Cell Therapy for Chronic Limb-Threatening Ischemia: Current Evidence and Future Directions

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SUMMARY
Cell-based therapies have gained interest as a potential treatment method in cardiovascular disease in the past two decades, peripheral artery disease amongst others. Initial pre-clinical and small pilot clinical studies showed promising effects of cell therapy in peripheral artery disease and chronic limb-threatening ischemia in particular. However, these promising results were not corroborated in larger high quality blinded randomized trials. This has led to a shift of the field towards more sophisticated cell products, especially mesenchymal stromal cells. Mesenchymal stromal cells have some important benefits, making these cells ideal for regenerative medicine, e.g., potential for allogeneic application, loss of disease-mediated cell dysfunction, reduced production costs, off-the-shelf availability. Future high quality and large clinical studies have to prove the efficacy of mesenchymal stromal cells in the treatment of peripheral artery disease.

SIGNIFICANCE STATEMENT
Chronic limb-threatening ischemia is the most severe form of peripheral artery disease, and a considerable number of patients with this condition are not eligible for conventional treatment strategies. Therapies that aim at neovascularization might provide an escape for these patients. Initial clinical studies for first generation cell therapies were quite disappointing; however, more sophisticated and better defined cell therapies—mesenchymal stromal cells in particular—seem promising. This article describes the future perspectives and challenges of cell therapy in limb ischemia.

INTRODUCTION
Peripheral artery disease arises from atherosclerosis of major arteries, with a predilection for the lower limbs. In its most severe manifestation, occlusion of limb arteries reaches a point where metabolic demands of the tissue can no longer be met; this stage is termed chronic limb-threatening ischemia (CLTI) or critical limb ischemia (CLI). CLTI poses a great unmet need for novel treatments, as 20%–40% of the CLTI patients are not eligible for conventional revascularization, ultimately leading to amputation, associated with an immense medical and socio-economic burden [1–3]. No-option status in these patients is due to extensive and diffuse, often infrapopliteal, atherosclerotic lesions, comorbidity, and/or lack of a suitable bypass graft [4, 5]. Novel approaches that target neovascularization provide a potential solution for these no-option patients. Cell-based therapies seem the most promising [6], although initial enthusiasm has abated after negative results in the first generation of progenitor cell trials. Here we will provide a concise review on the available evidence on and future directions for cell therapy in CLTI. In that context we will also briefly address literature regarding cell therapy in myocardial infarction (MI) because objectives in both MI and CLTI trials are to enhance revascularization through cell therapy.

CELL THERAPY FOR CLTI—THE PRESENT STATE OF AFFAIRS
The rationale behind using progenitor cell therapy as a treatment for ischemic cardiovascular disease was motivated by the discovery that human blood contains progenitor cells that home to ischemic tissues [7] and augment angiogenesis [8]. Relatively soon thereafter, a first generation of progenitor cell trials have been conducted using bone marrow mononuclear
cells (BM-MNCs), a direct BM isolate which contains a variety of different cell-types, mostly from the hematopoietic line. The primary hypothesis was that BM, as the reservoir of hematopoietic stem cells, also contains endothelial progenitor cells (EPCs) [9]. These putative EPCs were initially thought to promote angiogenesis through the formation of new vessels [7] as they actively homed to ischemic areas after injection. Early, uncontrolled clinical studies using BM-MNCs were promising, but placebo-controlled trials gave conflicting results. A large, double-blind, placebo-controlled randomized trial by our group, the JUVENTAS trial, showed no treatment effects of BM-MNC administration over placebo [10], which was corroborated by a meta-analysis [11]. Similar results were obtained with BM-MNC therapy for other indications such as MI, where an aggregated study comprising over 1,000 patients that were treated with BM-MNCs for MI failed to find a consistent positive effect [12].

Advancing insight into the biology of progenitor cells has in parallel, revealed that the mechanisms of effect involved in progenitor cell-therapy are different and more complex than initially thought. The use of cell surface markers to identify EPCs has been shown to be prone to isolation artifacts [13, 14], and several ontologically distinct cell populations display EPC markers. Furthermore it has been shown that BM-derived cells do not stably incorporate into newly formed vessels and only play an auxiliary role in neovascularization [15]. True vasculogenic ability has only been demonstrated for a single cell-type, designated the endothelial colony forming cell (ECFC) [16], which cannot be obtained from BM. While the auxiliary angiogenic effects of BM-MNCs have been demonstrated very consistently in animal models, it is likely that they only occur with BM isolates from comparatively young subjects without comorbidities [17, 18]. This restricts successful application of BM-MNCs in MI to specific subsets [19] of patients, and likely severely limits it in CLTI.

Collectively, these observations have precipitated a switch away from undefined raw cell isolates such as BM-MNCs, toward better-defined cell therapy products [20]. Whereas in the therapy for MI, the focus has shifted away from angiogenic cell therapy to cardiomyocyte regeneration [21], in CLTI the primary objective remains to augment angiogenesis.

Angiogenesis can be induced via different cell-based strategies (Fig. 1) by supplying endothelial-like cells, such as ECFCs directly, which will spontaneously organize into new vessels that integrate with the existing vasculature. Alternatively angiogenesis can be promoted indirectly by cells that secrete factors that remodel the extracellular matrix and recruit resident endothelial cells. In this category are circulating endothelial cells, which are of monocyte/myeloid origin, and may potentially act as bridging monocytes in angiogenesis [22]. Alternatively there are mesenchymal stromal cells (MSCs), which are of pericyte origin [23] and secrete a host of paracrine angiogenic and immunomodulatory factors [24]. However, it is debated that pericytes have the multilineage potential in vivo, which characterize MSCs in vitro and that the observed plasticity of MSCs result from manipulation ex vivo [25]. But irrespective of this it is likely that there are synergistic effects of combining these approaches, using a...
combination of ECFCs and a supportive cell-type [26, 27]. At present, however, translation to a clinical product has proven difficult as ex vivo expansion of the above-mentioned cells requires specialty cell culture additives that are difficult and costly to obtain for clinical grade production [28]. For this reason only MSCs, have advanced to a second generation of clinical trials, as they can be relatively easily obtained from different tissues such as BM, placenta and adipose tissue and reproducibly expanded ex vivo (see Table 1 for overview).

**MSC THERAPY FOR CLTI**

While MNC have proven to effectively enhance angiogenesis and neovascularization in preclinical studies, it has been suggested that the pro-angiogenic effect of MSCs is superior compared to MNCs in preclinical studies [34, 35]. In vitro and vivo studies have demonstrated that MSC can home to injured tissue and secrete beneficial factors that suppress inflammation and improve angiogenesis via paracrine pathways [36]. Several small exploratory clinical trials showed positive effects of MSCs in the treatment of CLTI compared to standard of care or placebo [30, 32, 37]. Clinical studies that directly compare BM-MSCs with MNCs for the treatment of CLTI are scarce. Only one study directly compared the two strategies in 41 diabetic CLTI patients, suggesting that MSC might be better tolerated and more effectively enhance perfusion and ulcer healing compared to MNC [31]. The disappointing results of clinical trials on MNC in CLTI, the promising effects of MSCs and several practical benefits of MSCs, in particular the potential for allogeneic application, have led to increased interest of MSCs as potential option for cell therapy in CLTI A similar switch is also observed for studies in cardiac disease [38].

MSCs, rather uniquely among transplanted cell grafts, are only minimally immunogenic [38] and display strong immunomodulatory properties [39]. This makes allogeneic application possible, at least in a single administration, as it is still unclear whether rejection occurs upon repeated administration [38]. At the present state of knowledge, allogeneic administration of MSCs is the most promising route to clinical application with the advantage of providing an off-the-shelf available product. An allogeneic product significantly reduces the burden on the patient, as patients will not have to undergo a (BM) harvesting procedure. Whereas in BM-MNCs it has been shown that patient-derived cell isolates show decreased pro-angiogenic effects [18], this does not necessarily apply to MSCs [40]. In a previous study we did not observe reduced angiogenic potency in CLTI MSC isolates in a murine hind limb ischemia model [40, 41]. In a clinical trial comparing efficacy of autologous versus allogeneic MSCs in non-ischemic dilated cardiomyopathy, however, allogeneic MSCs had a more favorable side-effect profile and a trend toward greater improvement in ejection fraction. Additionally the occurrence of serious adverse events was substantially lower in allogeneic than autologous MSCs; 28% versus 64%, respectively [38]. Furthermore, an advantage of allogeneic MSC therapy is that the (angiogenic) potency of the cell isolate can be tested in advance. Demonstrable potency will likely be of importance in the quality control of cell therapy products for clinical regulation [42]. As a single MSC isolate generally is sufficient to treat dozens of patients, a priori batch testing can conceivable improve clinical outcomes, provided that validated assays are developed [42, 43]. Last, allogeneic MSC administration is significantly less costly. Costs for expansion and quality testing of a BM isolate are high, which in autologous application were the per-patient cost, but which can be split over multiple patients in allogeneic application [44].

**Table 1. Overview of MSC trials in CLTI**

| Author          | Year | n   | Design                                  | Injection sites | Total dose and source                        |
|-----------------|------|-----|-----------------------------------------|----------------|---------------------------------------------|
| Kim et al. [29] | 2006 | 4   | No control group                        | NM             | $1 \times 10^9$ allogeneic HLA matched UCB-MSCs |
| Dash et al. [30]| 2009 | 24  | Open label; control group (1:1 randomization) | NM             | $45-60 \times 10^6$ autologous BM-MSCs       |
| Lu et al. [31]  | 2011 | 41  | Double blind study; randomly assigned treatment per leg; one leg treated with normal saline, the other treated with MNC or MSC | 20             | $9.3 \times 10^8$ autologous BM-MSCs         |
| Gupta et al. [32]| 2013 | 20  | Placebo controlled; double blind        | 40–60          | $2 \times 10^6$ allogeneic BM-MSCs per kilogram body weight |
| Gupta et al. [33]| 2016 | 90  | Nonrandomized; low dose, high dose or standard care | 40–60          | $(1 \ or \ 2) \times 10^5$ allogeneic BM-MSCs per kilogram body weight |

Abbreviations: BM, bone marrow; HLA, human leukocyte antigens; MSCs, mesenchymal stromal cells; NM, not mentioned; UCB, umbilical cord blood.

**CURRENT CHALLENGES AND FUTURE DIRECTIONS**

Allogeneic application makes MSC-therapy interesting for commercial parties, as a defined cell product can be comparably easily patented and produced by in-house companies, without the complications of harvesting donor material for each patient. Fifteen percentage of all clinical trials worldwide involving cell therapy are industry-sponsored and the vast majority of the remainder by leading academic centers. Additionally facilities and logistics involved in the development of cell therapy products are becoming more available and less expensive due to increased administration as standard of care or as investigational novel treatment in other diseases [45]. However, development of evidence-based accepted approaches remains challenging, due to high developmental costs, regulatory hurdles, and batch-per-batch product variation. Some of these factors may be less relevant for non-cellular...
cell-based therapies, such as exosome-based therapies, which could make commercialization less difficult [46]. Another important CLTI-specific limiting factor is that, historically, the design of high-quality studies for the treatment of CLTI has proven notoriously difficult [47]. Improved clinical management and technical advances in revascularization approaches, endovascular interventions in particular, have led to a near doubling of 1-year amputation-free survival for CLTI patients since the first trials with BM-MNCs. Therefore larger and better-designed trials are required to determine the potential added value of novel therapies in CLTI [48, 49]. In the light of these considerations, small phase I/II [31, 32] or pragmatically designed studies [33] have provided valuable first indications about potential efficacy of MSCs in CLTI. However, at no point can they substitute evidence from placebo-controlled double-blind randomized trials. Public demand for cell-based therapies has been such, that smaller commercial parties are offering cell treatments in the absence of evidence—positive or negative—potentially putting patients at risk [50], leading to public discussions with respect to ethical issues regarding regenerative medicine approaches [51].

We therefore would encourage increased openness and standardization, both in the use of the investigational cell product and trial design. Convincing evidence for efficacy of MSC therapy will only come from well-designed randomized controlled trials using hard and clinically relevant outcomes, which would be ideally related with future imaging methods to evaluate collateralization and neovascularization. It seems increasingly unlikely that single investigative centers will achieve sufficient statistical power to show efficacy. International collaborative efforts and data sharing are necessary to push the field forward and maintain scientific integrity.

**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

The authors indicated no potential conflicts of interest.

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