Review paper

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-

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ger) possible and best medical therapy is inefficient are defined as no-option CLI (N-O CLI) patients. In this group, novel strategies to promote vascular regeneration or neovascularization are critically needed [5–7]. Stem cell-based reparative and regenerative therapies have been gaining increasing attention.

In this paper we aim to provide an overview of the present knowledge on stem cell-based therapies in CLI, focusing on results of clinical trials and further directions.

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 and 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 next 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 (TcO_2), 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 mononuclear cells

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].

Table I. Minimal criteria for defining multipotent mesenchymal stromal cells according to International Society for Cellular Therapy, 2006 [27]

Plastic-adherent cells when maintained in standard culture conditions
Expression of CD105, CD73 and CD90 antigens Lack expression of CD45, CD34, CD14, CD11b, CD79α or CD19 and HLA-DR surface molecules
Trilineage differentiation capacity into osteoblasts, chondrocytes and adipocytes

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

Table II. Summary of individual trials assessing cellular therapies in N-O CLI

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	10^9 BM-MNCs vs. PB-MNCs	6 months	Improvement in ABI, TcO_2, pain reduction after BM-MNC administration
Powell *et al*. [22] RESTORE-CLI	Rutherford 4—6, toe sys. Pressure < 50 mm Hg, ankle sys. or Pressure < 70 mm Hg	72 (42 – Ixmyelocel-T, 24 – placebo)	Ixmyelocel-T (autologous, cultured BM-MSCs and HPCs)	IM, 20 injections	35–295 × 10^6 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 × 10^8 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 × 10^9/l 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, improved ulcer healing in BM-MNCs vs. control
Matoba *et al*. [55]	Rutherford 4–6	74	BM-MNCs vs. PB-MNCs	IM, 40 injections	10^9-MNCs	Up to 3 years	Improved rest pain and ulcer healing in BM-MNC group, no significant change in ABI and $TcPO_2$, 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)	10^9 BM-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 × 10^7/ml	6 months	Improvement in rest pain, ulcer healing and ABI in BM-MNC group, insignificant difference in amputation rate
Walter *et al*. [49] PROVASA	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)	10^8 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 × 10^9 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-MNCs	IM (2 cycles, 40 injections each)	10^9 G-CSF mPB-MNCs	3 months	Significant improvement in ulcer healing, pain reduction, blood perfusion of lower limbs in PB-MNC group

Table II. Cont.

Author/trial name	Study indication/ patient population	No. of subjects	Type of cells	Administration route	Dose	Follow-up	Main outcome
Huang *et al.* [59]	CLI, Rutherford 4–6	150 (76 – G-CSF mPB-MNCs, 74 – BM-MNCs)	G-CSF mPB-MNCs vs. BM-MNCs	IM (2 cycles, 40 injections each)	10^9 G-CSF mPB-MNCs vs. 10^8 BM-MNCs	3 months	Pain reduction, ABI increase, in both groups, significantly better in G-CSF mPB-MNCs vs. BM-MNCs. No difference in amputation rate
Ozturk *et al.* [60]	Diabetic patients with CLI	40 (20 – G-CSF mPB-MNCs, 20 – standard care)	G-CSF mPB-MNCs	IM (multiple injections, 1 ml each)	10^9 G-CSF mPB-MNCs	3 months	Significant increase of ABI, walking distance, decrease of pain score in mPB-MNC group vs. standard care. No significant difference in amputation rate
Lu *et al.* [61]	Type 2 diabetic patients with bilateral CLI, at least 1 foot ulcer	41 (20 – BM-MSCs, 21 – BM-MNCs)	BM-MSCs vs. BM-MNCs	IM (1 cycle, ~20 injections)	BM-MSCs 9.3 ±1.1 × 10^8 BM-MNCs 9.6 ±1.1 × 10^8	6 months	Ulcer healing rate significantly higher in BMMNC vs. BMMSC group (at 6 weeks). Significantly longer PFWD, higher ABI and $TcPO_2$ in BMMSC (after 6 months). No significant difference in amputation rate
Gupta *et al.* [62]	Rutherford 4–6	20 (10 – BM MSCs, 10 – placebo)	BM MSCs	IM	2 × 10^6 per kilo	6 months	Significant increase of ABI in cell group vs. placebo. No difference in amputations

ABI – ankle-brachial index, ASCs – adipose-derived stroma/stem cells, BM-MNCs – bone marrow mononuclear cells, BM-MSCs – bone marrow mesenchymal stromal cells, CLI – critical limb ischemia, G-CSF mPB MNCs – granulocyte colony-stimulating factor – mobilized peripheral blood mononuclear cells, HPC – hematopoietic progenitor cell, IA – intra-arterial, IM – intramuscular, NO-CLI – no option critical limb ischemia, PB-MNC – peripheral blood mononuclear cell, PFWD – pain-free walking distance, $TcPO_2$ – transcutaneous oxygen pressure.

included. Patients were randomly assigned to repetitive (3 times in 3 weeks intervals) intra-arterial infusion of 2 × 10^8 BM-MNCs or placebo. Follow-up time was 6 months. No significant differences between BM-MNCs and placebo groups were observed for the primary outcome of 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.

RESTORE-CLI assessed the efficacy and safety of intra-muscular injections of ixmyelocel-T, a patient-specific multicellular product derived from autologous bone marrow (biopsy) and produced in an automated closed-culture system [22]. The investigational product was composed of ~30–300 × 10^6 viable cells of primarily two types: mesenchymal stromal cells (CD90+) and a mixed population of hematopoietic cells (stem/progenitors and mature cells presenting the $CD45^+$ marker). Seventy-seven patients were enrolled with a diagnosis of CLI defined as persistent, recurring ischemic rest pain lasting ≥ 2 weeks and/or ulceration or gangrene of the toe/foot. Intramuscular injections were administered once, in over 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.

Procházka *et al.* conducted a trial assessing the efficacy of local application of bone marrow concentrate in treatment of ischemic foot ulcers [47]. A total of 96 patients with CLI and foot ulcers were randomized to two groups. Group I (n = 42) underwent a single procedure consisting of 40 intra-muscular injections (1 ml each) of autologous bone marrow concentrate (obtained from centrifugation of 240 ml of bone marrow aspirated from

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. 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, $TcPO_2$ 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 18% 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.
Moazzami *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-MNC.	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, $TcPO_2$ – 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 (TcO_2). 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, TcO_2 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 infusional toxicity, organ system complications, infections, death or malignancy has been reported [25].

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-

tract research organizations (CRO) monitoring, and statistically powered for rigorous objective endpoints such as Tc pO_2 and other tissue perfusion indices.

Conflict of interest

The authors declare no conflict of interest.

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