



Universiteit
Leiden
The Netherlands

Plaque angiogenesis and intraplaque hemorrhage in atherosclerosis

Parma, L.; Baganha, F.; Quax, P.H.A.; Vries, M.R. de

Citation

Parma, L., Baganha, F., Quax, P. H. A., & Vries, M. R. de. (2017). Plaque angiogenesis and intraplaque hemorrhage in atherosclerosis. *European Journal Of Pharmacology*, 816, 107-115. doi:10.1016/j.ejphar.2017.04.028

Version: Not Applicable (or Unknown)

License: [Leiden University Non-exclusive license](#)

Downloaded from: <https://hdl.handle.net/1887/95226>

Note: To cite this publication please use the final published version (if applicable).



Full length article

Plaque angiogenesis and intraplaque hemorrhage in atherosclerosis



Laura Parma¹, Fabiana Baganha¹, Paul H.A. Quax², Margreet R. de Vries^{*,2}

Department of Surgery and Einthoven Laboratory for Experimental Vascular Medicine, Leiden University Medical Center, Leiden, The Netherlands

ARTICLE INFO

Keywords:

Atherosclerosis
Angiogenesis
Plaque neovessels
Intraplaque hemorrhage
VEGF

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.

1. Introduction

The majority of acute cardiovascular events in patients is caused by occlusive thrombosis formed by rupture or erosion of an atherosclerotic plaque (Hansson et al., 2015). 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 (Yahagi et al., 2016). 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 (Chaabane et al., 2014). Also the role of calcification is more clarified, it has been shown that extended calcification can stabilize atherosclerotic plaques (Imoto et al., 2005), whereas spotty micro-calcifications contribute to plaque destabilization (Ehara et al., 2004; Ruiz et al., 2015; Hutcheson et al., 2016). Furthermore, it is becoming more and more clear that plaque angiogenesis and intraplaque hemorrhage (IPH) are important contributors to unstable lesions (Michel et al., 2011; Haasdijk et al., 2016). 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 mediators (de Vries and Quax, 2016).

This review focusses 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.

2. Angiogenesis-driving processes

2.1. Hypoxia

The molecular mechanism regulating angiogenesis in atherosclerosis involves signaling pathways that are mainly driven by the lack of oxygen (Sluimer and Daemen, 2009). Hypoxia occurs when oxygen supply is decreased and oxygen demand is increased. The ability to sense and respond to changes in O₂ 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 (Semenza, 2003; Pouyssegur et al., 2006).

Hypoxia promotes monocyte/ macrophage survival and oxLDL uptake by macrophages (Roiniotis et al., 2009). It also enhances the expression of matrix metalloproteases by a variety of cells in the plaque contributing to the instability of the plaque (Nakano et al., 2005). 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 (Fong, 2015). In contrast, when atherosclerosis prone mice were exposed to carbogen (95% O₂,

* Correspondence to: Department of Surgery, Einthoven Laboratory for Experimental Vascular Medicine, Leiden University Medical Center, PO Box 9600, 2300 RC Leiden, The Netherlands.

E-mail addresses: l.parma@lumc.nl (L. Parma), f.baganha_Carreiras@lumc.nl (F. Baganha), p.h.a.quax@lumc.nl (P.H.A. Quax), m.r.de_vries@lumc.nl (M.R. de Vries).

¹ Shared first author.

² Shared last author

<http://dx.doi.org/10.1016/j.ejphar.2017.04.028>

Received 15 December 2016; Received in revised form 31 March 2017; Accepted 20 April 2017

Available online 21 April 2017

0014-2999/ © 2017 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

5% CO₂) oxygenation, plaque growth was inhibited (Pouyssegur et al., 2006). Sluimer et al. showed extensive hypoxia in the center of advanced human carotid atherosclerotic plaques (Sluimer et al., 2008). Pimonidazole, a hypoxia marker, was co-localized with CD68 positive macrophages, HIF1- α and VEGF expression, suggesting the involvement of the HIF pathway in the regulation of human plaque angiogenesis and lesion progression (Sluimer, Gasc et al., 2008).

HIF is a heterodimeric protein composed of α and β subunits. The α chain confers oxygen regulation on the complex and its expression is hypoxia-dependent and has three isoforms, HIF-1 α , 2 α and 3 α of which only HIF-1 α and HIF-1 β are widely expressed in normal tissues (Tian et al., 1997; Wiesener et al., 1998; Pugh and Ratcliffe, 2003). The beta chain (three isoforms) is constitutively expressed and works as an aryl receptor nuclear translocator. Under normoxic conditions, the synthesized HIF-1 α is rapidly degraded and the co-activators are blocked by oxygen-dependent enzymes; the prolyl-hydroxylases domain (PHD) enzymes (Lando et al., 2002a, 2002b). During hypoxia, PHD activity is reduced, allowing the dimerization of HIF-1 α and HIF-1 β (Lee et al., 2004; Lijkwan et al., 2014). This active complex binds to DNA starting the transcription of downstream genes involved in angiogenesis and inflammation (Ho et al., 2006).

HIF-1 α mediates inflammation by promoting pro-inflammatory cytokine expression (stromal cell-derived factor 1, VEGF-A) and consequently inflammatory cell recruitment (Ramkhelawon et al., 2013; Folco et al., 2014). Aarup et al. have shown that HIF-1 α expression also modulates the macrophage glycolytic pathway, by increasing glucose uptake and glucose transporter 1 mRNA expression, enhancing oxygen consumption. In addition, HIF-1 α reduces the mRNA expression of the major cholesterol transporters (Aarup et al., 2016) and activates the lectin-like OxLDL receptor-1 scavenger receptors that mediates oxLDL uptake in macrophages (Cruet et al., 2013), 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 (Ramkhelawon et al., 2013). Thus, hypoxia induced overexpression of HIF-1 α not only regulates plaque angiogenesis, but also has additional effects stimulating the growth of atherosclerotic plaques.

Moreover, extensive crosstalk between HIF and nuclear factor- κ B (NF- κ B), two important molecular players in atherosclerosis, has been reported. They have common activating stimuli and share regulators and targets (Palazon et al., 2014; D'Ignazio et al., 2016). Only the canonical NF- κ B pathway is sensitive to hypoxia, while both the inhibitor of NF- κ B subunit alpha (IKK α) and the inhibitor of NF- κ B subunit beta (IKK β) can be hydroxylated by PHD (Cummins et al., 2006; Oliver et al., 2009). Marsch et al. shows that PHD1 knockout mice display a protective cardiovascular metabolic phenotype with lower plasma cholesterol levels and glucose tolerance improvement (Marsch et al., 2016). Furthermore, NF- κ B has been shown to play a role in basal and stimulated HIF-1 α mRNA expression. The p50 and p65 NF- κ B subunits can bind to the HIF-1 α promoter in response to hypoxia and when these subunits are overexpressed, an increase in HIF-1 α mRNA levels and promoter activity is observed (van Uden et al., 2008). The full mechanism between HIF and NF- κ B in hypoxia is not yet completely understood but these features highlight the complex and interrelated hypoxia and inflammatory signaling cascades in atherosclerosis.

2.2. 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 (Carmeliet and Jain, 2011; Eelen et al., 2013; Cantelmo et al., 2015; Coulon et al., 2010). 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 (De Smet et al., 2009).

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 (Herbert and Stainier, 2011). Interestingly, it was shown that Notch signaling promotes the progression of atherosclerosis in vivo (Herbert and Stainier, 2011). 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 (Herbert and Stainier, 2011), 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 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 (Van der Veken et al., 2016).

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 (Jaipersad et al., 2014). 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 (Fantin et al., 2010).

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

2.3. Hemodynamic forces

Blood flow plays crucial roles in angiogenesis by generating frictional force that develops between flowing blood and the vascular endothelium (Sewduth and Santoro, 2016). 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 (Ando and Yamamoto, 2013). Shear stress induces collateral artery growth as well as capillary growth and it was shown that endoglin played a crucial role in this process (Seghers et al., 2012). Furthermore, it is known that shear stress modulates the expression of thrombospondin 1 and its receptor CD36 during angiogenesis in vivo (Bongrazio et al., 2006). Impairment of the EC is response to shear

stress leads to activation of endothelial cells which ultimately can result in vascular diseases such as hypertension, and atherosclerosis (Ando and Yamamoto, 2013). 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 (Li et al., 2014), calcium (Qiu et al., 2001) and primary cilia (Goetz et al., 2014).

3. Angiogenesis in the atherosclerotic plaque

Pathological angiogenesis of the vessel wall is a consistent feature of atherosclerotic plaque development and progression of the disease (van Hinsbergh et al., 2015) 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. (Hellings et al., 2010).

Adventitial angiogenesis is thought to be the main source of neovessels. In addition, it has been observed that angiogenesis also occurs 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 LDL double 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 (Langheinrich et al., 2009).

Descriptive and cross-sectional studies in humans suggest a clear association between the neovessel density and atherosclerotic progression and vulnerability (Sluimer and Daemen, 2009). 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 (Hellings et al., 2010).

3.1. Intraplaque hemorrhage

Neovessels in vulnerable plaques are immature, irregular and fragile due to the compromised structural integrity (J.Y. Xu et al., 2015). In fact, they are characterized by a discontinuous basement membrane and a low number of tight junctions between the ECs (Sluimer et al., 2009). Moreover, these premature vessels are relatively poor in pericyte coverage and are highly susceptible to the leakage of circulating cells (Jeney et al., 2014) thus intraplaque hemorrhage (IPH). In the oncology field, newly formed vessels in tumors have been reported to have the same features. Tumor vessels are heterogeneous of appearance (J.A. Nagy et al., 2010), are organized in a chaotic fashion and do not follow the hierarchical branching pattern of normal vascular networks, seemingly lacking remodeling and pruning (Jain, 2003). Many of these vessels are hyper-permeable (Nagy et al., 2012). In their walls, there are inter-endothelial openings and trans-endothelial channels, resulting in a wide range of pore sizes (Martin et al., 2016). The hyper-permeability of tumor vessels allows plasma and cells to leak to the interstitial space (Martin et al., 2016).

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 (Giannoglou et al., 2009; Tziakas et al., 2011). This is further illustrated below.

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 (Carmeliet and Jain, 2011). 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 α 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 (Goel et al., 2012). 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 (Post et al., 2008). Also, PDGF-BB and its receptor (PDGFR)- β are known to be important in vessel permeability, fragility, and impaired perfusion (Van der Veken et al., 2016), due to their pivotal role of recruiting and stabilizing perivascular cells.

These findings suggest that the neovessels are subjected to frequent leakage in the form of extravasation of red blood cells, leucocytes and plasma lipids to the surrounding tissue.

3.1.1. 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 unesterified cholesterol, participate in both the cholesterol accumulation and the oxidative process (Michel et al., 2014). Once trapped in the highly oxidative environment of the atherosclerotic plaques, RBCs tend to lyse quickly (E. Nagy et al., 2010). The cytoplasm of RBCs is rich in hemoglobin, which can attract multiple monocytes and neutrophils to the plaque (Jeney et al., 2014).

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. RBC membranes have a high cholesterol content with a percentage of lipids up to 40% of the total weight of the cells (Kolodgie et al., 2007). 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 (Kolodgie et al., 2003).

3.2. 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 (de Vries and Quax, 2016). Monocytes differentiate into macrophages that engulf oxLDL and convert into lipid filled foam cells. Accumulation 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. 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 (Marsch et al., 2013). 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 (Khurana et al., 2005).

In advanced lesions, neovessel leakage constitutes the main entrance for inflammatory cells. RBCs can facilitate the extravasation of circulating inflammatory cells by increasing the numbers of rolling and adhering monocytes by enhancing the force and/or the frequency of collision of monocytes with the endothelium (Melder et al., 2000). Not

only monocytes are increasingly found around neovessels, also neutrophils and mast cells were found to be associated with neovessels. These cells can release their granular content rich in serine proteases and matrix metalloproteases that can digest components of elastic fibers (elastin) and of the basement membrane (collagen, laminin and fibronectin) (Michel et al., 2014). This high proteolytic activity can ultimately lead to fibrous cap thinning and plaque erosion (Leclercq et al., 2007; Dorweiler et al., 2008). 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 (phagocytosis of dying/dead cells) 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 (Schrijvers et al., 2005; Kojima et al., 2016). Additionally, their ability to efferocytose, 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 (Tabas and Bornfeldt, 2016). Furthermore, in endarterectomy samples obtained during surgery an accumulation of mast cells was observed in neovessel-rich areas of atherosclerotic plaques (Willems et al., 2013). 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 (Lappalainen et al., 2004). In line with these reports, it is observed that vasa vasorum vessel density in atherosclerotic lesions of ApoE KO mice highly correlates with the occurrence of inflammatory cells foci (Khurana et al., 2005; Eriksson, 2011; Rademakers et al., 2013; Butoi et al., 2016).

4. Imaging of angiogenesis

The detection of patients with atherosclerotic plaques at risk is a major challenge for the cardiovascular research field and led to 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 (Goncalves et al., 2015).

The most prominent imaging technologies are already used in clinical studies and their value to identify crucial characteristics of vulnerable plaques is undeniable (Taqueti et al., 2014). 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 (Alie et al., 2014; Calcagno et al., 2016; Perez-Medina et al., 2016). This high sensitive tool uses ^{18}F -fluorodeoxyglucose (FDG), a glucose analogue tracer. After intravenous injection, ^{18}F -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 ^{18}F -FDG uptake (Taqueti et al., 2014). 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) (Magnoni et al., 2015). 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 (Bourantas et al., 2016a). 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 (Matter et al., 2004). NIRF can also be used for the identification of specific plaque features, such as MMP activity (de Vries et al., 2012) or flow patterns (Wong, et al., 2014; Wong et al., 2016). 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 (Wang et al., 2015; Bourantas et al., 2016b).

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 (Tsuji et al., 2016). 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 (Bourantas et al., 2016b). Furthermore, the strong correlation between angiogenesis and plaque progression suggests a useful application of imaging technologies as a therapeutic approach for patients with atherosclerosis.

5. Angiogenesis targets

5.1. 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 (Goel et al., 2012; Jain, 2014). 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 (O'Reilly et al., 1997) 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 showed that prolonged treatment with endostatin reduced plaque growth (Moulton et al., 1999). More recently, Endostar has been tested in a swine model (X. Xu et al., 2015). 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 blockade 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 (Bogdanovich et al., 2016), it showed profound effects in a murine model by causing disruption of the endothelium and consequently accelerated atherosclerosis in ApoE KO mice (Winnik et al., 2013).

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 (Wang et al., 2011).

Another angiogenic target under study is Ang-2. Blockade of Ang-2 on experimental atherosclerosis in LDLR^{-/-} ApoB^{100/100} 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 (Theelen et al., 2015).

So far, the ideal anti-angiogenic target in atherosclerosis is yet to be found, but the potential of anti-angiogenic approaches in the tumor

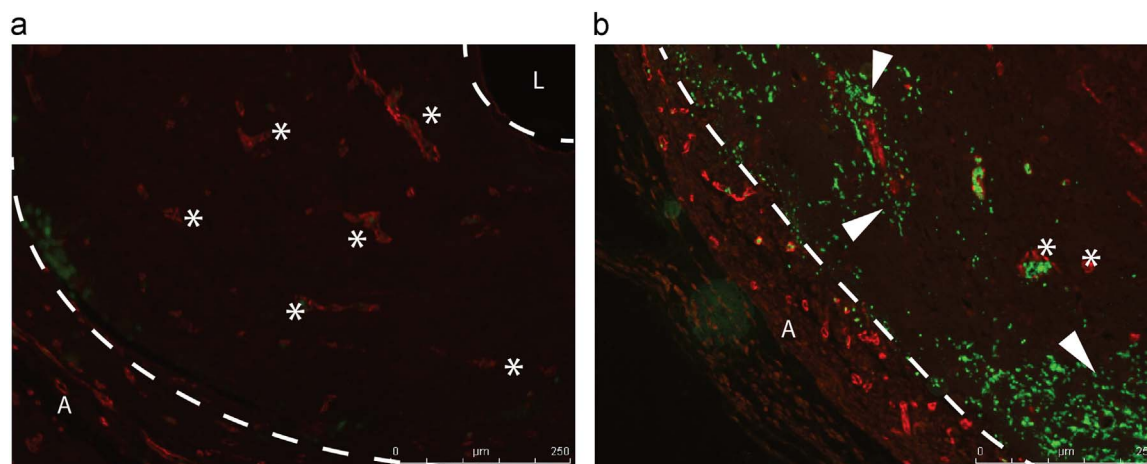


Fig. 1. Plaque angiogenesis. Once in the plaque macrophages engulf oxLDL and become lipid filled foam cells. Their accumulation activates cytokine production that promotes the influx of neutrophils, mast cells and monocytes. The high oxygen consumption of these inflammatory cells leads to hypoxia. HIF-1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

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.

5.2. 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 (Cruys et al., 2016). In atherosclerosis, plaque progression is associated with macro and micro endothelial dysfunction, which is attributed to EC metabolic maladaptation (Pircher et al., 2016). 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-bisphosphatase 3 (PFKFB3) (Pircher et al., 2016).

BH₄, an endothelial NOS (eNOS) cofactor, is metabolically 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 (Ozaki et al., 2002). However, clinical trials in patients with coronary heart disease and myocardial infarction have shown mixed results for BH₄ supplementation (Bendall et al., 2014).

NOX enzymes are another important source of ROS in atherosclerosis which strongly affect plaque angiogenesis. NOX use NADPH, another eNOS cofactor, for ROS production, compromising NO levels (Drummond and Sobey, 2014). In addition, NOX activate redox-sensitive transcriptional factors such as NF- κ B and HIF1 α , increasing inflammation and hypoxia in the plaque (Diebold et al., 2012; Menden et al., 2015). 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 (De Bock et al., 2013a, 2013b; Schoors et al., 2014). 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 (De Bock et al., 2013a, 2013b). 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 (Schoors, De Bock et al., 2014). Altogether, these findings illustrate the pivotal role of glycolysis in angiogenesis and the therapeutic potential of blocking glycolysis in plaque angiogenesis inhibition.

6. 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 (Van der Veken et al., 2016). 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 (Giannarelli et al., 2010).

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 (de Vries et al., 2016; Yahagi et al., 2016). 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 vasorum and consist of CD31 positive endothelial cells (de Vries et al., 2012). 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 (Fig. 1). Foam cell accumulation was even observed within seven days after vein bypass grafting, which illustrates the extreme fast initiation of this accelerated atherosclerosis (Lardenoye et al., 2002). Reducing inflammatory responses in this model could inhibit IPH, as well as erosions and plaque dissection thereby increasing plaque stability (Wezel et al., 2016).

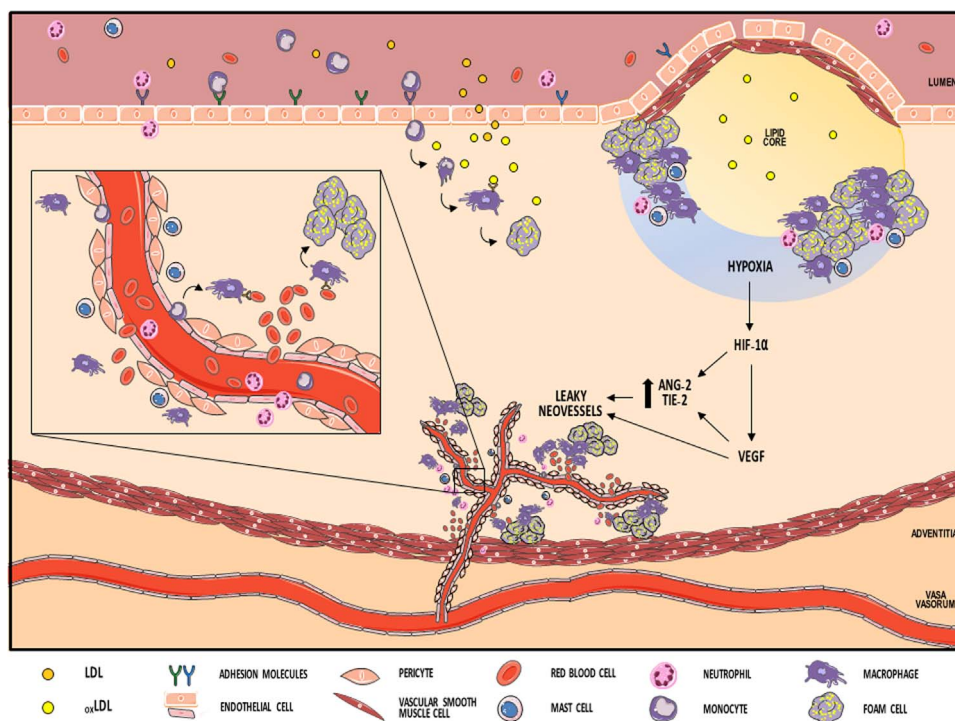


Fig. 2. Vein graft lesions in hypercholesterolemic ApoE3*Leiden mice 28 days after surgery. (a) Vein graft lesions show extensive neovessels (*). (b) Red blood cells dispersed in the extracellular matrix outside the neovessels demonstrate intraplaque hemorrhage (arrow head) in a vein graft lesion. CD31 positive endothelial cells (red), TER119 positive red blood cells (green) (A) adventitia, (L) lumen. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Another example of animal model with spontaneous plaque rupture 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 (Van der Veken et al., 2016). The effect of increased arterial stiffness, due to progressive elastic fiber degeneration, on atherosclerosis was studied in this model. ApoE KO Fbn1C1039G^{+/-} 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 (Van Herck et al., 2009). The atherosclerotic plaques of ApoE KO Fbn1C1039G^{+/-} mice contained highly leaky plaque neovessels and IPH, resulting in plaque rupture, myocardial infarction, stroke, and sudden death (Van der Donckt et al., 2015).

7. 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 (Fig. 2). 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.

Financial support and sponsorship

This work was supported by a grant from the European Union, Horizon 2020 MSCA joint doctoral project, MOGLYNET (Project 675527).

Conflicts of interest

There are no conflicts of interest.

Acknowledgements

None.

References

- Aarup, A., Pedersen, T.X., Junker, N., Christoffersen, C., Bartels, E.D., Madsen, M., Nielsen, C.H., Nielsen, L.B., 2016. Hypoxia-inducible factor-1alpha expression in macrophages promotes development of atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* 36 (9), 1782–1790.
- Alie, N., Eldib, M., Fayad, Z.A., Mani, V., 2014. Inflammation, atherosclerosis, and coronary artery disease: PET/CT for the evaluation of atherosclerosis and inflammation. *Clin. Med. Insights Cardiol.* 8 (Suppl 3), S13–S21.
- Ando, J., Yamamoto, K., 2013. Flow detection and calcium signalling in vascular endothelial cells. *Cardiovasc. Res.* 99 (2), 260–268.
- Bendall, J.K., Douglas, G., McNeill, E., Channon, K.M., Crabtree, M.J., 2014. Tetrahydrobiopterin in cardiovascular health and disease. *Antioxid. Redox Signal.* 20 (18), 3040–3077.
- Bogdanovich, S., Kim, Y., Mizutani, T., Yasuma, R., Tudisco, L., Cicatiello, V., Bastos-Carvalho, A., Kerur, N., Hirano, Y., Baffi, J.Z., Tarallo, V., Li, S., Yasuma, T., Arpitha, P., Fowler, B.J., Wright, C.B., Apicella, I., Greco, A., Brunetti, A., Ruvo, M., Sandomenico, A., Nozaki, M., Ijima, R., Kaneko, H., Ogura, Y., Terasaki, H., Ambati, B.K., Leusen, J.H., Langdon, W.Y., Clark, M.R., Armour, K.L., Bruhns, P., Verbeek, J.S., Gelfand, B.D., De Falco, S., Ambati, J., 2016. Human IgG1 antibodies suppress angiogenesis in a target-independent manner. *Signal Transduct. Target Ther.* 1.
- Bongrazio, M., Da Silva-Azevedo, L., Bergmann, E.C., Baum, O., Hinz, B., Pries, A.R., Zakrzewicz, A., 2006. Shear stress modulates the expression of thrombospondin-1 and CD36 in endothelial cells in vitro and during shear stress-induced angiogenesis in vivo. *Int. J. Immunopathol. Pharmacol.* 19 (1), 35–48.
- Bourantas, C.V., Garcia-Garcia, H.M., Torii, R., Zhang, Y.J., Westwood, M., Crake, T., Serruys, P.W., 2016a. Vulnerable plaque detection: an unrealistic quest or a feasible objective with a clinical value? *Heart* 102 (8), 581–589.
- Bourantas, C.V., Jaffer, F.A., Gijzen, F.J., van Soest, G., Madden, S.P., Courtney, B.K., Fard, A.M., Tenekcioglu, E., Zeng, Y., van der Steen, A.F., Emelianov, S., Muller, J., Stone, P.H., Marcu, L., Tearney, G.J., Serruys, P.W., 2016b. Hybrid intravascular imaging: recent advances, technical considerations, and current applications in the study of plaque pathophysiology. *Eur. Heart J.*
- Butoi, E., Gan, A.M., Tucureanu, M.M., Stan, D., Macarie, R.D., Constantinescu, C., Calin, M., Simionescu, M., Manduteanu, I., 2016. Cross-talk between macrophages and smooth muscle cells impairs collagen and metalloprotease synthesis and promotes angiogenesis. *Biochim. Biophys. Acta- Mol. Cell Res.* 1863 (7, Part A), 1568–1578.
- Calagno, C., Mulder, W.J.M., Nahrendorf, M., Fayad, Z.A., 2016. Systems biology and noninvasive imaging of atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* 36 (2), e1–e8.

- Cantelmo, A.R., Brajic, A., Carmeliet, P., 2015. Endothelial metabolism driving angiogenesis emerging concepts and principles. *Cancer J.* 21 (4), 244–249.
- Carmeliet, P., Jain, R.K., 2011. Molecular mechanisms and clinical applications of angiogenesis. *Nature* 473 (7347), 298–307.
- Chaabane, C., Coen, M., Bochaton-Piallat, M.L., 2014. Smooth muscle cell phenotypic switch: implications for foam cell formation. *Curr. Opin. Lipidol.* 25 (5), 374–379.
- Coulon, C., Georgiadou, M., Roncal, C., De Bock, K., Langenberg, T., Carmeliet, P., 2010. From vessel sprouting to normalization: role of the prolyl hydroxylase domain protein/hypoxia-inducible factor oxygen-sensing machinery. *Arterioscler. Thromb. Vasc. Biol.* 30 (12), 2331–2336.
- Cruet, M., Wust, S.J., Spielmann, P., Luscher, T.F., Wenger, R.H., Matter, C.M., 2013. Hypoxia enhances lipid uptake in macrophages: role of the scavenger receptors Lox1, SRA, and CD36. *Atherosclerosis* 229 (1), 110–117.
- Cruys, B., Wong, B.W., Kuchnio, A., Verdegem, D., Cantelmo, A.R., Conradi, L.-C., Vandekeere, S., Bouché, A., Cornelissen, I., Vinckier, S., Merks, R.M.H., Dejana, E., Gerhardt, H., Deweerth, M., Bentley, K., Carmeliet, P., 2016. Glycolytic regulation of cell rearrangement in angiogenesis. *Nat. Commun.* 7, 12240.
- Cummins, E.P., Berra, E., Comerford, K.M., Ginouves, A., Fitzgerald, K.T., Seeballuck, F., Godson, C., Nielsen, J.E., Moynagh, P., Poyussegur, J., Taylor, C.T., 2006. Prolyl hydroxylase-1 negatively regulates IkappaB kinase-beta, giving insight into hypoxia-induced NFkappaB activity. *Proc. Natl. Acad. Sci. USA* 103 (48), 18154–18159.
- D'Ignazio, L., Bandarra, D., Rocha, S., 2016. NF-kappaB and HIF crosstalk in immune responses. *Febs J.* 283 (3), 413–424.
- De Smet, F., Segura, I., De Bock, K., Hohensinner, P.J., Carmeliet, P., 2009. Mechanisms of vessel branching: filopodia on endothelial tip cells lead the way. *Arterioscler. Thromb. Vasc. Biol.* 29 (5), 639–649.
- de Vries, M.R., Niessen, H.W., Lowik, C.W., Hamming, J.F., Jukema, J.W., Quax, P.H., 2012. Plaque rupture complications in murine atherosclerotic vein grafts can be prevented by TIMP-1 overexpression. *PLoS One* 7 (10), e47134.
- de Vries, M.R., Quax, P.H., 2016. Plaque angiogenesis and its relation to inflammation and atherosclerotic plaque destabilization. *Curr. Opin. Lipidol.* 27 (5), 499–506.
- de Vries, M.R., Simons, K.H., Jukema, J.W., Braun, J., Quax, P.H., 2016. Vein graft failure: from pathophysiology to clinical outcomes. *Nat. Rev. Cardiol.* 13 (8), 451–470.
- De Bock, K., Georgiadou, M., Carmeliet, P., 2013a. Role of endothelial cell metabolism in vessel sprouting. *Cell Metab.* 18 (5), 634–647.
- De Bock, K., Georgiadou, M., Schoors, S., Kuchnio, A., Wong, Brian W., Cantelmo, Anna R., Quaegebeur, A., Ghesquière, B., Cauwenberghs, S., Eelen, G., Phng, L.-K., Betz, I., Tembuys, B., Brepoels, K., Welte, J., Gendens, L., Segura, I., Cruys, B., Bifari, F., Decimo, I., Blanco, R., Wyns, S., Vangindertael, J., Rocha, S., Collins, Russel T., Munck, S., Daelemans, D., Imamura, H., Devlieger, R., Rider, M., Van Veldhoven, Paul P., Schuit, F., Bartrons, R., Hofkens, J., Fraisl, P., Telang, S., DeBerardinis, Ralph J., Schoonjans, L., Vinckier, S., Chesney, J., Gerhardt, H., Deweerth, M., Carmeliet, P., 2013b. Role of PFKFB3-driven glycolysis in vessel sprouting. *Cell* 154 (3), 651–663.
- Diebold, I., Petry, A., Sabrane, K., Djordjevic, T., Hess, J., Grolach, A., 2012. The HIF1 target gene NOX2 promotes angiogenesis through urotensin-II. *J. Cell Sci.* 125 (Pt 4), 956–964.
- Dorweiler, B., Torzewski, M., Dahm, M., Kirkpatrick, C.J., Lackner, K.J., Vahl, C.F., 2008. Subendothelial infiltration of neutrophil granulocytes and liberation of matrix-destabilizing enzymes in an experimental model of human neo-intima. *Thromb. Haemost.* 99 (2), 373–381.
- Drummond, G.R., Sobey, C.G., 2014. Endothelial NADPH oxidases: which NOX to target in vascular disease? *Trends Endocrinol. Metab.* 25 (9), 452–463.
- Eelen, G., Cruys, B., Welte, J., De Bock, K., Carmeliet, P., 2013. Control of vessel sprouting by genetic and metabolic determinants. *Trends Endocrinol. Metab.* 24 (12), 589–596.
- Ehara, S., Kobayashi, Y., Yoshiyama, M., Shimada, K., Shimada, Y., Fukuda, D., Nakamura, Y., Yamashita, H., Yamagishi, H., Takeuchi, K., Naruko, T., Haze, K., Becker, A.E., Yoshikawa, J., Ueda, M., 2004. Spotty calcification typifies the culprit plaque in patients with acute myocardial infarction: an intravascular ultrasound study. *Circulation* 110 (22), 3424–3429.
- Eriksson, E.E., 2011. Intravital microscopy on atherosclerosis in apolipoprotein e-deficient mice establishes microvessels as major entry pathways for leukocytes to advanced lesions. *Circulation* 124 (19), 2129–2138.
- Fantin, A., Vieira, J.M., Gestri, G., Denti, L., Schwarz, Q., Prykhodchik, S., Peri, F., Wilson, S.W., Ruhrberg, C., 2010. Tissue macrophages act as cellular chaperones for vascular anastomosis downstream of VEGF-mediated endothelial tip cell induction. *Blood* 116 (5), 829–840.
- Folco, E.J., Sukhova, G.K., Quillard, T., Libby, P., 2014. Moderate hypoxia potentiates interleukin-1beta production in activated human macrophages. *Circ. Res.* 115 (10), 875–883.
- Fong, G.H., 2015. Potential contributions of intimal and plaque hypoxia to atherosclerosis. *Curr. Atheroscler. Rep.* 17 (6), 510.
- Giannarelli, C., Ibanez, B., Cimmino, G., Garcia Ruiz, J.M., Fajta, F., Bianchini, E., Zafar, M.U., Fuster, V., Garcia, M.J., Badimon, J.J., 2010. Contrast-enhanced ultrasound imaging detects intraplaque neovascularization in an experimental model of atherosclerosis. *JACC Cardiovasc. Imaging* 3 (12), 1256–1264.
- Giannoglou, G.D., Koskinas, K.C., Tziakas, D.N., Ziakas, A.G., Antoniadis, A.P., Tentis I. K., Parcharidis, G.K., Total cholesterol content of erythrocyte membranes and coronary atherosclerosis: an intravascular ultrasound pilot study. *Angiology*, 60(6), 2009 Dec–2010 Jan, 676–82.
- Goel, S., Wong, A.H., Jain, R.K., 2012. Vascular normalization as a therapeutic strategy for malignant and nonmalignant disease. *Cold Spring Harb. Perspect. Med.* 2 (3), a006486.
- Goetz, J.G., Steed, E., Ferreira, R.R., Roth, S., Ramsbacher, C., Boselli, F., Charvin, G., Liebling, M., Wyart, C., Schwab, Y., Vermot, J., 2014. Endothelial cilia mediate low flow sensing during zebrafish vascular development. *Cell Rep.* 6 (5), 799–808.
- Goncalves, I., den Ruijter, H., Nahrendorf, M., Pasterkamp, G., 2015. Detecting the vulnerable plaque in patients. *J. Intern. Med.* 278 (5), 520–530.
- Haasdijk, R.A., Den Dekker, W.K., Cheng, C., Tempel, D., Szulceck, R., Bos, F.L., Hermkens, D.M., Chirif, I., Brandt, M.M., Van Dijk, C., Xu, Y.J., Van De Kamp, E.H., Blonden, L.A., Bezui, J., Van Sluiter, J.C., Biessen, E.A., Van Nieuw Amerongen, G.P., Duckers, H.J., 2016. THSD1 preserves vascular integrity and protects against intraplaque haemorrhaging in ApoE^{-/-} mice. *Cardiovasc. Res.* 110 (1), 129–139.
- Hansson, G.K., Libby, P., Tabas, I., 2015. Inflammation and plaque vulnerability. *J. Intern. Med.* 278 (5), 483–493.
- Hellings, W.E., Peeters, W., Moll, F.L., Piers, S.R., van Setten, J., Van der Spek, P.J., de Vries, J.P., Seldenrijk, K.A., De Bruin, P.C., Vink, A., Velega, E., de Kleijn, D.P., Pasterkamp, G., 2010. Composition of carotid atherosclerotic plaque is associated with cardiovascular outcome: a prognostic study. *Circulation* 121 (17), 1941–1950.
- Herbert, S.P., Stainier, D.Y., 2011. Molecular control of endothelial cell behaviour during blood vessel morphogenesis. *Nat. Rev. Mol. Cell Biol.* 12 (9), 551–564.
- Ho, T.K., Abraham, D.J., Black, C.M., Baker, D.M., 2006. Hypoxia-inducible factor 1 in lower limb ischemia. *Vascular* 14 (6), 321–327.
- Hutcheson, J.D., Goetsch, C., Bertazzo, S., Maldonado, N., Ruiz, J.L., 2016. Genesis and growth of extracellular-vesicle-derived microcalcification in atherosclerotic plaques. *Nat. Mater.* 15 (3), 335–343.
- Imoto, K., Hiro, T., Fujii, T., Murashige, A., Fukumoto, Y., Hashimoto, G., Okamura, T., Yamada, J., Mori, K., Matsuzaki, M., 2005. Longitudinal structural determinants of atherosclerotic plaque vulnerability: a computational analysis of stress distribution using vessel models and three-dimensional intravascular ultrasound imaging. *J. Am. Coll. Cardiol.* 46 (8), 1507–1515.
- Jain, R.K., 2003. Molecular regulation of vessel maturation. *Nat. Med.* 9 (6), 685–693.
- Jain, R.K., 2014. Antiangiogenesis strategies revisited: from starving tumors to alleviating hypoxia. *Cancer Cell* 26 (5), 605–622.
- Jaiprasad, A.S., Lip, G.Y., Silverman, S., Shantsila, E., 2014. The role of monocytes in angiogenesis and atherosclerosis. *J. Am. Coll. Cardiol.* 63 (1), 1–11.
- Jeney, V., Balla, G., Balla, J., 2014. Red blood cell, hemoglobin and heme in the progression of atherosclerosis. *Front Physiol.* 5, 379.
- Khurana, R., Simons, M., Martin, J.F., Zachary, I.C., 2005. Role of angiogenesis in cardiovascular disease. *A Crit. Apprais.* 112 (12), 1813–1824.
- Kojima, Y., Volkmer, J.P., McKenna, K., Civelek, M., Lusic, A.J., Miller, C.L., Drenzo, D., Nanda, V., Ye, J., Connolly, A.J., Schadt, E.E., Quertermous, T., Betancur, P., Maegdefessel, L., Matic, L.P., Hedin, U., Weissman, L.L., Leeper, N.J., 2016. CD47-blocking antibodies restore phagocytosis and prevent atherosclerosis. *Nature* 536 (7614), 86–90.
- Kolodgie, F.D., Burke, A.P., Nakazawa, G., Cheng, Q., Xu, X., Virmani, R., 2007. Free cholesterol in atherosclerotic plaques: where does it come from? *Curr. Opin. Lipidol.* 18 (5), 500–507.
- Kolodgie, F.D., Gold, H.K., Burke, A.P., Fowler, D.R., Kruth, H.S., Weber, D.K., Farb, A., Guerrero, I.J., Hayase, M., Kutys, R., Narula, J., Finn, A.V., Virmani, R., 2003. Intraplaque hemorrhage and progression of coronary atheroma. *N. Engl. J. Med.* 349 (24), 2316–2325.
- Lando, D., Peet, D.J., Gorman, J.J., Whelan, D.A., Whitelaw, M.L., Bruick, R.K., 2002a. FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. *Genes Dev.* 16 (12), 1466–1471.
- Lando, D., Peet, D.J., Whelan, D.A., Gorman, J.J., Whitelaw, M.L., 2002b. Asparagine hydroxylation of the HIF transactivation domain a hypoxic switch. *Science* 295 (5556), 858–861.
- Langheinrich, A.C., Sedding, D.G., Kampschulte, M., Moritz, R., Wilhelm, J., Haberbusch, W.G., Ritman, E.L., Bohle, R.M., 2009. 3-Deazaadenosine inhibits vasasorum neovascularization in aortas of ApoE(-)/LDL(-) double knockout mice. *Atherosclerosis* 202 (1), 103–110.
- Lappalainen, H., Laine, P., Pentikainen, M.O., Sajantila, A., Kovanen, P.T., 2004. Mast cells in neovascularized human coronary plaques store and secrete basic fibroblast growth factor, a potent angiogenic mediator. *Arterioscler. Thromb. Vasc. Biol.* 24 (10), 1880–1885.
- Lardenoye, J.H., de Vries, M.R., Löwik, C.W., Xu, Q., Dhore, C.R., Cleutjens, J.P., van Hinsbergh, V.W., van Bockel, J.H., Quax, P.H., 2002. Accelerated atherosclerosis and calcification in vein grafts: a study in APOE*3 Leiden transgenic mice. *Circ. Res* 91 (7), 577–584.
- Leclercq, A., Houard, X., Philippe, M., Ollivier, V., Sebbag, U., Meilhac, O., Michel, J.B., 2007. Involvement of intraplaque hemorrhage in atherothrombosis evolution via neutrophil protease enrichment. *J. Leukoc. Biol.* 82 (6), 1420–1429.
- Lee, J.W., Bae, S.H., Jeong, J.W., Kim, S.H., Kim, K.W., 2004. Hypoxia-inducible factor (HIF-1)alpha: its protein stability and biological functions. *Exp. Mol. Med.* 36 (1), 1–12.
- Li, J., Hou, B., Tumova, S., Muraki, K., Bruns, A., Ludlow, M.J., Sedo, A., Hyman, A.J., McKeown, L., Young, R.S., Yuldasheva, N.Y., Majeed, Y., Wilson, L.A., Rode, B., Bailey, M.A., Kim, H.R., Fu, Z., Carter, D.A., Bilton, J., Imrie, H., Ajuh, P., Dear, T.N., Cubbon, R.M., Kearney, M.T., Prasad, K.R., Evans, P.C., Ainscough, J.F., Beech, D.J., 2014. Piezo1 integration of vascular architecture with physiological force. *Nature* 515 (7526), 279–282.
- Lijkwan, M.A., Hellingman, A.A., Bos, E.J., van der Bogt, K.E., Huang, M., Kooreman, N.G., de Vries, M.R., Peters, H.A., Robbins, R.C., Hamming, J.F., Quax, P.H., Wu, J.C., 2014. Short hairpin RNA gene silencing of prolyl hydroxylase-2 with a minicircle vector improves neovascularization of hindlimb ischemia. *Hum. Gene Ther.* 25 (1), 41–49.
- Magnoni, M., Ammirati, E., Camici, P.G., 2015. Non-invasive molecular imaging of vulnerable atherosclerotic plaques. *J. Cardiol.* 65 (4), 261–269.
- Marsch, E., Demandt, J.A., Theelen, T.L., Tullemans, B.M., Wouters, K., Boon, M.R., van

- Dijk, T.H., Gijbels, M.J., Dubois, L.J., Meex, S.J., Mazzone, M., Hung, G., Fisher, E.A., Biessen, E.A., Daemen, M.J., Rensen, P.C., Carmeliet, P., Groen, A.K., Sluimer, J.C., 2016. Deficiency of the oxygen sensor prolyl hydroxylase 1 attenuates hypercholesterolaemia, atherosclerosis, and hyperglycaemia. *Eur. Heart J.* 37 (39), 2993–2997.
- Marsch, E., Sluimer, J.C., Daemen, M.J., 2013. Hypoxia in atherosclerosis and inflammation. *Curr. Opin. Lipidol.* 24 (5), 393–400.
- Martin, J.D., Fukumura, D., Duda, D.G., Boucher, Y., Jain, R.K., 2016. "Reengineering the tumor microenvironment to alleviate hypoxia and overcome cancer heterogeneity. *Cold Spring Harb. Perspect. Med.* 6 (12).
- Matter, C.M., Schuler, P.K., Alessi, P., Meier, P., Ricci, R., Zhang, D., Halin, C., Castellani, P., Zardi, L., Hofer, C.K., Montani, M., Neri, D., Lüscher, T.F., 2004. Molecular imaging of atherosclerotic plaques using a human antibody against the extra-domain B of fibronectin. *Circ. Res.* 95 (12), 1225–1233.
- Melder, R.J., Yuan, J., Munn, L.L., Jain, R.K., 2000. Erythrocytes enhance lymphocyte rolling and arrest in vivo. *Microvasc. Res.* 59 (2), 316–322.
- Menden, H., Welak, S., Cossette, S., Ramchandran, R., Sampath, V., 2015. Lipopolysaccharide (LPS)-mediated angiotensin-2-dependent autocrine angiogenesis is regulated by NADPH oxidase 2 (Nox2) in human pulmonary microvascular endothelial cells. *J. Biol. Chem.* 290 (9), 5449–5461.
- Michel, J.B., Martin-Ventura, J.L., Nicoletti, A., Ho-Tin-Noe, B., 2014. Pathology of human plaque vulnerability: mechanisms and consequences of intraplaque haemorrhages. *Atherosclerosis* 234 (2), 311–319.
- Michel, J.B., Virmani, R., Arbustini, E., Pasterkamp, G., 2011. Intraplaque haemorrhages as the trigger of plaque vulnerability. *Eur. Heart J.* 32 (16), 1977–1985, (1985a, 1985b, 1985c).
- Moulton, K.S., Heller, E., Konerding, M.A., Flynn, E., Palinski, W., Folkman, J., 1999. Angiogenesis inhibitors endostatin or TNP-470 reduce intimal neovascularization and plaque growth in apolipoprotein E-deficient mice. *Circulation* 99 (13), 1726–1732.
- Nagy, E., Eaton, J.W., Jeney, V., Soares, M.P., Varga, Z., Galajda, Z., Szentmiklósi, J., Méhes, G., Csonka, T., Smith, A., Vercellotti, G.M., Balla, G., Balla, J., 2010. Red cells, hemoglobin, heme, iron, and atherogenesis. *Arterioscler. Thromb. Vasc. Biol.* 30 (7), 1347–1353.
- Nagy, J.A., Chang, S.H., Shih, S.C., Dvorak, A.M., Dvorak, H.F., 2010. Heterogeneity of the tumor vasculature. *Semin Thromb. Hemost.* 36 (3), 321–331.
- Nagy, J.A., Dvorak, A.M., Dvorak, H.F., 2012. Vascular hyperpermeability, angiogenesis, and stroma generation. *Cold Spring Harb. Perspect. Med.* 2 (2), a006544.
- Nakano, D., Hayashi, T., Tazawa, N., Yamashita, C., Inamoto, S., Okuda, N., Mori, T., Sohmiya, K., Kitaura, Y., Okada, Y., Matsumura, Y., 2005. Chronic hypoxia accelerates the progression of atherosclerosis in apolipoprotein E-knockout mice. *Hypertens. Res.* 28 (10), 837–845.
- O'Reilly, M.S., Boehm, T., Shing, Y., Fukai, N., Vasios, G., Lane, W.S., Flynn, E., Birkhead, J.R., Olsen, B.R., Folkman, J., 1997. Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. *Cell* 88 (2), 277–285.
- Oliver, K.M., Garvey, J.F., Ng, C.T., Veale, D.J., Fearon, U., Cummins, E.P., Taylor, C.T., 2009. Hypoxia activates NF-kappaB-dependent gene expression through the canonical signaling pathway. *Antioxid. Redox Signal* 11 (9), 2057–2064.
- Ozaki, M., Kawashima, S., Yamashita, T., Hirase, T., Namiki, M., Inoue, N., Hirata, K., Yasui, H., Sakurai, H., Yoshida, Y., Masada, M., Yokoyama, M., 2002. Overexpression of endothelial nitric oxide synthase accelerates atherosclerotic lesion formation in apoE-deficient mice. *J. Clin. Invest.* 110 (3), 331–340.
- Palazon, A., Goldrath, A.W., Nizet, V., Johnson, R.S., 2014. HIF transcription factors, inflammation, and immunity. *Immunity* 41 (4), 518–528.
- Perez-Medina, C., Binderup, T., Lobatto, M.E., Tang, J., Calcagno, C., Giesen, L., Wessel, C.H., Witjes, J., Ishino, S., Baxter, S., Zhao, Y., Ramachandran, S., Eldib, M., Sanchez-Gaytan, B.L., Robson, P.M., Bini, J., Granada, J.F., Fish, K.M., Stroes, E.S., Duivenvoorden, R., Tsimikas, S., Lewis, J.S., Reiner, T., Fuster, V., Kjaer, A., Fisher, E.A., Fayad, Z.A., Mulder, W.J., 2016. In vivo PET imaging of HDL in multiple atherosclerosis models. *JACC Cardiovasc Imaging* 9 (8), 950–961.
- Pircher, A., Treps, L., Bodrug, N., Carmeliet, P., 2016. Endothelial cell metabolism: a novel player in atherosclerosis? Basic principles and therapeutic opportunities. *Atherosclerosis* 253, 247–257.
- Post, S., Peeters, W., Busser, E., Lamers, D., Sluijter, J.P., Goumans, M.J., de Weger, R.A., Moll, F.L., Doevendans, P.A., Pasterkamp, G., Vink, A., 2008. Balance between angiotensin-1 and angiotensin-2 is in favor of angiotensin-2 in atherosclerotic plaques with high microvessel density. *J. Vasc. Res.* 45 (3), 244–250.
- Pouyssegur, J., Dayan, F., Mazure, N.M., 2006. Hypoxia signalling in cancer and approaches to enforce tumour regression. *Nature* 441 (7092), 437–443.
- Pugh, C.W., Ratcliffe, P.J., 2003. Regulation of angiogenesis by hypoxia: role of the HIF system. *Nat. Med.* 9 (6), 677–684.
- Qiu, W., Kass, D.A., Hu, Q., Ziegelstein, R.C., 2001. Determinants of shear stress-stimulated endothelial nitric oxide production assessed in real-time by 4,5-diaminofluorescein fluorescence. *Biochem. Biophys. Res. Commun.* 286 (2), 328–335.
- Rademakers, T., Douma, K., Hackeng, T.M., Post, M.J., Sluimer, J.C., Daemen, M.J., Biessen, E.A., Heeneman, S., van Zandvoort, M.A., 2013. Plaque-associated vascular vasomir in aged apolipoprotein E-deficient mice exhibit proatherogenic functional features in vivo. *Arterioscler. Thromb. Vasc. Biol.* 33 (2), 249–256.
- Ramkhalawon, B., Yang, Y., van Gils, J.M., Hewing, B., Rayner, K.J., Parathath, S., Guo, L., Oldebeken, S., Feig, J.L., Fisher, E.A., Moore, K.J., 2013. Hypoxia induces nectrin-1 and Unc5b in atherosclerotic plaques: a mechanism for macrophage retention and survival. *Arterioscler. Thromb. Vasc. Biol.* 33 (6), 1180–1188.
- Roiniotis, J., Dinh, H., Masendycz, P., Turner, A., Elsegood, C.L., Scholz, G.M., Hamilton, J.A., 2009. Hypoxia prolongs monocyte/macrophage survival and enhanced glycolysis is associated with their maturation under aerobic conditions. *J. Immunol.* 182 (12), 7974–7981.
- Ruiz, J.L., Hutcheson, J.D., Aikawa, E., 2015. Cardiovascular calcification: current controversies and novel concepts. *Cardiovasc. Pathol.* 24 (4), 207–212.
- Schoors, S., Bock, K. De, Cantelmo, Anna R., Georgiadou, M., Ghesquière, B., Cauwenberghs, S., Kuchnio, A., Wong, Brian W., Quaegebeur, A., Goveia, J., Bifari, F., Wang, X., Blanco, R., Tembuysen, B., Cornelissen, I., Bouché, A., Vinckier, S., Diaz-Moralli, S., Gerhardt, H., Telang, S., Cascante, M., Chesney, J., Dewerchin, M., Carmeliet, P., 2014. Partial and transient reduction of glycolysis by PFKFB3 blockade reduces pathological angiogenesis. *Cell Metab.* 19 (1), 37–48.
- Schrijvers, D.M., De Meyer, G.R., Kockx, M.M., Herman, A.G., Martinet, W., 2005. Phagocytosis of apoptotic cells by macrophages is impaired in atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* 25 (6), 1256–1261.
- Seghers, L., de Vries, M.R., Pardali, E., Hoefler, I.E., Hierck, B.P., ten Dijke, P., Goumans, M.J., Quax, P.H., 2012. Shear induced collateral artery growth modulated by endoglin but not by ALK1. *J. Cell Mol. Med.* 16 (10), 2440–2450.
- Semenza, G.L., 2003. Targeting HIF-1 for cancer therapy. *Nat. Rev. Cancer* 3 (10), 721–732.
- Sewduth, R., Santoro, M.M., 2016. "Decoding" angiogenesis: new facets controlling endothelial cell behavior. *Front. Physiol.* 7, 306.
- Sluimer, J.C., Daemen, M.J., 2009. Novel concepts in atherogenesis: angiogenesis and hypoxia in atherosclerosis. *J. Pathol.* 218 (1), 7–29.
- Sluimer, J.C., Gasc, J.M., van Wanroij, J.L., Kisters, N., Groeneweg, M., Sollewijn Gelpke, M.D., Cleutjens, J.P., van den Akker, L.H., Corvol, P., Wouters, B.G., Daemen, M.J., Bijmens, A.P., 2008. Hypoxia, hypoxia-inducible transcription factor, and macrophages in human atherosclerotic plaques are correlated with intraplaque angiogenesis. *J. Am. Coll. Cardiol.* 51 (13), 1258–1265.
- Sluimer, J.C., Kolodgie, F.D., Bijmens, A.P., Maxfield, K., Pacheco, E., Kutys, B., Duimel, H., Frederic, P.M., van Hinsbergh, V.W., Virmani, R., Daemen, M.J., 2009. Thin-walled microvessels in human coronary atherosclerotic plaques show incomplete endothelial junctions relevance of compromised structural integrity for intraplaque microvascular leakage. *J. Am. Coll. Cardiol.* 53 (17), 1517–1527.
- Tabas, I., Bornfeldt, K.E., 2016. Macrophage phenotype and function in different stages of atherosclerosis. *Circ. Res.* 118 (4), 653–667.
- Taqueti, V.R., Di Carli, M.F., Jeresch-Herold, M., Sukhova, G.K., Murthy, V.L., Folco, E.J., Kwong, R.Y., Ozaki, C.K., Belkin, M., Nahrendorf, M., Weissleder, R., Libby, P., 2014. Increased microvascularization and vessel permeability associate with active inflammation in human atheromata. *Circ. Cardiovasc. Imaging* 7 (6), 920–929.
- Theelen, T.L., Lappalainen, J.P., Sluimer, J.C., Gurbuz, E., Cleutjens, J.P., Gijbels, M.J., Biessen, E.A., Daemen, M.J., Alitalo, K., Ylä-Herttua, S., 2015. Angiopoietin-2 blocking antibodies reduce early atherosclerotic plaque development in mice. *Atherosclerosis* 241 (2), 297–304.
- Tian, H., McKnight, S.L., Russell, D.W., 1997. Endothelial PAS domain protein 1 (EPAS1), a transcription factor selectively expressed in endothelial cells. *Genes Dev.* 11 (1), 72–82.
- Tsujita, K., Kaikita, K., Araki, S., Yamada, T., Nagamatsu, S., Yamanaka, K., Sakamoto, K., Kojima, S., Hokimoto, S., Ogawa, H., 2016. In Vivo optical coherence tomography visualization of intraplaque neovascularization at the site of coronary vasospasm: a case report. *BMC Cardiovasc. Disord.* 16 (1), 235.
- Tziakias, D.N., Chalikias, G.K., Stakos, D., Tentes, I.K., Papazoglou, D., Grapsa, A., Gioka, G., Kaski, J.C., Boudoulas, H., 2011. Independent and additive predictive value of total cholesterol content of erythrocyte membranes with regard to coronary artery disease clinical presentation. *Int. J. Cardiol.* 150 (1), 22–27, (Jul 1).
- Van der Donck, C., Van Herck, J.L., Schrijvers, D.M., Vanhoutte, G., Verhoye, M., Bloxh, I., Van Der Linden, A., Bauters, D., Lijnen, H.R., Sluimer, J.C., Roth, L., Van Hove, C.E., Franssen, P., Knaapen, M.W., Hervent, A.S., De Keulenaer, G.W., Bult, H., Martinet, W., Herman, A.G., De Meyer, G.R., 2015. Elastin fragmentation in atherosclerotic mice leads to intraplaque neovascularization, plaque rupture, myocardial infarction, stroke, and sudden death. *Eur. Heart J.* 36 (17), 1049–1058.
- Van der Veken, B., De Meyer, G.R., Martinet, W., 2016. Intraplaque neovascularization as a novel therapeutic target in advanced atherosclerosis. *Expert Opin. Ther. Targets*, 1–11.
- Van Herck, J.L., De Meyer, G.R., Martinet, W., Van Hove, C.E., Foubert, K., Theunis, M.H., Apers, S., Bult, H., Vrints, C.J., Herman, A.G., 2009. Impaired fibrillin-1 function promotes features of plaque instability in apolipoprotein E-deficient mice. *Circulation* 120 (24), 2478–2487.
- van Hinsbergh, V.W.M., Eringa, E.C., Daemen, M., 2015. Neovascularization of the atherosclerotic plaque: interplay between atherosclerotic lesion, adventitia-derived microvessels and perivascular fat. *Curr. Opin. Lipidol.* 26 (5), 405–411.
- van Uden, P., Kenneth, N.S., Rocha, S., 2008. Regulation of hypoxia-inducible factor-1alpha by NF-kappaB. *Biochem. J.* 412 (3), 477–484.
- Wang, H., Gardecki, J.A., Ughi, G.J., Jacques, P.V., Hamidi, E., Tearney, G.J., 2015. Ex vivo catheter-based imaging of coronary atherosclerosis using multimodality OCT and NIRAF excited at 633 nm. *Biomed. Opt. Express* 6 (4), 1363–1375.
- Wang, Y., Zhou, Y., He, L., Hong, K., Su, H., Wu, Y., Wu, Q., Han, M., Cheng, X., 2011. Gene delivery of soluble vascular endothelial growth factor receptor-1 (sFlt-1) inhibits intra-plaque angiogenesis and suppresses development of atherosclerotic plaque. *Clin. Exp. Med.* 11 (2), 113–121.
- Wezel, A., de Vries, M.R., Maassen, J.M., Kip, P., Peters, E.A., Karper, J.C., Kuiper, J., Bot, I., Quax, P.H., 2016. Deficiency of the TLR4 analogue RP105 aggravates vein graft disease by inducing a pro-inflammatory response. *Sci. Rep.* 6, 24248.
- Wiesener, M.S., Turley, H., Allen, W.E., Willam, C., Eckardt, K.U., Talks, K.L., Wood, S.M., Gatter, K.C., Harris, A.L., Pugh, C.W., Ratcliffe, P.J., Maxwell, P.H., 1998. Induction of endothelial PAS domain protein-1 by hypoxia: characterization and comparison with hypoxia-inducible factor-1alpha. *Blood* 92 (7), 2260–2268.
- Willems, S., Vink, A., Bot, I., Quax, P.H.A., de Borst, G.J., de Vries, J.-P.P.M., van de Weg, S.M., Moll, F.L., Kuiper, J., Kovanen, P.T., de Kleijn, D.P.V., Hoefler, I.E.,

- Pasterkamp, G., 2013. Mast cells in human carotid atherosclerotic plaques are associated with intraplaque microvessel density and the occurrence of future cardiovascular events. *Eur. Heart J.* 34 (48), 3699–3706.
- Winnik, S., Lohmann, C., Siciliani, G., von Lukowicz, T., Kuschnerus, K., Kraenkel, N., Brokopp, C.E., Enseleit, F., Michels, S., Ruschitzka, F., Luscher, T.F., Matter, C.M., 2013. Systemic VEGF inhibition accelerates experimental atherosclerosis and disrupts endothelial homeostasis - implications for cardiovascular safety. *Int. J. Cardiol.* 168 (3), 2453–2461.
- Wong, C., Bezhaeva, T., Rothuizen, T.C., Metselaar, J.M., de Vries, M.R., Verbeek, F.P., Vahrmeijer, A.L., Wezel, A., van Zonneveld, A.J., Rabelink, T.J., Quax, P.H., Rotmans, J.I., 2016. Liposomal prednisolone inhibits vascular inflammation and enhances venous outward remodeling in a murine arteriovenous fistula model. *Sci. Rep.* 6, 30439.
- Wong, C.Y., de Vries, M.R., Wang, Y., van der Vorst, J.R., Vahrmeijer, A.L., van Zonneveld, A.J., Roy-Chaudhury, P., Rabelink, T.J., Quax, P.H., Rotmans, J.I., 2014. Vascular remodeling and intimal hyperplasia in a novel murine model of arteriovenous fistula failure. *J. Vasc. Surg.* 59 (1), 192–201, (e191).
- Xu, J.Y., Lu, X.T., Shi, G.P., 2015. Vasa Vasorum in atherosclerosis and clinical significance. *Int. J. Mol. Sci.* 16 (5), 11574–11608.
- Xu, X., Mao, W., Chai, Y., Dai, J., Chen, Q., Wang, L., Zhuang, Q., Pan, Y., Chen, M., Ni, G., Huang, Z., 2015. Angiogenesis Inhibitor, endostar, prevents vasa vasorum neovascularization in a swine atherosclerosis model. *J. Atheroscler. Thromb.* 22 (10), 1100–1112.
- Yahagi, K., Kolodgie, F.D., Otsuka, F., Finn, A.V., Davis, H.R., Joner, M., Virmani, R., 2016. Pathophysiology of native coronary, vein graft, and in-stent atherosclerosis. *Nat. Rev. Cardiol.* 13 (2), 79–98.