2018

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Oxford University Press (OUP)

Tieteelliset aikakauslehtiartikkelit

http://dx.doi.org/10.1093/cvr/cvy184

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: 8993

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: VASCULARISATION, ANGIOGENESIS AND 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 the embryo, but current understanding is more nuanced. Early embryonic angioblasts and haemoblasts share a very similar gene signature and hematopoietic stem cells and ECs display considerable plasticity[12, 13]. Notably, hematopoietic stem cells can be differentiated into endothelial cells [13] and these progenitors have shown therapeutic potential in several clinical and pre-clinical settings [14].

Angiogenesis is the creation of new vessels from pre-existing ones [15]. 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 [16]. To prevent ECs moving *en masse*, a particular type of ECs, known as tip cells, are selected to lead the advance [17]. 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 [18, 19]. 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 [20, 21].

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 [22].

2. NEO-VASCULARISATION: 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 hemodynamic forces within the vascular wall trigger vasculogenesis, angiogenesis and arteriogenesis which act in concert to establish a functional vascular network in ischaemic zones [23].

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 [24].

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 [25, 26]. Consistent with this relationship between angiogenesis and inflammation, several molecules that regulate inflammation have been implicated in new vessel formation [23]. 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 [27]. Recent studies suggest that flow dynamics control the activation of HIF1α[28] and the localisation of sprouting in vessels [29]. The location is not determined by on highest VEGF concentration, but by a combination of VEGF and biomechanical signals [30]. Thus, shear-induced mechanism appears to override pro-angiogenic signals such as VEGF [31]. 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 [21].

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 [23]. Thus, in the last decades, stimulation of vessel growth has emerged as a novel therapeutic option in patients with ischaemic diseases [32].

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 [33]. 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 [34, 35] . In experimental models plaque angiogenesis has been induced by stress [36, 37], treatment with pro-inflammatory mediators [38], pro-angiogenic growth factors [39] and viral gene delivery of pro-angiogenic factors [40-44], 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.[45] These ultrastructural characteristics suggest vascular leakage [46], that might be responsible for increased extravasation of immune cells and deposition of lipids and red blood cells in the plaques [47-49]. Therefore, these microvessels are thought to represent one of the main sources of intra-plaque haemorrhage, in addition to healed thrombi [50].

3. THERAPEUTIC VASCULARISATION

Growth factors, cells and non-coding RNA therapies

Multiple different approaches have been used to promote vascularization of ischaemic tissues.

Growth factors have been applied for therapeutic angiogenesis including VEGF [51], basic fibroblast growth factor (bFGF) [52], hepatocyte growth factor (HGF) [53], Angiopoietin 1 (ANG-1) [54] and insulin-like growth factor (IGF-1) [55] (Table 1). Preclinical studies in animal models using individual angiogenic factors have showed significant improvements in clinically relevant end points such as increased regional perfusion, improved exercise tolerance and tissue energy metabolism, improved myocardial function, and protection against ischemic damage [56]. 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 upregulated 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 [57]. 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 [58]. 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 [59]. 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 [60]. 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 [61] .

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, preconditioning and tissue engineering have been applied to improve the efficacy of stem cell therapy [62]. 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 [63] (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 [63]. 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 [64]. 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) [14]. 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 [14].

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 [65] . 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 [66] and the systemic delivery of miR-126 mimics rescued EC proliferation at vulnerable sites and inhibited atherosclerotic lesion progression [67]. 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 [68]. 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 [69]. 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 [70].

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 [71, 56] 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 [72]. 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.

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 [73, 74]. For applications like myocardial ischaemia, local targeted injections based on electromechanical mapping [75] or blood flow measurements using

positron emission tomography [75] 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 [76] but in larger animals and humans wide spread gene expression after any delivery method still remains a very challenging task [77].

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 [78]. 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 [79]. 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 [80]. 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 [81]. A novel concept of involving a biodegradable gelatin hydrogel carrying a sustained-release system of bFGF was studied in patients with CLI [82].

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. To make things more complicated, within one animal species, different strains show distinct naïve vascularization and even show opposite reactions [83].

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 [84]. 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 [85].

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.

For a long time, the dog [86, 87] , together with the rabbit [88, 16], 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 [32].

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 [89]. 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[91, 92].

Clinical trials for therapeutic vascularization: change of perspectives

Endpoints. Ongoing clinical gene and cell therapy trials have been reviewed elsewhere [71, 93]. 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, they do not have sufficient statistical power to capture endpoints like 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 [64].

Ideally, functional readouts based on imaging such as PET or MRI 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

[94]. 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 [95].

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 [96]. In multi-center studies, heterogeneity in patients and different cell preparations and products can influence the efficacy of cell therapy [97]. In addition, meta-regression showed that refinements in endovascular and surgical techniques leading to improved limb salvage reduces the potential impact of cell therapy [97] . 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.

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 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 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 [98]. 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.

4. VASCULARIZATION OF ATHEROSCLEROTIC PLAQUES

The therapeutic benefits of enhanced vascularization of ischemic tissues in ischemic tissues contrasts with the effects of vascularization in atherosclerotic lesions which can enhance plaque burden and also promote plaque rupture [45 99], potentially leading to myocardial infarction or stroke.

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 [100], TNP-470 [101], angiostatin [102], monoclonal antibody anti-VEGF-A [103] and VEGFR2 [104] or Tie2 inhibitors [105] (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 [106].

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 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 [107]. 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 [102]. This was more pronounced in ApoE fibrillin double knockout mice [108], 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 [109, 110]. 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 [110]. In addition, tissue contraction and deformation have been shown to induce VEGF-A expression [111]. 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 [112]. In murine atherosclerosis, experimental overexpression of VEGF-A increased signs of plaque vulnerability [39], 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 5). This is an important caveat because although adventitial vasa vasorum growth may precede atherosclerotic plaque development [113, 114], plaque rupture has been linked with increased intra-plaque angiogenesis rather than an increase in adventitial vasa vasorum in humans [45]. 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 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 CT 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 [100, 115], 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 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 [41, 42, 116, 117]. Interestingly, induction of diabetes accelerated atherogenesis and intraplaque angiogenesis in Watanabe heritable hyperlipidemic rabbits [118].

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 [119]. 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.

CONSENSUS STATEMENT

In this manuscript, 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 and 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.

Figure Legend

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

Funding: AC and PCE are funded by the British Heart Foundation. SS is supported by the Deutsche Forschungsgemeinschaft (DFG). JCS is funded by the Dutch heart foundation (2016T060) and member of a transatlantic network of excellence funded by the Ledcuq foundation. EL is funded by the NWO (VICI), the DFG (CRC1123), the European Research Council (ERC consolidator grant), and the EU (Horizon 2020, REPROGRAM). MB is supported by the Swedish Research Council Medicine, the Swedish Heart and Lung Foundation, and the AGing Innovation & Research (AGIR) Program. AFS is funded by the Max Planck Society and the Deutsche Forschungsgemeinschaft (DFG SI-1374/5-1) and DFG Cells-in-Motion Cluster of Excellence (EXC 1003-CIM), University of Münster, Germany. JJW is funded by the European Research Council (ERC starting grant). JT is funded by FIS (PI17/01615) and the Spanish Society of Cardiology.

Acknowledgements: We gratefully acknowledge Thomas Theelen for generation of tables.

Conflict of interest: None declared

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Table 1: Gene therapy in post-ischaemic vascularization

Growth factor	Model	Outcome	Ref
VEGF	pig MI	increase neoangiogenesis, improved regional myocardial function and myocardial perfusion.	51
bFGF	pig MI	enhanced arteriogenesis within the ischemic zone	52
HGF	rabbit HLI	increase of blood flow and arteriogenesis	53
Ang-1	mouse MI	increase in capillary density, reduction in infarct sizes, increase heart performance	54
IGF-1	mouse MI	increase in capillary density, increase heart performance	55
Disease/patient number	**Growth factor/Vector/Delivery**	**Primary Outcome**	**Trial**
PAD/60	FGF-2/ SeV/ i.m.	Walking performance	NCT02276937
PAD/500	HGF /Pl/ i.m.	Time to major amputation	NCT02144610
MI/41	VEGF-A116A /Ad / i.my	Time to 1mm ST depression during exercise stress testing	NCT01757223

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

Table 2: Cell therapy in post-ischaemic vascularization

Cell line	Model	Outcome	Ref.
BM-derived hematopoietic stem cells ($CD34^+$)	pig MI	greater vessel densities, and higher expressions of bFGF and SDF-1	120
BM-derived mesenchymal stem cells	pig MI	reduction in infarct size, increases in ejection fraction	121
Cardiac stem cells	pig MI	reduction in infarct size, increase in contractility	122
Disease/patient number	**Cell line/Delivery**	**Primary Outcome**	**Trial**
MI/142 MI/55	Cardiac stem cells/ i.c.	Infarct size by MRI Safety as measured by death and MACE in 12 months	ALLSTAR trial (NCT01458405). CAREMI trial (NCT02439398)
MI/3000	autologous BM-derived mono-nuclear cells/i.c.	Time from randomization to all-cause death	BAMI trial (NCT01569178)
Ischaemic heart failure/315	BM- derived mesenchymal stem cells/i.c.	Efficacy between groups post-index procedures	CHART-1 (NCT01768702) CHART-2 (NCT02317458)

i.c: intracoronary; MI: myocardial infarction

Table 3: Large animal models of post-ischaemic vascularization

Model	Readout	Ref.
Left anterior descending coronary artery ligation	Myocardial infarct size	88
Femoral artery ligation	Hind-limb perfusion	16
Femoral artery excision	Hind-limb perfusion	123
Coronary stenosis	Myocardial infarct size	91, 92
Left anterior descending coronary artery ligation	Myocardial infarct size	90
Femoral artery ligation	Hind-limb perfusion	89
Ameroid constrictors and coronary artery ligation	Myocardial infarct size	86
Ameroid constrictors	Myocardial infarct size	87

Table 4: Therapeutic strategies to reduce plaque angiogenesis

Animal Model	Treatment	Duration	Read out	Effect on plaque size	Intra-plaque angiogenesis	Adventitial angiogenesis	Ref
ApoE-/- LDLr-/- mouse	Thalidomide	39 wks chow	µCT	↓	ND	-	100
Collar placement + LDLr mouse	VEGFR2 vaccination	Not clear	Histo	↓	-	Present	105
ApoE -/- mouse	TNP-470	20 wks HCD	Histo	↓	-	-	101
Collar placement + LDLr mouse	Tie2 vaccination	8 wks HCD	Histo	↓	-	↓	104
Rabbit	Bevacizumab	3 wks HCD	Histo	↓	-	↓	103
Balloon Angioplasty Pig	Endostatin (Endostar)	12 wks HCD	Histo	↓	ND	↓	124

Table 5: Modelling effects of enhanced angiogenesis on mouse atherosclerotic plaque

	Mouse model	Treatment	Duration	Read out	Effect on plaque	Intra-plaque angiogenesis	Adventitial angiogenesis	Ref.
Short time diet	ApoE-/-	VEGF-A	7, 6, 5 wks HCD	Histo	↑	↑	ND	39
	ApoE-/-	VEGF-A	7, 6, 5 wks HCD	Histo	↑	↑	ND	39
	LDLR-/- ApoB38-/-	Time + VEGF-A, VEGF-B, VEGF-C, VEGF-D gene transfer	12 wks HCD	Histo	=	-	=	44
	ApoE-/- Coll XVIII-/-	Coll XVIII KO	24 wks HCD	Histo	↑	↑	↑	102
	ApoE-/- Fbn1 C1039G+/-	Fbn1 C1039G+/- KO	20 wks HCD	Histo	↑	↑	Present	108
Aged mice and/or prolonged diet time	ApoE-/-	Time	40-50 wks chow	Two photon microscopy	-	↑	↑	49
	ApoE-/-	bFGF	(I) 67-94 wks chow (II) 12 wks HCD	Histo	↑	ND	↑	125
	ApoE-/-	Time	40-96 wks HCD	Intravital microscopy	-	↑	↑	48
	ApoE-/- SV129-/-	Time + stress + SV129 KO	20 wks HCD	Histo	↑	↑	ND	36
Surgical Manipulation	ApoE-/-	Collar Placement + MMP9 gene therapy	Not clear	Histo	=	=	ND	40
	ApoE-/-	Collar placement + VEGF-A gene transfer	Not clear	Histo	↑	=	ND	40
	ApoE-/-	Tandem Stenosis	17, 13, 10, 8 wks HCD	Histo	↑	Present	Present	126
	ApoE-/-	Wire injury + alternative spliced Tissue Factor gene transfer	6 wks HCD	Histo	↑	↑	ND	43

Table 6: Large animal models of plaque angiogenesis

Animal Species	Anti/Pro Angiogenic	Treatment	Duration	Read out	Effect on plaque	Intra-plaque angiogenesis	Adventitial angiogenesis	Ref.
Rabbits	Pro	VEGF-A	6 wks HCD	Histo	↑	Increase but only total CD31 measured not density	ND	39
	Pro	Perivascular Collar + VEGF-A, VEGF-CNC, VEGF-D and VEGF-DNC gene transfer	3 wks HCD	Histo	↑	ND	↑	41
	Pro	Perivascular Collar + VEGF-E, VEGF-E+ soluble VEGFR2 gene transfer	10 days chow	Histo	↑	ND	↑	116
	Pro	Collar placement (rabbit)+ balloon angioplasty (rat) with VEGF and PR39 gene transfer	9 days (rabbit) and 14 days (rat) chow	Histo	↑	ND	↑	42
	Pro	Watanabe + Alloxan injection to induce diabetes		Histo NMR	↑	Total CD31 not density	ND	118
Pigs	-	PCSK9 Knock-in	46 wks HCD	Histo	-	Present	Present	119