VascularBiology

Vascular Biology (2024) **6** e240009
https://doi.org/10.1530/VB-24-0009

Received 27 June 2024
Accepted 4 October 2024
Available online 7 October 2024
Version of Record published 8 November 2024

REVIEW

Therapeutic angiogenesis for patients with chronic limb-threatening ischemia: promising or hoax?

Judith A H M Peeters[1,2], Abbey Schepers[1], Jaap F Hamming[1] and Paul H A Quax[1,2]

[1]Department of Vascular Surgery, Leiden University Medical Center, Leiden, The Netherlands
[2]Einthoven Laboratory for Experimental Vascular Medicine, Leiden University Medical Center, Leiden, The Netherlands

Correspondence should be addressed to J A H M Peeters: A.H.M.Peeters@lumc.nl

Abstract

Chronic limb-threatening ischemia (CLTI) is a critical end-stage disease that leads to high amputation rates. Over the past few decades, therapeutic angiogenesis has attracted a lot of attention as a means to reduce the necessity for amputations. Especially gene- and cell therapy are regarded to as possible treatment modalities to restore the hampered blood flow. So far, early-phase clinical trials often fail to prove a significant clinical improvement in mortality, amputation rate, and ulcer healing but still conclude that therapeutic angiogenesis might be promising as therapy. The subsequent phase III clinical trials based on these indecisive early trials fail consistently to demonstrate clinical benefits leaving the promising early results unvalidated. In this review we will illustrate that designing good trials for CLTI patients is challenging, not in the last place since patients are often not eligible due to strict inclusion criteria. Moreover, in this review, we advocate that clinical trials should be conducted with a low risk of bias and that it is of utmost importance to publish results, regardless of the outcome. It is definitely very concerning that many studies of a lower quality (due to small group size or high chance for bias) reporting positive outcomes are published while good quality trials (often with larger group sizes) are stopped prematurely due to lack of effects and remain unpublished. This keeps the 'promising but not yet proven' image of these therapeutic neovascularization studies alive, with still new groups starting similar trials.

Keywords: cell therapy; critical limb-threatening ischemia; gene therapy; publication bias; therapeutic angiogenesis

Burden of chronic limb threatening ischemia

Peripheral arterial disease (PAD) affects over 200 million individuals globally and presents as a significant and emerging condition associated with cardiovascular events and mortality (1). Advanced PAD can progress to chronic limb-threatening ischemia (CLTI), manifesting as rest pain and/or ischemic ulcers with potential gangrene development, resulting in the need for amputation if left untreated. CLTI incidence in Europe ranges from 360 to 1000 cases per million annually, impairing patients' health and quality of life due to frequent wound care, medication requirements, and impaired mobility (2, 3, 4).

Standard therapies aim to restore limb blood flow alongside drug therapy and cardiovascular risk management. However, success rates of conventional interventions like endovascular angioplasty and surgical procedures such as bypass grafting or

Published by Bioscientifica Ltd.
https://vb.bioscientifica.com

Vascular Biology (2024) **6** e240009
https://doi.org/10.1530/VB-24-0009

endarterectomies are highly variable and some have poor long-term outcomes. Additionally, some patients are ineligible for these procedures due to comorbidities, and anatomical or technical aspects, resulting in over 25% of CLTI patients undergoing amputations within a year of diagnosis (5). Amputations lead to functional disability and consequently severe psychological and social impact for patients and impose substantial economic burdens on healthcare systems worldwide due to subsequent procedures, reamputations, and readmissions (6, 7).

In order to reduce amputations and enhance the quality of life of CLTI patients, novel therapies like gene therapy and cell therapy emerge as promising alternatives or complements to traditional approaches. These therapy modalities aim at restoring blood flow by promoting neovascularization in the lower extremities. Neovascularization involves the formation of new blood vessels and includes the stimulation of both angiogenesis and arteriogenesis/collateral formation. Angiogenesis specifically entails sprouting and capillary growth from existing vessels, often triggered by hypoxia-induced growth factor expression. Arteriogenesis, in contrast, involves collateral vessel recruitment and remodeling to restore blood flow in response to increased shear stress by stabilizing sprouts with smooth muscle cells and pericytes. Therapeutic angiogenesis approaches targeting these processes aim to enhance vascularization in ischemic tissues, promoting angiogenesis and arteriogenesis to improve perfusion and diminish symptoms. These gene therapy approaches are usually based on the overexpression of pro-angiogenic factors and are based on the hypothesis that overexpressing one or two vascular growth factors using viral or plasmid vectors leads to more growth factors which subsequently promote angiogenesis.

An attractive alternative is cell therapy since the initial autologous bone marrow infusion experiments showed promising neovascularization effect. However, the exact underlying mechanism of different cell therapy strategies is still unknown (8, 9). So far, several options are considered, like a role for progenitor cells in the stimulation of neovascularization either angiogenesis or arteriogenesis, or more likely a paracrine effect by excreting factors that induces neovascularization (10). A lot of research has focused on unraveling the mechanisms of action of gene and cell therapy, in order to discover a possible treatment for patients with CLTI, however, this has not resulted in a reliable explanation yet.

The enthusiasm that was raised by the promising early-phase clinical trial outcomes resulted in many later phase II/III clinical trials as well as preclinical studies directed at unraveling the underlying mechanism. Unfortunately, so far no clear mechanism of action of angiogenic cell therapy has been demonstrated and phase III clinical trials failed to confirm efficacy. In this review the limitations and challenges of clinical trials in this research field, often leading to disappointing results and unpublished research, are discussed.

Gene therapy research in CLTI patients

Gene therapy is used in a spectrum of medical conditions including inherited disorders, malignancies, neurological conditions, and infectious diseases (11, 12, 13, 14, 15). Consequently, research into gene therapy is rapidly increasing, facilitating the development of novel therapeutic options for diverse pathologies. Gene therapy in the cardiovascular field is predominantly performed by administration of viral or plasmid vectors encoding proteins (mostly growth factors) that are overexpressed. It is still considered largely experimental and mainly studied in relation to clinical trials or to unravel pathophysiological processes. Over the past two decades, numerous randomized controlled gene therapy trials have been conducted to induce angiogenesis, using viral or plasmid vectors encoding vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and/or hepatocyte growth factor (HGF), as discussed below.

Vascular endothelial growth factor

In the first case published in The Lancet in 1996, a plasmid encoding VEGF was administered via an angioplasty balloon to the ischemic limb of a female patient, resulting in enhanced blood flow, augmented collateral vessel formation, and the emergence of spider angiomas with proliferative endothelium characteristics (16). This case raised optimism regarding the potential development of an efficacious treatment modality for patients with CLTI and subsequent investigations focused on gene therapy by growth factor encoding plasmids or adenoviruses. In the following decades, several randomized controlled trials have tried to replicate the findings of the Lancet article in double-blind randomized placebo-controlled trials across several different variations, with negative outcomes on the clinically relevant endpoints (Table 1). A study by Mäkinen *et al.* evaluating the safety and angiographic/hemodynamic responses to VEGF gene therapy revealed notable improvements in Rutherford class and ankle brachial index (ABI) within the VEGF gene-treated group. However, these enhancements were also observed in the control cohort, and intergroup comparisons did not yield significant differences in the ABI or Rutherford class (17). Kusumanto *et al.* evaluated the effect of intramuscular administration of phVEGF165, a VEGF gene-carrying plasmid, on CLTI, and did fail to show significant amputation reduction (18). They observed a significant improvement in ABI/toe brachial index (TBI) in the phVEGF165 group versus placebo, but patients who were not evaluable due to extensive ulceration or incompressible vessels due to an

Table 1 Overview of outcome measurements of illustrative trials in CLTI patients using gene therapy.

Reference	Vector	Injection site	Patients treated with gene therapy	Diabetic patients	Primary outcome	Secondary outcome	Endpoints met	Study design
Barć *et al*. (29)	VEGF + HGF enc. PL	IM	14 pIRES/ VEGF165/HGF; 14 untreated	All patients	No clear description	ABI, rest pain, angiography	Better wound healing, less rest pain, increased ABI, more collaterals	RNBNP-C
Belch *et al*. (25)	FGF enc. PL	IM	259 NV1FGF; 266 placebo	52% NV1FGF; 54% placebo	MAFS, study related death <1 year	Mortality, minor amputations, skin lesion status, pain, QOL	None	RDBP-C
Gu *et al*. (23)	HGF enc. PL	IM	50 low dose; 50 middle dose; 50 high dose; 50 placebo	34–38% per group	Rest pain Ulcer size	$TcPO_2$, ABI, TBI, amputation rate, mortality	Less rest pain, complete ulcer healing*	RDBP-C
Kusumanto *et al*. (18)	VEGF enc. PL	IM	27 phVEGF165; 27 placebo	All patients	Amputation	ABI, TBI, wound area, pain, mortality	TBI/ABI increased	RDBP-C
Mäkinen *et al*. (17)	VEGF enc. AV or PL	IA	17 plasmid; 18 adenoviral; 19 placebo	24% PL; 17% AV; 32% placebo	Vascularity (DSA) Major amputation, ulcer healing, rest pain	Restenosis rate, Rutherford class, ABI,	Vascularity increased (DSA)	RDBP-C
Nikol *et al*. (24)	FGF enc. Pl	IM	59 NV1FGF; 66 placebo	37% NV1FGF; 50% placebo	Ulcer healing	Amputation, mortality, hemodynamic parameters, pain	Amputation risk reduced	RDBP-C
Powell *et al*. (22)	HGF enc. PL	IM	21 HGF; 6 placebo	62% HGF; 50% placebo	Ulcer healing, major amputation, pain	ABI, TBI, mortality, QOL	TBI improved, pain improved	RDBP-C
Shigematsu *et al*. (21)	HGF enc. PL	IM	27 HGF; 13 placebo	52% HGF; 62% placebo	Ulcer healing, rest pain	Major amputation, ABI, QOL	Higher improvement rate**	RDBP-C

*in the high-dose group; **not further defined.

ABI, ankle brachial index; AV, adenovirus; enc., encoding; IA, intra-arterial; IM, intramuscular; MAFS, major amputation-free survival; Pl, plasmid; QOL, quality of life; RDBP-C, randomized double-blind placebo-controlled; RNBNP-C, randomized not blinded, not placebo-controlled; TBI, toe brachial index; $TcPO_2$, transcutaneous oxygen pressure.

Vascular Biology (2024) **6** e240009
https://doi.org/10.1530/VB-24-0009

advanced disease state were not included in the analysis, although these patients might probably have poor outcomes. Therefore, the improvement in ABI/TBI should be handled with care as it may presumably result in an overestimation of effect. Rajagopalan *et al.* performed a double-blind placebo-controlled study that was designed to test the efficacy of AdVEGF121, a replication-deficient adenovirus encoding the 121-isoform of VEGF, in patients with severe intermittent claudication and concluded that a single intramuscular injection of AdVEGF121 was not associated with improved exercise performance or quality of life (19). However, as AdVEGF121 has only been studied in patients with severe intermittent claudication conclusions regarding the lack of effects on resolving blood circulation issues could not be extrapolated to patients with critical limb ischemia. In conclusion, gene therapy studies with overexpression of VEGF do not show clinically relevant improvements in CLTI patients. In addition to the extensively studied overexpression of VEGF, researchers have also investigated the overexpression of other factors.

Hepatocyte growth factor

HGF has emerged as another intriguing pro-angiogenic factor that has been studied thoroughly for its role in angiogenesis including endothelial cell proliferation, promotion of cellular migration, induction of vascular tubulogenesis, and activation of angiogenic signaling cascades via the PI3K/AKT and MAPK/ERK pathways (20). Application in a clinical setting was also performed. The phase II AnGes trial investigated the injection of a naked HGF encoding plasmid in 44 CTLI patients demonstrating a significant improvement in rest pain, ulcer size, and quality of life such as bodily pain and mental health compared to placebo (21). Powell *et al.* studied HGF gene therapy in 27 CTLI patients and showed significantly improved TBI and decreased rest pain compared to placebo (22). Recently, Gu *et al.* performed a randomized double-blind placebo-controlled phase II study with 200 participating patients showing that after administration of NL003, a plasmid that expresses two isoforms of HGF, pain severity was significantly decreased and ulcer healing was higher in the treatment group. No differences were observed in transcutaneous oxygen pressure ($TcPO_2$), ABI, or toe brachial index (23). These trials show promising results but without a doubt, larger phase III studies are necessary to assess the efficacy on clinically relevant outcomes. In 2014 a large international worldwide phase III trial, the AnGes trial, was set up, but unfortunately, this trial ended prematurely and the results were not published (unpublished data, EudraCT 2014-001129-34). At this moment, there is only one phase III clinical trial reported in ClinicalTrial.gov that recruits CLTI patients and uses HGF gene therapy (search 25 March 2024). Thus HGF gene therapy for angiogenesis has shown promising results in phase II clinical trials, and these results should be validated in phase III clinical trials.

Fibroblast growth factor

Like HGF, FGF was identified as an interesting target for gene therapy in patients with CLTI. In the TALISMAN trial, the efficacy and safety of intramuscular administration of NV1FGF, a plasmid-based angiogenic gene delivery system aimed at local expression of FGF-1, was compared to placebo in patients with CLTI (24). Hundred and twenty-five CLTI patients with nonhealing ulcers and no other viable treatment options were included. The primary endpoint was the occurrence of complete ulcer healing, with secondary endpoints including ABI, amputation rates, and mortality. The results showed similar improvements in ulcer healing in both groups but a significant reduction in the risk of amputations in the NV1FGF group compared to placebo. Additionally, there was a noticeable trend towards a reduced mortality risk associated with NV1FGF use. These findings suggested a potential benefit of NV1FGF and a phase III multicenter, international clinical trial, the TAMARIS trial, was set up to confirm these promising results. Unfortunately, the efficacy of NV1FGF in the phase II trial was not confirmed in the phase III trial involving 525 CLTI patients (25, 26). No significant differences in primary endpoints major amputation and death between treatment and placebo groups were observed. The abovementioned research serves as an illustration among numerous studies wherein promising outcomes observed in phase II trials fail to be confirmed in well-designed low-bias phase III trials.

Combined gene overexpression therapy

Overall, many studies using one angiogenic growth factor gene lacked the desired efficacy in an advanced clinical setting (27). Recognizing the multifactorial processes in angiogenesis, researchers have postulated that the unsatisfying outcomes of gene therapy trials could be explained by focusing on one specific angiogenic factor, whilst the expression of multiple genes could induce effective angiogenesis. This hypothesis directed researchers towards the use of combined VEGF and HGF gene therapy, as demonstrated in a small study by Barć *et al.* involving 12 CLTI patients who received injections of pIRES/VEGF165/HGF plasmid encoding both VEGF and HGF separated by an internal ribosome entry site (28). This intervention led to improved ABI and ulcer healing; however, 25% of the patients ultimately required amputation. A recent small-scale randomized controlled trial on double VEGF/HGF gene therapy versus standard care was performed by the same research group, involving 28 CLTI patients with diabetes, and resulted in raised levels of pro-angiogenic factors in affected tissues and improvements in vascularization as assessed by CT-angiography. Clinically, a significant increase in ABI and alleviation of rest pain were observed (29). These early-phase results again seem promising, but no large phase III trials have been conducted yet using double

gene therapy to validate that overexpressing multiple growth factors is effective in the treatment of CLTI.

In conclusion, gene therapy is regarded as very promising, and randomized controlled trials confirmed the safety of gene therapy. However, the overall efficacy outcomes across numerous clinical trials have failed to demonstrate significant alterations in mortality, amputation rates, amputation-free survival duration, or ulcer healing in patients with CLTI (30, 31). In addition, studies in this field have relatively low patient numbers, are predominantly not randomized, and several studies are not published, contributing to the risk of performance and reporting bias (32). In conclusion, research efforts fail to prove that pro-angiogenic gene therapy is effective for the treatment of CLTI.

Cell therapy research in CLTI patients

Since the discovery of endothelial progenitor cells (EPCs) in the late 1990s the interest in regenerative medicine raised, particularly in the context of therapeutic angiogenesis (33). This interesting discovery led to enthusiasm for cell-based therapies aimed at promoting new blood vessel formation, with potential applications ranging from peripheral vascular disorders, such as CLTI, to ischemic heart disease. Cell-based therapy involves the transplantation of stem cells or progenitor cells and has gathered significant attention alongside gene therapy. Since the start of its use in the early 21st century it particularly focused on (Granulocyte colony-stimulating factor mobilized) bone marrow-derived mononuclear cells (G-CSF BM-MNCs or BM-MNCs), mesenchymal stem cells (MSCs), and EPCs (34, 35, 36, 37). Bone marrow-derived mononuclear cells are a heterogeneous population of cells that include lymphocytes, monocytes, hematopoietic stem cells, and EPCs. G-CSF is a cytokine that stimulates bone marrow to produce and release specific types of cells, particularly granulocytes. G-CSF is commonly used to increase the number of hematopoietic stem cells in peripheral blood circulation. MSCs are a specific type of adult stem cell found in various tissues and have the capacity to differentiate into multiple cell types of mesodermal origin, such as bone, cartilage, adipose tissue, and muscle. They also possess immunomodulatory properties, making them attractive for therapeutic purposes. Numerous studies have delved into identifying the optimal cell transplantation source, dosage, administration method, and delivery route (38). Phase I/II clinical trials have consistently demonstrated the safety of these aforementioned cell therapies and show varying results considering efficacy (39). Also for cell therapy the results of phase III clinical trials are not unambiguous, as we will discuss in more detail below. Trials that investigated the effects of BM-MNCs are discussed in this part of the review (Table 2).

In 2002, the first clinical study (TACT trial) suggested that BM-MNCs could be both safe and efficacious in treating CLTI (35). Long-term follow-up of the TACT trial revealed prolonged amputation-free survival and higher overall survival rates among patients who received BM-MNCs (40). Subsequent research efforts aimed to validate the encouraging outcomes of the TACT trial, leading to the initiation of multiple clinical trials. Among these trials, the JUVENTAS trial, a randomized, double-blind, placebo-controlled study involving 160 CLTI patients who received either BM-MNCs or placebo, failed to demonstrate significant clinical benefit (41). No reduction in major amputation rates was observed and the improvement in secondary outcomes in both the BM-MNC and the placebo group points out the importance of placebo-controlled design. Similarly, in another randomized controlled phase III clinical trial by Lindeman *et al.* evaluating outcomes following intramuscular injections with BM-MNCs or placebo, no discernible clinical benefit was observed in various outcome parameters such as Rutherford class, amputation-free survival time, pain-free walking distance, rest pain, and ulcer healing (42). In the PROVASA study, BM-MNCs were administered intra-arterially in 19 patients and no difference in the primary outcome, ABI, was noted. However, improvements in ulcer healing and reduced rest pain were observed within three months post-therapy, although amputation rates and amputation-free survival did not differ between groups (43). In this study BM-MNC or placebo was administered at baseline and after three months the study proceeded as an open-label study and all patients, including patients that originally were in the placebo group, received BM-MNC after 3 months. The rationale for this unusual study set up has not been explained. A more recent trial, the BALI trial in 2017, which included 38 patients receiving BM-MNCs or placebo via intramuscular injections, presented results 6 months and 12 months after treatment. No differences between groups were observed in major amputation, minor amputation, and revascularization. In addition, no significant difference was observed in any group concerning the ABI and the frequency of ulcers (44). Lastly, the unpublished SALAMANDER trial administering autologous stem cells to CLTI patients with Rutherford 5 as a potential angiogenic therapy was terminated prematurely due to insufficient evidence of efficacy (unpublished data, EudraCT 2016-003980-21). The primary endpoint of this study was complete ulcer healing, and this endpoint was not achieved. There were no safety issues reported.

The initial efficacy of BM-MNCs observed in the TACT trial was not consistently supported by subsequent randomized, blinded, placebo-controlled studies (41, 42, 43, 44). Nevertheless, some studies did identify beneficial effects such as improved ABI and/or $TcPO_2$ of BM-MNC treatment (45, 46).

A randomized, double-blind, placebo-controlled trial by Sharma *et al.* was performed in patients with

Table 2 Overview of the outcome parameters and results of clinical studies performed using BM-MNC in CLTI patients.

Reference	Cells	Injection site	Patients treated	Diabetic patients	Primary Outcome	Secondary Outcome	Endpoints met	Study design
Lindeman *et al*. (42)	BM-MNC	IM	28 BM-MNC; 26 placebo	None	Limb salvage, pain-free walking distance	ABI, QoL, pain	None	RCDBP-C
Malyar *et al*. (46)	BM-MNC	IA + IM	16 BM-MNC	50%	ABI, $TcPO_2$	Pain-free walking distance	14/16 limb salvage after 6 months	NRNBNP-C
Pignon *et al*. (44)	BM-MNC	IM	17 BM-MNC; 19 placebo	55% of BM-MNC; 35% of placebo	Major amputation, death	Pain, ulcers, ABI, $TcPO_2$	Decreased risk of major amputation	RDBP-C
Sharma *et al*. (45)	BM-MNC	IA	27 BM-MNC; 29 placebo	30% of BM-MNC; 28% of placebo	ABI, $TcPO_2$	Rest pain, ulcer size, major amputation	Improved ABI, improved $TcPO_2$	RDBP-C
Tateishi *et al*. (35)	BM-MNC and PB-MNC	IM	22 patients; 1 leg BM-MNC; 1 leg PB-MNC	69%	ABI, rest pain	$TcpO_2$; pain-free walking time DSA	ABI, $TcpO_2$, rest pain, pain-free walking time	DB
Teraa *et al*. (41)	BM-MNC	IA	81 BM-MNC; 79 placebo	36% of BM-MNC; 39% of placebo	Major amputation <6 months	Mortality, minor amputations, ulcer size, rest pain, pain-free walking distance, ABI, TcpO2, QOL	None	RDBP-C
Walter *et al*. (43)	BM-MNC	IA	19 BM-MNC; 21 placebo	53% of BM-MNC; 48% of placebo	ABI	Ulcer healing, amputation-free survival, mortality, rest pain	Ulcer area decreased, decreased pain	RDBP-C

ABI, ankle brachial index; DB, double-blind; DSA, digital subtraction angiography; IA, intra-arterial; IM, intramuscular; NRNBNP-C, not randomized, not blinded, not placebo-controlled; QOL, quality of life; RCDBP-C, randomized controlled, double-blind, placebo-controlled; RDBP-C, randomized, double-blind, placebo-controlled; TBI, toe brachial index; $TcpO_2$, transcutaneous oxygen pressure.

severe PAD, CLTI or severe claudication, who received either a sham injection or intra-arterial autologous bone marrow cells (45). The treatment group showed more improvement in ABI and $TcPO_2$ compared to the control group. No difference was observed in pain relief, ulcer size, or pain-free walking distance. The trial team concluded that intra-arterial delivery of autologous bone marrow cells is safe and effective in the management of severe PAD despite the lack of effect on pain relief, ulcer size, or pain-free walking distance. Malyar *et al.* reported positive results from a trial that was conducted among patients with Rutherford class 3–6 with BM-MNCs and showed increased ABI, enhanced transcutaneous oxygen pressure, and a rise in maximum walking distance (46). These results seem promising, but on a critical note, this study was not randomized and not placebo-controlled and therefore prone to bias. There are a lot of studies in this area with a high risk of bias that report positive outcomes while well-designed studies with low bias risk fail to achieve positive results (47).

To summarize, there is a tremendous amount of research performed in cell therapy for the treatment of CLTI. However, despite some promising preclinical findings and initial clinical trials, a definitive breakthrough in the application of cell therapy for CLTI patients remains elusive (48). The results of the early-phase clinical trials have been mixed, with modest improvements in surrogate endpoints such as ABI and pain relief, but limited evidence of substantial long-term clinical benefits, such as reduced amputation rates or improved limb salvage.

Challenges in angiogenic therapy clinical trials

Successful introduction of cell therapy for CLTI in daily clinical practice still faces lots of challenges and this research field knows many limitations including issues related to cell potency, delivery methods, patient selection, and trial design.

Patient recruitment

Recruiting patients for clinical trials involving bone marrow-derived mononuclear cell therapy or gene therapy for the treatment of CLTI can be challenging due to several factors. First of all, the window of opportunity is relatively small. Most CLTI patients initially still have conventional treatment options. After such an intervention, most clinical trial protocols exclude these patients for at least a few months because it can be difficult to distinguish the effect of the trial therapy from the effect of the conventional therapy. If conventional therapy fails, disease progression is often too fast, and patients proceed in disease severity beyond the study inclusion criteria.

Another complicating factor is that CLTI is an advanced stage of disease and these patients often have multiple comorbidities and lower life expectancy. These are sometimes exclusion criteria for clinical trials. Furthermore, longer-term follow-up for these patients is therefore challenging, and clinical studies that aim for longer follow-up periods risk having a high proportion of patients with incomplete follow-up data, compromising the results of the trial. Indeed, more patient-focused outcomes may be more relevant for these trials involving patients with CLTI.

Moreover, the prevalence of no-option CLTI patients who meet the often very strict inclusion criteria is relatively low, while surgeons are constantly trying more options for surgical interventions. And compared to less severe stages of PAD the CLTI patient population is a rather limited subgroup. Patient heterogeneity such as age, ethnicity, anatomic location of the occlusion, sex, comorbidities, disease severity, and the extent of tissue damage further complicate the selection of appropriate study candidates.

Cell product composition, administration, and expected effect size

Besides the challenges in patient recruitment, the composition of the (bone marrow-derived) cell product to be used is a challenge. The heterogeneity of the various cell products used for treatment, such as the autologous BM-MNCs, comprises mixtures of various cell types with distinct functions, complicating standardization and consistency across patients (49). The heterogeneity of BM-MNCs necessitates precise identification and enrichment techniques to isolate the desired cell types effectively. Achieving such purification without compromising cell viability or functionality remains a technical hurdle. Despite selecting specific cells and cell subpopulations, not all cells within a specific population may exert the desired pro-angiogenic effects uniformly and the yield may vary across patients. Factors such as patient age, health status, and other individual variability influence the potency and efficacy of cells promoting neovascularization (50).

Subsequently, the optimal dose is hard to determine, regarding the heterogeneity of cell composition between subjects, and differences in patient characteristics. Moreover, the optimal timing, frequency, and location of administration are yet to be defined. Additionally, the delivery of cells to the ischemic tissue remains an obstacle, with challenges related to cell retention, viability, and homing to target sites (51, 52).

In gene therapy, challenges are more in the expected effect size. Expecting an effect of overexpression of one or two factors is ambitious considering that the process of neovascularization is complex and multifactorial with lots of downstream pathways.

Vascular Biology (2024) **6** e240009
https://doi.org/10.1530/VB-24-0009

Outcome parameters

The aforementioned complexity also influences a wide variety of outcome parameters. There is a lack of standardized outcome measurements and the reliance on surrogate endpoints poses challenges in assessing the clinical efficacy. Frequently used primary and secondary efficacy endpoints are (major) amputations, amputation-free survival time, ulcer healing, improvements in $TcPO_2$, ABI, rest pain, and pain-free walking distance. Setting default endpoints is pivotal for a successful study. The intricate pathophysiology of CLTI, the diverse clinical presentations, and the impossibility to predict disease course make it exceedingly challenging to determine which parameters to select as efficacy endpoints. Efficacy endpoints can be based on external features (e.g. wound healing), based on patient experience (e.g. pain and quality of life) or based on measurements (e.g. $TcPO_2$, ABI, walking distance). Amputation and death are well-defined endpoints but endpoints such as $TcPO_2$, ABI, and wound data assessment are sensitive to interobserver and intra-observer variability, and all measurement endpoints should be highly standardized. Furthermore, it should be taken into account that there are large interpatient differences in the disease course and that certain measurements or analyses still cannot reliably predict future outcomes or progression of the disease in most cases.

Geography

An underestimated study parameter is the effect of geography. The study using NV1FGF has shown increased amputation-free survival in a phase II trial. An international pivotal phase III trial was set up recruiting more than 500 patients with CLTI and diabetes from 170 sites worldwide. Study results showed variations by region of origin, a surprising and unexpected result (26). Demographic information can have a major influence on the course of disease and geographic differences may possibly contribute to understanding why most phase III trials cannot confirm promising results of phase II trials. Geographic differences can be caused due to differences in patient characteristics, such as socioeconomic status, genetics, cultural background, and lifestyle but also due to differences in healthcare costs, accessibility, and protocols (53, 54).

Preclinical studies

Difficulties in study design arise partly from the unclear molecular pathways controlling the therapeutic effects of cell therapy leading to neovascularization or restoring the blood flow to the affected limb. Determining the optimal cell type to use and understanding the specific cell subset responsible for the observed effects is of critical importance. So far, in preclinical studies, the molecular mechanism underlying the efficacy of cell therapy has not yet been elucidated despite many reported pro-angiogenic or pro-arteriogenic effects in preclinical studies.

Shortcomings and limitations in the research field

Clinical studies performed in the angiogenic therapy field are mostly early-phase trials that are open-label, not placebo-controlled, not double-blind, or not randomized. For example, Huang *et al.* report improved ABI and rest pain after cell therapy and Franz *et al.* report improved ABI, and prevention of major amputation after cell therapy (55, 56), thus positive efficacy outcomes. However, these studies are non-randomized, non-controlled trials and therefore of low value. In order to ensure the reliability of data, clinical trials should be accurately designed to minimize bias risk, incorporating features such as double-blinding, placebo controls, and randomization. Many published studies report encouraging outcomes despite the high risk of bias study designs, thereby casting doubt on the reliability of the conclusions.

Publication of clinical trial outcomes plays a pivotal role in advancing medical knowledge and informing evidence-based practice. Trials serve as critical avenues for evaluating the safety and efficacy of interventions. However, a significant challenge within the field of clinical trial publication is the presence of publication bias, wherein studies with positive or statistically significant results are more likely to be published than those with neutral or negative findings. This bias can distort the overall understanding of the safety and effect of treatments, leading to wrong conclusions and potentially influencing clinical practice inappropriately. Moreover, they may add to maintaining an optimistic and positive attitude towards future applications and larger trials, whereas the negative results advocating against this optimism stay undisclosed. Recognizing and addressing publication bias is essential for maintaining the integrity of scientific literature. Therefore, efforts are made such as preregistration of trials, transparency in reporting, and encouraging publication of negative or inconclusive results. However, despite these efforts, the results of clinical trials are not always reported in the literature (57). Reasons for not publishing include lack of time or low priority, incomplete study, unimportant or negative results, poor study quality or design, fear of rejection et cetera. In the angiogenic therapy field, this is mainly the case in large phase III clinical trials that followed up on promising phase II trials but were stopped due to futility at interim analysis (unpublished data, EudraCT 2016-003980-21, EudraCT 2010-019774-33/NCT02287974 and EudraCT 2014-001129-34/NCT02144610). Although brief study reports were uploaded to the clinical database, it is very concerning that (negative) data is not submitted for publication

in a scientific paper. Additionally, the ethical issue is that patients believe that they contribute to scientific knowledge by participating in clinical trials and are not updated about the lack of publication. The trial registry is not managed by specific laws, but many countries have regulations or guidelines requiring clinical trial registration. The importance of trial registration is to encourage transparency and accountability in research, prevent duplication of efforts, and reduce publication bias. The latter is not successful given that around 29% of all trial results of trials registered on ClinicalTrials.gov remain unpublished in scientific journals (58). This number is profoundly concerning as physicians base their clinical practice guidelines on published data and this information might be incomplete or incorrect.

A comprehensive search of the PubMed database was conducted to identify clinical review papers focusing on therapeutic angiogenesis in PAD/CLTI resulting in over 250 review papers (PubMed search on 3 April 2024). This underscores the great interest in this subject, and maybe also the hope that exists that the promising preliminary results eventually will lead to a reliable broadly applicable new treatment option for a group of patients in desperate need of new therapeutic options. In that light, it is extremely frustrating to see that none of the larger (phase III) clinical trials has been reported to be successful, or even worse, disappear in the silence of the radar without decent reporting. It would be relevant if the question of whether gene or cell therapy for the treatment of severe PAD or CLTI patients is still promising or is to be regarded as a hoax could be settled.

Future studies should include study set ups that result in relevant and trustworthy outcomes, that are reported in an unbiased manner. Trial outcome registration is the most important issue that is addressed in this review. The underreported or non-reported results still leave the question unanswered as to why early-phase trials lead to promising results while these results can not be reproduced in larger pivotal trials. Understanding the issues that contribute to this can be very helpful in designing successful future trials. Due to the hurdles in CLTI research, changing focus to earlier disease stages like rest pain or claudication and/or a more realistic endpoint such as improvement in Rutherford/Fontaine class can be beneficial and less challenging in achieving study time endpoints. Current gene therapy studies focus on platelet-derived growth factor, stromal derived factor 1 (SDF-1), EPCs, or endothelial nitric oxygen species besides the ongoing research on well-known gene targets such as VEGF, HGF and FGF (59, 60). All these genes are involved in processes such as wound healing, tissue repair, enhanced vascular repair, recruitment of cells to ischemic tissues, and promoting neovascularization, all related to angiogenesis. Changing the treatment focus to earlier disease stages, as suggested above, consequently may have effects on the choice of potential gene targets or cell therapies.

Conclusion

CLTI is an emerging problem and current therapies are insufficiently effective. Many studies have been performed in the therapeutic angiogenesis field with the aim to increase blood flow to the limb and consequently reduce the need for amputations. The study outcomes are highly variable and promising early-phase clinical trials are often not confirmed in larger and more valid phase III clinical trials. Concerningly, many clinical trials that are terminated early due to different reasons do not end up published in scientific papers. After almost 30 years of research in this field, no groundbreaking results have yet been achieved proving that therapeutic angiogenesis works in patients with CLTI. When considering starting a new trial, one should bear in mind that the results obtained so far have not been convincing and that trial design and setting the right outcome parameters are of utmost importance. We hope to have fueled the discussion whether gene or cell therapy for the treatment of severe PAD or CLTI patients is still promising or is to be regarded as a hoax. In conclusion, considering the collective body of lack of efficacy evidence, to our opinion it may be prudent to discontinue research in this direction and redirect focus toward exploring alternative therapeutic options to treat patients with CLTI.

Declaration of interest

All authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this study. Paul Quax serves as Editor-in-Chief of ***Vascular Biology***. He was not involved in the review or editorial process for this paper, on which he is listed as an author.

Funding

This work did not receive any specific grant from any funding agency in the public, commercial, or not-for-profit sector.

References

1 Tsao CW, Aday AW, Almarzooq ZI, Alonso A, Beaton AZ, Bittencourt MS, Boehme AK, Buxton AE, Carson AP, Commodore-Mensah Y, *et al.* Heart disease and stroke statistics—2022 update: a report from the American Heart Association. *Circulation* 2022 **145** e153–e639. (https://doi.org/10.1161/cir.0000000000001052)

2 Nehler MR, Duval S, Diao L, Annex BH, Hiatt WR, Rogers K, Zakharyan A & Hirsch AT. Epidemiology of peripheral arterial disease and critical limb ischemia in an insured national population. *Journal of Vascular Surgery* 2014 **60** 686-95.e2. (https://doi.org/10.1016/j.jvs.2014.03.290)

3 Hart O, Xue N, Davis-Havill B, Pottier M, Prakash M, Reimann S-A, King J, Xu W & Khashram M. The incidence of chronic limb-threatening ischemia in the midland region of New Zealand over a 12-year period. *Journal of Clinical Medicine* 2022 **11** 3303. (https://doi.org/10.3390/jcm11123303)

4 Norgren L, Hiatt WR, Dormandy JA, Nehler MR, Harris KA, Fowkes FGR & TASC II Working Group. Inter-society consensus for the management of peripheral arterial disease (TASC II). *Journal of Vascular Surgery* 2007 **45**(Supplement S) S5–S67. (https://doi.org/10.1016/j.jvs.2006.12.037)

5 Ezzatvar Y & García-Hermoso A. Global estimates of diabetes-related amputations incidence in 2010–2020: a systematic review and meta-analysis. *Diabetes Research and Clinical Practice* 2023 **195** 110194. (https://doi.org/10.1016/j.diabres.2022.110194)

6 Atherton R & Robertson N. Psychological adjustment to lower limb amputation amongst prosthesis users. *Disability and Rehabilitation* 2006 **28** 1201–1209. (https://doi.org/10.1080/09638280600551674)

7 Stewart CC, Berhaneselase E & Morshed S. The burden of patients with lower limb amputations in a community safety-net hospital. *Journal of the American Academy of Orthopaedic Surgeons* 2022 **30** e59–e66. (https://doi.org/10.5435/JAAOS-D-21-00293)

8 Peeters JAHM, Peters HAB, Videler AJ, Hamming JF, Schepers A & Quax PHA. Exploring the effects of human bone marrow-derived mononuclear cells on angiogenesis in vitro. *International Journal of Molecular Sciences* 2023 **24**. (https://doi.org/10.3390/ijms241813822)

9 Teraa M, Sprengers RW, Westerweel PE, Gremmels H, Goumans MJTH, Teerlink T, Moll FL, Verhaar MC & JUVENTAS study group. Bone marrow alterations and lower endothelial progenitor cell numbers in critical limb ischemia patients. *PLoS One* 2013 **8** e55592. (https://doi.org/10.1371/journal.pone.0055592)

10 Silvestre JS, Smadja DM & Lévy BI. Postischemic revascularization: from cellular and molecular mechanisms to clinical applications. *Physiological Reviews* 2013 **93** 1743–1802. (https://doi.org/10.1152/physrev.00006.2013)

11 Andtbacka RHI, Kaufman HL, Collichio F, Amatruda T, Senzer N, Chesney J, Delman KA, Spitler LE, Puzanov I, Agarwala SS, *et al.* Talimogene laherparepvec improves durable response rate in patients with advanced melanoma. *Journal of Clinical Oncology* 2015 **33** 2780–2788. (https://doi.org/10.1200/JCO.2014.58.3377)

12 Maude SL, Laetsch TW, Buechner J, Rives S, Boyer M, Bittencourt H, Bader P, Verneris MR, Stefanski HE, Myers GD, *et al.* Tisagenlecleucel in children and young adults with B-cell lymphoblastic leukemia. *New England Journal of Medicine* 2018 **378** 439–448. (https://doi.org/10.1056/NEJMoa1709866)

13 Papadouli I, Mueller-Berghaus J, Beuneu C, Ali S, Hofner B, Petavy F, Tzogani K, Miermont A, Norga K, Kholmanskikh O, *et al.* EMA review of axicabtagene ciloleucel (Yescarta) for the treatment of diffuse large B-cell lymphoma. *Oncologist* 2020 **25** 894–902. (https://doi.org/10.1634/theoncologist.2019-0646)

14 Day JW, Finkel RS, Chiriboga CA, Connolly AM, Crawford TO, Darras BT, Iannaccone ST, Kuntz NL, Peña LDM, Shieh PB, *et al.* Onasemnogene abeparvovec gene therapy for symptomatic infantile-onset spinal muscular atrophy in patients with two copies of SMN2 (STR1VE): an open-label, single-arm, multicentre, phase 3 trial. *Lancet. Neurology* 2021 **20** 284–293. (https://doi.org/10.1016/S1474-4422(21)00001-6)

15 Locatelli F, Thompson AA, Kwiatkowski JL, Porter JB, Thrasher AJ, Hongeng S, Sauer MG, Thuret I, Lal A, Algeri M, *et al.* Betibeglogene autotemcel gene therapy for non-β(0). *New England Journal of Medicine* 2022 **386** 415–427. (https://doi.org/10.1056/NEJMoa2113206)

16 Isner JM, Pieczek A, Schainfeld R, Blair R, Haley L, Asahara T, Rosenfield K, Razvi S, Walsh K & Symes JF. Clinical evidence of angiogenesis after arterial gene transfer of phVEGF165 in patient with ischaemic limb. *Lancet* 1996 **348** 370–374. (https://doi.org/10.1016/s0140-6736(96)03361-2)

17 Mäkinen K, Manninen H, Hedman M, Matsi P, Mussalo H, Alhava E & Ylä-Herttuala S. Increased vascularity detected by digital subtraction angiography after VEGF gene transfer to human lower limb artery: a randomized, placebo-controlled, double-blinded phase II study. *Molecular Therapy* 2002 **6** 127–133. (https://doi.org/10.1006/mthe.2002.0638)

18 Kusumanto YH, van Weel V, Mulder NH, Smit AJ, van den Dungen JJ, Hooymans JM, Sluiter WJ, Tio RA, Quax PH, Gans RO, *et al.* Treatment with intramuscular vascular endothelial growth factor gene compared with placebo for patients with diabetes mellitus and critical limb ischemia: a double-blind randomized trial. *Human Gene Therapy* 2006 **17** 683–691. (https://doi.org/10.1089/hum.2006.17.683)

19 Rajagopalan S, Mohler ER, 3rd, Lederman RJ, Mendelsohn FO, Saucedo JF, Goldman CK, Blebea J, Macko J, Kessler PD, Rasmussen HS, *et al.* Regional angiogenesis with vascular endothelial growth factor in peripheral arterial disease: a phase II randomized, double-blind, controlled study of adenoviral delivery of vascular endothelial growth factor 121 in patients with disabling intermittent claudication. *Circulation* 2003 **108** 1933–1938. (https://doi.org/10.1161/01.CIR.0000093398.16124.29)

20 Kaga T, Kawano H, Sakaguchi M, Nakazawa T, Taniyama Y & Morishita R. Hepatocyte growth factor stimulated angiogenesis without inflammation: differential actions between hepatocyte growth factor, vascular endothelial growth factor and basic fibroblast growth factor. *Vascular Pharmacology* 2012 **57** 3–9. (https://doi.org/10.1016/j.vph.2012.02.002)

21 Shigematsu H, Yasuda K, Iwai T, Sasajima T, Ishimaru S, Ohashi Y, Yamaguchi T, Ogihara T & Morishita R. Randomized, double-blind, placebo-controlled clinical trial of hepatocyte growth factor plasmid for critical limb ischemia. *Gene Therapy* 2010 **17** 1152–1161. (https://doi.org/10.1038/gt.2010.51)

22 Powell RJ, Goodney P, Mendelsohn FO, Moen EK, Annex BH & HGF-0205 Trial Investigators. Safety and efficacy of patient specific intramuscular injection of HGF plasmid gene therapy on limb perfusion and wound healing in patients with ischemic lower extremity ulceration: results of the HGF-0205 trial. *Journal of Vascular Surgery* 2010 **52** 1525–1530. (https://doi.org/10.1016/j.jvs.2010.07.044)

23 Gu Y, Cui S, Wang Q, Liu C, Jin B, Guo W, Liu C, Chu T, Shu C, Zhang F, *et al.* A randomized, double-blind, placebo-controlled phase II study of hepatocyte growth factor in the treatment of critical limb ischemia. *Molecular Therapy* 2019 **27** 2158–2165. (https://doi.org/10.1016/j.ymthe.2019.10.017)

24 Nikol S, Baumgartner I, Van Belle E, Diehm C, Visoná A, Capogrossi MC, Ferreira-Maldent N, Gallino A, Wyatt MG, Wijesinghe LD, *et al.* Therapeutic angiogenesis with intramuscular NV1FGF improves amputation-free survival in patients with critical limb ischemia. *Molecular Therapy* 2008 **16** 972–978. (https://doi.org/10.1038/mt.2008.33)

25 Belch J, Hiatt WR, Baumgartner I, Driver IV, Nikol S, Norgren L, Van Belle E & TAMARIS Committees and Investigators. Effect of fibroblast growth factor NV1FGF on amputation and death: a randomised placebo-controlled trial of gene therapy in critical limb ischaemia. *Lancet* 2011 **377** 1929–1937. (https://doi.org/10.1016/S0140-6736(11)60394-2)

26 Van Belle E, Nikol S, Norgren L, Baumgartner I, Driver V, Hiatt WR & Belch J. Insights on the role of diabetes and geographic variation in patients with critical limb ischaemia. *European Journal of Vascular and Endovascular Surgery* 2011 **42** 365–373. (https://doi.org/10.1016/j.ejvs.2011.04.030)

27 Sanada F, Fujikawa T, Shibata K, Taniyama Y, Rakugi H & Morishita R. Therapeutic angiogenesis using HGF plasmid. *Annals*

of Vascular Diseases 2020 **13** 109–115. (https://doi.org/10.3400/avd.ra.20-00035)

28 Barć P, Antkiewicz M, Śliwa B, Baczyńska D, Witkiewicz W & Skóra JP. Treatment of critical limb ischemia by pIRES/VEGF165/HGF administration. *Annals of Vascular Surgery* 2019 **60** 346–354. (https://doi.org/10.1016/j.avsg.2019.03.013)

29 Barć P, Antkiewicz M, Śliwa B, Frączkowska K, Guziński M, Dawiskiba T, Małodobra-Mazur M, Witkiewicz W, Kupczyńska D, Strzelec B, *et al.* Double VEGF/HGF gene therapy in critical limb ischemia complicated by diabetes mellitus. *Journal of Cardiovascular Translational Research* 2021 **14** 409–415. (https://doi.org/10.1007/s12265-020-10066-9)

30 Hammer A & Steiner S. Gene therapy for therapeutic angiogenesis in peripheral arterial disease - a systematic review and meta-analysis of randomized, controlled trials. *Vasa* 2013 **42** 331–339. (https://doi.org/10.1024/0301-1526/a000298)

31 Annex BH. Therapeutic angiogenesis for critical limb ischaemia. *Nature Reviews. Cardiology* 2013 **10** 387–396. (https://doi.org/10.1038/nrcardio.2013.70)

32 Forster R, Liew A, Bhattacharya V, Shaw J & Stansby G. Gene therapy for peripheral arterial disease. *Cochrane Database of Systematic Reviews* 2018 **10** CD012058. (https://doi.org/10.1002/14651858.CD012058.pub2)

33 Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G & Isner JM. Isolation of putative progenitor endothelial cells for angiogenesis. *Science* 1997 **275** 964–967. (https://doi.org/10.1126/science.275.5302.964)

34 Arai M, Misao Y, Nagai H, Kawasaki M, Nagashima K, Suzuki K, Tsuchiya K, Otsuka S, Uno Y, Takemura G, *et al.* Granulocyte colony-stimulating factor: a noninvasive regeneration therapy for treating atherosclerotic peripheral artery disease. *Circulation Journal* 2006 **70** 1093–1098. (https://doi.org/10.1253/circj.70.1093)

35 Tateishi-Yuyama E, Matsubara H, Murohara T, Ikeda U, Shintani S, Masaki H, Amano K, Kishimoto Y, Yoshimoto K, Akashi H, *et al.* Therapeutic angiogenesis for patients with limb ischaemia by autologous transplantation of bone-marrow cells: a pilot study and a randomised controlled trial. *Lancet* 2002 **360** 427–435. (https://doi.org/10.1016/S0140-6736(02)09670-8)

36 Losordo DW, Kibbe MR, Mendelsohn F, Marston W, Driver VR, Sharafuddin M, Teodorescu V, Wiechmann BN, Thompson C, Kraiss L, *et al.* A randomized, controlled pilot study of autologous CD34+ cell therapy for critical limb ischemia. *Circulation. Cardiovascular Interventions* 2012 **5** 821–830. (https://doi.org/10.1161/CIRCINTERVENTIONS.112.968321)

37 Shirbaghaee Z, Heidari Keshel S, Rasouli M, Valizadeh M, Hashemi Nazari SS, Hassani M & Soleimani M. Report of a phase 1 clinical trial for safety assessment of human placental mesenchymal stem cells therapy in patients with critical limb ischemia (CLI). *Stem Cell Research and Therapy* 2023 **14** 174. (https://doi.org/10.1186/s13287-023-03390-9)

38 Huerta CT, Voza FA, Ortiz YY, Liu ZJ & Velazquez OC. Mesenchymal stem cell-based therapy for non-healing wounds due to chronic limb-threatening ischemia: a review of preclinical and clinical studies. *Frontiers in Cardiovascular Medicine* 2023 **10** 1113982. (https://doi.org/10.3389/fcvm.2023.1113982)

39 Beltrán-Camacho L, Rojas-Torres M & Durán-Ruiz MC. Current status of angiogenic cell therapy and related strategies applied in critical limb ischemia. *International Journal of Molecular Sciences* 2021 **22**. (https://doi.org/10.3390/ijms22052335)

40 Idei N, Soga J, Hata T, Fujii Y, Fujimura N, Mikami S, Maruhashi T, Nishioka K, Hidaka T, Kihara Y, *et al.* Autologous bone-marrow mononuclear cell implantation reduces long-term major amputation risk in patients with critical limb ischemia: a comparison of atherosclerotic peripheral arterial disease and Buerger disease. *Circulation. Cardiovascular Interventions* 2011 **4** 15–25. (https://doi.org/10.1161/CIRCINTERVENTIONS.110.955724)

41 Teraa M, Sprengers RW, Schutgens REG, Slaper-Cortenbach ICM, van der Graaf Y, Algra A, van der Tweel I, Doevendans PA, Mali WPTM, Moll FL, *et al.* Effect of repetitive intra-arterial infusion of bone marrow mononuclear cells in patients with no-option limb ischemia: the randomized, double-blind, placebo-controlled rejuvenating endothelial progenitor cells via transcutaneous intra-arterial supplementation (JUVENTAS) trial. *Circulation* 2015 **131** 851–860. (https://doi.org/10.1161/CIRCULATIONAHA.114.012913)

42 Lindeman JHN, Zwaginga JJ, Kallenberg-Lantrua G, van Wissen RC, Schepers A, van Bockel HJ, Fibbe WE & Hamming JF. No clinical benefit of intramuscular delivery of bone marrow-derived mononuclear cells in nonreconstructable peripheral arterial disease: results of a phase-III randomized-controlled trial. *Annals of Surgery* 2018 **268** 756–761. (https://doi.org/10.1097/SLA.0000000000002896)

43 Walter DH, Krankenberg H, Balzer JO, Kalka C, Baumgartner I, Schlüter M, Tonn T, Seeger F, Dimmeler S, Lindhoff-Last E, *et al.* Intraarterial administration of bone marrow mononuclear cells in patients with critical limb ischemia: a randomized-start, placebo-controlled pilot trial (PROVASA). *Circulation. Cardiovascular Interventions* 2011 **4** 26–37. (https://doi.org/10.1161/CIRCINTERVENTIONS.110.958348)

44 Pignon B, Sevestre MA, Kanagaratnam L, Pernod G, Stephan D, Emmerich J, Clement C, Sarlon G, Boulon C, Tournois C, *et al.* Autologous bone marrow mononuclear cell implantation and its impact on the outcome of patients with critical limb ischemia - results of a randomized, double-blind, placebo-controlled trial. *Circulation Journal* 2017 **81** 1713–1720. (https://doi.org/10.1253/circj.CJ-17-0045)

45 Sharma S, Pandey NN, Sinha M, Kumar S, Jagia P, Gulati GS, Gond K, Mohanty S & Bhargava B. Randomized, double-blind, placebo-controlled trial to evaluate safety and therapeutic efficacy of angiogenesis induced by intraarterial autologous bone marrow-derived stem cells in patients with severe peripheral arterial disease. *Journal of Vascular and Interventional Radiology* 2021 **32** 157–163. (https://doi.org/10.1016/j.jvir.2020.09.003)

46 Malyar NM, Radtke S, Malyar K, Arjumand J, Horn PA, Kröger K, Freisinger E, Reinecke H, Giebel B & Brock FE. Autologous bone marrow mononuclear cell therapy improves symptoms in patients with end-stage peripheral arterial disease and reduces inflammation-associated parameters. *Cytotherapy* 2014 **16** 1270–1279. (https://doi.org/10.1016/j.jcyt.2014.05.001)

47 Rigato M, Monami M & Fadini GP. Autologous cell therapy for peripheral arterial disease: systematic review and meta-analysis of randomized, nonrandomized, and noncontrolled studies. *Circulation Research* 2017 **120** 1326–1340. (https://doi.org/10.1161/CIRCRESAHA.116.309045)

48 Gupta NK, Armstrong EJ & Parikh SA. The current state of stem cell therapy for peripheral artery disease. *Current Cardiology Reports* 2014 **16** 447. (https://doi.org/10.1007/s11886-013-0447-2)

49 Merkulova-Rainon T, Broquères-You D, Kubis N, Silvestre JS & Lévy BI. Towards the therapeutic use of vascular smooth muscle progenitor cells. *Cardiovascular Research* 2012 **95** 205–214. (https://doi.org/10.1093/cvr/cvs097)

50 Thej C, Ramadasse B, Walvekar A, Majumdar AS & Balasubramanian S. Development of a surrogate potency assay to determine the angiogenic activity of Stempeucel®, a pooled, ex-vivo expanded, allogeneic human bone marrow mesenchymal

stromal cell product. *Stem Cell Research and Therapy* 2017 **8** 47. (https://doi.org/10.1186/s13287-017-0488-3)

51 Ullah M, Liu DD & Thakor AS. Mesenchymal stromal cell homing: mechanisms and strategies for improvement. *iScience* 2019 **15** 421–438. (https://doi.org/10.1016/j.isci.2019.05.004)

52 De Becker A & Riet IV. Homing and migration of mesenchymal stromal cells: how to improve the efficacy of cell therapy? *World Journal of Stem Cells* 2016 **8** 73–87. (https://doi.org/10.4252/wjsc.v8.i3.73)

53 Søgaard R, Londero LS & Lindholt J. Geographical variation in the management of peripheral arterial occlusive disease: a nationwide danish cohort study. *European Journal of Vascular and Endovascular Surgery* 2022 **63** 72–79. (https://doi.org/10.1016/j.ejvs.2021.10.037)

54 Parcha V, Kalra R, Suri SS, Malla G, Wang TJ, Arora G & Arora P. Geographic variation in cardiovascular health among american adults. *Mayo Clinic Proceedings* 2021 **96** 1770–1781. (https://doi.org/10.1016/j.mayocp.2020.12.034)

55 Huang PP, Yang XF, Li SZ, Wen JC, Zhang Y & Han ZC. Randomised comparison of G-CSF-mobilized peripheral blood mononuclear cells versus bone marrow-mononuclear cells for the treatment of patients with lower limb arteriosclerosis obliterans. *Thrombosis and Haemostasis* 2007 **98** 1335–1342. (https://doi.org/10.1160/th07-02-0137)

56 Franz RW, Shah KJ, Pin RH, Hankins T, Hartman JF & Wright ML. Autologous bone marrow mononuclear cell implantation therapy is an effective limb salvage strategy for patients with severe peripheral arterial disease. *Journal of Vascular Surgery* 2015 **62** 673–680. (https://doi.org/10.1016/j.jvs.2015.02.059)

57 Song F, Loke Y & Hooper L. Why are medical and health-related studies not being published? A systematic review of reasons given by investigators. *PLoS One* 2014 **9** e110418. (https://doi.org/10.1371/journal.pone.0110418)

58 Jones CW, Handler L, Crowell KE, Keil LG, Weaver MA & Platts-Mills TF. Non-publication of large randomized clinical trials: cross sectional analysis. *BMJ* 2013 **347** f6104. (https://doi.org/10.1136/bmj.f6104)

59 Asahara T. Cell therapy and gene therapy using endothelial progenitor cells for vascular regeneration. *Handbook of Experimental Pharmacology* 2007 (180) 181–194. (https://doi.org/10.1007/978-3-540-68976-8_8)

60 Abunasra HJ, Smolenski RT, Morrison K, Yap J, Sheppard MN, O'Brien T, Suzuki K, Jayakumar J & Yacoub MH. Efficacy of adenoviral gene transfer with manganese superoxide dismutase and endothelial nitric oxide synthase in reducing ischemia and reperfusion injury. *European Journal of Cardio-Thoracic Surgery* 2001 **20** 153–158. (https://doi.org/10.1016/s1010-7940(01)00704-7)