysfunction, which is attributed to EC metabolic

maladaptation.<sup>107</sup> Therefore, targeting the endothelial glycolytic metabolism might be a promising therapeutic approach. To date, no treatments are available yet, however some targets have been described: tetrahydrobiopterin (BH4), NADPH oxidase 1 (NOX1) and NADPH oxidase 2 (NOX2) and 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3).<sup>107</sup>

BH4, an endothelial NOS (eNOS) cofactor, is metabolic inactivated during endothelial dysfunction. Strategies to restore vascular BH4 availability is being tested in ongoing studies. In ApoE KO mice with reduced nitric oxide (NO) synthesis, supplementation with BH4 precursors reduces reactive oxygen species (ROS) production and fosters NO synthesis.<sup>108</sup> However, clinical trials in patients with coronary heart disease and myocardial infarction have shown mixed results for BH4 supplementation.<sup>109</sup>

NOX enzymes are another important source of ROS in atherosclerosis which strongly affects plaque angiogenesis. NOX use NADPH, another eNOS cofactor, for ROS production, compromising NO levels.<sup>110</sup> In addition, NOX activate redox-sensitive transcriptional factors such as NF- $\kappa$ B and HIF1 $\alpha$ .<sup>111, 112</sup> Based on those findings, NOX antagonists have been developed for the treatment of cardiovascular diseases and are currently in preclinical testing.

PFKFB3, a key activator of glycolysis (the main pathway source for energy in EC), is highly expressed in proliferating ECs. Interestingly, genetic or pharmacological inhibition of PFKFB3 impairs the ability of ECs to sprout *in vitro* and to form vessels *in vivo*.<sup>113, 114</sup> Pharmacological PFKFB3 blockade, leads to a partial and transient reduction of glycolysis, capable of reducing pathological angiogenesis in inflamed skin, colon and eye disease, without evoking systemic effects.<sup>115</sup> In addition, overexpression of PFKFB3 overrules the pro-stalk cell

effect of Notch signaling, thereby making the stalk cell more competitive for the tip position.<sup>114</sup> Altogether, these findings illustrate the pivotal role of glycolysis in angiogenesis and the therapeutic potential of blocking glycolysis in plaque angiogenesis inhibition.

## **ANIMAL MODELS**

Among the different animal models used to study atherosclerosis, pigs and rats are rarely suitable for exploring plaque neovascularization in the atherosclerotic plaque because they seldom display plaque neovessels.<sup>43</sup> In contrast, induced advanced atherosclerotic plaques in the thoracic descending aorta of New Zealand white rabbits show intra plaque angiogenesis, as detected using contrast-enhanced ultrasound.<sup>116</sup>

Mice are a preferred model to study atherosclerosis since there are all kinds of transgenic strains available. Unfortunately most of the traditionally used strains, ApoE KO, LDLR KO and ApoE3\*Leiden, do not develop extensive neovessels in their atherosclerotic plaques. Recently two models have been developed with atherosclerotic lesions that are more unstable and prone to rupture.

One model is based on murine vein graft atherosclerosis. Human atherosclerotic lesions in saphenous vein bypass grafts are vulnerable and have a higher risk to disrupt than native atherosclerotic lesions.<sup>2, 117</sup> The murine vein graft model is performed by the interposition of a caval vein from a donor mouse into the carotid artery of an atherosclerosis prone recipient mouse. Hypercholesterolemia in ApoE3\*Leiden mice resulted in a significant increase in accelerated atherosclerosis in vein grafts with profound vein graft thickening within 4 weeks

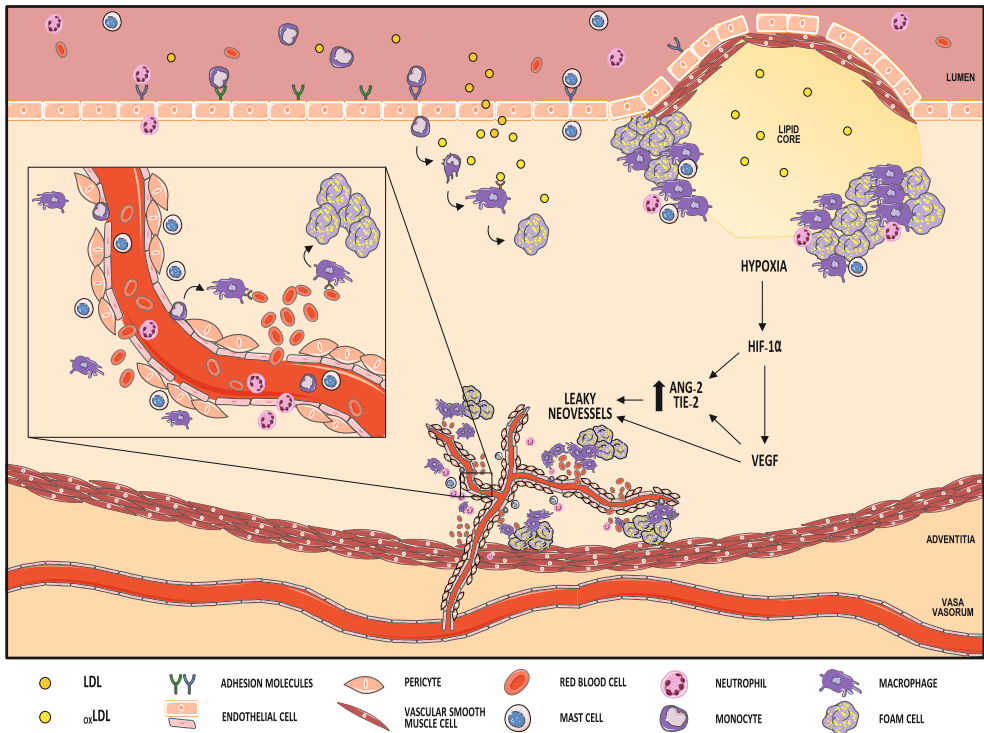
after surgery. These lesions are rich in neovessels and are most likely formed through angiogenesis from the vasa vasora and consist of CD31 positive endothelial cells.<sup>91</sup> In a substantial number of vein grafts, a considerable amount of RBCs could be found in the extracellular matrix, adjacent to the neovessels, suggesting leakiness and intraplaque hemorrhage (Figure. 1). Foam cell accumulation was even observed within seven days after vein bypass grafting, which illustrates the extreme fast initiation of this accelerated atherosclerosis<sup>118</sup>. Reducing inflammatory responses in this model could inhibit IPH, as well as erosions and plaque dissection thereby increasing plaque stability.<sup>119</sup>

Another example of animal model with spontaneous plaque rupture is the ApoE KO Fbn1C1039G<sup>+/-</sup> mice model. A heterozygous mutation C1039G<sup>+/-</sup> in the Fbn1 gene results in the fragmentation of elastic fibers in the media of the vessel wall.<sup>43</sup> The effect of increased arterial stiffness, due to progressive elastic fiber degeneration, on atherosclerosis was studied in this model. ApoE KO Fbn1C1039G<sup>+/-</sup> mice fed with a Western diet for 20 weeks, show sign of plaque destabilization, such as increased number of fibrous caps and enlargement of the necrotic core.<sup>120</sup> The atherosclerotic plaques of ApoE KO Fbn1C1039G<sup>+/-</sup> mice contained highly leaky plaque neovessels and IPH, resulting in plaque rupture, myocardial infarction, stroke, and sudden death.<sup>121</sup>

## CONCLUSIONS

In this review, we described the pathological processes associated with angiogenesis in atherosclerotic plaques and illustrate how plaque neovascularization and IPH are strongly correlated with atherosclerotic plaque progression, instability and rupture. The established impact of plaque

neovascularization on the evolution of atherothrombotic events, together with improved animal models and new imaging technologies, provide a new basis for the development of anti-angiogenic strategies to prevent atherosclerotic plaque progression and instability.



**Figure 2. Plaque angiogenesis.** Once in the plaque macrophages engulf oxLDL and become lipid filled foam cells. Their accumulation activates cytokine production that promotes the influx of neutrophils, mast cells and monocytes. The high oxygen consumption of these inflammatory cells leads to hypoxia. HIF-1 $\alpha$  and VEGF, together with an unbalanced presence of the destabilizing factor Ang2 bound to its receptor Tie2, trigger the formation of leaky neovessels. (Magnification) Intraplaque hemorrhage, with extravasation of red blood cells and inflammatory cells, is due to immature neovessels, lacking proper enveloping pericytes and poor tight junctions between endothelial cells. Macrophages accumulate cholesterol to the point of becoming foam cells by phagocytizing extravasated red blood cells.



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