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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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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 towards 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.

Keywords

Post-ischaemic angiogenesis • Pre-clinical studies • Gene and cell delivery • Clinical trials
1. Basic principles: vascularization, angiogenesis, and arteriogenesis

Vasculogenesis describes the coalescence of mesoderm-derived angioblasts into the first primitive blood vessels. The process was first observed in quail embryos and subsequently, shown to be conserved in other vertebrates including mouse and zebrafish. These studies revealed many similarities not only between the morphogenetic processes of early blood vessel formation, but also between the molecules co-ordinating these processes. Several signalling pathways such as Notch and Sonic Hedgehog, were shown to influence the early differentiation of arterial and venous endothelial cells (ECs) from angioblasts. Vasculogenesis was initially thought to be limited to the embryo, but current understanding is more nuanced. Early embryonic angioblasts and haemoblasts share a very similar gene signature and hematopoietic differentiation of arterial and venous endothelial cells (ECs) from angioblasts. The establishment of flow in newly formed vessels leads to mechanical signals (shear stress) that feedback to reduce and consolidate the vessel walls. This specification of tip and stalk cells is governed by the Notch signalling pathway. Notably, HSC can be differentiated into ECs, and these progenitors have shown therapeutic potential in several clinical and pre-clinical settings.

Angiogenesis is the creation of new vessels from pre-existing ones. Hypoxia is one of the key drivers of the process. It activates ECs to become more motile and produce filopodia. Further angiogenic factors such as vascular endothelial growth factor (VEGF) strongly dictate 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. To prevent ECs moving en masse, a particular type of ECs, known as tip cells, are selected to lead the advance. 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. 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.

Once stenosis in a large main artery becomes haemodynamically significant, the elevation of shear stress against the wall of these arteries 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.

2. Neo-vascularization: physiology and pathophysiology

2.1 Post-ischaemic vascularization

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 haemodynamic forces within the vascular wall trigger vasculogenesis, angiogenesis, and arteriogenesis, which act in concert to establish a functional vascular network in ischaemic zones.

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.

During ischaemia, inflammatory cells release angiogenic factors (e.g. VEGF) and cytokines (e.g. TNFα), which decrease EC junctions and enhance vascular permeability to promote the recruitment of inflammatory cells. Consistent with this relationship between angiogenesis and inflammation, several molecules that regulate inflammation have been implicated in new vessel formation. Changes in haemodynamic forces (mechanical forces linked to pressure and flow rate) occurring in collateral vessels in response to arterial occlusion also contribute to post-ischaemic vascularization. Recent studies suggest that flow dynamics control the activation of HIF1α and the localization of sprouting in vessels. The location is not determined by highest VEGF concentration, but by a combination of VEGF and biomechanical signals. Thus, shear-induced mechanism appears to override pro-angiogenic signals such as VEGF. 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.

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. Thus, in the last decades, stimulation of vessel growth has emerged as a novel therapeutic option in patients with ischaemic diseases.

2.2 Vascularization of atherosclerotic plaques

Under physiological circumstances, microvessels originate from the adventitia and provide the media of large arteries with oxygen and nutrients. 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. In experimental models, plaque angiogenesis has been induced by stress, treatment with pro-inflammatory mediators, pro-angiogenic growth factors, and viral gene delivery of pro-angiogenic factors, 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. These ultrastructural characteristics suggest vascular leakage, which might be responsible for increased extravasation of immune cells and deposition of lipids and red blood cells in the plaques. Therefore, these microvessels are thought to represent one of the main sources of intra-plaque haemorrhage, in addition to healed thrombi.

3. Therapeutic vascularization

3.1 Growth factors, cells, and non-coding RNA therapies

Multiple different approaches have been used to promote vascularization of ischaemic tissues.
3.1.1 Growth factors
Growth factors have been applied for therapeutic angiogenesis including VEGF, basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), Angiopoietin 1 (ANG-1), and insulin-like growth factor (IGF-1) (Table 1). Pre-clinical studies in animal models using individual angiogenic factors have shown significant improvements in clinically relevant endpoints such as increased regional perfusion, improved exercise tolerance and tissue energy metabolism, improved myocardial function, and protection against ischaemic damage. Among these, VEGF, bFGF, and HGF are the best studied and have reached human clinical trials (Table 1). However, apart from demonstration of increased vascularity, very few results with clinical significance have been obtained.

VEGF is a critically important regulator of physiological angiogenesis during growth, healing and in response to hypoxia. VEGF is up-regulated by HIF1α more than any other inducible angiogenic factor during ischaemia. However, when administered alone, VEGF can increase endothelial permeability, which leads to the formation of leaky-capillaries and tissue oedema. Platelet Derived Growth Factors (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. Basic fibroblast growth factor is among the first discovered angiogenic factors to have both angiogenic and arteriogenic properties, which may facilitate formation of a mature blood vessel network. The HGF family induces potent angiogenic responses by binding to the c-MET receptor, which is expressed on ECs, vascular smooth muscle cells, and HSC. HGF is known to have mitogenic, angiogenic, anti-apoptotic, and anti-fibrotic activities in various cells. 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.

3.1.2 Cell therapy
Cell-based therapy has been demonstrated to have the capability of tissue repair in many animal studies and in ongoing clinical trials (Table 2). Cell transplantation in ischaemic tissue may attenuate severity of tissue damage and accelerate the regeneration process. Genetic modification, pre-conditioning, and tissue engineering have been applied to improve the efficacy of stem 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 stem, progenitor, and stromal cells (Table 2).

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 periphery artery disease (PAD) severity. However, most of clinical trials have primarily focused on CLI patients in small Phase I or II studies. A variety of cell types have been studied as potential PAD treatments including unselected bone marrow mononuclear cells (BM-MNC) or peripheral blood mononuclear cells (PB-MNC), marker-specific cells selected from the marrow or blood, mesenchymal stem cells (MSCs), and adipose tissue-derived regenerative cells. In clinical studies of neovascularization considerable progress in the use of adult stem cells for cell transplantation has been made using HSC, bone marrow-derived dendritic cells, MSC, and endothelial progenitor cells. 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.

3.1.3 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 including atherosclerosis. Emerging data show that several miRNAs are linked to both adaptive and maladaptive vascular remodeling processes. Mir-126, one of the most abundantly expressed microRNAs in ECs, has a pro-angiogenic as well as anti-atherosclerotic role and the systemic delivery of miR-126 mimics rescued EC proliferation at vulnerable sites and inhibited atherosclerotic lesion progression. 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. Notably, miR-503 expression is increased in ischaemic limb muscles and ECs of diabetic mice. Inhibition of miR-503 by adenoval delivery to the ischaemic adductor muscles of diabetic mice corrected diabetes-induced impairment of post-ischaemia angiogenesis and blood flow recovery. Even though the

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**Table 1 Gen therapy in post-ischaemic vascualrization**

<table>
<thead>
<tr>
<th>Disease/patient number</th>
<th>Growth factor/vector/delivery</th>
<th>Outcomes</th>
<th>Primary outcomes</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAD/100</td>
<td>FGF-2/SeV/i.m.</td>
<td>Walking performance</td>
<td>NCT02276937</td>
<td></td>
</tr>
<tr>
<td>PAD/500</td>
<td>HGF/pl/i.m.</td>
<td>Time to major amputation</td>
<td>NCT02144610</td>
<td></td>
</tr>
<tr>
<td>MI/41</td>
<td>VEGF-A116A/Ad/li.my</td>
<td>Time to 1 mm ST depression during exercise stress testing</td>
<td>NCT01757223</td>
<td></td>
</tr>
</tbody>
</table>

Growth factors Models Outcomes

<table>
<thead>
<tr>
<th>VEGF</th>
<th>Pig MI</th>
<th>Increase neoangiogenesis, improved regional myocardial function, and myocardial perfusion</th>
<th>51</th>
</tr>
</thead>
<tbody>
<tr>
<td>bFGF</td>
<td>Pig MI</td>
<td>Enhanced arteriogenesis within the ischaemic zone</td>
<td>52</td>
</tr>
<tr>
<td>HGF</td>
<td>Rabbit HLI</td>
<td>Increase of blood flow and arteriogenesis</td>
<td>53</td>
</tr>
<tr>
<td>Ang-1</td>
<td>Mouse MI</td>
<td>Increase in capillary density, reduction in infarct sizes, and increase heart performance</td>
<td>54</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Mouse MI</td>
<td>Increase in capillary density and increase heart performance</td>
<td>55</td>
</tr>
</tbody>
</table>

Ad, adenovirus; HLI, hind-limb ischaemia; i.m., intramuscular; i.my., intramyocardial; MI, myocardial infarction; PAD, peripheral artery disease; Pl, plasmid; SeV, sendaivirus.
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.73

### 3.2 Gene and cell delivery

Delivery of therapies 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 been too low.56,74 Several factors contribute to this problem: the human heart is a very large muscle when compared with mice and rats and vectors or cell solutions cannot easily penetrate deep into the myocardium. The adenovirus associated virus for instance, bind tightly to heparan sulphate proteoglycans and they do not easily escape from the intraluminal space into the myocardium.73 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 had poor success for gene and cell delivery.

#### 3.2.1 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-A165, delivered by transgenes, bind strongly to heparan sulphate 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.76,77 For applications such as myocardial ischaemia, local targeted injections based on electromechanical mapping,78 or blood flow measurements using positron emission tomography79 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 mice79 but in larger animals and humans widespread gene expression after any delivery method still remains a very challenging task.80

#### 3.2.2 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.81 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.82 Gene delivery using lipid formulations has been applied in ischaemic tissues for therapeutic angiogenesis. Jeon et al.83 reported that VEGF-A gene delivery using heparin-conjugated Polyethyleneimine significantly up-regulated VEGF-A expression, resulting in extensive neovascularization in mouse ischaemic limbs. 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.84 A novel concept of involving a biodegradable gelatin hydrogel carrying a sustained-release system of bFGF was studied in patients with CLI.85

### 3.3 Animal models

Models to investigate post-ischaemic angiogenesis have been established in rodents and larger animals such as rabbits, pigs, or dogs (Table 3). They exhibit considerable variation because each species differs in the extent of naïve vascularization and thus reacts differently to vascular growth stimuli (Figure 1). To make things more complicated, within one

<table>
<thead>
<tr>
<th>Disease/patient number</th>
<th>Cell line/delivery</th>
<th>Primary outcomes</th>
<th>Trial</th>
</tr>
</thead>
<tbody>
<tr>
<td>MI/142 MI/55</td>
<td>Cardiac stem cells/i.c.</td>
<td>Infarct size by MRI Safety as measured by death and MACE in 12 months</td>
<td>ALLSTAR trial (NCT01458405). CAREMI trial (NCT02439398)</td>
</tr>
<tr>
<td>MI/3000</td>
<td>Autologous BM-derived mono-nuclear cells/i.c.</td>
<td>Time from randomization to all-cause death</td>
<td>BAM trial (NCT01569178)</td>
</tr>
<tr>
<td>Ischaemic heart failure/315</td>
<td>BM-derived mesenchymal stem cells/i.c.</td>
<td>Efficacy between groups post-index procedures</td>
<td>CHART-1 (NCT01768702)CHART-2 (NCT02317458)</td>
</tr>
</tbody>
</table>

i.c, intracoronary; MI, myocardial infarction.
animal species, different strains show distinct naïve vascularization and even show opposite reactions.94

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.95 Compared with 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.96

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 with 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.

For a long time, the dog,92,93 together with the rabbit,16,86 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.72

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.91 In contrast, the pig was long considered to have insufficient capabilities to compensate for coronary ischaemia by neovascularization.90 In the past decade, however, several groups succeeded in establishing also pig coronary neovascularization models by inducing progressive coronary stenosis rather than acute occlusions.88,89

### 3.4.1 Endpoints

Ongoing clinical gene and cell therapy trials have been reviewed elsewhere.74,97 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 when compared with large pharmaceutical Phase II/III trials, they do not have sufficient statistical power to capture endpoints such as overall mortality or MACE. For example, 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.67

Ideally, functional readouts based on imaging such as positron emission tomography or magnetic resonance imaging should be obtained in parallel with 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.98 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.99

### 3.4.2 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.100 In multi-centre studies, heterogeneity in patients and different cell preparations and products can influence the efficacy of cell therapy.101 In addition, meta-regression showed that refinements in endovascular and surgical techniques leading

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**Table 3 Large animal models of post-ischaemic vascularization**

<table>
<thead>
<tr>
<th>Models</th>
<th>Readout</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Left anterior descending</td>
<td>Myocardial infarct size</td>
<td>86</td>
</tr>
<tr>
<td>coronary artery ligation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Femoral artery ligation</td>
<td>Hind-limb perfusion</td>
<td>16</td>
</tr>
<tr>
<td>Femoral artery excision</td>
<td>Hind-limb perfusion</td>
<td>87</td>
</tr>
<tr>
<td>Coronary stenosis</td>
<td>Myocardial infarct size</td>
<td>88,89</td>
</tr>
<tr>
<td>Left anterior descending</td>
<td>Myocardial infarct size</td>
<td>90</td>
</tr>
<tr>
<td>coronary artery ligation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Femoral artery ligation</td>
<td>Hind-limb perfusion</td>
<td>91</td>
</tr>
<tr>
<td>Ameroid constrictors and</td>
<td>Myocardial infarct size</td>
<td>92</td>
</tr>
<tr>
<td>coronary artery ligation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ameroid constrictors</td>
<td>Myocardial infarct size</td>
<td>93</td>
</tr>
</tbody>
</table>

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to improved limb salvage reduces the potential impact of cell therapy. 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 when compared with 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.

3.4.3 Growth factor 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 because they increase vascular permeability and thrombosis. Instead, growth factors with more appropriate signalling kinetics for improving cardiac function 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. Signalling kinetics for VEGF-D are also longer lasting than VEGF-A. Therefore, it may be better suited for therapeutic applications than the previously tested growth factors. Recent Phase III/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 1 year after the treatment. Also, the trial suggests that patients with high Lp(a) benefit most from the adeno virus VEGF-D therapy. Therefore, we can expect improved therapeutic applications in the future after learning important lessons from the previous trials.

### 4. Vascularization of atherosclerotic plaques

The therapeutic benefits of enhanced vascularization of ischaemic tissues in ischaemic tissues contrasts with the effects of vascularization in atherosclerotic lesions, which can enhance plaque burden and also promote plaque rupture, potentially leading to myocardial infarction or stroke.

#### 4.1 Therapies

Investigations using animal models have shown that inhibiting vascular growth factors can preserve vascular integrity and reduce plaque angiogenesis. Notably, most of the intervention strategies to manipulate angiogenesis in atherosclerosis have been restricted to mouse models using molecules such as thalidomide, TNP-470, angiostatin, monoclonal antibody anti-VEGF-A, and VEGFR2 or Tie2 inhibitors (effects summarized in Table 4). However, since VEGFs are involved in important physiological processes, it is not surprising that multiple trials with VEGF inhibiting compounds show also cardiovascular harmful effects.

#### 4.2 Animal models

Many studies of atherosclerosis use murine models, however, there are several limitations in their applicability to analyse plaque vascularization (Table 5). Notably, atherosclerotic plaques developing in hypercholesterolaemic 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. Therefore, future cardiovasculargene and cell therapy trials should focus on improved limb salvage reduces the potential impact of cell therapy. 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

### Table 4 Therapeutic strategies to reduce plaque angiogenesis

<table>
<thead>
<tr>
<th>Animal model</th>
<th>Treatment</th>
<th>Duration</th>
<th>Readout</th>
<th>Effect on plaque size</th>
<th>Intra-plaque angiogenesis</th>
<th>Adventitial angiogenesis</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApoE-/- LDLr/- mouse</td>
<td>Thalidomide</td>
<td>39 weeks HCD</td>
<td>μCT</td>
<td>↓</td>
<td>ND</td>
<td>–</td>
<td>104</td>
</tr>
<tr>
<td>Collar placement + LDLr mouse</td>
<td>VEGFR2 vaccination</td>
<td>Not clear</td>
<td>Histo</td>
<td>↓</td>
<td>–</td>
<td>Present</td>
<td>107</td>
</tr>
<tr>
<td>ApoE-/- mouse</td>
<td>TNP-470</td>
<td>20 weeks HCD</td>
<td>Histo</td>
<td>↓</td>
<td>–</td>
<td>–</td>
<td>105</td>
</tr>
<tr>
<td>Collar placement + LDLr mouse</td>
<td>Tie2 vaccination</td>
<td>8 weeks HCD</td>
<td>Histo</td>
<td>↓</td>
<td>–</td>
<td>↓</td>
<td>108</td>
</tr>
<tr>
<td>Rabbit</td>
<td>Bevacizumab</td>
<td>3 weeks HCD</td>
<td>Histo</td>
<td>↓</td>
<td>–</td>
<td>↓</td>
<td>107</td>
</tr>
<tr>
<td>Balloon angioplasty pig</td>
<td>Endostatin (Endostar)</td>
<td>12 weeks HCD</td>
<td>Histo</td>
<td>↓</td>
<td>ND</td>
<td>–</td>
<td>110</td>
</tr>
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</table>
## Table 5 Modelling effects of enhanced angiogenesis on mouse atherosclerotic plaque

<table>
<thead>
<tr>
<th>Mouse model</th>
<th>Treatment</th>
<th>Duration</th>
<th>Readout</th>
<th>Effect on plaque</th>
<th>Intra-plaque angiogenesis</th>
<th>Adventitial angiogenesis</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Short time diet</td>
<td>ApoE-/- VEGF-A</td>
<td>7, 6, 5 weeks HCD</td>
<td>Histo</td>
<td>↑</td>
<td>↑</td>
<td>ND</td>
<td>39</td>
</tr>
<tr>
<td>ApoE-/- VEGF-A</td>
<td>7, 6, 5 weeks HCD</td>
<td>Histo</td>
<td>↑</td>
<td>↑</td>
<td>ND</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>LDLR-/- ApoB38-/-</td>
<td>Time + VEGF-A, VEGF-B, VEGF-C, VEGF-D gene transfer</td>
<td>12 weeks HCD</td>
<td>Histo</td>
<td>=</td>
<td>=</td>
<td>Present</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>ApoE-/- Coll XVIII-/- Fbn1 C1039G +/- KO</td>
<td>24 weeks HCD</td>
<td>Histo</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>106</td>
</tr>
<tr>
<td></td>
<td>ApoE-/- Fbn1 C1039G +/- KO</td>
<td>20 weeks HCD</td>
<td>Histo</td>
<td>↑</td>
<td>↑</td>
<td>Present</td>
<td>112</td>
</tr>
<tr>
<td>Aged mice and/or</td>
<td>ApoE-/- Time</td>
<td>40–50 weeks chow</td>
<td>Two photon microscopy</td>
<td>–</td>
<td>↑</td>
<td>↑</td>
<td>49</td>
</tr>
<tr>
<td>prolonged diet time</td>
<td>ApoE-/- bFGF (I) 67–94 weeks chow</td>
<td>Histo</td>
<td>↑</td>
<td>ND</td>
<td>↑</td>
<td>↑</td>
<td>113</td>
</tr>
<tr>
<td></td>
<td>ApoE-/- Time</td>
<td>40–96 weeks HCD</td>
<td>Intravital microscopy</td>
<td>–</td>
<td>↑</td>
<td>↑</td>
<td>48</td>
</tr>
<tr>
<td>Surgical Manipulation</td>
<td>ApoE-/- Collar Placement + MMP9 gene transfer</td>
<td>Not clear</td>
<td>Histo</td>
<td>=</td>
<td>=</td>
<td>ND</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>ApoE-/- Collar placement + VEGF-A gene transfer</td>
<td>Not clear</td>
<td>Histo</td>
<td>↑</td>
<td>=</td>
<td>ND</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>ApoE-/- Tandem Stenosis</td>
<td>17, 13, 10, 8 weeks HCD</td>
<td>Histo</td>
<td>↑</td>
<td>Present</td>
<td>Present</td>
<td>114</td>
</tr>
<tr>
<td></td>
<td>ApoE-/- Wire injury + alternative spliced Tissue Factor gene transfer</td>
<td>6 weeks HCD</td>
<td>Histo</td>
<td>↑</td>
<td>↑</td>
<td>ND</td>
<td>43</td>
</tr>
</tbody>
</table>

## Table 6 Large animal models of plaque angiogenesis

<table>
<thead>
<tr>
<th>Animal Species</th>
<th>Anti/Pro Angiogenic</th>
<th>Treatment</th>
<th>Duration</th>
<th>Readout</th>
<th>Effect on plaque</th>
<th>Intra-plaque angiogenesis</th>
<th>Adventitial angiogenesis</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbits</td>
<td>Pro VEGF-A</td>
<td>6 weeks HCD</td>
<td>Histo</td>
<td>↑</td>
<td>Increase but only total CD31 measured not density</td>
<td>ND</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>Pro Perivascular Collar + VEGF-A, VEGF-CNC, VEGF-D and VEGF-DNC gene transfer</td>
<td>3 weeks HCD</td>
<td>Histo</td>
<td>↑</td>
<td>ND</td>
<td>↑</td>
<td>41</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pro Perivascular Collar + VEGF-E, VEGF-E+ soluble VEGFR2 gene transfer</td>
<td>10 days chow</td>
<td>Histo</td>
<td>↑</td>
<td>ND</td>
<td>↑</td>
<td>123</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pro Collar placement (rabbit) + balloon angioplasty (rat) with VEGF and PR39 gene transfer</td>
<td>9 days (rabbit) and 14 days (rat) chow</td>
<td>Histo</td>
<td>↑</td>
<td>ND</td>
<td>↑</td>
<td>42</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pro Watanabe + Alloxan injection to induce diabetes</td>
<td>Histo NMR</td>
<td>↑</td>
<td>Total CD31 not density</td>
<td>ND</td>
<td>↑</td>
<td>124</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pigs</td>
<td>– PCSK9 knock-in</td>
<td>46 weeks HCD</td>
<td>Histo</td>
<td>–</td>
<td>–</td>
<td>Present</td>
<td>Present</td>
<td>125</td>
</tr>
</tbody>
</table>
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 mm²), microvessel count (per section or per mouse), CD31 positive adventitial area, or vasa vasorum volume have been used (Table 5). 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 computed tomography have been used to visualize adventitial microvessels (Table 5). Moreover, the experimental design often limits the translatability of the findings. In two studies, induction/manipulation of angiogenesis was started together with atherosclerosis induction, whereas pre-existing plaques represent the treatment target in human atherosclerosis.

In addition to mice and rats, rabbits and pigs have been used to study angiogenesis in atherosclerosis (Table 6). 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 mm². In some studies, adventitial angiogenesis was specifically targeted using a hollow perivascular collar together with a relatively short post-operation time of 9–21 days. Interestingly, induction of diabetes accelerated atherogenesis and intra-plaque angiogenesis in Watanabe heritable hyperlipidaemic rabbits. In pigs, atherosclerosis was induced by high-cholesterol diet and/or surgical interventions (balloon angioplasty or stenting). However, intra-plaque angiogenesis was not detected in all studies except for one. 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. 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. Consensus statement

In this article, the ESC Working Group for Atherosclerosis and Vascular Biology provides guidance for the development of treatments to target the vasculature in post-ischaemic disease, for their delivery to ischaemic tissues and for their assessment in pre-clinical and clinical studies:

- 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.
- Tissue specific delivery of pro-angiogenic therapies is advantageous, because it avoids the potential deleterious side effects associated with systemic delivery of growth factors such as the promotion of atherosclerosis. In the setting of PAD or coronary artery disease, 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 vectors should be developed to allow precise temporal control of inducible transgene expression, thus avoiding detrimental effects due to continuous overexpression.
- Endpoints of clinical trials of therapeutic vascularization have varied between studies. We propose that functional, metabolic, and imaging readouts should be further developed to capture therapeutic efficacy and biological activity of treatments, thereby support clinical hard endpoints.
- 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 Canadian Cardiovascular Society (CCS) Class 2–3 for refractory angina, in the future will be justified. Finally, further genetic characterization of non-responder patient groups in neovascularization clinical trials would help to identify factors affecting treatment responsiveness.

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