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Future directions for therapeutic strategies in post-ischaemic vascularization: a position paper from European Society of Cardiology Working group on Atherosclerosis and Vascular Biology

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Words:

Abstract

Modulation of vessel growth holds great promise for treatment of cardiovascular disease. Strategies to promote vascularization can potentially restore function in ischaemic tissues. On the other hand, plaque neovascularization has been shown to associate with vulnerable plaque phenotypes and adverse events. The current lack of clinical success in regulating vascularization illustrates the complexity of the vascularization process, which involves a delicate balance between pro- and anti-angiogenic regulators and effectors. This is compounded by limitations in the models used to study vascularization that do not reflect the eventual clinical target population. Nevertheless, there is a large body of evidence that validate the importance of angiogenesis as a therapeutic concept. The overall aim of this Position Paper of the ESC Working Group of Atherosclerosis and Vascular biology is to provide guidance for the next steps to be taken from pre-clinical studies on vascularization toward clinical application. To this end, the current state of knowledge in terms of therapeutic strategies for targeting vascularization in post-ischaemic disease is reviewed and discussed. A consensus statement is provided on how to optimize vascularization studies for the identification of suitable targets, the use of animal models of disease and the analysis of novel delivery methods.

1. Basic principles – vasculogenesis, angiogenesis, arteriogenesis

Vasculogenesis describes the coalescence of mesoderm-derived angioblasts into the first primitive blood vessels [1]. The process was first observed in quail embryos [2, 3] and subsequently shown to be conserved in other vertebrates including mouse [4, 5] and zebrafish [6, 7]. These studies revealed many similarities not only between the morphogenetic processes of early blood vessel formation, but also between the molecules coordinating these processes [8]. Several signalling pathways, such as Notch[9] [10] and Sonic Hedgehog [11], were shown to influence the early differentiation of arterial and venous endothelial cells (ECs) from angioblasts. Vasculogenesis was initially thought to be limited to embryo, but current understanding is more nuanced. Early embryonic angioblasts and hemoblasts share a very similar gene signature and the endothelium is hemogenic during development, meaning that hematopoietic stem cells differentiate from endothelial cells [12]. The opposite is also true, hematopoietic stem cells can be differentiated into endothelial cells [13].

Angiogenesis is the creation of new vessels from pre-existing ones [14]. Hypoxia is one of the key drivers of the process. It activates ECs to become more motile and protrude filopodia. Further angiogenic factors such as vascular endothelial growth factor (VEGF) strongly dilates small arteries and capillaries which is the primary mode of VEGF action at low concentrations (intussusception angiogenesis). At high concentrations of VEGF, sprouting angiogenesis is the preferred mode of action [15]. To prevent ECs moving en masse, a particular type of ECs, known as tip cells, are selected to lead the advance [16]. Neighbouring cells assume an ancillary role as stalk cells, which divide to elongate the new vessel and establish a lumen. This specification of tip and stalk cells is governed by the Notch signalling pathway [17, 18]. The establishment of flow in newly formed vessels leads to mechanical signals (shear stress) that feedback to reduce angiogenic sprouting thereby preventing excessive vascular growth [19, 20].

Once stenosis in a large main artery becomes hemodynamically significant, the elevation of shear stress against the wall of these arterioles induces their enlargement. This is described as arteriogenesis. The collateral circulation may subsequently develop into a functional vascular structure to ensure regional perfusion after the

ischaemic event, thus protecting the tissues against necrosis. Simultaneously, arterioles, venules, and arteriovenous anastomoses are formed, following the production of smooth muscle cells and of the extracellular matrix (ECM), which consolidates the walls of these vascular structures [21].

2. Neo-vascularization: physiology and pathophysiology

2.1 Post-ischaemic angiogenesis: a physiological adaptation

After the onset of ischaemia, cardiac or skeletal muscle undergoes a continuum of molecular, cellular, and extracellular responses that determine the function and the remodelling of the ischaemic tissue. Hypoxia-related pathways, the alterations in immunoinflammatory balance, as well as changes in hemodynamic forces within the vascular wall trigger vasculogenesis, angiogenesis and arteriogenesis which act in concert to establish a functional vascular network in ischaemic zones [22].

The principal signalling pathway induced by hypoxia involves activation of hypoxia-induced factor (HIF1α), which induces the expression of a set of genes appropriate to respond to this situation. Indeed, HIF1α controls the expression of numerous major players involved in angiogenesis and vascular remodelling, including VEGF. Moreover, the target genes of HIF1α are involved in metabolism, erythropoiesis, pH homeostasis, and autophagy [23].

During ischaemia, inflammatory cells release angiogenic factors (e.g. VEGF) and cytokines (e.g. TNFα), that decrease EC junctions and enhance vascular permeability to promote the recruitment of inflammatory cells [24, 25]. Consistent with this relationship between angiogenesis and inflammation, several molecules that regulate inflammation have been implicated in new vessel formation [22]. Changes in hemodynamic forces (mechanical forces linked to pressure and flow rate) occurring in collateral vessels in response to arterial occlusion also contribute to post-ischaemic vascularization [26]. Recent studies suggest that flow dynamics control the localisation of sprouting in vessels [27]. The location is not determined by on highest VEGF concentration, but by a combination of VEGF and biomechanical signals [28]. Thus, shear-induced mechanism appears to override pro-angiogenic signals such as VEGF [29]. These pathways can also participate in vascular pathology; for example, the

mechanosensitive transcription factor TWIST1 promotes angiogenesis in the embryo and is also required for plaque formation in atherosclerosis models [20].

In patients with ischaemic diseases in the presence of comorbidities such as diabetes, hypertension and obesity, most of the cellular and molecular mechanisms involved in the activation of vessel growth and vascular remodelling are markedly impaired [22]. Thus, in the last decades, stimulation of vessel growth has emerged as a novel therapeutic option in patients with ischaemic diseases [30].

2.2 Plaque vascularization and pathophysiology

Under physiological circumstances, microvessels originate from the adventitia and provide the media with oxygen and nutrients **[31]**. However, microvessels in atherosclerotic plaques have been implicated in progression of the disease and adverse outcomes.

It is postulated that plaque angiogenesis is driven by plaque hypoxia and inflammation **[32, 33 34]**. In experimental models plaque angiogenesis has been induced by stress [35, 36], treatment with pro-inflammatory mediators [37], pro-angiogenic growth factors [38] and viral gene delivery of pro-angiogenic factors [39-43], and was shown to increase plaque burden. Besides an increase in the number of microvessels, the physiological properties (quality) of the microvessel are also associated with risk for human plaque rupture. Microvessels of ruptured plaques in coronary arteries displayed detachments of the endothelial junctions, endothelial membrane blebs and a thin or absent endothelial basement membrane, and surrounding pericytes were found to be absent in a majority of microvessels in ruptured plaques.[44] These ultrastructural characteristics suggest vascular leakage [45], that might be responsible for increased extravasation of immune cells and deposition of lipids and red blood cells in the plaques [46-48]. Therefore, these microvessels are thought to represent one of the main sources of intra-plaque haemorrhage, in addition to healed thrombi [49].

3. Vascularization therapeutics

3.1 Growth factor therapy

Various growth factors have been applied for therapeutic angiogenesis including VEGF, basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), Platelet-derived growth factor (PDGF), stromal derived factor 1 (SDF-1),

Angiopoietin 1 (ANG-1) and insulin-like growth factor (IGF-1). Among these, VEGF and bFGF are the best studied and have reached human clinical trials. VEGF is the most important regulator of physiological angiogenesis during growth, healing and in response to hypoxia. VEGF is upregulated 30-fold by HIF1α, which is more than any other inducible angiogenic factor during ischaemia. However, when administered alone, VEGF could increase endothelial permeability which leads to the formation of leaky capillaries and tissue oedema [50].

PDGF can help stabilize nascent blood vessels by recruiting mesenchymal progenitors, and co-delivery of VEGF and PDGF has been shown to lead to early formation of mature vessels in animal models [51]. bFGF is among the first discovered angiogenic factors to have both angiogenic and arteriogenic properties, which may facilitate formation of a mature blood vessel network [52]. The HGF family induces potent angiogenic responses by binding to the c-MET receptor, which is expressed on ECs, vascular smooth muscle cells and hematopoietic stem cells. HGF is known to have mitogenic, angiogenic, anti-apoptotic, and anti-fibrotic activities in various cells [53]. Clinical trials of SDF-1 in critical limb ischaemia (CLI) patients are underway and a better understanding of the mechanisms of chemokines, especially SDF-1, is crucial in filling the missing link in growth factor studies in therapeutic angiogenesis [54] .

Notably, most of the intervention strategies to manipulate angiogenesis in atherosclerosis have been restricted to mouse models by using antiangiogenic or proangiogenic molecules such as halidomide [55], TNP-470 [56], angiostatin [57, 58], anti-VEGF-A [59], bFGF [38] and VEGF-A [60].

3.2 Cell therapy

Since the first pilot clinical study to evaluate treatment of peripheral vascular disease with stem cell therapy in 2002, over 50 clinical studies have been reported with progenitor cells [61]. Therapeutic details such as patient selection, effective cell type selection and processing, optimal dosage, and delivery route are constantly improved.

Studies have included patients of varying peripheric artery disease (PAD) severity. However, most of clinical trials have primarily focused on CLI patients in small phase I or II studies [61]. A variety of cell types have been studied as potential PAD treatments, including unselected bone marrow mononuclear cells (BM-MNC) or peripheral blood MNC (PB-MNC), marker-specific cells selected from the marrow or

blood, mesenchymal stem cells (MSCs), and adipose tissue–derived regenerative cells [62].

In clinical studies of neovascularization considerable progress in the use of adult stem cells for cell transplantation has been made using hematopoietic stem cells (HSC), bone marrow-derived dendritic cells (BMDC), MSC, and endothelial progenitor cells (EPC) [63]. Neovascularization in infarcted heart can be mediated by the incorporation of vascular progenitor cells into the capillary or by the paracrine factors released from stem cells and progenitor cells. In relation to the effectiveness of the use of adult stem cells for cell transplantation, the variability in the reported findings may be partly explained by differences in the delivery methods, treatment logistics, and target diseases [63].

3.3 Non-coding RNA therapy

Short (microRNAs; miRNAs) or longer (long non-coding RNA (lncRNAs) non-coding RNAs play important roles in several physiological and pathological conditions such as cancer and cardiovascular diseases (CVD) including atherosclerosis [64] . Emerging data show that several miRNAs are linked to both adaptive and maladaptive vascular remodelling processes. Mir-126, one of the most abundantly expressed microRNAs in ECs, has a pro-angiogenic as well as anti-atherosclerotic role [65] and the systemic delivery of miR-126 mimics rescued EC proliferation at vulnerable sites and inhibited atherosclerotic lesion progression [66]. On the other hand, the 17-92 miRNA cluster is anti-angiogenic but pro-atherosclerotic. Recent studies described that the endothelial-specific deletion of miR-17-92 in mice enhanced arterial density and improved post-ischaemia blood flow recovery [67]. Notably, miR-503 expression is increased in ischaemic limb muscles and ECs of diabetic mice. Inhibition of miR-503 by adenoviral delivery to the ischaemic adductor muscles of diabetic mice corrected diabetes-induced impairment of post-ischaemic angiogenesis and blood flow recovery [68]. Even though the functions of individual microRNAs in angiogenesis are not yet completely elucidated, because a single microRNA could regulate several growth factors at the same time, miRNA-derived therapy could replace single-factor angiogenic gene therapy [69].

4. Animal models of angiogenesis

4.1 Pre-clinical studies of therapeutic angiogenesis

Models to investigate post-ischaemic angiogenesis have been established in rodents and larger animals such as rabbits, pigs or dogs. They exhibit considerable variation because each species differs in the extent of naïve vascularization and thus reacts differently to vascular growth stimuli. To make things more complicated, within one animal species, different strains show distinct naïve vascularization and even show opposite reactions [70].

So far, most studies have been performed in mice, because of the availability of a wide range of genetic knockout strains and the ease of introducing new genetic manipulations, including knock-in and temporal or tissue-specific manipulations. Moreover, the breeding is relatively fast and less expensive than experimentation with large animals and data obtained in mouse models are still necessary to justify experiments in large animal.

A commonly used method in mice to induce post-ischaemic angiogenesis is the hind-limb ischaemia model, which is based on ligation of the femoral artery [71]. Compared to the coronary or carotid artery, the femoral artery is easier to access and the method is accompanied by lower mortality rates. Moreover, live imaging of blood flow in ischaemic areas can be easily performed by laser Doppler imaging. Nevertheless, many of the mechanisms underlying neovascularization in response to ischaemia in peripheral arteries are not directly transferable to angiogenic processes in the heart. Experimental models of cardiac ischaemia are based on transient or permanent occlusion of the left descending coronary artery, induced by a highly invasive surgical procedure requiring thoracotomy. Moreover, in vivo imaging of coronary arteries by for instance intravital microscopy is complicated by the rapid movements due to cardiac and respiratory cycles [72].

Rat models are also frequently used due to the ease of breeding and their extended lifespan. The methods and readouts normally applied do not differ essentially from those used in mice. Their major advantage compared to mice therefore lies in their size, without improving translatability into humans. Moreover, larger animals require a longer time to restore vessel function by neovascularization. Of course, this is an oversimplification, but it partly explains why larger animal models

are often regarded to have added value for translation of angiogenic therapies into human medicine (Table 1).

For a long time, the dog [73, 74] , together with the rabbit [75, 15], were the animals of choice for investigation of neovascularization. Amongst other reasons such as easy handling, dogs are well known for their extended myocardial vascularization that allows performing coronary artery occlusions with low complication rates. Much of our current knowledge on the role of various angiogenic and arteriogenic growth factors is based on experiments performed in dogs. However, ethical considerations have led to a significant decrease in the use of dogs for animal experimentation.

The occlusion pathophysiology and tissue recovery that occur after an acute arterial ligation are very different in animal models than in human chronic ischaemic diseases. Experimental acute vessel occlusion results in an immediate vascular response in animals which reflects the situation in a limited subgroup of patients (such as young patients with traumatic injuries), who require immediate medical interventions and are not typically enrolled in angiogenic therapy clinical trials. Another crucial difference between the experimental models and patients is that the patients, owing to their comorbidities, do not have sufficient growth of collaterals, showing decreased endogenous angiogenic stimuli and reduced angiogenic signalling [30].

The search for an adequate replacement with potentially even higher translational value has resulted in an increasing number of pig models. Hind-limb ischaemia in pigs can be safely performed without leading to limb necrosis [76]. In contrast, the pig was long considered to have insufficient capabilities to compensate for coronary ischaemia by neovascularization [77]. In the past decade, however, several groups succeeded in establishing also pig coronary neovascularization models by inducing progressive coronary stenosis rather than acute occlusions[78, 79].

4.2 Pre-clinical studies of plaque angiogenesis

Many studies of atherosclerosis use murine models, however there are several limitations in their applicability to analyse plaque vascularization. Notably, atherosclerotic plaques developing in hypercholesterolemic murine models contain fewer microvessels than human atherosclerotic plaques. The reason for this remains uncertain but it may be due to differences in the transport of oxygen between human versus murine atherosclerotic plaques, ECM turnover and different biomechanics between mice and man [80]. A role for ECM was implicated by studies of knockout mice

lacking collagen XVIII which had enhanced intra-plaque vascularization in response to hypercholesterolemia compared to controls [57]. This was more pronounced in ApoE fibrillin double knockout mice [81], suggesting that lack of proper ECM components in the media and plaque might mediate angiogenesis. Besides ECM degradation, different biomechanical properties between mice and man might also explain the lack of plaque angiogenesis [82, 83]. Lower fibrotic material stiffness (cellular and hypocellular) and a fundamental difference in plaque morphology (dome-like) together with a smaller vessel size as well as lower peak cap stress are present in murine compared to human plaques [83]. In addition, tissue contraction and deformation have been shown to induce VEGF-A expression [84]. Lower biomechanical stresses might account for lower VEGF-A levels in mice versus humans. Indeed, ruptured human plaques express higher levels of VEGF-A compared to stable plaques [85]. In murine atherosclerosis, experimental overexpression of VEGF-A increased signs of plaque vulnerability [38], showing that endogenous VEGF-A expression is not sufficient to evoke signs of plaque rupture.

Another limitation relates to the site of microvessel formation. While a minority of studies report intra-plaque angiogenesis in murine atherosclerosis models, most focus on plaque-associated vasa vasorum of the adventitia as a surrogate for intra-plaque microvessels (Table 2). This is an important caveat because although adventitial vasa vasorum growth may precede atherosclerotic plaque development [86, 87], plaque rupture has been linked with increased intra-plaque angiogenesis rather than an increase in adventitial vasa vasorum in humans [44]. Thus far, this discrepancy limits the extrapolation of murine adventitial angiogenesis as an outcome parameter to human studies.

Moreover, several methodological limitations hamper the comparability of murine and human studies. Firstly, while murine models usually examine on various regions (e.g. aortic root, ascending aorta, descending aorta, brachiocephalic artery, and carotid artery) they often ignore other clinically-relevant vessels such as the coronary and renal arteries. In addition to this, the parameters measured to assess vascularization vary considerably between studies: for example, microvessel density (number of microvessels per mm^2), microvessel count (per section or per mouse), CD31 positive adventitial area or vasa vasorum volume have been used (Table 2). Moreover, also the imaging method varied between studies: most of them used histology, but also intra-vital microscopy, two photon microscopy, confocal microscopy

and micro CT have been used to visualize adventitial microvessels (Table 2). Moreover, the experimental design often limits the translatability of the findings. In two studies, induction/manipulation of angiogenesis was started together with atherosclerosis induction [55, 88], whereas pre-existing plaques represent the treatment target in human atherosclerosis.

In addition to mice and rats, rabbits and pigs (Table 3) have been used to study angiogenesis in atherosclerosis. In rabbit models, atherosclerosis was mostly induced by a combination of balloon angioplasty and high cholesterol diet, leading to plaques with a baseline microvascular density between 15 and 80 vessels per mm^2. In some studies, adventitial angiogenesis was specifically targeted using a hollow perivascular collar together with a relatively short post-operation time of 9 to 21 days [40, 41, 89, 90]. Interestingly, induction of diabetes accelerated atherogenesis and intraplaque angiogenesis in Watanabe heritable hyperlipidemic rabbits [91] (Table 3).

In pigs, atherosclerosis was induced by high cholesterol diet and/or surgical interventions (balloon angioplasty or stenting) (Table 3). However, intra-plaque angiogenesis was not detected in all studies except for one (Table 3). Here, a genetically engineered Yucatan mini pig was used, which develops hypercholesterolemia due to pro-protein convertase subtilisin/kexin type 9 (PCSK9) overexpression, when fed a high cholesterol diet [92]. The resulting plaques show a human like morphology including intra-plaque and adventitial angiogenesis. However, data on microvascular density were unfortunately not provided. Practically, larger animal models allow for the use of clinical diagnostic tools such as magnetic resonance imaging to detect microvessels. Therefore, it will be easier to translate the study results to the human situation.

5. Gene and cell delivery

5.1 Viral delivery

Gene and cell delivery into the myocardium has been a major challenge over the past decade. Efficient therapeutic approaches developed in animal models have not been successful in human clinical trials because gene and cell transfer efficiency in cardiac muscle has remained too low [93, 94] Several factors contribute to this problem: the human heart is a very large muscle as compared to mice and rats and vectors or cell solutions cannot easily penetrate deep into the myocardium. The adeno

associated virus (AAVs) for instance, bind tightly to heparansulphate proteoglycans and they do not easily escape from the intraluminal space into the myocardium [95]. In previous trials, intracoronary injections, intramyocardial injections from the left ventricle and intramyocardial injections during thoracotomy or bypass surgery have been tested. However, because occluded coronary arteries do not get adequate perfusion, fail to deliver substances into the ischaemic areas. Thus, it is not surprising that intracoronary injections have not been very successful for gene and cell delivery.

5.2 Mechanical delivery

Intramyocardial injections lead to better transduction efficiencies but diffusion of viral vectors in the myocardium is still limited and the binding to ECM components further limits vector spreading in the myocardium. Protein, such as VEGF-A_{165}, delivered by transgenes, bind strongly to heparansulphate proteoglycans which reduces their diffusion in ischaemic and fibrotic myocardium. Similar obstacles exist for successful cell delivery into the myocardium. Intracoronary injections seldom lead to viable, engrafted cells in the heart. Intramyocardial injections cause significant mechanical stress on the cells during injections. Most cells seem to die within hours or during the first days and paracrine factors seem to contribute to the potential therapeutic effects [96, 97]. For applications like myocardial ischaemia, local targeted injections based on electromechanical mapping [98] or blood flow measurements using positron emission tomography [98] have recently improved the situation and targeted injections into hibernating myocardium can now be achieved with 10-20% efficiency around the needle track. Multiple injections are still needed to cover larger areas in ischaemic myocardium. To improve myocardial function in heart failure, the effects of gene or cell transfer should be very global to transduce as many cardiomyocytes as possible. At the moment, this can be achieved with some vectors in mice [99] but in larger animals and humans wide spread gene expression after any delivery method still remains a very challenging task [100, 101].

5.3 Non-viral delivery

Several methods of non-viral gene transfer have been utilized to deliver genes of interest to ischaemic tissues to stimulate therapeutic angiogenesis. Genes encoding pro-angiogenic proteins have been administered by cationic polymers, lipids, liposomes and three-dimensional scaffolds [102]. Targeting strategies using polymers or

lipids modified with specific ligands for the receptors on target tissues could improve the efficacy of current gene delivery systems by facilitating cellular uptake of genes via receptor-mediated endocytosis [103]. Gene delivery using lipid formulations has been applied in ischaemic tissues for therapeutic angiogenesis. Jeon et al. reported that VEGF-A gene delivery using heparin-conjugated Polyethylenimine (PEI) significantly upregulated VEGF-A expression, resulting in extensive neovascularization in mouse ischaemic limbs [104]. Nanoparticles composed of biocompatible and biodegradable polymers (e.g., poly (lactic-co-glycolic acid; PLGA) are considered to serve as gene carriers for the treatment of ischaemic tissues due to the efficient delivery mechanism and low toxicity. Indeed, VEGF-A delivery using PLGA nanoparticles resulted in higher VEGF-A expression and more extensive angiogenesis in mouse ischaemic limbs [105]. A novel concept of involving a biodegradable gelatin hydrogel carrying a sustained-release system of bFGF was studied in patients with CLI. The delivery improved transcutaneous oxygen tension, increased walking time, and decreased ischaemic pain [106]. Despite this progress, the ability to introduce new genes into ischaemic tissue remains an important challenge and a limiting factor in the field of therapeutic angiogenesis.

6. Clinical trials for therapeutic vascularization: change of perspectives

6.1 Endpoints

Ongoing clinical gene and cell therapy trials have been reviewed elsewhere [93, 107]. In most ongoing trials, very stringent endpoints have been selected, such as overall mortality, major adverse cardiovascular events (MACE), improvement in exercise test, or various quality of life endpoints. However, since most gene and cell therapy trials are still quite small as compared to large pharmaceutical phase II/III trials, endpoints like overall mortality or MACE cannot easily capture potentially significant treatment effects, such as reduced frequency of hospital admissions and lower number of multiple additional interventions in chronically ill patients. For example, endpoints like ST segment decline of 1 mm is not useful for refractory angina patients who usually have infarction scars, ECG alterations and the ST segment changes cannot be reliably detected. Small phase I and phase II clinical trials for CLI have shown that cell-based therapies are safe and improve wound healing, but the trials were not large enough to detect any improvements in delaying amputation [62].

Ideally, functional readouts based on imaging such as PET or MRI should be obtained ahead of hard clinical endpoints to validate the biological effects of the intervention along the way. It would be especially important to measure functional improvements in the myocardial function and extend analysis to various sensitive imaging and metabolic measurements. In cancer trials for example, it is well accepted that drugs can be approved based on imaging-derived complete or partial responses and/or timelines to recurrence even though there are no effects on survival or mortality [108]. In addition, it is likely that only some patient populations will be responding positively to gene and cell therapies and therefore it would be important to identify biomarkers, which could differentiate responders from non-responder populations [109].

6.2 Patient populations

So far, while non-controlled, non-randomized gene and cell therapy trials in cardiovascular diseases have provided positive outcomes, most randomized, controlled, blinded studies have not achieved any clinically relevant effects in heart and limb muscles [110]. In multi-center studies, heterogeneity in patients and different cell preparations and products can influence the efficacy of cell therapy [111]. In addition, meta-regression showed that a refinement in endovascular and surgical techniques leading to improved limb salvage is expected to reduce the potential incremental benefit of cell therapy [111] . Therefore, future cardiovascular gene and cell therapy trials should focus more on randomized, blinded and controlled study designs where less severely affected patients are treated as compared to so called no-option patients which have been frequently targeted in previous non-randomized trials. It is likely that these no-option patients have already lost at least some of their regenerative capacity and therefore are not optimal for testing new biological therapeutic approaches.

6.3 Growth factors development

To achieve better outcomes, an optimal profile of growth factors should be identified for clinical testing since some of the previously tested factors, such as VEGF-A, are problematic in respect to their fast and very strong effects. They also induce harmful side effects like increased vascular permeability and thrombosis. Despite promising effects of anti VEGF therapy on tumor angiogenesis, serious adverse effects on cardiovascular events (angina pectoris, arterial thrombosis, cerebral or

myocardial ischaemia and infarction) have been shown for the VEGF-A inhibitor bevacizumab in a meta-review study [112].

Instead, growth factors with more appropriate signaling kinetics for improving cardiac condition should be taken into clinical testing. A possible example is VEGF-D, which is both angiogenic and lymphangiogenic and therefore can improve fluid drainage from myocardium after inducing angiogenic effects. Signaling kinetics for VEGF-D are also slower, less aggressive and longer lasting than VEGF-A. Therefore, it may be better suited for therapeutic applications than the previously tested growth factors. Recent phase I/IIa clinical trial results in refractory angina patients have indeed supported this approach. The trial results showed improved myocardial perfusion reserve in the treated ischaemic, hibernating myocardium one year after the treatment [113]. Also, the trial suggests that patients with high Lp(a) benefit most from the adenovirus VEGF-D therapy. Therefore, we can expect improved therapeutic applications in the future after learning important lessons from the previous trials.

Consensus statement:

The design of therapeutic strategies for angiogenesis is crucial to the success of the vascularization studies. In this section, the ESC Working Group for Atherosclerosis and Vascular Biology provides guidance for the development of treatments to target the vasculature in post-ischaemic disease and for their assessment in pre-clinical and clinical studies:

- Research during the last decade has identified an intricate genetic network of molecules that control the assembly of the first blood vessels from individual angioblasts prior to the onset of circulation. Moreover, large-scale changes in transcriptomes as well as changes in non-coding RNAs that regulate the angiogenic process have been investigated. The potential to harness these novel mechanisms to drive therapeutic angiogenesis should now be tested.
- Although murine models have underpinned a wealth of basic biology studies, they also have certain limitations (reviewed extensively above). Standardization of animal models for cardiovascular research and inclusion of comorbidities are necessary to reach the standard for clinical translation. It is our view that large animal models, including novel transgenic pig models, can be useful for long-term experimentation because their close similarity with human size, anatomy

and metabolism enhances their relevance for clinical translation. Main limitations for the use of large animal models could be the costs and ethics behind such studies.

- Efficacy and tissue specific delivery is a key point to consider in planning neovascularization studies. It appears that physical/mechanical interventions (i.e., by surgery or via a catheter) currently provides better control for the delivery procedure compared to systemic injection. In the setting of PAD or coronary artery disease (CAD), local cell or gene therapy to promote post-ischaemic angiogenesis could be combined with systemic pharmacological therapy to reduce risk factors for atherosclerosis. A new generation of inducible viral vectors should be developed to allow precise temporal control of inducible transgene expression, thus avoiding detrimental effects due to continuous overexpression. As an alternative, non-viral gene transfer, has become a possible option for transgene delivery.
- An analysis of the clinical trial of the last decades has shown that endpoints have been inconsistently used in clinical trials. We propose that functional and metabolic readouts should be further developed to capture therapeutic efficacy and biological activity of the treatments and support clinical hard endpoints. To this end, imaging readouts represent one avenue for the future that will require further standardization.
- Patient selection is critical, given the influence that comorbidities, aging and medications may have on the results of the trials. Since safety of gene and cell therapy has been very good in almost all reported trials, moving towards trials of less severe patients, such as CCS class 2-3 for refractory angina, in the future will be justified. Biomarkers and scores that would enable appropriate identification of specific target populations that benefit most from gene or cell therapy need to be proposed. Finally, further genetic characterization of non-responder patient groups in neovascularization clinical trials would help to identify factors affecting treatment responsiveness.

Figure Legend

Figure 1: Difference in heart vascularization and response to ischaemia between animals and humans

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References

1. Risau W, Flamme I. Vasculogenesis. Annu Rev Cell Dev Biol 1995;**11**:73-91.
2. Coffin JD, Poole TJ. Embryonic vascular development: immunohistochemical identification of the origin and subsequent morphogenesis of the major vessel primordia in quail embryos. Development 1988;**102**:735-748.
3. Poole TJ, Coffin JD. Vasculogenesis and angiogenesis: two distinct morphogenetic mechanisms establish embryonic vascular pattern. J Exp Zool 1989;**251**:224-231.
4. Drake CJ, Fleming PA. Vasculogenesis in the day 6.5 to 9.5 mouse embryo. Blood 2000;**95**:1671-1679.
5. Walls JR, Coultas L, Rossant J, Henkelman RM. Three-dimensional analysis of vascular development in the mouse embryo. PLoS One 2008;**3**:e2853.
6. Brown LA, Rodaway AR, Schilling TF, Jowett T, Ingham PW, Patient RK, Sharrocks AD. Insights into early vasculogenesis revealed by expression of the ETS-domain transcription factor Fli-1 in wild-type and mutant zebrafish embryos. Mech Dev 2000;**90**:237-252.
7. Jin SW, Beis D, Mitchell T, Chen JN, Stainier DY. Cellular and molecular analyses of vascular tube and lumen formation in zebrafish. Development 2005;**132**:5199-5209.
8. Ellertsdottir E, Lenard A, Blum Y, Krudewig A, Herwig L, Affolter M, Belting HG. Vascular morphogenesis in the zebrafish embryo. Developmental biology 2010;**341**:56-65.
9. Krebs LT, Shutter JR, Tanigaki K, Honjo T, Stark KL, Gridley T. Haploinsufficient lethality and formation of arteriovenous malformations in Notch pathway mutants. Genes Dev 2004;**18**:2469-2473.
10. Lawson ND, Scheer N, Pham VN, Kim CH, Chitnis AB, Campos-Ortega JA, Weinstein BM. Notch signaling is required for arterial-venous differentiation during embryonic vascular development. Development 2001;**128**:3675-3683.
11. Lawson ND, Vogel AM, Weinstein BM. sonic hedgehog and vascular endothelial growth factor act upstream of the Notch pathway during arterial endothelial differentiation. Dev Cell 2002;**3**:127-136.
12. Gritz E, Hirschi KK. Specification and function of hemogenic endothelium during embryogenesis. Cell Mol Life Sci 2016;**73**:1547-1567.
13. Romagnani P, Annunziato F, Liotta F, Lazzeri E, Mazzinghi B, Frosali F, Cosmi L, Maggi L, Lasagni L, Scheffold A, Kruger M, Dimmeler S, Marra F, Gensini G, Maggi E, Romagnani S. CD14+CD34low cells with stem cell phenotypic and functional features are the major source of circulating endothelial progenitors. Circ Res 2005;**97**:314-322.
14. Risau W. Mechanisms of angiogenesis. Nature 1997;**386**:671-674.
15. Rissanen TT, Korpisalo P, Markkanen JE, Liimatainen T, Orden MR, Kholova I, de Goede A, Heikura T, Grohn OH, Yla-Herttuala S. Blood flow remodels growing vasculature during vascular endothelial growth factor gene therapy and determines between capillary arterialization and sprouting angiogenesis. Circulation 2005;**112**:3937-3946.
16. Gerhardt H, Golding M, Fruttiger M, Ruhrberg C, Lundkvist A, Abramsson A, Jeltsch M, Mitchell C, Alitalo K, Shima D, Betsholtz C. VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. J Cell Biol 2003;**161**:1163-1177.

17. Hasan SS, Tsaryk R, Lange M, Wisniewski L, Moore JC, Lawson ND, Wojciechowska K, Schnittler H, Siekmann AF. Endothelial Notch signalling limits angiogenesis via control of artery formation. Nat Cell Biol 2017;**19**:928-940.
18. Pitulescu ME, Schmidt I, Giaimo BD, Antoine T, Berkenfeld F, Ferrante F, Park H, Ehling M, Biljes D, Rocha SF, Langen UH, Stehling M, Nagasawa T, Ferrara N, Borggrefe T, Adams RH. Dll4 and Notch signalling couples sprouting angiogenesis and artery formation. Nat Cell Biol 2017;**19**:915-927.
19. Chouinard-Pelletier G, Jahnsen ED, Jones EA. Increased shear stress inhibits angiogenesis in veins and not arteries during vascular development. Angiogenesis 2013;**16**:71-83.
20. Mahmoud MM, Kim HR, Xing R, Hsiao S, Mammoto A, Chen J, Serbanovic-Canic J, Feng S, Bowden NP, Maguire R, Ariaans M, Francis SE, Weinberg PD, van der Heiden K, Jones EA, Chico TJ, Ridger V, Evans PC. TWIST1 Integrates Endothelial Responses to Flow in Vascular Dysfunction and Atherosclerosis. Circ Res 2016;**119**:450-462.
21. Carmeliet P. Mechanisms of angiogenesis and arteriogenesis. Nature medicine 2000;**6**:389-395.
22. Silvestre JS, Smadja DM, Levy BI. Postischemic revascularization: from cellular and molecular mechanisms to clinical applications. Physiol Rev 2013;**93**:1743-1802.
23. Semenza GL. Hypoxia-inducible factors in physiology and medicine. Cell 2012;**148**:399-408.
24. Grivennikov SI, Greten FR, Karin M. Immunity, Inflammation, and Cancer. Cell 2010;**140**:883-899.
25. Costa C, Incio J, Soares R. Angiogenesis and chronic inflammation: cause or consequence? Angiogenesis 2007;**10**:149-166.
26. Hoefer IE, den Adel B, Daemen MJ. Biomechanical factors as triggers of vascular growth. Cardiovascular research 2013;**99**:276-283.
27. Ghaffari S, Leask RL, Jones EA. Flow dynamics control the location of sprouting and direct elongation during developmental angiogenesis. Development 2015;**142**:4151-4157.
28. Ghaffari S, Leask RL, Jones EAV. Blood flow can signal during angiogenesis not only through mechanotransduction, but also by affecting growth factor distribution. Angiogenesis 2017;**20**:373-384.
29. Song JW, Munn LL. Fluid forces control endothelial sprouting. Proc Natl Acad Sci U S A 2011;**108**:15342-15347.
30. Dragneva G, Korpisalo P, Yla-Herttuala S. Promoting blood vessel growth in ischemic diseases: challenges in translating preclinical potential into clinical success. Dis Model Mech 2013;**6**:312-322.
31. Buerk DG, Goldstick TK. Arterial wall oxygen consumption rate varies spatially. The American journal of physiology 1982;**243**:H948-958.
32. Gössl M, Versari D, Lerman LO, Chade AR, Beighley PE, Erbel R, Ritman EL. Low vasa vasorum densities correlate with inflammation and subintimal thickening: potential role in location--determination of atherogenesis. Atherosclerosis 2009;**206**:362-368.
33. Sluimer JC, Gasc J-M, van Wanroij JL, Kisters N, Groeneweg M, Sollewijn Gelpke MD, Cleutjens JP, van den Akker LH, Corvol P, Wouters BG, Daemen MJ, Bijnens A-PJ. Hypoxia, hypoxia-inducible transcription factor, and macrophages in human atherosclerotic plaques are correlated with

intraplaque angiogenesis. Journal of the American College of Cardiology 2008;**51**:1258-1265.
34. Cheng C, Chrifi I, Pasterkamp G, Duckers HJ. Biological mechanisms of microvessel formation in advanced atherosclerosis: the big five. Trends in cardiovascular medicine 2013;**23**:153-164.
35. Najafi AH, Aghili N, Tilan JU, Andrews JA, Peng X, Lassance-Soares RM, Sood S, Alderman LO, Abe K, Li L, Kolodgie FD, Virmani R, Zukowska Z, Epstein SE, Burnett MS. A new murine model of stress-induced complex atherosclerotic lesions. Disease models & mechanisms 2013;**6**:323-331.
36. Roth L, Rombouts M, Schrijvers DM, Lemmens K, De Keulenaer GW, Martinet W, De Meyer GRY. Chronic intermittent mental stress promotes atherosclerotic plaque vulnerability, myocardial infarction and sudden death in mice. Atherosclerosis 2015;**242**:288-294.
37. Haghighat A, Weiss D, Whalin MK, Cowan DP, Taylor WR. Granulocyte colony-stimulating factor and granulocyte macrophage colony-stimulating factor exacerbate atherosclerosis in apolipoprotein E-deficient mice. Circulation 2007;**115**:2049-2054.
38. Celletti FL, Waugh JM, Amabile PG, Brendolan A, Hilfiker PR, Dake MD. Vascular endothelial growth factor enhances atherosclerotic plaque progression. Nature medicine 2001;**7**:425-429.
39. Lucerna M, Zernecke A, de Nooijer R, de Jager SC, Bot I, van der Lans C, Kholova I, Liehn Ea, van Berkel TJC, Yla-Herttuala S, Weber C, Biessen EaL. Vascular endothelial growth factor-A induces plaque expansion in ApoE knock-out mice by promoting de novo leukocyte recruitment. Blood 2007;**109**:122-129.
40. Bhardwaj S, Roy H, Heikura T, Ylä-Herttuala S. VEGF-A, VEGF-D and VEGF-D(DeltaNDeltaC) induced intimal hyperplasia in carotid arteries. European journal of clinical investigation 2005;**35**:669-676.
41. Khurana R, Zhuang Z, Bhardwaj S, Murakami M, De Muinck E, Yla-Herttuala S, Ferrara N, Martin JF, Zachary I, Simons M. Angiogenesis-dependent and independent phases of intimal hyperplasia. Circulation 2004;**110**:2436-2443.
42. Giannarelli C, Alique M, Rodriguez DT, Yang DK, Jeong D, Calcagno C, Hutter R, Millon A, Kovacic JC, Weber T, Faries PL, Soff GA, Fayad ZA, Hajjar RJ, Fuster V, Badimon JJ. Alternatively spliced tissue factor promotes plaque angiogenesis through the activation of hypoxia-inducible factor-1α and vascular endothelial growth factor signaling. Circulation 2014;**130**:1274-1286.
43. Leppänen P, Koota S, Kholová I, Koponen J, Fieber C, Eriksson U, Alitalo K, Ylä-Herttuala S. Gene transfers of vascular endothelial growth factor-A, vascular endothelial growth factor-B, vascular endothelial growth factor-C, and vascular endothelial growth factor-D have no effects on atherosclerosis in hypercholesterolemic low-density lipoprotein. Circulation 2005;**112**:1347-1352.
44. Sluimer JC, Kolodgie FD, Bijnens AP, Maxfield K, Pacheco E, Kutys B, Duimel H, Frederik PM, van Hinsbergh VW, Virmani R, Daemen MJ. 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 2009;**53**:1517-1527.
45. Carmeliet P. Angiogenesis in health and disease. Nature medicine 2003;**9**:653-660.
46. Virmani R, Kolodgie FD, Burke AP, Finn AV, Gold HK, Tulenko TN, Wrenn SP, Narula J. Atherosclerotic plaque progression and vulnerability to rupture:

angiogenesis as a source of intraplaque hemorrhage. Arteriosclerosis, thrombosis, and vascular biology 2005;**25**:2054-2061.
47. Eriksson EE. Intravital microscopy on atherosclerosis in apolipoprotein e-deficient mice establishes microvessels as major entry pathways for leukocytes to advanced lesions. Circulation 2011;**124**:2129-2138.
48. Rademakers T, Douma K, Hackeng TM, Post MJ, Sluimer JC, Daemen MJAP, Biessen EAL, Heeneman S, van Zandvoort MAMJ. Plaque-associated vasa vasorum in aged apolipoprotein E-deficient mice exhibit proatherogenic functional features in vivo. Arteriosclerosis, thrombosis, and vascular biology 2013;**33**:249-256.
49. Michel J-B, Martin-Ventura JL, Nicoletti A, Ho-Tin-Noé B. Pathology of human plaque vulnerability: mechanisms and consequences of intraplaque haemorrhages. Atherosclerosis 2014;**234**:311-319.
50. Yla-Herttuala S, Rissanen TT, Vajanto I, Hartikainen J. Vascular endothelial growth factors: biology and current status of clinical applications in cardiovascular medicine. J Am Coll Cardiol 2007;**49**:1015-1026.
51. Deveza L, Choi J, Yang F. Therapeutic angiogenesis for treating cardiovascular diseases. Theranostics 2012;**2**:801-814.
52. Yu P, Wilhelm K, Dubrac A, Tung JK, Alves TC, Fang JS, Xie Y, Zhu J, Chen Z, De Smet F, Zhang J, Jin SW, Sun L, Sun H, Kibbey RG, Hirschi KK, Hay N, Carmeliet P, Chittenden TW, Eichmann A, Potente M, Simons M. FGF-dependent metabolic control of vascular development. Nature 2017;**545**:224-228.
53. Losordo DW, Dimmeler S. Therapeutic angiogenesis and vasculogenesis for ischemic disease. Part I: angiogenic cytokines. Circulation 2004;**109**:2487-2491.
54. Ho TK, Shiwen X, Abraham D, Tsui J, Baker D. Stromal-Cell-Derived Factor-1 (SDF-1)/CXCL12 as Potential Target of Therapeutic Angiogenesis in Critical Leg Ischaemia. Cardiol Res Pract 2012;**2012**:143209.
55. Kampschulte M, Gunkel I, Stieger P, Sedding DG, Brinkmann A, Ritman EL, Krombach GA, Langheinrich AC. Thalidomide influences atherogenesis in aortas of ApoE(-/-)/LDLR (-/-) double knockout mice: a nano-CT study. The international journal of cardiovascular imaging 2014;**30**:795-802.
56. Moulton KS, Heller E, Konerding Ma, Flynn E, Palinski W, Folkman J. Angiogenesis inhibitors endostatin or TNP-470 reduce intimal neovascularization and plaque growth in apolipoprotein E-deficient mice. Circulation 1999;**99**:1726-1732.
57. Moulton KS, Olsen BR, Sonn S, Fukai N, Zurakowski D, Zeng X. Loss of collagen XVIII enhances neovascularization and vascular permeability in atherosclerosis. Circulation 2004;**110**:1330-1336.
58. Ganaha F, Kao EY, Wong H, Elkins CJ, Lee J, Modanlou S, Rhee C, Kuo MD, Yuksel E, Cifra PN, Waugh JM, Dake MD. Stent-based controlled release of intravascular angiostatin to limit plaque progression and in-stent restenosis. Journal of vascular and interventional radiology : JVIR 2004;**15**:601-608.
59. Stefanadis C, Toutouzas K, Stefanadi E, Lazaris A, Patsouris E, Kipshidze N. Inhibition of plaque neovascularization and intimal hyperplasia by specific targeting vascular endothelial growth factor with bevacizumab-eluting stent: an experimental study. Atherosclerosis 2007;**195**:269-276.
60. Tanaka K, Nagata D, Hirata Y, Tabata Y, Nagai R, Sata M. Augmented angiogenesis in adventitia promotes growth of atherosclerotic plaque in apolipoprotein E-deficient mice. Atherosclerosis 2011;**215**:366-373.

61. Raval Z, Losordo DW. Cell therapy of peripheral arterial disease: from experimental findings to clinical trials. Circulation research 2013;**112**:1288-1302.
62. Samura M, Hosoyama T, Takeuchi Y, Ueno K, Morikage N, Hamano K. Therapeutic strategies for cell-based neovascularization in critical limb ischemia. J Transl Med 2017;**15**:49.
63. Fernandez-Aviles F, Sanz-Ruiz R, Climent AM, Badimon L, Bolli R, Charron D, Fuster V, Janssens S, Kastrup J, Kim HS, Luscher TF, Martin JF, Menasche P, Simari RD, Stone GW, Terzic A, Willerson JT, Wu JC, Group TW, Authors/Task Force Members C, Basic Research S, Translational Research S, Challenges of Cardiovascular Regenerative Medicine S, Tissue Engineering S, Delivery NT, Assessment S, Clinical Trials S, Regulatory, funding strategies s, Delivery NT, Assessment S. Global position paper on cardiovascular regenerative medicine. Eur Heart J 2017;**38**:2532-2546.
64. Poller W, Dimmeler S, Heymans S, Zeller T, Haas J, Karakas M, Leistner DM, Jakob P, Nakagawa S, Blankenberg S, Engelhardt S, Thum T, Weber C, Meder B, Hajjar R, Landmesser U. Non-coding RNAs in cardiovascular diseases: diagnostic and therapeutic perspectives. Eur Heart J 2017.
65. Schober A, Nazari-Jahantigh M, Wei Y, Bidzhekov K, Gremse F, Grommes J, Megens RT, Heyll K, Noels H, Hristov M, Wang S, Kiessling F, Olson EN, Weber C. MicroRNA-126-5p promotes endothelial proliferation and limits atherosclerosis by suppressing Dlk1. Nature medicine 2014;**20**:368-376.
66. Zernecke A, Bidzhekov K, Noels H, Shagdarsuren E, Gan L, Denecke B, Hristov M, Koppel T, Jahantigh MN, Lutgens E, Wang S, Olson EN, Schober A, Weber C. Delivery of microRNA-126 by apoptotic bodies induces CXCL12-dependent vascular protection. Science signaling 2009;**2**:ra81.
67. Landskroner-Eiger S, Qiu C, Perrotta P, Siragusa M, Lee MY, Ulrich V, Luciano AK, Zhuang ZW, Corti F, Simons M, Montgomery RL, Wu D, Yu J, Sessa WC. Endothelial miR-17 approximately 92 cluster negatively regulates arteriogenesis via miRNA-19 repression of WNT signaling. Proc Natl Acad Sci U S A 2015;**112**:12812-12817.
68. Caporali A, Meloni M, Vollenkle C, Bonci D, Sala-Newby GB, Addis R, Spinetti G, Losa S, Masson R, Baker AH, Agami R, le Sage C, Condorelli G, Madeddu P, Martelli F, Emanueli C. Deregulation of microRNA-503 contributes to diabetes mellitus-induced impairment of endothelial function and reparative angiogenesis after limb ischemia. Circulation 2011;**123**:282-291.
69. Mellis D, Caporali A. MicroRNA-based therapeutics in cardiovascular disease: screening and delivery to the target. Biochemical Society transactions 2017.
70. Marques SM, Campos PP, Castro PR, Cardoso CC, Ferreira MA, Andrade SP. Genetic background determines mouse strain differences in inflammatory angiogenesis. Microvasc Res 2011;**82**:246-252.
71. Niiyama H, Huang NF, Rollins MD, Cooke JP. Murine Model of Hindlimb Ischemia. Journal of Visualized Experiments : JoVE 2009:1035.
72. Vinegoni C, Aguirre AD, Lee S, Weissleder R. Imaging the beating heart in the mouse using intravital microscopy techniques. Nature protocols 2015;**10**:1802-1819.
73. Unger EF, Banai S, Shou M, Jaklitsch M, Hodge E, Correa R, Jaye M, Epstein SE. A model to assess interventions to improve collateral blood flow: continuous administration of agents into the left coronary artery in dogs. Cardiovasc Res 1993;**27**:785-791.

74. Unger EF, Banai S, Shou M, Lazarous DF, Jaklitsch MT, Scheinowitz M, Correa R, Klingbeil C, Epstein SE. Basic fibroblast growth factor enhances myocardial collateral flow in a canine model. Am J Physiol 1994;**266**:H1588-1595.
75. Morales C, Gonzalez GE, Rodriguez M, Bertolasi CA, Gelpi RJ. Histopathologic time course of myocardial infarct in rabbit hearts. Cardiovasc Pathol 2002;**11**:339-345.
76. Buschmann IR, Voskuil M, van Royen N, Hoefer IE, Scheffler K, Grundmann S, Hennig J, Schaper W, Bode C, Piek JJ. Invasive and non-invasive evaluation of spontaneous arteriogenesis in a novel porcine model for peripheral arterial obstructive disease. Atherosclerosis 2003;**167**:33-43.
77. Iwanaga K, Takano H, Ohtsuka M, Hasegawa H, Zou Y, Qin Y, Odaka K, Hiroshima K, Tadokoro H, Komuro I. Effects of G-CSF on cardiac remodeling after acute myocardial infarction in swine. Biochem Biophys Res Commun 2004;**325**:1353-1359.
78. Hinkel R, Lebherz C, Fydanaki M, Wuchrer A, El-Aouni C, Thormann M, Thein E, Kupatt C, Boekstegers P. Angiogenetic potential of Ad2/Hif-1alpha/VP16 after regional application in a preclinical pig model of chronic ischemia. Current vascular pharmacology 2013;**11**:29-37.
79. Rissanen TT, Nurro J, Halonen PJ, Tarkia M, Saraste A, Rannankari M, Honkonen K, Pietila M, Leppanen O, Kuivanen A, Knuuti J, Yla-Herttuala S. The bottleneck stent model for chronic myocardial ischemia and heart failure in pigs. American journal of physiology Heart and circulatory physiology 2013;**305**:H1297-1308.
80. Camare C, Pucelle M, Negre-Salvayre A, Salvayre R. Angiogenesis in the atherosclerotic plaque. Redox Biol 2017;**12**:18-34.
81. Van der Donckt C, Van Herck JL, Schrijvers DM, Vanhoutte G, Verhoye M, Blockx I, Van Der Linden A, Bauters D, Lijnen HR, Sluimer JC, Roth L, Van Hove CE, Fransen P, Knaapen MW, Hervent A-S, De Keulenaer GW, Bult H, Martinet W, Herman AG, De Meyer GRY. Elastin fragmentation in atherosclerotic mice leads to intraplaque neovascularization, plaque rupture, myocardial infarction, stroke, and sudden death. European heart journal 2015;**36**:1049-1058.
82. Riou LM, Broisat A, Ghezzi C, Finet G, Rioufol G, Gharib AM, Pettigrew RI, Ohayon J. Effects of mechanical properties and atherosclerotic artery size on biomechanical plaque disruption - mouse vs. human. Journal of biomechanics 2014;**47**:765-772.
83. van der Heiden K, Hoogendoorn A, Daemen MJ, Gijsen FJH. Animal models for plaque rupture: a biomechanical assessment. Thrombosis and haemostasis 2015;**115**.
84. Rivron NC, Vrij EJ, Rouwkema J, Le Gac S, van den Berg A, Truckenmüller RK, van Blitterswijk Ca. Tissue deformation spatially modulates VEGF signaling and angiogenesis. Proceedings of the National Academy of Sciences of the United States of America 2012;**109**:6886-6891.
85. Pelisek J, Well G, Reeps C, Rudelius M, Kuehnl A, Culmes M, Poppert H, Zimmermann A, Berger H, Eckstein H-H. Neovascularization and angiogenic factors in advanced human carotid artery stenosis. Circulation journal : official journal of the Japanese Circulation Society 2012;**76**:1274-1282.
86. Kumamoto M, Nakashima Y, Sueishi K. Intimal neovascularization in human coronary atherosclerosis: its origin and pathophysiological significance. Human pathology 1995;**26**:450-456.

87. Stenmark KR, Yeager ME, El Kasmi KC, Nozik-Grayck E, Gerasimovskaya EV, Li M, Riddle SR, Frid MG. The adventitia: essential regulator of vascular wall structure and function. Annual review of physiology 2013;**75**:23-47.
88. Petrovan RJ, Kaplan CD, Reisfeld RA, Curtiss LK. DNA vaccination against VEGF receptor 2 reduces atherosclerosis in LDL receptor-deficient mice. Arteriosclerosis, thrombosis, and vascular biology 2007;**27**:1095-1100.
89. Bhardwaj S, Roy H, Babu M, Shibuya M, Yla-Herttuala S. Adventitial gene transfer of VEGFR-2 specific VEGF-E chimera induces MCP-1 expression in vascular smooth muscle cells and enhances neointimal formation. Atherosclerosis 2011;**219**:84-91.
90. Bhardwaj S, Roy H, Kärpänen T, He Y, Hi Y, Jauhiainen S, Hedman M, Alitalo K, Ylä-Herttuala S. Periadventitial angiopoietin-1 gene transfer induces angiogenesis in rabbit carotid arteries. Gene therapy 2005;**12**:388-394.
91. Roy H, Bhardwaj S, Babu M, Kokina I, Uotila S, Ahtialansaari T, Laitinen T, Hakumaki J, Laakso M, Herzig KH, Yla-Herttuala S. VEGF-A, VEGF-D, VEGF receptor-1, VEGF receptor-2, NF-kappaB, and RAGE in atherosclerotic lesions of diabetic Watanabe heritable hyperlipidemic rabbits. FASEB J 2006;**20**:2159-2161.
92. Al-Mashhadi RH, Sørensen CB, Kragh PM, Christoffersen C, Mortensen MB, Tolbod LP, Thim T, Du Y, Li J, Liu Y, Moldt B, Schmidt M, Vajta G, Larsen T, Purup S, Bolund L, Nielsen LB, Callesen H, Falk E, Mikkelsen JG, Bentzon JF. Familial hypercholesterolemia and atherosclerosis in cloned minipigs created by DNA transposition of a human PCSK9 gain-of-function mutant. Science translational medicine 2013;**5**:166ra161.
93. Yla-Herttuala S, Bridges C, Katz MG, Korpisalo P. Angiogenic gene therapy in cardiovascular diseases: dream or vision? Eur Heart J 2017.
94. Yla-Herttuala S, Baker AH. Cardiovascular Gene Therapy: Past, Present, and Future. Mol Ther 2017;**25**:1095-1106.
95. Pajusola K, Kunnapuu J, Vuorikoski S, Soronen J, Andre H, Pereira T, Korpisalo P, Yla-Herttuala S, Poellinger L, Alitalo K. Stabilized HIF-1alpha is superior to VEGF for angiogenesis in skeletal muscle via adeno-associated virus gene transfer. Faseb J 2005;**19**:1365-1367.
96. Fisher SA, Doree C, Taggart DP, Mathur A, Martin-Rendon E. Cell therapy for heart disease: Trial sequential analyses of two Cochrane reviews. Clin Pharmacol Ther 2016;**100**:88-101.
97. Banovic M, Loncar Z, Behfar A, Vanderheyden M, Beleslin B, Zeiher A, Metra M, Terzic A, Bartunek J. Endpoints in stem cell trials in ischemic heart failure. Stem Cell Res Ther 2015;**6**:159.
98. Hassinen I, Kivela A, Hedman A, Saraste A, Knuuti J, Hartikainen JE, Yla-Herttuala S. Intramyocardial gene therapy directed to hibernating heart muscle using a combination of electromechanical mapping and positron emission tomography. Human gene therapy 2016.
99. Merentie M, Lottonen-Raikaslehto L, Parviainen V, Huusko J, Pikkarainen S, Mendel M, Laham-Karam N, Karja V, Rissanen R, Hedman M, Yla-Herttuala S. Efficacy and safety of myocardial gene transfer of adenovirus, adeno-associated virus and lentivirus vectors in the mouse heart. Gene Ther 2016;**23**:296-305.
100. Lahteenvuo JE, Lahteenvuo MT, Kivela A, Rosenlew C, Falkevall A, Klar J, Heikura T, Rissanen TT, Vahakangas E, Korpisalo P, Enholm B, Carmeliet P, Alitalo K, Eriksson U, Yla-Herttuala S. Vascular endothelial growth factor-B induces myocardium-specific angiogenesis and arteriogenesis via vascular

endothelial growth factor receptor-1- and neuropilin receptor-1-dependent mechanisms. Circulation 2009;**119**:845-856.
101. Hulot JS, Ishikawa K, Hajjar RJ. Gene therapy for the treatment of heart failure: promise postponed. Eur Heart J 2016;**37**:1651-1658.
102. Park HJ, Yang F, Cho SW. Nonviral delivery of genetic medicine for therapeutic angiogenesis. Adv Drug Deliv Rev 2012;**64**:40-52.
103. Pack DW, Hoffman AS, Pun S, Stayton PS. Design and development of polymers for gene delivery. Nat Rev Drug Discov 2005;**4**:581-593.
104. Jeon O, Yang HS, Lee TJ, Kim BS. Heparin-conjugated polyethylenimine for gene delivery. J Control Release 2008;**132**:236-242.
105. Kang SW, Lim HW, Seo SW, Jeon O, Lee M, Kim BS. Nanosphere-mediated delivery of vascular endothelial growth factor gene for therapeutic angiogenesis in mouse ischemic limbs. Biomaterials 2008;**29**:1109-1117.
106. Kumagai M, Marui A, Tabata Y, Takeda T, Yamamoto M, Yonezawa A, Tanaka S, Yanagi S, Ito-Ihara T, Ikeda T, Murayama T, Teramukai S, Katsura T, Matsubara K, Kawakami K, Yokode M, Shimizu A, Sakata R. Safety and efficacy of sustained release of basic fibroblast growth factor using gelatin hydrogel in patients with critical limb ischemia. Heart Vessels 2016;**31**:713-721.
107. Cambria E, Pasqualini FS, Wolint P, Gunter J, Steiger J, Bopp A, Hoerstrup SP, Emmert MY. Translational cardiac stem cell therapy: advancing from first-generation to next-generation cell types. NPJ Regen Med 2017;**2**:17.
108. O'Connor JP, Aboagye EO, Adams JE, Aerts HJ, Barrington SF, Beer AJ, Boellaard R, Bohndiek SE, Brady M, Brown G, Buckley DL, Chenevert TL, Clarke LP, Collette S, Cook GJ, deSouza NM, Dickson JC, Dive C, Evelhoch JL, Faivre-Finn C, Gallagher FA, Gilbert FJ, Gillies RJ, Goh V, Griffiths JR, Groves AM, Halligan S, Harris AL, Hawkes DJ, Hoekstra OS, Huang EP, Hutton BF, Jackson EF, Jayson GC, Jones A, Koh DM, Lacombe D, Lambin P, Lassau N, Leach MO, Lee TY, Leen EL, Lewis JS, Liu Y, Lythgoe MF, Manoharan P, Maxwell RJ, Miles KA, Morgan B, Morris S, Ng T, Padhani AR, Parker GJ, Partridge M, Pathak AP, Peet AC, Punwani S, Reynolds AR, Robinson SP, Shankar LK, Sharma RA, Soloviev D, Stroobants S, Sullivan DC, Taylor SA, Tofts PS, Tozer GM, van Herk M, Walker-Samuel S, Wason J, Williams KJ, Workman P, Yankeelov TE, Brindle KM, McShane LM, Jackson A, Waterton JC. Imaging biomarker roadmap for cancer studies. Nat Rev Clin Oncol 2017;**14**:169-186.
109. Jokerst JV, Cauwenberghs N, Kuznetsova T, Haddad F, Sweeney T, Hou J, Rosenberg-Hasson Y, Zhao E, Schutt R, Bolli R, Traverse JH, Pepine CJ, Henry TD, Schulman IH, Moye L, Taylor DA, Yang PC. Circulating Biomarkers to Identify Responders in Cardiac Cell therapy. Sci Rep 2017;**7**:4419.
110. Yla-Herttuala S, Bridges C, Katz MG, Korpisalo P. Angiogenic gene therapy in cardiovascular diseases: dream or vision? Eur Heart J 2017;**38**:1365-1371.
111. Rigato M, Monami M, Fadini GP. Autologous Cell Therapy for Peripheral Arterial Disease: Systematic Review and Meta-Analysis of Randomized, Nonrandomized, and Noncontrolled Studies. Circ Res 2017;**120**:1326-1340.
112. Scappaticci FA, Skillings JR, Holden SN, Gerber HP, Miller K, Kabbinavar F, Bergsland E, Ngai J, Holmgren E, Wang J, Hurwitz H. Arterial thromboembolic events in patients with metastatic carcinoma treated with chemotherapy and bevacizumab. Journal of the National Cancer Institute 2007;**99**:1232-1239.

113. Hartikainen J, Hassinen I, Hedman A, Kivelä A, Saraste A, Knuuti J, Husso M, Mussalo H, Hedman M, Rissanen TT, Toivanen P, Heikura T, Witztum JL, Tsimikas S, Ylä-Herttuala S. Adenoviral intramyocardial VEGF-DΔNΔC gene transfer increases myocardial perfusion reserve in refractory angina patients: a phase I/IIa study with 1-year follow-up. . European Heart Journal 2017;**38**: 2547-2555.
114. Rissanen TT, Markkanen JE, Arve K, Rutanen J, Kettunen MI, Vajanto I, Jauhiainen S, Cashion L, Gruchala M, Narvanen O, Taipale P, Kauppinen RA, Rubanyi GM, Yla-Herttuala S. Fibroblast growth factor 4 induces vascular permeability, angiogenesis and arteriogenesis in a rabbit hindlimb ischemia model. FASEB J 2003;**17**:100-102.
115. Hauer AD, van Puijvelde GHM, Peterse N, de Vos P, van Weel V, van Wanrooij EJA, Biessen EaL, Quax PHa, Niethammer AG, Reisfeld Ra, van Berkel TJC, Kuiper J. Vaccination against VEGFR2 attenuates initiation and progression of atherosclerosis. Arteriosclerosis, thrombosis, and vascular biology 2007;**27**:2050-2057.
116. Hauer AD, Habets KLL, van Wanrooij EJA, de Vos P, Krueger J, Reisfeld RA, van Berkel TJC, Kuiper J. Vaccination against TIE2 reduces atherosclerosis. Atherosclerosis 2009;**204**:365-371.
117. Chen Y, Song YX, Wang ZN. The microRNA-148/152 family: multi-faceted players. Molecular cancer 2013;**12**:43.
118. ten Dijke P, van Dam H. 14-3-3zeta turns TGF-beta to the dark side. Cancer cell 2015;**27**:151-153.

Table 1: Large animal models of post-ischaemic angiogenesis

Animal Species	Model	Outcome	Ref.
Rabbit	Left anterior descending coronary artery ligation	Myocardial Infarction	75
	Femoral artery ligation	Hind-limb ischaemia	15
	Femoral artery excision	Hind-limb ischaemia	114
Pigs	Coronary stenosis	Myocardial Infarction	78, 79
	Left anterior descending coronary artery ligation	Myocardial Infarction	77
	Femoral artery ligation	Hind-limb ischaemia	76
Dog	Ameroid constrictors and coronary artery ligation	Myocardial Infarction	73
	Ameroid constrictors	Myocardial Infarction	74

Table 2: Model of angiogenesis in mouse atherosclerotic plaque

	Animal Species	Anti/Pro Angiogenic	Mechanism	Duration	Read out	Effect on plaque	Intra-plaque	Incidence	Adventitial	Incidence	Ref.
Short time diet	ApoE-/-	Pro	Time + hr VEGF-A	7, 6, 5 wks HCD	Histo	↑	↑	ND	ND	-	[38]
	LDLR-/-	Anti	Time + VEGF R2 vaccination	20 wks HCD	Histo	↓	ND	-	↓	ND	[88]
	LDLR-/- ApoB38-/-	Pro	Time + VEGF-A, VEGF-B, VEGF-C, VEGF-D genetransfer	12 wks HCD	Histo	=	-	-	=	5 vessels/section	[43]
	ApoE-/- Coll XVIII-/-	Pro	Time + CollXVIII KO	24 wks HCD	Histo	↑	↑	Incidence, baseline 13% and KO 53%	↑	Baseline 17% vessels/area and KO 32% vessels/area	[57]
	ApoE-/- Fbn1 C1039G+/-	Pro	Time + Fbn1 C1039G+/- KO	20 wks HCD	Histo	↑	↑	Incidence, baseline 4% and KO 90%, baseline MVD 0 and KO MVD 6 vessels/10^7 µm2	Present	ND	[81]
Aged mice and/or prolonged diet time	ApoE-/-	-	Time	40-50 wks chow	Two photon micro.	-	↑	Incidence 30%	↑	Incidence 88.2%	[48]
	ApoE-/-	Pro	Time + rbFGF	(I) 67-94 wks chow (II) 12 wks HCD	Histo	↑	ND	-	↑	ND	[60]
	ApoE-/-	-	Time	40-96 wks HCD	Intravital micro.	-	↑	5% 2 of 39 studied advanced plaques (but total mice imaged 168 mice	↑	Incidence 77%	[47]
	ApoE-/- LDLr-/-	Anti	Time + Thalidomide	39 wks chow	µCT	↓	ND	-	↓	Baseline 8 vessels/section, treatment 5.5 vessels/section	[55]
	ApoE-/- SV129-/-	Pro	Time + stress + SV129 KO	20 wks HCD	Histo	↑	↑	ND	ND	-	[35]
Surgical Manipulation	ApoE-/-	-	Collar Placement + MMP9 gene therapy	Not clear	Histo	=	=	ND	ND	-	[39]
	LDLr	Anti	Collar placement + VEGFR2 vaccination	Not clear	Histo	↓	-	-	Present	ND	[115]
	ApoE-/-	Pro	Collar placement + VEGF-A genetransfer	Not clear	Histo	↑	=	ND =	ND	-	[39]
	LDLr	Anti	Collar placement + Tie2 vaccination	8 wks HCD	Histo	↓	-	-	↓	Baseline 22 vessels/section, treatement 7.5 vessels/section	[116]
	ApoE-/-	-	Tandem Stenosis	17, 13, 10, 8 wks HCD	Histo	↑	Present	0.03 microvessels/mm2	Present	ND	[117]

	ApoE-/-	Pro	Wireinjury + asTF genetransfer	6 wks HCD	Histo	↑	↑	Baseline 0.5 vessels/mm2, treatment 1.75 vessels/mm2	ND	-	[42]

Table 3: Large animal models of plaque angiogenesis

Animal Species	Anti/Pro Angiogenic	Mechanism	Duration	Read out	Effect on plaque	Intra-plaque	Adventitial	Ref.
Rabbits	Pro	Time +hrVEGF-A	6 wks HCD	Histo	↑	Increase but only total CD31 measured not density	ND	[38]
	Pro	Perivascular Collar + VEGF-A, VEGF-CNC, VEGF-D and VEGF-DNC gene transfer	3 wks HCD	Histo	↑	ND	↑	[40]
	Pro	Perivascular Collar + VEGF-E, VEGF-E+(s)VEGF-R-2 gene transfer	10 days chow	Histo	↑	ND	↑	[89]
	Pro	Collar placement (rabbit)+ balloon angioplasty (rat) with VEGF and PR39 gene transfer	9 days (rabbit) and 14 days (rat) chow	Histo	↑	ND	↑	[41]
	Pro	Watanabe + Alloxan injection to induce diabetes		Histo NMR	↑	Total CD31 not density	ND	[91]
Pigs	Anti	Time + Thalidomide 4mg/kg	12 wks HCD	µCT	↓	ND	↓	[32]
	Anti	Balloon Angioplasty + Endostar	12 wks HCD	Histo	↓	ND	↓	[118]
	-	Time + PCSK9 KI	46 wks HCD	Histo	-	Present	Present	[92]