Cellular therapies in no-option critical limb ischemia: present status and future directions

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Abstract

Critical limb ischemia – an advanced stage of lower extremity arterial disease with presence of rest pain and/or ischemic ulcers – remains an important cause of major amputations and disability in developed societies. Novel treatment strategies are urgently needed to prevent (or delay) amputations in particular for patients in whom effective revascularization is no longer feasible for anatomic and/or technical reasons (no-option critical limb ischemia – N-O CLI). Cellular therapies have been gaining the growing attention of researchers and clinicians in the last two decades. Several cell types have been used in pre-clinical and clinical studies, and a number of mechanisms have been proposed to contribute to vascular reparation and regeneration in N-O CLI. Although early trials suggested clinical improvement with use of cell-based therapies in N-O CLI, meta-analyses that included randomized controlled trials have not provided definitive conclusions. Fundamental limitations have involved poorly defined cell lines/populations, limited numbers of study participants and limited follow-up periods, and enrolling patients “too sick to benefit” (such as those in Rutherford class 6). Recent advances include standardized “unlimited” sources of therapeutic cells and better understanding of mechanisms that may contribute to vascular reparation and regeneration. Furthermore, based on recent pre-clinical and clinical studies, cell-free preparations (such as microvesicle-based) are being increasingly developed along with advanced therapy medical products consisting of engineered cells and pro-angiogenic factors.

Key words: critical limb ischemia, cell transplantation, stem cells,stromal cells, regenerative medicine, Wharton’s jelly mesenchymal stem cells.

Introduction

Critical limb ischemia (CLI) is an advanced stage of lower extremity arterial disease (LEAD) with the presence of ischemic rest pain and/or ischemic ulceration or gangrene (stages III–IV according to the Fontaine classification and 4–6 in Rutherford’s classification) [1, 2]. The incidence of CLI is approximately 220–1000 new cases per million population per year [2, 3]. It is estimated that 5% to 10% of all patients with LEAD will develop CLI [4]. Natural history of the condition is presently clouded by several forms of treatment, including revascularization attempts in the majority (50–90%) of patients [2, 4]. Despite pharmacologic management and (if/until feasible) revascularization, CLI is still inextricably associated with a high rate of major amputations and mortality. Within 1 year from diagnosis, 22% to 25% of patients die, 22% to 30% undergo major limb amputation, 20% remain alive but still present symptoms of CLI and only 25% of cases resolve [2, 4].

Endovascular and surgical revascularization combined with medical therapy remain the gold standard of treatment [2, 5], whose efficacy, however, is in many patients not sufficient to prevent limb loss. Patients with CLI in whom effective revascularization is not (or no lon-
The concept of vascular regeneration and fundamentals of stem cell regenerative therapies

Several different processes have been proposed to contribute to vascular reparation and regeneration of the ischemic tissue [8].

Angiogenesis is formation of new capillary networks from preexisting vessels. It is triggered by local tissue ischemia via the activity of hypoxia inducible factor-1α (HIF-1α) that induces production of vascular endothelial growth factor (VEGF) and other pro-angiogenic cytokines. Subsequently, endothelial cell migration, proliferation and luminogenesis lead to generation of new capillaries [8].

Vasculogenesis is de novo synthesis of new blood vessels by endothelial precursor cells (EPC). It was proved that vasculogenesis is not restricted to embryonic development, when Asahara et al. described circulating EPC that homed in ischemic tissue and were able to form new vessels [9].

Arteriogenesis is enlargement of previously existing collateral channels into functional arterioles to form a natural bypass in the ischemic limb [8, 10, 11]. When blood flow in a major artery is occluded (for instance by atherosclerotic plaque), flow to collaterals is increased. That induces their remodeling (triggered by recruited monocytes and macrophages mediating matrix reconstructing) and stabilization by supportive smooth muscle cells or pericytes.

The above processes of vascular proliferation and regeneration are governed by several lineages of stem and progenitor cells. Myeloid hematopoietic progenitor cells (HPCs) secrete cytokines to promote angiogenesis. Circulating and vessel-derived endothelial precursor cells (EPCs) form new vessels in vasculogenesis. Mesenchymal stem cells (MSCs) secrete chemokines to recruit accessory cells and differentiate into wrapping pericytes enabling arteriogenesis [11]. In summary, according to current knowledge, stem and/or progenitor cells contribute to vascular regeneration by physically integrating into the tissues, by secreting growth factors, or by both means [7, 12, 13]. Therefore regenerative strategies based on stem cells transplantation in patients with ischemic diseases seemed to be a promising research direction. The results of the first in-human trial concerning therapeutic angiogenesis induced by cell transplantation in patients with limb ischemia was published in 2002 [14]. To date, after two decades of pre-clinical and clinical trials evaluating different populations of stem and progenitor cells administered in a variety of routes, doses and protocols, definitive evidence to assess efficacy of these therapies is still lacking [5, 6, 15, 16]. Nevertheless, prior work has made significant contributions to understanding how therapeutic stem cells may co-ordinate the myriad of host cells and signals required for effective angiogenesis in the ischemic tissue.

Stem cell exhaustion and its impact on native vascular regeneration failure

Atherosclerosis and diabetes mellitus (frequently co-existing) lead to oxidative stress, chronic inflammation and glucotoxicity and thereby have a negative impact on vascular regeneration [11]. Stem cell exhaustion has been defined as acceleration of cellular aging in adult stem cells causing progenitor cell dysfunction including aberrant proliferation, differentiation, migration, mobilization and signaling [17]. The number and migratory function of EPC have been found to be reduced in patients with coronary disease, type 1 and 2 diabetes and metabolic syndrome [18]. Hill et al. reported a significant inverse correlation between the Framingham score and endothelial-progenitor-cell counts with higher scores associated with diminished counts [19]. These findings have at least two implications in patients with CLI, especially in the context of cellular regenerative therapies.

First, spontaneous angiogenic, vasculogenic, and arteriogenic mechanisms are severely compromised, or in some cases absent among patients with CLI in whom chronic arterial injury overwhelms the ability of EPC to maintain homeostasis [11, 20]. Secondly, trials using autologous cells to treat CLI may have transferred cells with impaired function. We believe that it is crucial to underline this aspect, because a vast majority of clinical trials to date have assessed autologous mononuclear stem cells [5, 6, 11, 15, 16, 21, 22]. Indeed, a recent review of 19 clinical and preclinical studies on cellular therapies in N-O CLI by Qadura et al. suggested that in the majority of trials to date the transferred autologous cells were affected by chronic disease and demonstrated poor survival in the ischemic environment as well as impaired function [11]. Therefore, recent attention has been directed to using allogenic cells derived from healthy donors (bone marrow, adipose tissue, umbilical blood, Wharton’s jelly of the umbilical cord). Research in this area is needed to determine whether progenitor cells less burdened by chronic morbidities would indeed be more effective in vascular regeneration [11]. Pilot work has assessed transplantation of allogenic MSCs derived from Wharton’s jelly (Wharton’s jelly mesenchymal stem cells – WJMSCs) to stimulate myocardial regeneration [23] and a similar pilot report concerns use of WJMSCs in N-O CLI [24].
Fundamental limitations in methodology of previous trials

In 2002, Tateishi-Yuyama et al. [14] published in The Lancet the results of the TACT trial investigating the efficacy of intramuscular injection of bone marrow mononuclear cells (BM-MNCs) and peripheral blood mononuclear cells (PB-MNCs) in patients with N-O CLI. Their paper remains a landmark article in this field and the majority of subsequent trials for nearly the two decades had similar inclusion criteria and end points and used similar cell populations. The most common inclusion criterion in past trials was CLI (Rutherford 4–6) with no option of further endovascular/surgical or hybrid revascularization. Only in 3 trials published to date did the follow-up period exceed 1 year, whereas in the vast majority of studies the patients were followed for 3–6 months [5, 6, 11]. The most common primary end point was major amputation and/or death. Other assessed outcomes were: ulceration healing, occurrence of new gangrene, transcutaneous oxygen pressure (TcO2), ankle-brachial index (ABI), pain-free walking distance (PFWD), and score of rest pain [5–7, 11, 15, 25, 26]. Nevertheless, convincing evidence for efficacy of cell-based therapeutic approaches in CLI patients is still lacking [5, 6, 11, 15, 26]. Developing more efficient regenerative strategies for CLI is likely to require novel cell sources, rectification of harvesting and cell conditioning methods, rectification of administration routes and doses and (perhaps) repeated administrations [5].

Cell populations in regenerative therapies in N-O CLI

We believe it is important to note that that many cell preparations used in clinical studies to date have not met the actual “stem cell” criteria such as those of the International Society of Cellular Therapy (Table I). In particular, surface antigens and morphological features that are typical for cell lineages more mature than those in “stem cells” have been present [27].

Bone marrow mononuclear cells and peripheral blood–derived MNCs

Bone marrow (BM-MNCs) and peripheral blood-derived MNCs (PB-MNCs) are the two cell populations most widely investigated for therapeutic angiogenesis [5, 6, 11, 15, 16, 21, 22]. Meta-analyses of cell therapies in CLI hardly provide any information on cell populations different than BM-MNCs and PB-MNCs [5, 6, 11]. Initial selection of BM-MNCs and PB-MNCs for regenerative strategies in CLI seemed natural for several reasons. First, autologous transplantation is free of the need for histocompatibility matching and post-procedural immunosuppression. However, early trials used predominantly unpurified, heterogeneous cell products with a low percentage of “active” cells with documented pro-angiogenic functions [11]. Because pro-angiogenic progenitor cells are rare in human bone marrow (approx. 1 pro-angiogenic HPC per 10,000 mononuclear cells), a large number of cells are required. Therefore, several MNC harvesting and purification methods have been developed. MNCs can be efficiently purified by CD34 or CD133 antigen expression and further harvested in expansion media (serum-free and xeno-free) under defined conditions [11]. More recently, automated systems and large-scale bio-reactors provide safe, effective and more cost-efficient expansion of lineage-restricted progenitor cells [28, 29]. Nevertheless, it is important to note that extended culture negatively impacts the regenerative function of cells. Further reselection of cells is therefore required to isolate cells with enhanced pro-vascular functions (based on higher aldehyde dehydrogenase activity correlated with cell immaturity) [30].

Another important issue related to the use of autologous BM-MNCs and PB-MNCs is that transplanted cells are affected by oxidative stress connected with advanced-stage vascular disease (stem cell exhaustion theory that was already discussed above). Bone marrow biopsy restricts cell numbers whereas G-CSF stimulated mobilization leads to harvests including cells of limited angiogenic potential [11]. Simultaneous transplantation of different cell populations may play an important role in developing future therapies [11].

Adipose-derived stem cells

Adipose-derived stem cells (ASCs) are plastic-adherent, multipotent cells isolated from adipose tissue [31, 32]. This cell population can be obtained from subcutaneous adipose tissue [32]. However, the ASC isolation process requires manipulation of large volumes of lipid-laden cells; thus several devices enabling automated cell isolation to make the process more efficient have been developed [32–35]. Interestingly, the surface immunophenotype of ASCs is >90% identical with human BM-MSCs [36]. Nevertheless, several potentially important differences in surface protein expression have also been reported. For instance, glycoprotein CD34, which is not present on MSCs, was found on human ASCs in early passages. Identification of ASC surface antigens provided a mechanism to enrich or purify the cell population from...
the heterogeneous stromal vascular fraction separated from fat tissue [32]. ASCs can differentiate into different cell lineages, including endothelial cells and smooth muscle cells that are crucial for angiogenesis [7]. In addition, the potential therapeutic effect of ASCs in ischemic diseases may rely on paracrine secretion. Rehman et al. found that ASCs promote angiogenesis by producing VEGF, HGF, and TGF-β, and that VEGF secretion increases fivefold when ASCs are cultured in hypoxic conditions [37]. As for BM-MSCs and PB-MSCs, chronic diseases – thromboangiitis obliterans (TAO, Burger’s disease) and diabetes – may impair pro-angiogenic function of ASCs. Lee et al. found that in a colony-forming unit assay, the stromal vascular fraction of TAO and diabetic patients yielded fewer colonies than that of healthy donors [38]. To date, several phase I/II clinical trials assessing ASC administration in patients with ischemic diseases including CLI have been attempted, but only a few have been completed and published [7].

Lee et al. enrolled fifteen patients with CLI lasting 6 months or longer (12 with TAO and 3 with diabetic foot) unsuitable for endovascular intervention or bypass operation. The patients were administered multiple intramuscular injections of autologous ASCs [38]. During follow-up (mean time 6 months) clinical improvement occurred in 66.7% of patients. Five patients required minor amputations during follow-up and all amputation sites healed completely. At 6 months, significant improvement was noted on pain rating scales and in claudication distance. Digital subtraction angiography suggested formation of numerous vascular collateral networks across affected arteries.

Another ASC study was conducted by Bura et al. [39], who included 7 patients with N-O CLI and, similar to Lee et al., intramuscular administration of autologous ASCs. An increase in trans-cutaneous oxygen pressure was reported in most patients, along with an improvement in ulcer healing [39]. In both studies no serious safety issues were reported.

A randomized, placebo-controlled, multi-center study with single-dose intramuscular administration of ASCs from healthy donors in diabetic N-O CLI patients is ongoing [40].

**Wharton’s jelly mesenchymal stem cells**

Due to several unique properties, MSCs may be more effective than other cell types for cardiovascular regeneration [41]. Wharton’s jelly mesenchymal stem cells (WJMSCs) seem to be particularly attractive for regenerative therapy in cardio-vascular diseases. First, WJMSCs express all surface antigens typical for MSCs, are easy to isolate (without invasive procedure as in the case of BM-MSCs) and harvest without ethical concerns [10, 22, 23, 41–43]. WJMSCs spontaneously secrete pro-angiogenic factors, such as vascular endothelial growth factor (VEGF), angiopoietin-1, transforming growth factor β1 (TGF-β1) and hepatocyte growth factor (HGF) [17]. Because WJMSCs do not express major histocompatibility complex class II (HLA-DR) antigens or surface antigens CD40, CD 80, CD86, they do not elicit an allogenic immune response or transplant rejection [10]. WJMSCs possess stemness properties that last several passages in vitro and are multipotent, but do not induce tumorigenesis, even though they have some embryonic stem cell markers [22, 42]. Furthermore, expansion of WJMSCs is not associated with loss of genetic stability, as these cells are not susceptible to spontaneous malignant transformation [25]. The above-mentioned features encourage efforts to create regenerative therapy for N-O CLI based on an “off-the-shelf” WJMSC product. Such attempts have already been made for myocardial regeneration after acute myocardial infarction, demonstrating feasibility and procedural safety [23]. The regenerative potential of WJMSC-derived advanced therapy medical products (ATMP) was recently demonstrated in an animal model of hindlimb ischemia [10]. A randomized placebo-controlled study in humans with N-O CLI is underway (NCT03423732).

**Exosomes and microvesicles containing pro-angiogenic mediators: a new direction in regenerative medicine**

A significant proportion of the benefits of stem and progenitor cell administration may arise from their paracrine secretion rather than proliferation and multi-differentiation [7]. Microvesicles/exosomes, plasma-membrane derived vesicles released from various cell types, may target distant sites with potent pro-angiogenic stimuli [11]. Pre-clinical studies demonstrated that human mesenchymal stem cells and CD34+ cell-derived exosomes improved limb perfusion and promoted angiogenesis [44–46]. Therapeutic application of microvesicle administration in CLI patients is drawing increasing interest but in-human data are lacking [7, 11].

**Cellular therapies in N-O CLI to date: overview of largest clinical trials and meta-analyses**

Table II summarizes controlled (i.e., including a placebo/sham group or a different therapeutic agent group) clinical trials in at least 20 patients, assessing cellular therapies in N-O CLI.

Several cell-therapy trials that deserve particular attention are briefly discussed below.

Rejuvenating Endothelial Progenitor Cells via Transcutaneous Intra-arterial Supplementation (JUVENTAS) [21] was a randomized, double blinded placebo-controlled trial with the largest number of enrolled individuals among the CLI randomized controlled trials (RCTs) published to date [5, 11, 21]. 160 patients with severe (rest pain and/or ulcers), nonrevascularizable limb ischemia were...
| Author/trial name | Study indication/patient population | No. of subjects | Type of cells | Administration route | Dose | Follow-up | Main outcome |
|------------------|------------------------------------|-----------------|---------------|---------------------|------|-----------|--------------|
| Tateishi-Yayuma et al. [14] TACT Trial | Rutherford 4–6, ABI < 0.6, bilateral leg ischemia | 22 (group B with randomization) | BM-MNCs vs. PB-MNCs | IM, 40 injections | 10⁶ BM-MNCs vs. PB-MNCs | 6 months | Improvement in ABI, TcO₂, pain reduction after BM-MNC administration |
| Powell et al. [22] RESTORE-CLI | Rutherford 4–6, toe syst. Pressure < 50 mm Hg, ankle syst. or Pressure < 70 mm Hg | 72 (42 – kmyoecyt-T, 24 – placebo) | kmyoecyt-T (autologous, cultured BM-MSCs and HPCs) | IM, 20 injections | 35-295 × 10⁶ BM-MSCs and HPCs | 12 months | Significant reduction of mortality and gangrene. Non-significant reduction of amputation rate |
| Teraa et al. [21] JUVENTAS | Rutherford 4–6 | 160 (81 – BM-MNCs, 79 – placebo) | BM-MNCs | IA infusion (repetitive 3 times in 3-week intervals) | 2 × 10⁶ BM-MNCs | 6 months | No differences in mortality and amputation rates |
| Procházka et al. [47] | CLI with foot ulcers | 96 (42 – BM stem cells, 54 – standard care) | BM-MNCs (poor characterization of cell product) | IM, 40 injections | 40 ml of product containing 0.49 ± 0.05 × 10⁷/1 CD34+ cells | 4 months | Significant reduction of amputation rate |
| Barć et al. [54] | N-O CLI (> 12 weeks of rest pain and/or ulcer), ABI < 0.5 | 29 (14 – BM-MNCs, 15 – standard care) | BM-MNC | IM (1 cycle, 4–12 injections) + IA (1 infusion via angiography catheter, 30–50 ml) | Unspecified | 6 months | No difference in ABI, 7/15 amputations in control, 3/14 amputations in BM-MNCs vs. control |
| Matoba et al. [55] | Rutherford 4–6 | 74 | BM-MNCs vs. PB-MNCs | IM, 40 injections | 10⁶-MNCs | Up to 3 years | Improved rest pain and ulcer healing in BM-MNC group, no significant change in ABI and TcPO₂, amputations not reported |
| Benoit et al. [50] | Rutherford 4–5 | 48 (34 – BM-MNCs, 14 – blood as placebo) | BM-MNCs | IM (1 cycle, 40 injections) | 10⁷BM-MNCs | 6 months | Reduced amputation rate in BM-MNC group (not statistically significant) |
| Li et al. [56] | CLI – rest pain, ABI < 0.6 | 58 (29 – BM-MNCs, 29 – placebo) | BM-MNCs | IM, 1 cycle, multiple injections, 0.5 ml each | 1 × 10⁷/ml | 6 months | Improvement in rest pain, ulcer healing and ABI in BM-MNC group, insignificant difference in amputation rate |
| Walter et al. [49] | NO CLI – Rutherford 4–6 | 40 (21 – BM-MNCs, 19 – placebo) | BM-MNCs vs. placebo in randomized start phase, BM-MNCs in all patients in open label phase | IA (1 administration, catheter based) | 10⁶ BM-MNCs per single dose | 6 months | Significantly improved ulcer healing and pain reduction in BM-MNC group, no difference in amputation rate. ABI not increased with BM-MNCs |
| Van Tongeren et al. [57] | NO CLI, Rutherford 4–6 | 27 (12 – IA + IM administration, 15 – sole IM) | BM-MNCs | IM (1 cycle, 40 injections) or IM + IA (20 ml infusion via angiographic catheter) | 1.23 ± 0.49 × 10⁶ bone marrow cells | 12 months | Statistically insignificant difference in amputation rate between IA + IM vs. IM groups, significant increase of ABI, PFWD and pain reduce |
| Huang et al. [58] | Diabetic patients with CLI, Rutherford 4–5 | 28 (14 – PB-MNC, 14 – placebo) | G-CSF mPB-MNC | IM (2 cycles, 40 injections each) | 10⁷ G-CSF mPB-MNCs | 3 months | Significant improvement in ulcer healing, pain reduction, blood perfusion of lower limbs in PB-MNC group |
| Author/trial name | Study indication/patient population | No. of subjects | Type of cells | Administration route | Dose | Follow-up | Main outcome |
|-------------------|-----------------------------------|----------------|--------------|---------------------|------|-----------|-------------|
| Huang et al. [59]  | Diabetic patients with CLI and foot ulcers | 150/76 | G-CSF mPB-MNCs vs. BM-MNCs | IM (2 cycles, 40 injections each) | 10 G-CSF mPB-MNCs vs. BM-MNCs | 3 months | Pain reduction, ABI increase, in both groups, IM (2 cycles, 109 G-CSF mPB-MNCs vs. 108 BM-MNCs). CLI, Rutherford 4–6 150 (76 – G-CSF vs. 108 BM-MNCs). No significant difference in amputation rate between G-CSF mPB-MNCs vs. BM-MNCs. No significant difference in pain score in MNC vs. BM-MNC groups. Significant increase of ABI and TcPO2 in G-CSF mPB-MNC group (at 6 weeks) vs. BM-MNCs. No significant difference in amputation rate. Significantly lower PFWD, higher ABI and TcPO2 in BM-MNC group vs. BM-MNCs. No significant difference in amputation rate. Ulcer healing rate significantly higher in BM-MNC group vs. BM-MNCs, BM-MNCs vs. standard care. No significant difference in amputation rate. |
| Ozturk et al. [60]  | Diabetic patients with bilateral CLI | 41/20-BM-MNCs, 21-BM-MCNs | BM-MSCs vs. BM-MNCs | IM (1 cycle, ~20 injections) | BM-MSCs/BM-MNCs 9.3 ±1.1 × 10^8 vs. BM-MNCs 9.6 ±1.1 × 10^8 | 6 months | Ulcer healing rate significantly higher in BM-MNC group vs. BM-MNCs. Major amputation rate at 6 months (19% in BM-MNCs vs. 13% in placebo group; relative risk 1.46; 95% CI: 0.62–3.42). Similarly, the rate all-cause mortality or hospitalization due to infection did not statistically significantly differ between BM-MNC and placebo groups. Secondary outcomes (rest pain, quality of life, ankle-brachial index, transcutaneous oxygen pressure) improved during follow-up in both groups, with no significant differences between the groups [21]. In essence, JUVENTAS provided level-1 data that intra-arterial infusion of BM-MNCs is unlikely to affect the course of CLI. |
| Lu et al. [61]  | Diabetic patients with CLI and foot ulcers | 20 (10 – BM MSCs, 10 – placebo) | BM-MSCs | IM | BM-MSCs/BM-MNCs 2 × 10^6 per kilo | 6 months | Significant increase of ABI in cell groups vs. placebo. No difference in amputations. |
| Gupta et al. [62]  | Giant cell arteritis with bilateral CLI, at least 1 foot ulcer | 20 locations in the lower thigh, calf and foot. Follow-up was 12 months. Efficacy assessment included time to first occurrence of treatment failure (TTF, including major amputation, all-cause mortality, doubling of total wound surface area from baseline, de novo gangrene) and amputation-free survival (AFS). AFS was defined as the number of days from injection of ixmyelocel-T to the first trial day on which a major amputation of the injected leg or death occurred. Major amputation was defined as an amputation at or above the talus. TTF was significantly extended for patients treated with the cellular product when compared with controls (p = 0.0032). Furthermore, there was non-significantly higher amputation-free survival in the ixmyelocel-T treated patients than in the placebo group. In addition, the treatment effect for both TTF and AFS was more pronounced in patients who entered the trial with baseline wounds. No major safety issues related to ixmyelocel-T treatment were reported. |
each individual). No detailed characteristics of the bone marrow concentrate used were provided other than that it contained CD34+ cells (0.49 ± 0.05 × 10^9/l), platelets, white blood cells, lymphocytes, monocytes and neutrophils. The bone marrow concentrate was injected along the posterior and anterior tibial arteries of the ischemic limb. Patients in group II (n = 54) received standard medical care. The frequency of major limb amputation in groups I and II was 21% vs. 44% respectively (p < 0.05). In this trial, intra-muscular administration of bone marrow concentrate in patients with ischemic foot ulcers appeared to improve limb salvage [47]. The two important weak points of this trial seem to be a relatively short follow-up (4 months) and a poor characterization of bone marrow concentrate. (NB. The study indicates use of “autologous bone marrow stem cells.”)

Table III provides a list of meta-analyses and systematic reviews of studies and trials using stem and progenitor cells in N-O CLI.

Abdul Wahid et al. provided a review of N-O CLI therapies based on autologous cells derived from different sources and administered using different regimens [5]. They included seven RCTs with a total of 359 participants. Not only did the authors try to assess “global” efficacy of cellular therapies in N-O CLI but they also compared bone marrow mononuclear cells (BM-MNCs) vs. mobilized peripheral blood stem cells (mPBSCs), BM-MNCs vs. bone marrow-mesenchymal stem cells (BM-MSCs), high versus low doses, and routes of product administration – intraarterial (IA) vs. intramuscular (IM). Overall, no clear differences between different stem cell sources and treatment regimens were found for the outcomes all-cause mortality, amputation rate, ulcer healing, and rest pain (with mostly low and very low quality of evidence). Similarly, no clear difference in efficacy was observed between IA and IM administration. No significant short-term adverse effects were reported. As a general conclusion, the authors stated that high-quality evidence

Table III. Summary of meta-analyses and reviews of trials assessing cellular therapies in N-O CLI

| Author          | Study type and number of included trials and studies | Objective Assessed cell population | Main findings, conclusions, limitations |
|-----------------|------------------------------------------------------|-----------------------------------|------------------------------------------|
| Abdul Wahid et al. [5] | Review. 7 RCTs with a total of 359 participants. | Comparison of different autologous cell sources, routes of administration and doses for NO CLI patients. BM-MNCs, mPBSCs, BM-MSCs. | Mostly low and very low-quality evidence. No difference in amputation rate, pain reduction, ulcer healing, TcPO2, and between IM and IA administration and cell doses. Improved ulcer healing in BM-MSC group vs. BM-MNC (moderate-quality evidence). ABI higher in BM-MSC vs. BM-MNC (low-quality evidence). |
| Gao et al. [6]   | Systematic review and meta-analysis. 27 RCTs with a total of 1186 included. | Evaluation of efficacy and safety of autologous cell transplantation in patients with CLI. BM-MSCs, BM-MNCs, PBSCs. | Low quality of evidence. Majority of studies showed high risk of bias. Improved ulcer healing in stem cell group vs. conventional therapy. Significant improvement in ABI and PFWD. Significant reduction of amputation rate and rest pain scores. No serious side effects related to stem cells reported. |
| Rigato et al. [15] | Systematic review and meta-analysis. 19 RCTs (837 patients), 7 non-randomized trials (338 patients), 41 uncontrolled trials (1177 patients). | Evaluation of efficacy and safety of autologous cell transplantation in patients with PAD. Primary outcome assessed: major amputation. BM-MNCs, BM-MSCs, PB-MNCs. | High heterogeneity of studies, risk of bias. Primary analysis (all RCTs) showed risk of amputation reduced by 37%, improved amputation-free survival by 38% and improved wound healing by 59% in cell therapy group. Efficacy of cell therapy on all end points was no longer significant in placebo-controlled RCTs and disappeared when only RCTs with low risk of bias were analyzed. |
| Zhao et al. [7] | Review. 27 phase I/II trials on ASCs in therapeutic angiogenesis for ischemic diseases (11 in CLI). | Evaluation of efficacy and safety of ASCs based therapy in patients with ischemic diseases. ASCs. | Improved rest pain scores and collateral vessel formation. |
| Moazami et al. [26] | Review. 2 RCTs with a total of 57 patients. | Evaluation of efficacy and safety of local intramuscular transplantation of autologous adult BM-MNCs as a treatment for CLI. BM-MSCs. | Moderate quality of evidence. Insufficient evidence to support cell-based therapies for CLI. |

ABI – ankle-brachial index, ASCs – adipose-derived stem cells, BM-MNCs – bone marrow mononuclear cells, BM-MSCs – bone marrow mesenchymal stromal cells, IA – intra-arterial, IM – intramuscular, mPBSCs – mobilized peripheral blood stem cells, N-O CLI – no option critical limb ischemia, PB-MNC – peripheral blood mononuclear cell, PAD – peripheral artery disease, PFWD – pain-free walking distance, RCTs – randomized controlled trials, TcPO2 – transcutaneous oxygen pressure.
is lacking to confirm the efficacy and long-term safety of autologous cell transplantation in N-O CLI.

An integrated review of pre-clinical and clinical studies by Qadura et al. [11] focused on mechanisms of vascular regeneration and the role that stem and progenitor cells play in these processes. Furthermore, 15 RCTs using cellular therapy for CLI were reviewed. This work concluded that, despite promising preclinical studies in animal models, transplantation of bone marrow derived cells in N-O CLI patients shows limited benefits.

Rigato et al. [15] published a systematic review and meta-analysis of studies on autologous cell therapy for LEAD. They included 19 randomized controlled trials (837 patients), 7 nonrandomized trials (338 patients) and 41 non-controlled studies (1,177 patients). Primary analysis of the randomized controlled trials (n = 19) indicated that “cell therapy” may reduce the risk of amputation (by ~40%), improve amputation-free survival (by ~20%), and improve wound healing (in ~60% active-treatment patients), but it did not affect mortality. Among secondary end-points, “cell therapy” improved ABI, reduced pain score and increased transcutaneous oxygen pressure (TcO2). However, in sub-analysis including RCTs with a low risk of bias (n = 11), “cell therapy” was not associated with significant improvements in amputation rate, amputation-free survival and wound healing. This important observation suggests that trials lacking randomization or blinding were strongly and systematically biased in favor of cell therapy. A fundamental conclusion from this work is that high-quality RCTs are needed.

A meta-analysis published by Gao et al. in 2019 that included 27 RCTs (with a total of 1186 patients) showed that autologous stem cell therapy was more effective than conventional therapy as regards the ulcer healing rate, and significantly improved ABI, TcO2, and pain-free walking distance [6]. While significant reductions were observed in the general amputation rate and rest pain scores, no significant improvement in major limb salvage was reported. The authors underlined that the quality of evidence for all outcomes was low. As in all above-mentioned reviews and meta-analyses, the researchers concluded that NO-CLI patients “may benefit from stem cell therapy” but larger, randomized, double-blinded, placebo-controlled, multicenter trials with long term follow-up are still needed.

**Safety aspects of cellular therapies in CLI**

Most studies published to date indicate a good safety profile of cell-based therapies in CLI [6, 15, 21, 22, 25]. Fever has been reported after administration of stem or progenitor cells (both autologous and allogenic) [23, 25]. A transient, responsive to paracetamol, temperature rise was also reported following WJMSC administration [23]. No association between MSC injection and acute infu-

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**Lessons for patient selection in future trials**

Patients affected with CLI, and especially those unsuitable for further revascularization procedures (N-O CLI), are characterized by a high index of comorbidities including diabetes, coronary artery disease, and chronic cardiac failure. 25% of patients diagnosed with CLI die within 12 months from CLI diagnosis, and an additional 30% will undergo major limb amputation [2, 4]. Thus, recruitment of patients with CLI to clinical trials and sustaining them for follow-up are challenging, even though morbidity from peripheral artery disease is high and is increasing [48]. As a consequence, the evidence level on regenerative strategies in CLI remains low. Furthermore, some physicians may be reluctant to refer patients with advanced limb ischemia to trials, believing that trial participation will only slightly delay the “inevitable” major amputation. Indeed, published data may be perceived as providing some substance for that concept. Overall, cell therapies seem more likely beneficial in patients without extensive gangrene but are probably inefficient in individuals who have crossed the “point of no return” (Rutherford 6 class) [49, 50].

Most studies concerning regenerative therapies in CLI have assessed atherosclerosis-related ischemia. There are very limited data on stem-cell therapies in limb ischemia of another etiology – particularly TAO [7, 51–53]. Including patients at LEAD stages less advanced than CLI seems important for future studies. Patients with less advanced consequences of atherosclerotic tissue damage may be more responsive to regenerative strategies.

**Conclusions**

Pre-clinical studies have indicated that several lines of stem/progenitor cells may partly reverse lower-limb ischemic injury through different mechanisms. In patients with N-O CLI, cell-based therapies have been shown to be generally safe (including allogenic cell use). While large-scale trials are lacking, meta-analyses of studies including ~20–80 patients subjected to cell therapies suggest potential benefits of some cell lines. Analysis of the data available today indicates that patients with severe ischemic tissue loss (such as Rutherford 6) may be “too sick to benefit” from the therapy, clouding potential therapeutic effects in clinical studies. New protocols should attempt to enroll patients without excessive necrosis. Furthermore, using repeated administrations of the therapeutic agent(s) and combined delivery routes (such as intra-muscular and intra-arterial) should be considered.

Further directions include use of “unlimited” (mostly allogenic) cell sources, cell-free preparations (such as microvesicles), engineered cells and mixtures of cells and pro-angiogenic factors. Clinical trials should be scientifically rigorous, including double blinding, external con-
Cellular therapies in no-option critical limb ischemia: present status and future directions

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Conflict of interest
The authors declare no conflict of interest.

References
1. Mills JL, Conte MS, Armstrong DG, et al. The society for vascular surgery lower extremity threatened limb classification system: risk stratification based on wound, ischemia, and foot infection (WIfI). J Vasc Surg 2014; 59: 220-34.
2. Norgren L, Hiatt WR, Dormandy JA, et al. TASC II Working Group. Inter-Society consensus for the management of peripheral arterial disease (TASC II). J Vasc Surg 2007; 45 Suppl S: S5-67.
3. Rothwell PM, Eliasziw M, Gutnikov SA, et al.; Carotid Endarterectomy Trialists Collaboration. Endarterectomy for symptomatic carotid stenosis in relation to clinical subgroups and timing of surgery. Lancet 2004; 20: 915-24.
4. Abu Dabrh AM, Steffen MW, Undavalli C, et al. The natural history of untreated severe or critical limb ischemia. J Vasc Surg 2015; 62: 1642-51.
5. Abdul Wahid SF, Ismail NA, Wan Jamaludin WF, et al. Autologous cells derived from different sources and administered using different regimens for 'no-option' critical lower limb ischaemia patients. Cochrane Database Syst Rev 2018; 29: CD010747.
6. Gao W, Chen D, Liu G, Ran X. Autologous stem cell therapy for peripheral arterial disease: a systematic review and meta-analysis of randomized controlled trials. Stem Cell Res Ther 2019; 21: 140.
7. Zhao L, Johnson T, Liu D. Therapeutic angiogenesis of adipose-derived stem cells for ischemic diseases. Stem Cell Res Ther 2017; 5: 125.
8. Semenza GL. Vasculogenesis, angiogenesis, and arteriogenesis: mechanisms of blood vessel formation and remodeling. J Cell Biochem 2007; 102: 840-7.
9. Ashara T, Murohara T, Sullivan A, et al. Isolation of putative progenitor endothelial cells for angiogenesis. Science 1997; 275: 964-7.
10. Musial-Wysoccka A, Kot M, Sulkowski M, Majka M. Regenerative potential of the product "CardioCell" derived from the wharton's jelly mesenchymal stem cells for treating hindlimb ischemia. Int J Mol Sci 2019; 20: 4632.
11. Norgren L, Hiatt WR, Dormandy JA, et al. TASC II Working Group. Inter-Society consensus for the management of peripheral arterial disease (TASC II). J Vasc Surg 2007; 45 Suppl S: S5-67.
12. Rothwell PM, Eliasziw M, Gutnikov SA, et al.; Carotid Endarterectomy Trialists Collaboration. Endarterectomy for symptomatic carotid stenosis in relation to clinical subgroups and timing of surgery. Lancet 2004; 20: 915-24.
13. Swamynathan P, Venugopal P, Kannan S, et al. Are serum-free and xeno-free culture conditions ideal for large scale clinical grade expansion of Wharton’s jelly derived mesenchymal stem cells? A comparative study. Stem Cell Res Ther 2014; 5: 88.
14. Vasa M, Fichtlscherer S, Aicher A, et al. Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. Circ Res 2001; 89: E1-7.
15. Hill JM, Zalos G, Halcox JP, et al. Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. N Engl J Med 2003; 348: 593-600.
16. Perin EC, Murphy MP, March KL, et al. Evaluation of cell therapy on exercise performance and limb perfusion in peripheral artery disease: the CCTRN PACE Trial (Patients With Intermittent Claudication Injected With ALDH Bright Cells). Circulation 2017; 135: 1417-28.
17. Rigato M, Monami M, Fadini GP. Autologous cell therapy for peripheral arterial disease: systematic review and meta-analysis of randomized, nonrandomized, and noncontrolled studies. Circ Res 2017; 120: 1326-340.
18. Powell RJ, Marston WA, Berceli SA, et al. Cellular therapy with Ixmyelocel-T to treat critical limb ischemia: the randomized, double-blind, placebo-controlled Rejuvenating Endothelial Progenitor Cells via Transcutaneous Intra-arterial Supplementation (JUVENTAS) trial. Circulation 2015; 131: 851-60.
19. Hill JM, Zalos G, Halcox JP, et al. Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. N Engl J Med 2003; 348: 593-600.
20. Goldschmidt-Clermont PJ, Soo DM, Wang L, et al. Inflammation, stem cells and atherosclerosis genetics. Curr Opin Mol Ther 2010; 12: 712-23.
21. Teraa M, Sprengers RW, Schutgens RE, 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: 851-60.
22. Powell RJ, Marston WA, Berceli SA, et al. Cellular therapy with Ixmyelocel-T to treat critical limb ischemia: the randomized, double-blind, placebo-controlled RESTORE-CLI trial. Mol Ther 2012; 20: 1280-6.
23. Musialek P, Mazurek A, Jarocha D, et al. Myocardial regeneration strategy using Wharton’s jelly mesenchymal stem cells as an off-the-shelf ‘unlimited’ therapeutic agent: results from the Acute Myocardial Infarction First-in-Man Study. Adv Interv Cardiol 2015; 11: 100-7.
24. Kwiatkowski T, Zbierska-Rubinkiewicz K, Krzywoń JW, Majka M. Combined intra-arterial and intra-muscular transfer of Wharton’s jelly mesenchymal stem/stromal cells in no-option critical limb ischemia — the CIRCULATE N-O CLI Pilot Study. Adv Interv Cardiol 2015; 11: 100-7.
25. Tang Q, Chen Q, Lai X, et al. Malignant transformation potentials of human umbilical cord mesenchymal stem cells both spontaneously and via 3-methycholanthrene induction. PLoS One 2013; 8: e81844.
26. Moazzami K, Moazzami B, Roohi A, et al. Local intramuscular transplantation of autologous mononuclear cells for critical lower limb ischaemia. Cochrane Database Syst Rev 2014; 2014: CD008347.
27. Dominici M, Le Blanc K, Mueller I, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 2006; 8: 315-7.
28. Csaaszar E, Kirouac DC, Yu M, et al. Rapid expansion of human hematopoietic stem cells by automated control of inhibitory feedback signaling. Cell Stem Cell 2012; 10: 218-29.
29. Kirouac DC, Zandstra PW. The systematic production of cells for cell therapies. Cell Stem Cell 2008; 3: 369-81.
30. Sherman SE, Kuljanin M, Cooper TT, et al. High aldehyde dehydrogenase activity identifies a subset of human mesenchymal stromal cells with vascular regenerative potential. Stem Cells 2017; 35: 1542-53.

31. Si Z, Wang X, Sun C, Kang Y, et al. Adipose-derived stem cells: sources, potency, and implications for regenerative therapies. Biomed Pharmacother 2019; 114: 108765.

32. Gimble JM, Katz AJ, Bunnell BA. Adipose-derived stem cells for regenerative medicine. Circ Res 2007; 100: 1249-60.

33. Katz AJ, Hedrick MH, Lulli R, Futrell JW. A novel device for the simple and efficient refinement of liposuctioned tissue. Plast Reconstr Surg 2001; 107: 595-7.

34. Cleveland EC, Albano NJ, Hazen A. Roll, spin, wash, or filter? processing of liposapirate for autologous fat grafting: an updated, evidence-based review of the literature. Plast Reconstr Surg 2015; 136: 706-13.

35. Zielins ER, Brett EA, Longaker MT, Wan DC. Autologous fat grafting: the science behind the surgery. Aesthet Surg J 2016; 36: 488-96.

36. Zuk PA, Zhu M, Ashjian P, et al. Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell 2002; 13: 4279-95.

37. Rehman J, Traktuev D, Li J, et al. Secretion of angiogenic and antiapoptotic factors by human adipose stromal cells. Circulation 2004; 109: 1292-8.

38. Lee HC, An SG, Lee HW, et al. Safety and effect of adipose tissue-derived stem cell implantation in patients with critical limb ischemia: a pilot study. Circ J 2012; 76: 1750-60.

39. Bura A, Planat-Beauregard V, Bourin P, et al. Phase I trial: the use of autologous cultured adipose-derived stroma/stem cells to treat patients with non-revascularizable critical limb ischemia. Cytotherapy 2014; 16: 245-57.

40. Soria-Juan B, Garcia-Arranz M, Llanos Jiménez L, et al. Efficacy and safety of intramuscular administration of allogeneic adipose tissue derived and expanded mesenchymal stromal cells in diabetic patients with critical limb ischemia with no possibility of revascularization: study protocol for a randomized controlled double-blind phase II clinical trial (The NOMA Trial). Trials 2021; 22: 595.

41. Majka M, Sulikowski M, Badyra B, Musialek P. Concise review: mesenchymal stem cells in cardiovascular regeneration: emerging research directions and clinical applications. Stem Cells Transl Med 2017; 6: 1859-67.

42. Musial-Wysocza A, Kot M, Majka M. The pros and cons of mesenchymal stem-cell-based therapies. Cell Transplant 2019; 28: 801-12.

43. Bongso A, Fong CY. The therapeutic potential, challenges and future clinical directions of stem cells from the Wharton’s jelly of the human umbilical cord. Stem Cell Rev Rep 2013; 9: 226-40.

44. Mathiyalagan P, Liang Y, Kim D, et al. Angiogenic mechanisms of human CD34+ stem cell exosomes in the repair of ischemic hindlimb. Circ Res 2017; 120: 1466-76.

45. Ranghino A, Cantaluppi V, Grange C, et al. Endothelial progenitor cell-derived microvesicles improve neovascularization in a murine model of hindlimb ischemia. Int J Immunopathol Pharmacol 2012; 25: 75-85.

46. Kang T, Jones TM, Naddell C, et al. Adipose-derived stem cells induce angiogenesis via microvesicle transport of miRNA-31. Stem Cells Transl Med 2016; 5: 440-50.

47. Procházková V, Gumenec J, Jalůvka F, et al. Cell therapy, a new standard in management of chronic critical limb ischemia and foot ulcer. Cell Transplant 2010; 19: 1413-24.

48. Criqui MH, Aboyans V. Epidemiology of peripheral artery disease. Circ Res 2015; 116: 1509-26.

49. Walter DH, Krankenberg H, Balzer JO, et al. Intraarterial administration of bone marrow mononuclear cells in patients with critical limb ischemia: a randomized-start, placebo-controlled pilot trial (PROVASC). Circ Cardiovasc Interv 2011; 4: 26-37.

50. Benoit E, O’Donnell TF Jr, Ifarrat MD, et al. The role of amputation as an outcome measure in cellular therapy for critical limb ischemia: implications for clinical trial design. J Transl Med 2011; 9: 165.

51. Cacione DG, do Carmo Novaes F, Moreno DH. Stem cell therapy for treatment of thromboangiitis obliterans (Buerger’s disease). Cochrane Database Syst Rev 2018; 10: CD012794.

52. Szabó GV, Kövesd Z, Cseresnjev J, et al. Peripheral blood-derived autologous stem cell therapy for treatment of patients with late-stage peripheral artery disease-results of the short- and long-term follow-up. Cytotherapy 2013; 15: 1245-52.

53. Lee KB, Kang ES, Kim AK, et al. Stem cell therapy in patients with thromboangiitis obliterans: assessment of the long-term clinical outcome and analysis of the prognostic factors. Int J Stem Cells 2011; 4: 88-98.

54. Bart R, Skóra J, Pupka A, et al. Bone-marrow cells in therapy of critical limb ischaemia of lower extremities – own experience. Acta Angiologica 2006; 12: 155-66.

55. Matoba S, Satsumi T, Murohara T, et al. Long-term clinical outcome after intramuscular implantation of bone marrow mononuclear cells (Therapeutic Angiogenesis by Cell Transplantation [TACT] trial) in patients with chronic limb ischemia. Am Heart J 2008; 156: 1010-8.

56. Li M, Zhou H, Jin X, et al. Autologous bone marrow mononuclear cells transplant in patients with critical leg ischemia: preliminary clinical results. Exp Clin Transplant 2013; 11: 435-9.

57. Van Tongeren RB, Hamming H, Fibbe WE, et al. Intramuscular or combined intramuscular/intra-arterial administration of bone marrow mononuclear cells: a clinical trial in patients with advanced limb ischemia. J Cardiovasc Surg 2008; 49: 51-8.

58. Huang P, Li S, Han M, et al. Autologous transplantation of granulocyte colony-stimulating factor-mobilized peripheral blood mononuclear cells improves critical limb ischemia in diabetes. Diabetes Care 2005; 28: 2155-60.

59. Huang PP, Yang XF, Li SZ, et al. 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. Thromb Haemost 2007; 98: 1335-42.

60. Ozturk A, Kucukkardali Y, Tangi F, et al. Therapeutic potential of autologous peripheral blood mononuclear cell transplantation in patients with type 2 diabetic critical limb ischemia. J Diabetes Complications 2011; 26: 29-33.

61. Lu D, Chen B, Liang Z, et al. Comparison of bone marrow mesenchymal stem cells with bone marrow-derived mononuclear cells for treatment of diabetic critical limb ischemia and foot ulcer: a double-blind, randomized, controlled trial. Diabetes Res Clin Pract 2011; 92: 26-36.

62. Gupta PK, Chullikana A, Parakh R, et al. A double blind randomized placebo controlled phase I/II study assessing the safety and efficacy of allogeneic bone marrow derived mesenchymal stem cell in critical limb ischemia. J Transl Med 2013; 11: 143.