Autologous cell therapy in diabetes-associated critical limb ischemia: From basic studies to clinical outcomes (Review)

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Received May 4, 2020; Accepted September 30, 2020

DOI: 10.3892/ijmm.2021.5006

Abstract. Cell therapy is becoming an attractive alternative for the treatment of patients with no-option critical limb ischemia (CLI). The main benefits of cell therapy are the induction of therapeutic angiogenesis and neovascularization that lead to an increase in blood flow in the ischemic limb and tissue regeneration in non-healing cutaneous trophic lesions. In the present review, the current state of the art of strategies in the cell therapy field are summarized, focusing on intra-operative autologous cell concentrates in diabetic patients with CLI, examining different sources of cell concentrates and their mechanisms of action. The present study underlined the detrimental effects of the diabetic condition on different sources of autologous cells used in cell therapy, and also in delaying wound healing capacity. Moreover, relevant clinical trials and critical issues arising from cell therapy trials are discussed. Finally, the new concept of cell therapy as an adjuvant therapy to increase wound healing in revascularized diabetic patients is introduced.

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Abbreviations: Ang-1, angiopoietin; ADRCs, adipose-derived regenerative cells; ADSCs, adipose-derived stem/progenitor cells; AD-SVF, adipose-derived stromal vascular fraction; AFS, amputation-free survival; AT, Adipose tissue; BM, bone marrow; BMAC, bone marrow aspirate concentrate; BM-MNCs, bone marrow-derived mononuclear cells; CLI, critical limb ischemia; EPCs, endothelial progenitor cells; EGF, endothelial growth factor; G-CSF, granulocyte-colony stimulating factor; IFATS, International Federation of Adipose Therapeutics and Science; IGF-1, insulin growth factor; IL-1β, interleukin 1β; ISCT, International Society for Cellular Therapy; KGF, keratinocyte growth factor; HSCs, hematopoietic stem cells; PAD, peripheral artery disease; PB, peripheral blood; PB-MNCs, peripheral blood mononuclear cells; MCP-1, monocyte chemoattractant protein-1; MIP-1, macrophage inflammatory protein 1; MSCs, mesenchymal stem cells; SDF-1, stromal-derived factor 1; SVF, stromal vascular fraction; TAO, thromboangiitis obliterans; T2DM, type 2 diabetes mellitus; TGFβ, transforming growth factor β; TNC, total nuclear cell; VM, vascular mimicry; VEGF, vascular endothelial growth factor

Key words: cell therapy, critical limb ischemia, wound healing, diabetic foot ulcers, vascular disease

Contents

1. Introduction

Peripheral artery disease (PAD) is characterized by lower limb ischemia due to atherosclerotic plaque formation (1). Critical limb ischemia (CLI) is the most severe form of PAD and is often associated with diabetes, age, hypercholesterolemia and smoking (2). The incidence of PAD is predicted to increase due to the increasing rates of diabetes and obesity in the population, together with aging (3). CLI, in fact, promotes the development of non-healing ulcers with consequent tissue necrosis with gangrene and rest pain. Patients with CLI are mainly treated with surgical bypass or endovascular procedures in order to restore perfusion, thus preventing limb amputation (4).

Notwithstanding the increase in the use of these procedures, up to 30% of patients with CLI cannot be revascularized and the mortality rate remains high (5). The wound healing process is impaired in diabetic foot ulcers and plays a causative role in limb amputations (6). In total, 75% of amputations out of one million individuals per year are performed on patients with type 2 diabetes mellitus (T2DM) (7). The mechanisms of skin wound healing impairment in T2DM remain poorly known, despite the high incidence rate.

Recently, cell therapy has become an attractive alternative for the treatment of patients with no-option CLI (8-10), as well as for patients with diabetic ulcers (8,10-12). The primary goal of cell therapy is to induce therapeutic angiogenesis and neovascularization, promoting collateral vessel formation, and tissue regeneration in non-healing skin lesions (13).

The present study reviews the current state of the cell therapy field, focusing on intra-operative autologous cell concentrates in CLI in diabetic patients. Moreover, the clinical indications that are moving from the no-option for revascularization for patients with CLI to the new concept of cell therapy as an adjuvant therapy to also increase healing in revascularized patients are discussed.

2. Cell therapy: Unfractioned mixed cell population concentrate or pure stem/progenitor cell concentrate

Increasing interest in cell-based therapy for the treatment of CLI has arisen, although there is confusion as per the use of the term 'stem cell' therapy in clinical research, regarding the cell population used. The term 'stem' or more appropriately 'progenitor' cell is used both for homogeneous cells produced by culture expansion (in authorized cell factory facilities) and, in some cases, for cell concentrates, which are heterogeneous, mixed population, containing only a small fraction of multipotent cells produced intra-operatory, resulting in misunderstanding and confusion.

To correctly use the term 'stem cell therapy', the cells should have been isolated from a pellet of cell concentrate, followed by culture expansion or selective concentration of a pure stem/progenitor cell population (14) and then characterized for their self-renewal capacity, the expression of specific cell surface markers and for their multilineage differentiation capacity (15). In this case, the obtained cell population can be referred to as 'mesenchymal stem cells (MSCs) used for stem cell therapy'.

In contrast to pure MSC therapy, cell concentrates produced intra-operatory are generally derived by point of care devices (summarized in Table I) and should be described as 'cell concentrate' or 'mixed cells populations', specifying the source of tissue used, which in CLI are commonly the following: Bone marrow (BM), adipose tissue (AT), or peripheral blood (PB).

The lack of a standardized description of cell therapies creates confusion and difficulty of comparison between the different basic research and published papers. Recently, the DOSES tool was developed by International Expert Consensus to improve standardization and transparency in describing cell therapies (16). 'D' stands for donor (i.e., autologous, allogenic, xenogeneic); 'O' for origin of the tissue (i.e., BM, PB, AT, or other); 'S' for the separation method (minimal manipulation such as centrifugation or filtration, laboratory culture or purified through affinity separation); 'E' for exhibited cell characteristics (including, but not limited to the expression of cell surface markers, functional/performance attributes); and 'S' for the site of delivery (i.e., intra-muscular, intravenous and intra-articular) (16).

A mixed cell concentrate, that includes a combination of different cell types, instead of a pure population concentrate, is easier to produce and may offer enhanced benefits, since it provides different lineage precursors or a crosstalk between different cell populations (17).

The aim of the present review was to summarize and clarify the differences in tissue sources, type of cells used in cell therapies, and to provide further clinical indications and outcomes of current autologous cell-based therapies in CLI.

Cells derived from BM. BM contains blood cells at different differentiation stages (18). The nucleated cells [BM-derived mononuclear cells (BM-MNCs)] are heterogeneous population containing endothelial progenitors cells (EPCs), MSCs and hematopoietic stem cells (HSCs), that increase angiogenesis and can be exploited to ameliorate poor tissue perfusion (19).

Although MSCs in BM are only a small percentage of the total nucleated cells, they can be expanded 100- to 10,000-fold over a period of several weeks in culture (20). As previously described, MSCs can also be isolated from various tissue sources, including AT. BM-MSCs were the first identified MSCs, and consequently are the most well-characterized and have been studied extensively. MSCs for cell therapy according to the International Society for Cellular Therapy ISCT) criteria should be: i) Plastic-adherent fibroblastic-like shape; ii) positive for CD90, CD73, CD105, CD34, CD11b and CD45, and negative for HLA-DR; iii) capable of differentiating into three mesodermal lineages: Chondroblasts, osteoblasts and adipocytes (15).

BM-MSCs are a promising cell type for cell therapy; however, they require *in vitro* culture expansion that entails several limitations, such as high costs, two-step surgery and safety in human treatments.

In vitro expanded cells, in fact, necessitate genetic stability, sterility and culture expansion cytokine removal. For this reason, they are currently under the regulatory authorities strict guidelines for human use (21).

Conversely, BM-MNCs [also known as bone marrow aspirate concentrate (BMAC)] can be prepared simply by BM harvest and centrifugation in a one-step intra-operatory implant with minimal cell manipulation (Table I). Therefore, the BM-MNC concentrate seems to be an attractive alternative to BM-MSCs, although it contains only a small percentage of stem cells (Fig. 1A).

Mechanisms of action of cells derived from BM

a) MSCs. MSCs have been the subject of scientific investigation since their discovery in the late 1960s. The first hypothesis was based on the migration of MSCs, following administration, to the injury sites, their implant and differentiation into cells capable of regenerating damaged or not functional connective tissues. As shown by the results from hundreds of animal studies and numerous human trials, it has become clear that the cells do not engraft in the percentage or time to adequately explain the results in terms of tissue replacement (22-24). The new hypotheses indicates that MSCs heal injured tissue by enhancing cell viability and/or proliferation, reducing cell apoptosis and sometimes modulating the immune responses (25). The healing capacity of MSCs is based on paracrine activity through secreted growth factors, cytokines, hormones, extracellular vesicles (i.e., exosomes)

Table I. Point of care (POC) devices used to produce autologous cell concentrate.

Tissue sources	Withdrawal volume (ml)	Device name	Cell populations	Procedure	Cell characterization (Refs.)
Bone marrow	60	MarrowStim Zimmer Biomet	MNC, CD34+/ MSC	Single use device kit to be centrifuged	Yes (170)
	240	BMAC-Terumo	MNC, CD34+/ MSC	Single use device kit to be centrifuged	Yes (171)
Blood and bone marrow	40-180	Angel-Arthrex	cPRP	Automated system based on centrifugation and sensors technology	No
	120	Sepax	MNC, TNC	Fully automated GMP compliant system for processing of umbilical cord blood, BM, PB.	Yes (172)
Peripheral blood	120	Pall Celeris/ MonoCells Athena/Hematrate Cook Regentec	MNC, TNC	Single use selective filter-based technology on cell membrane potential. No equipment needed.	Yes (65)
Adipose tissue	100-130	Lipogems	SVF micro-fragmented fat	Mechanical fragmentation through metal beads stainless steel marbles followed by centrifugation	Yes (58)
	25	Adiprep TERUMO	Nucleated Cells and ASCs	Adipose cell concentrates from AT centrifugation	No
	20	HyTissue FIDIA	SVF micro-fragmented fat	Adipose cell fragmented concentrate from AT centrifugation after filtration	No

MNCs, mononuclear cells; MSCs, mesenchymal cells; TNCs, total nuclear cells; cPRP, concentrate PRP from blood or blood/bone marrow; SVF, stromal vascular fraction; ASCs, adipose stem cells; AT, adipose tissue; BM, bone marrow.

containing peptides/proteins, mRNAs and microRNAs (miRNAs or miRs), that may have immunomodulatory and anti-inflammatory effects and pro-survival effects (25). Recently, it was demonstrated that the success of MSC therapy is not due to cell engraftment and replacement efficiency (26). MSCs modulate the local immune responses and facilitate tissue repair through a paracrine release, but do not reconstitute damaged tissue.

Since MSCs are environmentally sensitive, improving their biological activities will help to ameliorate their therapeutic features (27). Therefore, it is important to define the environment in which they are implanted in order to avoid unexpected behaviors (28). Moreover, successful therapies should take the paracrine effects of MSCs into account (29,30). Indeed, it has been shown that inflammation modulates the multilineage potential, immunomodulation, the immunophenotyped and hematopoietic features of MSCs, and should be tightly controlled in cell therapy in order to increase efficiency (26).

For example, in ischemic tissue, despite limited cell survival, BM-MSCs secrete higher levels of vascular endothelial growth factor (VEGF) compared to fibroblasts, thus angiogenic properties of MSCs occur through paracrine and autocrine effects, and depend on tissue source (31).

As previously demonstrated, the administration of MSCs or conditioned media does not improve revascularization immediately, but only when administered one day following the induction of ischemia in a mouse model (32). Thus, inflammatory processes impair the therapeutic efficacy of MSCs, and administration timing has been proven to be crucial (26,33,34).

The 'immune centric revolution' or 'immune centric approach' suggests that the regenerative capacity of stem cells is controlled and orchestrated by the local immune system consequently to tissue damage, with macrophages being the main actors and coordinators of the injury response, able to promote endogenous repair (35). It has recently been demonstrated that the immune system plays a critical role in tissue healing in addition to stem cells and growth factors (36). Actual regenerative strategies may be reinforced by the control of the immune-mediated tissue repair and regeneration mechanisms as an alternative to stem cells and growth factors therapies (37). Immune cells have recently emerged as key components of the niche microcosm and prominent effectors of stem cell behaviors (37). During tissue damage, stem cells communicate with the frontline of resident immune sentinels to organize

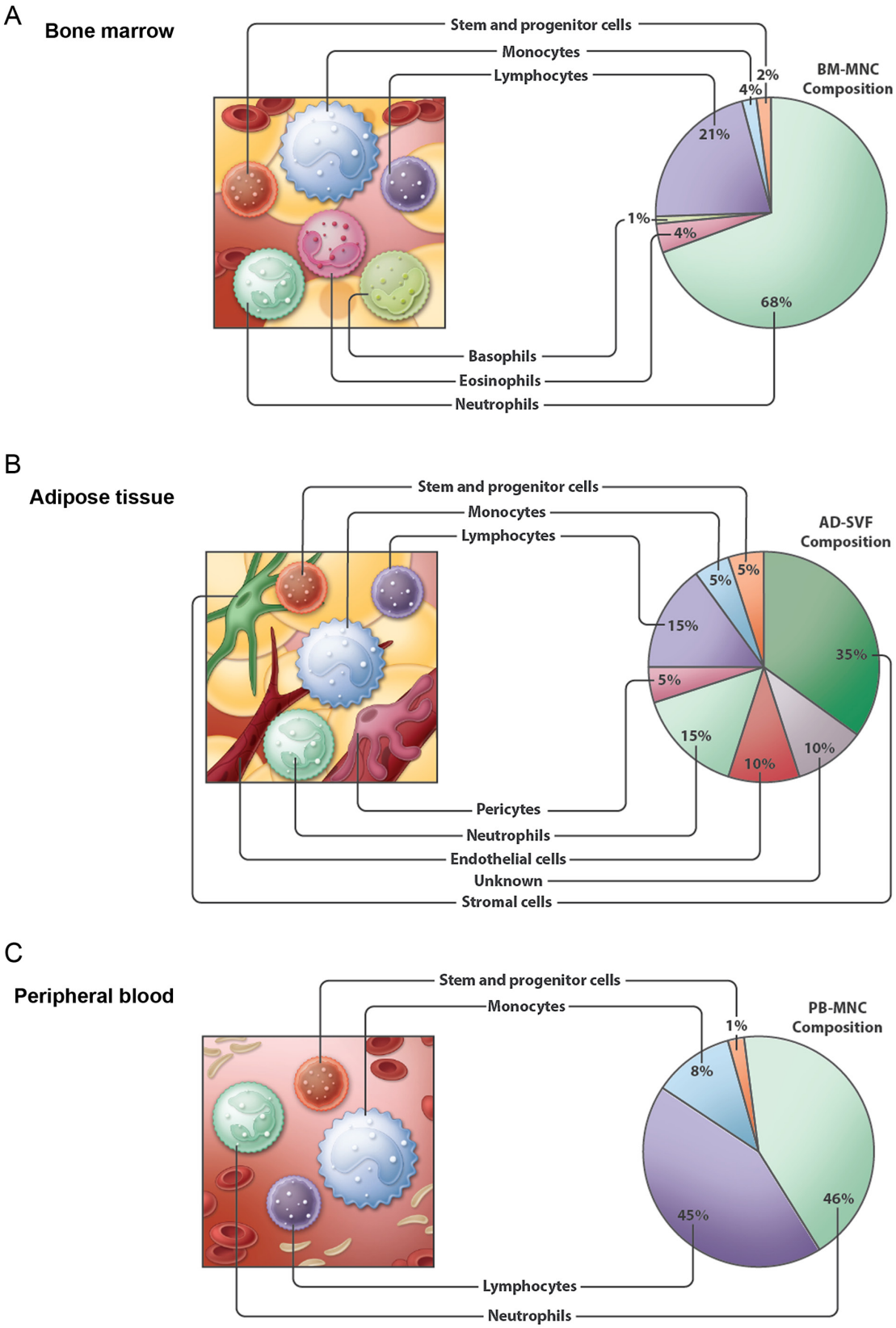

Figure 1. Tissue-specific composition of different cell concentrates. (A) Bone marrow-derived cell composition expressed as % of total BM-MNCs; (B) adipose-derived cell composition expressed as % of total AD-SVF; (C) peripheral blood-derived cell composition expressed as % of total PB-MNCs. BM-MNCs, bone marrow-derived mesenchymal cells; AD-SVF, adipose-derived stromal vascular fraction; PB-MNCs, peripheral blood mononuclear cells.

the response: Immune effectors, as monocyte/macrophages, quickly enter from circulation, infiltrating the stressed tissue to remove pathogens, to start the repair process and restore homeostasis. Briefly, stem cells sense, communicate with, and co-opt resident immune cells to preserve the tissue homeostasis (37).

MSCs are positively regulated by macrophages. Indeed, it has been demonstrated *in vitro* that MSC proliferation, vitality and paracrine functions are improved by macrophage-derived growth factors (38). Indeed, the crosstalk between macrophages and MSCs is not unidirectional. Implanted MSCs provoke a shift to an anti-inflammatory M2-like macrophage phenotype (see paragraph below entitled 'a) Monocytes/macrophages' under 'Mechanisms of action of cells derived from PB') in different tissues, such as the injured myocardium (33).

Hypoxia has been shown to decrease both M1 to M2 macrophage transition and anti-inflammatory interleukin (IL)-10 macrophage production, suggesting a possible reduced therapeutic effects of bone marrow MSCs in a hypoxic environment (39).

b) BM-MNCs. The implantation of autologous BM-MNCs into ischemic skeletal muscles has been proven successful in developing angiogenesis and collateral vessel formation in human trials (40-42). The mechanisms of angiogenesis by BM-MNC implantation have been evaluated in several studies, suggesting that the differentiation of BM-MNCs into endothelial cells (ECs) is a rare condition in skeletal muscle tissues (43). However, cytokines and chemokines released from MNCs are able to activate pre-existing EC sprouting through either a direct or an indirect mechanism. It has been demonstrated that cytokines released from BM-MNCs implanted in ischemic sites are the main players in angiogenesis induction (44) (Fig. 2).

Moreover, the acute phase of ischemia is the optimum stage for cell-based therapeutics with BM-MNCs, because of the robust upregulation of IL-1β expression, and a consequent positive effect on the angiogenic potential of BM-MNCs (45).

Cutaneous wound healing is a multi-step complicated process in which the skin repairs itself after injury. BM-MNCs participate in this process with various cell populations, such as MSCs, inflammatory cells, fibrocytes, and not only release cytokines, promoting wound repair, but also differentiate into keratinocytes and dermal myofibroblasts (46). BM-MNCs in fact, release endothelial growth factor (EGF), insulin growth factor (IGF-1) and keratinocyte growth factor (KGF) to promote keratinocyte proliferation. VEGF, angiopoietin 1 (Ang-1) and macrophage inflammatory protein (MIP)-1, which are all proangiogenic cytokines, recruit monocytes in the wound, while stromal-derived factor 1 (SDF-1) and granulocyte-colony stimulating factor (G-CSF) secretion recruit EPCs (46) (Fig. 2).

In a macrophage depleted mouse model, it was demonstrated that the major contribution of BM-MNCs to wound healing was linked to the proangiogenic effects of macrophages contained in the BM (47).

Finally, BMNCs act as a repair source, by supporting angiogenesis and vasculogenesis, and producing several growth factors through a paracrine effect (17,19,40,48) (Fig. 2). Similar to MCSs, BM-MNCs also act via different mechanisms; however, it is not clear which BM-MNC cellular population is optimal for CLI treatment (49,50).

Cells derived from adipose tissue. There are two classes of cells in the AT: Stromal cells also known as the stromal vascular fraction (SVF) and mature adipocytes (MAs), the major component of the AT volume.

According to the ISCT and the International Federation of Adipose Therapeutics and Science (IFATS), the main cell types in the AD-SVF are blood and stromal cells, such as red cells, platelets, ECs and EPCs, a heterogeneous population of nucleated cells, including leukocytes ($CD45^+$), lymphocytes ($CD4^+$) and monocytes/macrophages ($CD14^+$) (51-53) (Fig. 1B). The SVF also contains multi-potent mesenchymal stem/progenitor cells able to differentiate into, chondrocytes, osteoblasts, myocytes, adipocytes, pericytes and fibroblasts (14). Indeed, it has been reported that AT contains adipose-derived regenerative cells (ADRCs) and adipose-derived stem/progenitor cells (ADSCs), which are multipotent mesenchymal stem cells, able to regenerate damaged tissues (54).

ADSCs are becoming important in the field of regenerative medicine and stem cell research. However, the isolation of ADSCs requires extensive manipulation, consisting of an *in vitro* selection following a collagenase digestion of the adipose derived stromal vascular fraction (AD-SVF) and an expansion process, that prevents an immediate use in the clinical practice. ADSCs are more suitable than BM-MSCs in the clinical use since the AT can be repeatedly obtained by liposuction, a well-established and minimally invasive procedure (50). Moreover, the AT stem cell concentration is approximately 500-fold greater than the concentration obtained from an equivalent amount of BM aspirate (55).

In contrast to ASCs, the AD-SVF represents a minimally processed cell population which can be used immediately (14). However, the term ADSC is sometimes improperly used to refer to AD-SVF, increasing confusion in clinical results of stem cell-based therapies (56).

Moreover, it has been proven that harvesting site, lipo-aspiration, and reinjection techniques strongly influence the quality of AD-SVF cells (57) and should be considered when using an AT-based cell-therapy.

AD-SVF cell therapy has been proposed as a novel therapy for damaged tissue regeneration and repair. Since fat tissue is abundant, easy to isolate, and rich in stem/progenitor cells able to secrete angiogenic growth factors, AT-based therapy has been considered one of the favorite candidates for non-healing wounds. For this reason recently, point of care devices have been placed on the market for the production of micro-fragmented adipose tissue (58), also known as the nanograft and adipose cells concentration systems that contain AD-SVF (Table I).

Mechanisms of action of cells derived from adipose tissue. The therapeutic efficacy of the AD-SVF is based on immunomodulatory, anti-inflammatory regenerative and angiogenic effects, and on the interaction of different cell populations present in the AD-SVF (Fig. 2).

A superior angiogenic effect of fresh AD-SVF compared to cultured ADSCs has been observed, in mice with hindlimb ischemia (59). *In vitro*, ASCs secrete higher levels of VEGF, hepatocyte growth factor and, transforming growth factor (TGF)β (60) compared to BM-MSCs, and exhibit a greater vascularization potency in a mouse model of hindlimb ischemia (61).

The nucleated cell therapeutic potential of AD-SVF is largely unknown. Recently, the AT-resident monocyte role in tissue vascularization has been shown (62). Regarding the

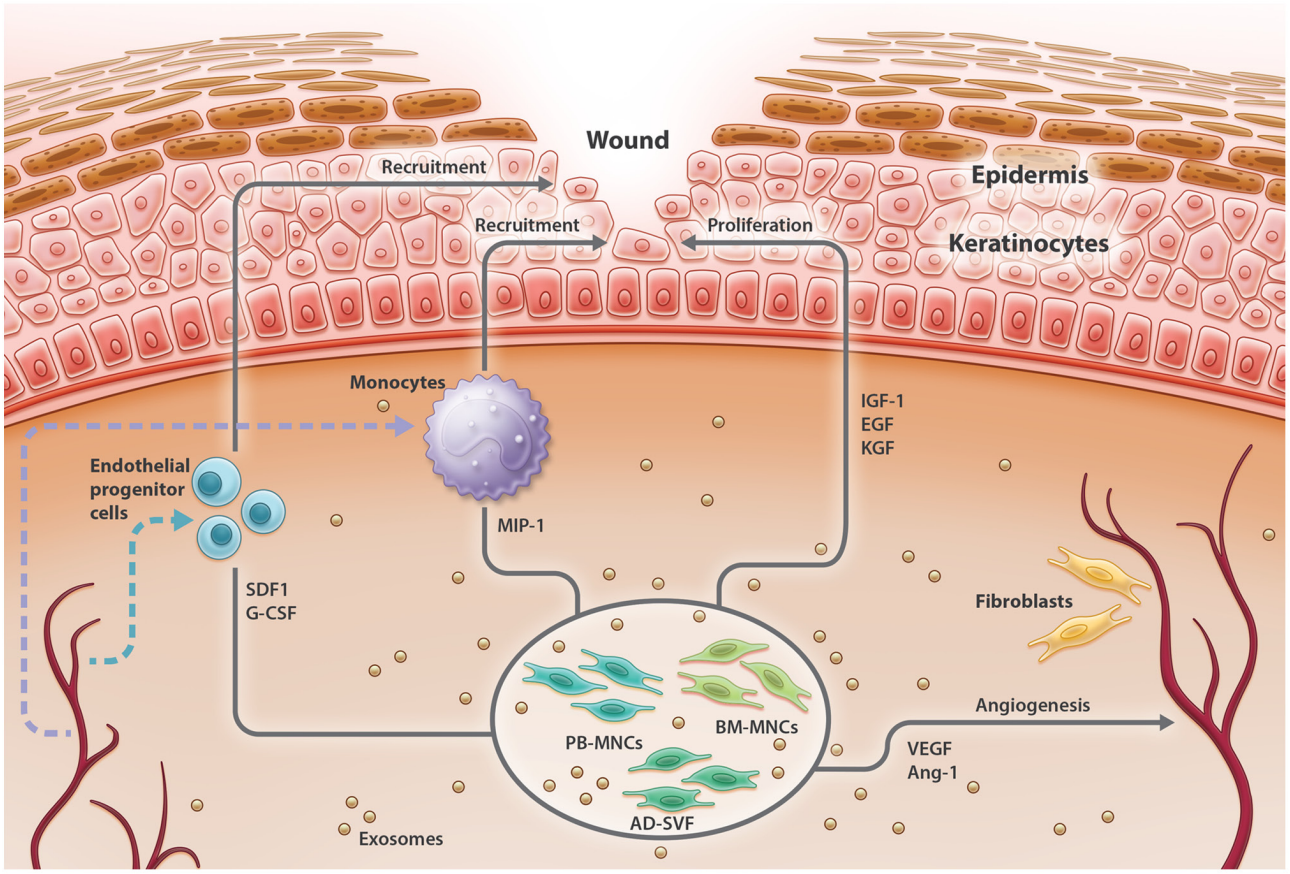

Figure 2. Paracrine effects of different autologous cell therapies in diabetic foot ulcers healing. This figure summarizes the paracrine effects of different sources used for cell therapies: PB-MNCs, BM-MNCs and AD-SVF. These cells are administered intramuscular or intra-arterial and act through paracrine mechanisms that target different tissue cells (endothelial cells, keratinocytes, fibroblasts and monocytes) indicated in the figure, concurring to the amelioration of wound healing. In a cutaneous wound the administered cells release cytokines and chemokines that enhance EPCs and monocytes recruitment into the wound, keratinocyte, fibroblast proliferation and angiogenesis. Additionally, healing capacity is accelerated by paracrine activity of exosomes secreted by all cellular types allowing intercellular communication. BM-MNCs, bone marrow-derived mesenchymal cells; AD-SVF, adipose-derived stromal vascular fraction; PB-MNCs, peripheral blood mononuclear cells; Ang-1, angiopoietin-1; EGF, epidermal growth factor; EPC, endothelial progenitor cell; G-CSF, granulocyte colony-stimulating factor; IGF-1, insulin-like growth factor 1; KGF, keratinocyte growth factor; MCP-1, monocyte chemoattractant protein 1; MIP-1, macrophage inflammatory protein-1; SDF1, stromal cell-derived factor-1; VEGF, vascular endothelial growth factor.

angiogenic cell population present in the adipose tissue, it has been shown that SVF monocytes ($CD14^+$) are more efficient in inducing angiogenesis than ADSCs derived from AD-SVF, suggesting that adipose monocytes may represent a new angiogenesis cell-based therapy (62).

Of note, these data are in line with the observation that macrophages in fat grafts are strongly responsible for tissue regeneration (63). The depletion of macrophages impairs strongly fat graft survival and angiogenesis, reduces stem cell recruitment, as well as, the rate of retention. Conversely, an upregulation/activation of macrophages allows survival and angiogenesis increase, indicating that macrophages are crucial for tissue revascularization and regeneration (63).

Pericytes of AD-SVF regulate vessel stability and vascular contractility (64), by contributing to angiogenesis, promoting EC survival and endothelial spreading (Fig. 2). Thus far, the AD-SVF nucleated cells clinical outcome is not known when implanted in subcutaneous tissue in humans. The paracrine effects of cytokines released by AD-SVF cells can be innovative, and is strictly associated to cell survival in the implanted tissue (52).

Accordingly, it has been shown that SVF cells mainly contain blood-derived cells, ADSCs, and ECs (Fig. 1B), and most of them do not survive 4 days following transplantation, although $CD34^+$ ADSCs remain viable after 14 days when implanted in ischemic tissue (64).

Cells derived from PB. PB-MNCs used in autologous cell therapy are a heterogeneous population composed of both $CD34^-$ (lymphocytic and monocytic) and $CD34^+$ HSCs and EPCs (Fig. 1C) (65).

The therapeutic properties of the MNC population were initially attributed to the $CD34^+$ EPC component, since EPCs are considered to be protective in both acute and chronic vascular injury (66). EPCs present in the adult human PB $CD34^+$ stem-cell population play a major role in postnatal neo-vascularization following BM mobilization (48). The immune system, as already described, is fundamental in tissue homeostasis, development, and wound repair. Immune system cells and secretomes are able to repair damaged tissues (67-70) as observed by Metchnikoff in the late 1800s (71). Recent studies have confirmed earlier observations and have demonstrated that the immune system regulates and control tissue regeneration (35-37,72). Since then, our understanding of immune cell role in tissue

regeneration, in particular macrophages and T-lymphocytes, has improved (67,72). Moreover, PB-MNCs can be produced easily by point of care system based on selective filtration (65) (Table I).

Mechanisms of action of cells derived from PB. Initially, EPCs were considered the only BM-derived cells capable to differentiate into vascular endothelium and to induce angiogenesis. Therefore, PB that contains only a small fraction of EPC compared to BM, were used following mobilization with G-CSF in order to increase the stem cells concentration in PB (73).

It is now clear that both monocytes and the lymphocyte itself, forming the PB-MNC mixed cell concentrate, are also involved in angiogenesis (74,75), arteriogenesis (76) and in tissue regeneration (77,78).

The angiogenic potency of human PB-MNCs isolated by a selective filtration-based point-of-care device has been proven both *in vitro* and *in vivo* (65). Human PB-MNCs isolated secrete a panel of angiogenic molecules and are capable of migrating in response to SDF-1 and VEGF gradient. It has been shown that PB-MNC injection induces neovascularization (i.e., capillary, arteriole and regenerative fiber numbers increase) upon hindlimb ischemia in mice (65). Notably, human PBMNCs can be detected in the murine ischemic tissue after 7 days. A similar effect has been shown in a *in vivo* potency assay, using BMNCs in comparison with expanded bone marrow cells enriched in $CD90^+$ stem cells (79).

In agreement, PB-MNCs exhibit a comparable or superior angiogenic capacity to that of BM-MNCs in a hindlimb ischemia mouse model, suggesting that EPCs do not play a pivotal role in the PB-MNC mediated limb ischemia treatment (80). Due to these considerations, an increasing number of clinical studies are using non mobilized PB-MNCs for the treatment of CLI (75,81-84).

a) Monocytes/macrophages. The marked angiogenic and arteriogenic ability of monocytes is well described and known for years (85-87).

Studies on the role of monocytes in arteriogenesis have been accomplished since 1970. Schaper *et al* described that monocytes adhere to ECs using electron microscopy (88). Subsequently, different studies demonstrated that monocytes are recruited to the collateral artery during arteriogenesis in rabbit and murine models of hindlimb ischemia (85,89). Moreover, mice with monocyte deficiency exhibit decreased blood flow and arteriogenesis upon femoral artery ligation compared to the controls (90). Monocyte recruitment requires the presence of chemokines, such as monocyte chemoattractant protein-1 (MCP-1), that is overexpressed in hypoxic tissue. Monocyte recruitment to ischemic sites induces angiogenesis in a MCP-1-dependent manner, suggesting a physiological and homo-functional role of monocytes in neovascularization (17,87). It has been shown that MCP-1 injection provokes collateral artery growth, as well as, monocyte accumulation around the arterial walls in ischemic porcine and rabbit and hindlimb ischemia, suggesting that monocyte recruitment is a required condition for high collateralization, thus explaining monocyte accumulation in targeted vessels (91).

Monocytes and macrophages play a key role in promoting a collateral circulation following arterial occlusion through the production of proangiogenic factors and the formation of a vascular plexus by bridging to sprouting ECs (92). Recently, it has been shown in live imaging that macrophages are immediately recruited to the wound area after injury and are strongly involved in the entire repair phase (74) (Fig. 2). Moreover, macrophage ablation results in impaired neoangiogenesis. It has been recently observed that inflammatory macrophages are sufficient to drive vessel sprouting via VEGF signaling during wound repair (74).

Macrophages form primitive, non-endothelial 'vessels' terned vascular mimicry (VM) channels, structurally and functionally connected to the systemic vasculature and that hypoxia is an important mediator of VM formation (93).

In 2016, an unexpected role of macrophages in the repair of brain vascular ruptures was described, where macrophages mediated the repair by adhesion and mechanical forces creation (94). In that study, the authors suggested that this mechanism was potentially conserved to PB vessel repair. Another study demonstrated that macrophages from recruited monocytes promoted arteriogenesis and tissue repair following ischemia (76).

Macrophages promote angiogenesis by secreting pro-angiogenic factors and modulating angiogenesis via cell-to-cell contacts with ECs, that promote the differentiation of pro-angiogenic macrophages (95). Macrophages and ECs communicate through secreted microvesicles, such as exosomes, in the angiogenesis process (95) (Fig. 2).

Apart from the angiogenic and arteriogenic ability, monocytes are involved in tissue regeneration via soluble factors (96-99), also in diabetic lesions (100-102). Tissue macrophages increase following injury, due to active monocyte recruitment from PB and to their differentiation into macrophages (103). Physiological wound healing consists of three phases: i.e., Acute inflammation, angiogenesis/proliferation and remodeling. Monocytes/macrophages are the cells majorly required during the wound healing process and can polarize either into the M1 (inflammatory) or in M2 (anti-inflammatory) phenotype, depending on specific signals during tissue recruitment (98,104,105).

During the process of wound healing, macrophages evolve with the stages. In the normal healing process, the macrophage population switches from a pro-inflammatory (M1) to an anti-inflammatory phenotype (M2), promoting the migration and proliferation of fibroblasts and keratinocytes to repair cutaneous tissue. Moreover, in this phase, the proliferation of ECs to repair vasculature is also observed. By contrast, in chronic wounds and diabetic wounds, macrophages retain a pro-inflammatory characteristic, and the ulcers remain indefinitely in the first inflammatory stage of wound healing (98).

b) Lymphocytes. Apart from monocytes, lymphocytes are also relevant to adult vascular repair. T-lymphocytes play a key role in collateral vessel formation as suggested by the $CD4^+$-T-helper lymphocyte knockout murine model (106). Indeed, in $CD4^+$-deficient mice, reduced numbers of monocytes/macrophages have been observed in the ischemic muscles; thus, a crosstalk between monocytes and T-lymphocytes is required (106).

Pre-stimulated T-cell monocytes induce neovascularization in hindlimb ischemia in mice, suggesting that monocytes may be used as novel potential candidates for regenerative cell

therapy in patients with PAD (107). It has been shown that CD4-knockout mice have an impaired arteriogenic response to acute hindlimb ischemia (108) and that $CD8^+$ T-lymphocytes are necessary for arteriogenesis and for $CD4^+$ cell recruitment via the release of IL-16 expression (109). Zouggari *et al* confirmed that regulatory T-cells (Tregs) are important for neovascularization following ischemia (110), and that natural killer cells also play a role in angiogenesis by the secretion of interferon-γ (111).

There are significantly more $CD4^+$ Th1 cells, but fewer Tregs in ischemic tissues from patients with T2D than from normoglycemic patients with PAD (112). It has been shown that Th1 cells impair vascular regeneration in individuals with T2D in a paracrine manner, while Tregs potentiate regeneration (112). Recently, it has been observed that Treg lymphocytes are able to polarize macrophages and activate satellite cells in ischemic tissue (113).

Further studies are required to ameliorate and strengthen Treg function in tissue regeneration for clinical use (70).

c) $CD34^+$ cells and EPCs. The therapeutic benefits of administering $CD34^+$ cells to patients with CLI have been largely attributed to the angiogenic capacity of EPCs. The discrepancy in treatment outcome of using $CD34^+$ cells in previous studies may refer to the fact that EPCs comprise only a minor percentage of $CD34^+$ cells that also include several subpopulations of HSCs. In fact, EPCs account for merely 1% of all circulating MNCs (114) (Fig. 1C).

The percentage of circulating EPCs that are able to differentiate in ECs and form collateral vessel formation is very small (115). Moreover, the pro-angiogenic role of circulating EPCs is based on paracrine effects and not on differentiation into ECs (116).

The true EPCs are $CD14^+CD34$ low cells and are effectively able to differentiate in ECs *in vitro* but their role *in vivo* is not confirmed (117). Conversely, the co-culture of $CD34^+$ cells with $CD34^-$ cells induces a strong neovascularization improvement *in vitro* compared to that with only $CD34^+$ cells, indicating that a mixed MNC population behaves more efficiently than purified $CD34^+$ cells (118).

This result was also observed in clinical trials where mixed PB-MNCs induced a better therapeutic effect compared to pure $CD34^+$ cells (119).

3. Diabetes impairs the angiogenic and regenerative capacity of autologous cell therapy

T2DM induces a shortage of vascular regenerative cells and angiogenic capacity, increasing the risk of cardiovascular diseases. T2DM causes several impairments that delay wound healing (Fig. 3) and also affects the autologous cell therapy of the different cell population deriving from BM, AT and PB (summarized in Table II).

In fact, T2DM causes functional BM impairment (120). Indeed, in response to G-CSF, the mobilization and angiogenic ability of $CD34^+$ cells is increased in healthy subjects, but not in patients with T2DM (121,122). Therefore, in T2DM, tissue repair is impaired, increasing the possibility of cardiovascular complications (122).

Moreover, T2DM is also associated with a decrease in BM-derived EPCs, compromising their mobilization,

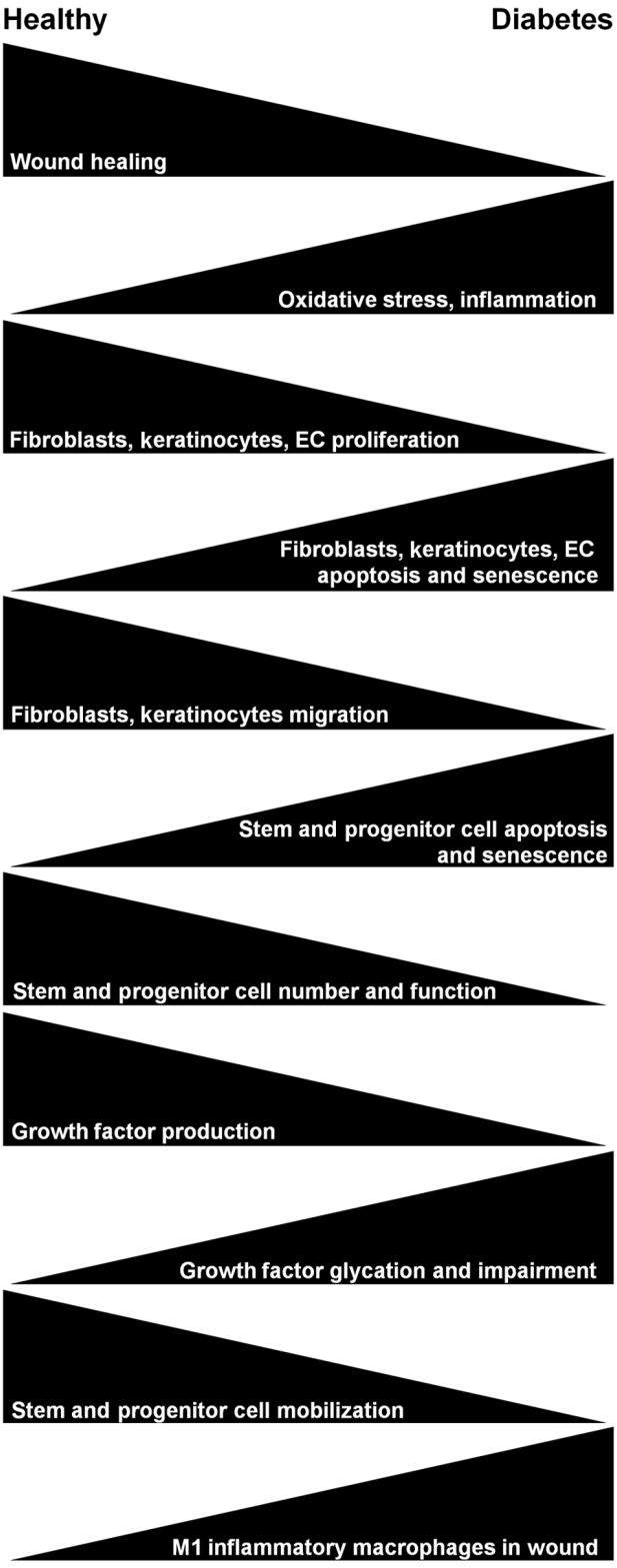

Figure 3. Schematic outline of diabetic effect on wound healing. Diabetes has a detrimental effect on wound healing capacity, increases oxidative stress and inflammation and causes proliferation decrease, apoptosis and increased senescence of endothelial cells, cutaneous keratinocytes, and fibroblasts. Moreover, the migration of cutaneous keratinocytes and fibroblasts is decreased in T2DM. Similarly, stem and progenitor cells are decreased in number due to an increase in apoptosis and senescence and exhibit reduced migration. This effect is aggravated by growth factor demise and functional impairment, caused by growth factor glycation. Finally, there is an increase of inflammatory macrophages (M1), since in T2DM the macrophage population retains a pro-inflammatory characteristic and do not switch to the anti-inflammatory phenotype M2 useful for tissue repair. All the above-described features combine to delay wound healing in T2DM.

Table II. Effects of T2DM on different sources of autologous cell concentrate.

Autologous cell concentrate source	Effects of T2DM	(Refs.)
Bone marrow	Reduced stem cell mobilization	(120-122)
	Decreased EPC number	(123,124,126)
	Reduced $CD34^+$ and EPC sensitivity to hypoxia	(127)
	Hostile microenvironment for resident SCs, induced by microvessels, sensory neuron rarefaction, fat accumulation	(122)
	Reduction of angiogenic factors (VEGF, SDF-1, HIF1α), functional impairment of growth factors	(122,125)
	Decreased wound macrophages number and increased M1 polarization	(7)
Adipose tissue	Reduced ADSC production of growth factors	(134)
	Reduced ADSC angiogenic potential due to depletion of subpopulations	(66)
	ADSC dysfunction due to apoptosis and impaired autophagy	(141)
	Vascular smooth muscle cell and pericyte dysfunction	(122)
Peripheral blood	Reduced $CD34^+$ proliferation, migration, production of growth factors and sensitivity to hypoxia	(127)
	Reduced monocyte migration towards VEGF-A	(128)

ADRCs, adipose-derived regenerative cells; EPC, endothelial progenitor cell; VEGF, vascular endothelial growth factor; SDF-1, stromal-derived factor 1; ADSC, adipose-derived stem/progenitor cell.

recruitment and function due to increased apoptosis (123,124). It has been shown that in T2DM, there is also a reduction of angiogenic factors, i.e., VEGF, SDF-1, HIF1α, necessary for the recruitment of PB-MNCs to ischemic sites in T2DM (122); post-translational modifications such as glycation can modify growth factor angiogenic capacity; indeed, fibroblast growth factor-2 (FGF-2) glycation inhibits the angiogenic capacity compared to unmodified FGF-2 (125).

Notably, BM-derived EPCs from diabetic mice have been shown to exhibit a decreased ability to induce capillary density formation *in vivo*. Furthermore, EPCs derived from p66Shc knockout mice display less oxidative stress in diabetes and behave like wild-type EPCs (126). These data demonstrate that the diabetic EPC impairment of angiogenic properties and survival is partially associated with increased oxidative stress and decreased nitric oxide (NO) bioavailability (126).

Furthermore, EPCs derived from diabetic patients do not respond to hypoxia and display an impairment in paracrine function and in angiogenic potential (127). Indeed, $CD34^+$ cells of patients with T2DM release lower concentrations of hepatocyte growth factor, stem cell factor, thrombopoietin and HIF1α, whereas they release higher levels of inflammatory cytokines (i.e., TNF-alfa and IL-1β) compared to $CD34^+$ cells from healthy subjects. As a consequence, these cells demonstrate an impairment in migration under hypoxic conditions, as well as a vasoreparative dysfunction (127).

By contrast, T2DM monocytes are less affected than $CD34^+$ stem cells (102). A comparative study of the angiogenic potential of $CD34^+$ stem cells or monocytic-like ($CD14^+$) endothelial progenitors of PB-MNCs in diabetes demonstrated that monocytic progenitors stimulate vascular growth and healing in diabetes, although not as rapidly or effectively as $CD34^+$ cell treatment (102). Thus, in diabetic conditions with compromised $CD34^+$ cells, $CD14^{++}$ cells can provide a good therapeutic option. Most probably, $CD14^+$ cells mediate healing, since they exhibit an increased sensitivity to MCP-1 and VEGF, by their capacity to induce angiopoietins in diabetic patients (102).

Conversely, another demonstrated that monocytes have a reduced migratory ability towards VEGF-A in patients with T2DM (128). The increase in oxidative stress and advanced glycation end products in diabetic monocytes induces VEGFR-1 signaling pathway activation, leading to a desensitization of the VEGFR-1 response. Furthermore, monocytes in a T2DM contest are dysfunctional and exhibit a VEGF resistance, causing cellular dysfunction (128).

T2DM-affected wounds are characterized by an increase in inflammation due to the excessive presence of M1 macrophages (129,130), probably due to hyperglycemia and the dysregulation of hematopoietic stem cell differentiation towards macrophages.

During the inflammatory initial phase of wound healing, macrophages display both M1 and M2 phenotypes, although in healed wounds, the predominant switch is towards the M2 phenotype in the proliferative phase. In diabetic and chronic ulcers, this switch is highly reduced (7,131).

It has been demonstrated that the higher recruitment of monocytes/macrophages in a muscle lesion polarizes the inflammatory M1 into the anti-inflammatory M2 phenotype, inducing myogenic differentiation and repair (132). Furthermore, as previously demonstrated, in a diabetic wound not healed after 2 months from revascularization, the PB-MNC implant leads to efficient angiogenesis, the inhibition of the inflammatory marker, NF-κB, and polarizes macrophages from the M1 to the M2 phenotype, inducing a complete healing (133).

Recently, it was observed that a novel epigenetic mechanism in diabetic HSCs causes macrophage number decrease and their polarization towards M1 (7). Diabetes has been demonstrated to impair adipose tissue-derived stem cell wound healing capacity in mice (134). ADSCs from diabetic patients exhibit a reduction in VEGF secretion and proliferation, as well as an impaired angiogenic capacity (66,135,136).

4. Clinical trials in no-option patients

In 15-20% of patients with CLI, revascularization is not possible or not effective, with a consequent increased risk of major amputation. This condition is defined as no-option CLI, it is most frequent in diabetic patients and also in patients with end-stage renal disease, who could have heavily calcified arteries below the knee and below the ankle.

Over the past decade, various meta-analyses on cell therapy in no-option patients have been published mainly on BM-MNCs and PB-MNCs, including both patients with arteriopathy and diabetic patients with CLI.

Benoit *et al* evaluated 45 clinical trials, of which 7 were randomized for a total of 1,272 treated patients with a significant reduction in amputations in patients treated with autologous cell therapy (PB-MNC and BM-MNC) compared to patients treated with medical therapy (137). Liew *et al,* in a meta-analysis of 16 randomized and controlled trials (RCTs) considering 774 patients, reported a significant reduction in major amputations, a complete healing of the lesions, and an increased ankle arm index (ABI) (138). Both PB-MNCs and BM-MNCs significantly reduced the risk of major amputation. PB-MNCs also significantly improve wound healing.

Jiang *et al* defined wound healing as the primary end-point in a meta-analysis on RCTs (139). Autologous cellular therapies with BM-MNCs or PB-MNCs were significantly associated with improved wound healing in 12 clinical studies of 290 patients. No differences in wound healing were found between BM-MNCs and mobilized PB-MNCs, and autologous cell therapies were not associated with any increased risk for side-effects (139).

Ai *et al* analyzed 25 trials that included both PB-MNCs and BM-MNCs, and reported that cell therapy significantly reduced the amputation rate and increased amputation-free survival (AFS) (140). Cell-based therapy significantly ameliorated the ABI, increased the rate of ulcer healing and transcutaneous oximetry ($TcPO_2$), reducing limb pain and improving the movement. No significant association between the injected cell number and the therapeutic effect has been described (140).

Rigato *et al* performed a meta-analysis on 19 RCTs (837 patients), 8 non-randomized trials (338 patients) and 41 uncontrolled studies (1,177 patients), including studies with BM-MNCs and PB-MNCs, mobilized and non-mobilized in patients without indications for revascularization (8). However, Rigato *et al* (8) observed that considering a limited number of studies with a better quality of controls, there was a very poor effect of cell therapy. The primary analysis of RCTs revealed that cell therapy significantly reduced the risk of amputations by 37%, improved amputation-free survival by 18% and increased the rate of ulcer healing by 59%. Furthermore, cell therapy significantly improved the ABI, $TcPO_2$, pain free walking distance and reduced pain at rest. The analysis on non-RCTs revealed that cell therapy can prevent amputation even in 50% of treated patients (8).

When different cell types were compared, the meta-analysis also revealed that PB-MNCs, but not other cell types, significantly decreased the amputation rate and the AFS (8). BM-MNCs only significantly improved wound healing, whereas both BM and PB-MNCs significantly improvedthe ABI, $TcPO_2$ and the rest pain score (8). Accordingly, Peeters Weem *et al*, in a meta-analysis of placebo controlled trials, demonstrated that BM-derived cell therapy did not give any advantage on the primary outcome survival, measures of amputation and AFS in CLI-affected patients (141).

In the MOBILE randomized double-blind study, 152 patients (155 limbs) with Rutherford 4 or Rutherford 5 critical limb ischemia were randomized to receive an injection of BM-MNCs or the placebo (142). At one year, there was no significant difference in the rate of AFS between the two groups. However, a post hoc analysis at two years revealed that there was a significant difference between the BM-MNC group and the placebo, with a hazard ratio of 0.49 in favor of cell therapy at 52 weeks. The analysis also revealed that while BM-MNCs did provide a significant benefit for patients without diabetes at Rutherford stage 4, they did not provide any benefit for diabetic patients and/or those with Rutherford stage 5. Therefore, these results would suggest a negative impact of diabetes on BM-MNC therapy for peripheral ischemia.

Accordingly, a retrospective study on 367 patients revealed that Rutherford's stage 5 was the best indication for autologous cell therapy in PAD; 50% of Rutherford's stage 6 patients who initially had major tissue loss, overstepping metatarsus phalangeal level (Rutherford's stage 6), all went through a major amputation, suggesting that treatment was performed in a too advanced stage in some patients (143).

In a recent meta-analysis on a limited number of cases (7 studies on 224 patients) of diabetic foot with lesions classified by the Wagner scale, Shu *et al* demonstrated a benefit of autologous cell therapies on both complete and partial healing of injuries (144).

Guo *et al*, in a meta-analysis including 6 eligible RCTs on diabetic foot treatments, revealed that autologous stem cell administration (one study with BM-MSCs, two studies with BMMNCs, one with PBMNCs, one with BNMNC enriched in $CD90^+$ cells, and one with SVF) was significantly effective in ulcer healing. Subgroup analyses indicated that stem cells exerted beneficial effects on ulcer sizes of ≥ 5 cm^2 and <5 cm^2, as well as cell on patients aged ≥70 years and <70 years, suggesting a positive role for stem cells in the treatment of diabetic foot ulcers (145).

Recently, a systematic review and meta-analysis on 27 RCTs, involving 1,186 patients and 1,280 limbs, revealed that autologous stem cell therapy (including BM-MSCs, BM-MNCs, PB-MNC, $CD34^+$ cells and $CD133^+$ cells), ameliorated clinical outcomes in terms of the ulcer healing rate, ABI, pain free walking distance improvement, amputation rate and rest pain score reduction, but not in major limb salvage improvement, compared to conventional therapies (146). On the other hand, cell therapy

reduced amputations and increased the ulcer healing rate in diabetic patients. Larger double-blinded, randomized, placebo-controlled, multi-center trials with the long-term follow-up of high quality are warranted to confirm efficacy and safety of autologous cell therapy for PAD (146).

As regards the use of cellular therapies from AT in the treatment of CLI, there are only limited studies on animal models (147) and a limited number of clinical trials.

The first clinical study by Lee *et al* included 12 thrombo-angiitis obliterans (TAO), 3 diabetic patients with PAD with ischemic resting pain in one limb with/without non-healing ulcers and necrotic foot treated by multiple intramuscular ASC injections (148). Of note, the stromal vascular fraction of diabetic patients and TAO produced lesser colonies compared to SVF obtained from abdominal liposuction of 3 healthy donors in a colony forming unit assay (148). Moreover, SVF from diabetic patients exhibited a lower proliferative ability than the SVF from TAO and healthy patients. Conversely, the SVF from diabetic patients exhibited a similar angiogenic factor expression to the healthy control patients. After 6 months of ADSC implantation, 66.7% of patients exhibited improved pain rating scales and in claudication or walking distance. Moreover, the vascular collateral network increased during the 6-months follow-up period. Thus, multiple intramuscular SVF injections may represent a safe alternative to therapeutic angiogenesis obtainment in patients with CLI refractory to other treatments (148).

The Cell-DREAM phase I trial evaluated the safety and feasibility of intramuscular injections of autologous ASCs, cultured for 2 weeks and then injected into the ischemic leg. The results revealed an increase in $TcPO_2$ in the majority of patients and wound healing improvement in both non-diabetic and diabetic patients (149). However, that study presents a major size limitation, since only 7 patients were treated.

The study by Darinskas *et al* included 15 patients with rest pain and some with ulcerations; SVF was injected once or twice in the ischemic limb along the arteries (150). Clinical improvement in terms of pain, meters of claudication and ABI occurred in 86.7% of patients, but only 6 patients out of 15 had ulcers and two patients underwent major amputation, although the amputation sites healed completely. Moreover, the vascular collateral network formation of arteries was observed by digital subtraction angiography upon SVF cell therapy (150).

Recently, Moon *et al* published a pilot study on SVF cell treatment around the wounds of 1o diabetic feet (151). $TcPO_2$ values increased at 12 weeks after the SVF injection, and cutaneous microvascular blood flow also increased without any adverse event; none of the patients had CLI, as indicated from an initial TcPO2 >30 mmHg (151).

To date, to the best of our knowledge, only one randomized trial on 114 patients comparing standard care to micro-fragmented adipose tissue implants has been published (152). At 6 months of follow-up, 80% of micro-fragmented adipose tissue-treated feet healed compared to the control group, where only 46% healed. Wound healing was improved in the treatment group; however, no effect was observed on the pain scale between the two groups. These observations suggest that autologous micro-fragmented adipose tissue local injection can improve the healing rate after minor amputations in diabetic foot. In that study, all patients had adequate perfusion, as assessed by $TcPO_2 \geq 30$ mmHg, as in the study by Moon *et al* (151), and micro-fragmented adipose tissue injection was used as an adjuvant of stump healing and not for its angiogenic property.

In conclusion, the above-described clinical studies on AT-derived cells are all controlled pilot studies performed on a limited number of patients. Well-designed RCTs are necessary to verify reliability, safety, and efficacy on diabetic patients with CLI.

Critical issues in clinical trials. Trials and meta-analyses are not exempt to some limitations; the latter will be discussed in the following few paragraphs.

a) Definition of no-option patients. This definition may sometimes change across countries and within different centers depending on the surgeons' expertise, and this can create confusion and inconsistency in the results obtained in multicentric studies and meta-analyses.

b) Type of administration. The majority of cell therapy trials for CLI have utilized intramuscular cell delivery although intra-arterial injection has also been used (153,154). The meta-analysis by Rigato *et al* indicated that the intra-muscular injection was associated with better results compared to intra-arterial delivery (8).

c) *Cell number and frequency of implants*. The clinical trials reported thus far use variable number of cells implanted and different frequencies of implants for cell therapy. In a pilot randomized controlled trial, patients who received 4 repeated BM-MNC injections vs. 1 single treatment exhibited an increase in pain-free walking distance, whereas the ABI and pain were not altered at 24 weeks after the injections (155). Accordingly, Kang *et al* confirmed that several treatments were more effective than a higher number of cells administered in one single treatment (156). Moreover, Beugels *et al*, in a rat model, demonstrated that the centrifuged human BM suspension containing low and medium concentrations of mesenchymal and hematopoietic stem cells significantly improved vascularization in limb ischemia, but that the effect was almost lost with higher stem cell doses (157).

d) G-CSF mobilization. The cellular mobilization in regenerative therapy is a long-debated topic. Mobilization has been widely used; however, it has been suggested that the mobilization of $CD34^+$ stem cells from the BM through the administration of G-CSF is not efficient in diabetic patients (121). Particularly, the mobilization potentially useful for the activity of stem cells does not appear useful in PB-MNC treatments; in fact, no-option patients with CLI treated with no-mobilized PB-MNCs have been shown to respond positively to the treatment (75,81-84). Moreover, in a previous study, there were no differences in AFS in no-option patients treated with pure $CD34^+$ or PB-MNCs (119). Furthermore, the SCELTA TRIAL suggests the 'non-inferiority' of non-mobilized PB-MNCs compared to BM-MNCs (84).

From no-option patients to adjuvant therapy. The clinical trials described thus far involve no-option patients; however, cellular therapy can also be considered as adjuvant therapy in patients undergoing revascularization (158). These therapies can be useful in diabetic patients, where the evolution

of ulcerative lesions is not always closely related to proper revascularization (159-161). Arterial revascularization has been recognized a condition necessary for the obtainment of a positive clinical outcome. However, in a retrospective trial, Lehalle *et al* demonstrated that revascularization was not sufficient for wound healing in diabetic patients (143). Moreover, the same study also observed that 20% of wounds healed of diabetic patients were going to develop new ulcers after 3 years. Okazaki *et al,* in 304 revascularized patients, observed that only 56.3, 63.4 and 64.0% of wounds healed at 1, 2 and 3 years, respectively (161).

Shiraki *et al*, in a retrospective study on 871 patients, of which 734 with trophic lesions and 553 diabetic patients, revealed that 33% patients did not heal at 1 year following successful PTA revascularization (160). These data were confirmed by Robinson *et al*, who that demonstrated a mean of 209 days healing time following revascularization (162).

Another retrospective study on 179 patients with CLI and tissue loss demonstrated that a higher rate of major amputation was recorded in non-healing patients within 4 months from revascularization, while patients with wounds which healed in 3 months exhibited the lowest amputation rate (163). All these studies clearly suggest that successful revascularization not always ensures wound healing and that can determine an amputation, especially in diabetic patients.

BM-MNC cell therapy as an adjuvant of revascularization has already been described with encouraging results (158). More recently, Persiani *et al* observed that diabetic patients at Fontaine stage IV treated with PB-MNCs as adjuvant therapy in revascularization, exhibited an improvement trend in the amputation rate, ulcerative lesions, $TcPO_2$ and pain reduction (82).

5. Conclusions and future perspectives

The studies reported in the present review describe cell therapy in vascular limb rescue surgery in diabetic patients and its usefulness in those patients who are either not eligible for revascularization or in revascularization failures.

Currently, the optimal cellular source for angiogenic therapies remains undefined, since most of the trials of BM-MNCs and PB-MNCs have confirmed comparable clinical results in line with the major meta-analyses, where a single implant for each cell concentrate was compared.

Clinical studies on cell therapy from AT for the treatment of CLI and diabetic patients are still limited. The selection between BM-MNCs and PB-MNCs can be affected by the cellular concentrate, reliability of the treatment and the characteristics of the patients.

The higher concentration of stem cells in BM-MNCs has led to an extensive use of this technique compared to PB-MNCs, where the number of $CD34^+$ cells is much lower. Moreover, cell therapy with PB-MNCs leads to a good angiogenic and wound healing effect, with the great advantage of minor withdrawal invasiveness, which allows one to repeat cell implants several times.

It is now clear that T2DM affects cell functionality both in BM and in AT. Moreover, while $CD34^+$ stem cells are considered compromised, PB-MNCs seem to be less affected in functionality, albeit not all the studies agree on this point (128). Thus, in T2DM with CLI, PB-MNC cell therapy could guarantee a better therapeutic outcome. Furthermore, a meta-analysis demonstrated a decrease in amputation rates and an increase in AFS periods and clinical parameters such as ABI, $TcPO_2$, and the VAS score, indicating that this approach could be effective in no-option diabetic patients (8).

Ultimately, different studies described in the present review suggest the use of autologous cell therapies as promising tools of revascularization adjuvant for wound healing in diabetic patients with no-option CLI; however, further analyses are necessary to confirm preliminary positive outcomes.

Although over the past years autologous cell therapies were mainly based on the stem cell populations for treating patients with no-option CLI, recently several studies have observed that the capacity of stem cells is influenced and orchestrated by local immune responses to tissue damage, with monocytes/macrophages as key players of tissue repair (35-37). Moreover, diabetes strongly impairs stem cell populations in BM, PB and AT. The paradigm shift, from stem cells to immune cell-based therapy, is known as the immune-centric revolution, that was recently confirmed by a study that found that stem cell therapy did not improve heart function by producing new cardiomyocytes, but by inducing an immune response due to the activation of macrophages (164). Those authors observed that the benefit was likely to be related to the local and acute immune response rather than to the regenerative capacity of the stem cells themselves, clearly demonstrating that the immune-modulation triggered by the immune system is at the basis of the repair mechanism. In line with this immune centric vision, a randomized, controlled, multi-center study using autologous peripheral blood total nuclear cells (TNCs) that will enroll up to 350 diabetic patients with no-option CLI, is ongoing (HT-CLI pivotal trial NCT03809494). The results of this trial will give a clear vision of the importance of immune cells population in angiogenesis and wound healing. A number of research groups are focusing on immune cell regenerative effects on different tissues, such as skeletal muscle (165,166).

Furthermore, given the increasing number of diabetic patients and the significant percentages of patients with non-healing lesions even following effective revascularization, adjuvant cell therapy may soon take hold. However, more controlled clinical trials will be needed to prove this hypothesis.

Finally, a promising approach for therapeutic angiogenesis and wound healing resides in the study of exosomes derived by MNCs. Recently, it was demonstrated that MSC-derived exosomes played a pivotal role in enhancing the proliferation and migration of fibroblasts of both normal donors and patients with chronic wound (167). Moreover, these exosomes induce angiogenesis *in vitro*, since ECs can uptake them. Exosomes are fundamental for angiogenic improvement and similar to miRNAs, are useful in activating signaling pathways involved in angiogenesis. The exosomes can also contain active transcription factors, such as STAT3, able to induce the transcriptional upregulation of different growth factors; i.e., SDF1, IL-6, HGF and nerve growth factor (NGF) that are all compromised in chronic wounds, particularly in diabetic patients. Further, it was shown that exosome-depleted conditioned medium had impaired angiogenesis response (167).

Recently, exosomes of human induced pluripotent stem cell-derived mesenchymal stem cells (hiPSC-MSCs) have demonstrated to be efficacious and to be an interesting alternative for cell transplantation therapy. MSCs exosomes have been shown to be efficacious in repairing injured tissues in a rat skin wound model (168).

The therapeutic approach based on exosomes has been demonstrated to be effective also in different diseases, and an interesting approach has been developed in cancer therapy, in which ADSCs overexpressing a miR-125b bearing a specific ExoMotif sequence tag enhance the loading into extracellular vesicles. These vesicles have then been used to deliver this anti-metastatic miRNA in hepatocellular carcinoma cells, reducing their proliferation (169).

A similar method can be used with exosomes as a therapeutic approach in chronic wounds, using autologous MNCs engineered to overexpress a specific miRNA or a transcription factor useful for angiogenesis and wound healing improvement. The latter could be injected subcutaneously around wound sites to ameliorate healing.

Acknowledgements

The authors apologize to the many researchers whose work was not cited in the present review. The authors would like to thank Dr Robert Monticone of the Laboratory of Cardiovascular Science, National Institute on Aging (NIA), National Institutes of Health (NIH), Baltimore, MD, USA, for the English proofreading.

Funding

The present study was partly supported by the Italian Ministry of Health RF-02362708 grant to AM, the AFM Telethon 22522, Idi Farmaceutici s.r.l. grant to AM, and by the intramural research Program of the NIH, National Institute on Aging to MCF.

Availability of data and materials

Not applicable.

Authors' contributions

AM, MCF, MR and SF were involved in the conception and design of the manuscript. AM, MCF and SF drafted the manuscript. AM, MCF, MR and SF revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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