

Stem cell and progenitor cell therapy in peripheral artery disease

A critical appraisal

Holger Lawall¹; Peter Bramlage²; Berthold Amann³

¹SRH-Klinikum Karlsbad-Langensteinbach, Angiology / Diabetology, Karlsbad, Germany; ²Institute for Cardiovascular Pharmacology und Epidemiology, Mahlow, Germany;

³Department of Internal Medicine, Franziskus Krankenhaus, Berlin Vascular Center, Berlin, Germany

Summary

Atherosclerotic peripheral artery disease (PAD) is a common manifestation of atherosclerosis. The occlusion of large limb arteries leads to ischaemia with claudication which can progress to critical limb ischaemia (CLI) with pain at rest, and to tissue loss. At present, common therapy for CLI is either surgical or endovascular revascularisation aimed at improving blood flow to the affected extremity. However, major amputation and death are still frequent complications. Exploring new strategies for revascularisation of ischaemic limbs is thus of major importance. Bone marrow (BM)-derived stem and progenitor cells have been identified as a potential new therapeutic option to induce therapeutic angiogenesis. Encouraging results of preclinical studies have rapidly led to several small clinical trials, in which BM-derived mononuclear cells were administered to patients with limb ischaemia. Clinical benefits were reported from these trials including improvement of ankle-brachial index (ABI), transcutaneous partial pressure of oxygen (TcPO₂), re-

duction of pain, and decreased need for amputation. Nonetheless, large randomised, placebo-controlled, double-blind studies are necessary and currently ongoing (BONMOT-CLI, JUVENTUS and NCT00498069). Further research relates to the optimal cell type and dosage, the isolation method, the role of colony-stimulating factors, administration route, and the supportive stimulation of cells with reduced functioning due to advanced PAD. Autologous stem cell therapy for ischaemic peripheral disease seems to be a promising new tool for the treatment of severe limb ischaemia. Preliminary evidence has established its safety, feasibility and effectiveness on several important endpoints. Several large endpoints studies are underway to further consolidate this evidence.

Keywords

Critical limb ischaemia, bone marrow transplantation, angiogenesis, progenitor cell therapy, peripheral arterial disease

Correspondence to:

Holger Lawall, MD
Angiology / Diabetology
SRH-Klinikum Karlsbad-Langensteinbach
Guttmanstraße 1, 76307 Karlsbad, Germany
Tel.: +49 7202 613357, Fax: +49 7202 616167
E-mail: holger.lawall@kkl.srh.de

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Introduction

Peripheral artery disease (PAD) is a common manifestation of generalised atherosclerosis. In Mediterranean and Asian countries, thromboangiitis obliterans (TAO, Buerger's disease) is an additional cause of PAD (1). Risk factors for atherosclerotic PAD are mainly, but not exclusively, smoking and diabetes, and are therefore identical with those for atherosclerosis in the cerebrovascular and coronary circulation. Comorbid PAD substantially increases the mortality risk conferred by coronary artery disease (CAD) and/or cardiovascular disease (CVD) alone (2).

The occlusion of large limb arteries may lead to ischaemia with claudication, and can progress to critical limb ischaemia (CLI) with pain at rest and tissue loss in atherosclerotic PAD. For all stages of the disease, minimisation of risk factors is mandatory. The mainstay of therapy for severe, limb-threatening ischaemia is either surgical or endovascular revascularisation aiming to improve blood flow to the affected extremity. If revascularisation has

failed or is not possible, major amputation is often necessary. This relates to about 30% of all cases of severe limb ischaemia (3), corresponding to about 100,000 major leg amputations in the EU, and to 120,000 in the US. Leg amputation in atherosclerotic PAD causes an acute mortality rate of around 30% and a grim five-year prognosis with a survival rate of less than 30% (4). In the case of TAO, life expectancy is not as severely limited, but the major amputations necessary often result in severe handicaps, in the mostly younger patients.

Consequently, exploring new strategies for revascularisation of ischaemic limbs is of major importance. Bone marrow (BM)-derived stem and progenitor cells have been identified as a potential new therapeutic option to induce therapeutic angiogenesis. This approach aims at improving the vascularisation of the ischaemic leg so that perfusion increases sufficiently for wound healing to occur, and at resolving of pain at rest, this ultimately allowing limb salvage.

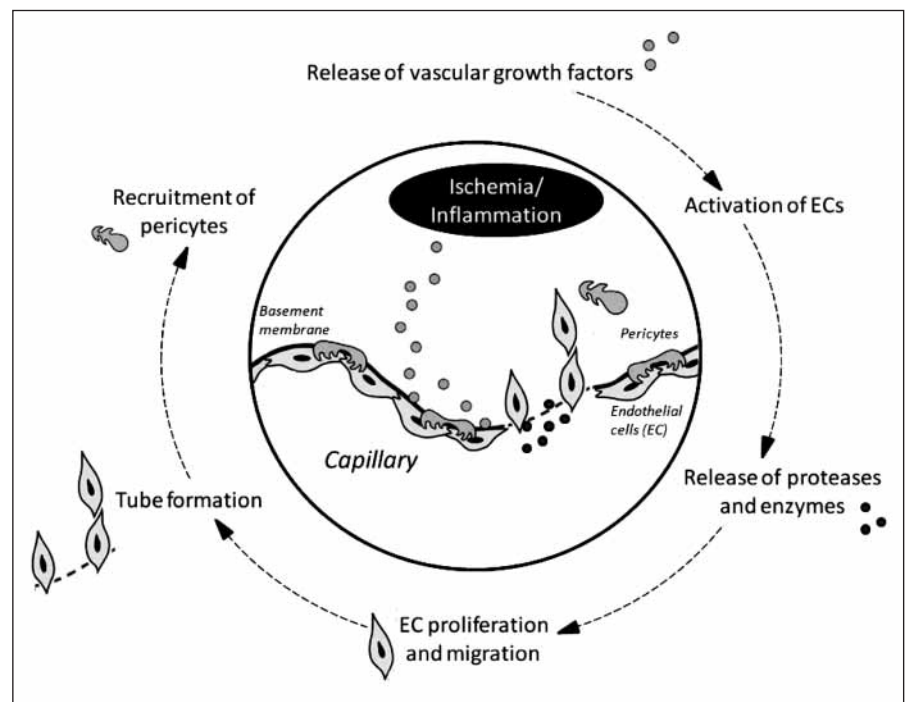


Figure 1: Schema of possible events in the angiogenic process. EC, endothelial cell.

Angiogenesis: collateral vs. capillary growth

Development of PAD in the legs is characterised by narrowing and occlusion of arterial vessels, eventual reduction in distal perfusion, and by local compensating mechanisms which include *capillary growth* and the development of collateral arterial vessels (*arteriogenesis* [5, 6]). Both collateral artery and capillary growth are sometimes collectively called *angiogenesis* which reflects common therapeutic implications. It is necessary to distinguish these two fundamentally different processes of neovascularisation, however:

Capillary growth occurs as a sprouting of small endothelial tubes from preexisting capillary beds in response to local hypoxia. It is mediated by hypoxia-induced release of chemo- and cytokines (vascular endothelial growth factor [VEGF] and related growth factors). No influx of non-tissue resident cells is needed (7). The resulting capillaries are small, with a diameter of about 10–20 μm , and cannot sufficiently compensate/substitute a large occluded transport artery due to Hagen-Poiseuille's law (► Fig. 1).

Arteriogenesis leads to enlargement of preexisting collateral arterioles in parallel orientation with the obstructed vessel. The original diameter of a small, initially non-perfused arteriole may increase up to 20 times (8, 9) during the process of arteriogenesis. Experimental evidence indicates that endogenous arteriogenesis can almost fully restore a normal vascular conductance in animal models of limb ischaemia induced by large vessel occlusion. This correlates well with the clinical observation that many patients with PAD and e.g. femoral artery occlusion are free of ischaemic symptoms because their collateral network delivers enough blood to meet the perfusion need of the lower limb (10). We will focus on arteriogenesis in the following overview (► Fig. 2).

Physiology of collateral artery growth (arteriogenesis)

Arteriogenesis (see [11–13] for recent reviews) is initiated by increasing shear forces against the vessel wall induced by narrowing or occlusion of the transport artery. Blood flow is therefore redirected to the small collateral branches (14). Fluid shear stress is the primary and strongest arteriogenic stimulus (15). Shear responsive elements in the endothelial lining of the very small pre-existing collaterals activate synthesis of chemotactic proteins (chemoattractants). In the first, inflammatory-like phase of arteriogenesis, several chemoattractants for circulating monocytic precursor cells create a chemotactic gradient (16). Adhesion molecules are up-regulated followed by adhesion and invasion of circulating cells of monocytic lineage (17). The invading monocytes, which migrate to the perivascular space and which rarely – if at all – incorporate into the collateral vessel wall, are BM-derived cells with monocytic and/or macrophagic surface markers (18). They activate matrix proteases in the pericollateral space which destruct the tissue surrounding the growing vessel, creating space in which the collateral arterial wall can expand. These physiologically preformed arterio-arterial collateral connections increase in size and diameter in a temporally and spatially well defined cascade of events until a fully working three-layered collateral artery restores blood flow four to six weeks after the occlusion of the large artery (19). Heil et al. have therefore suggested that the role of BM-derived monocytes in physiological arteriogenesis is to act as small perivascular “cytokine factories” which “deliver software and not hardware” (20). The concept of BM-derived monocytes as the main effectors of arteriogenesis is further reinforced by the recent discovery that so-called “circulating endothelial progenitor cells” (EPC) (21) also

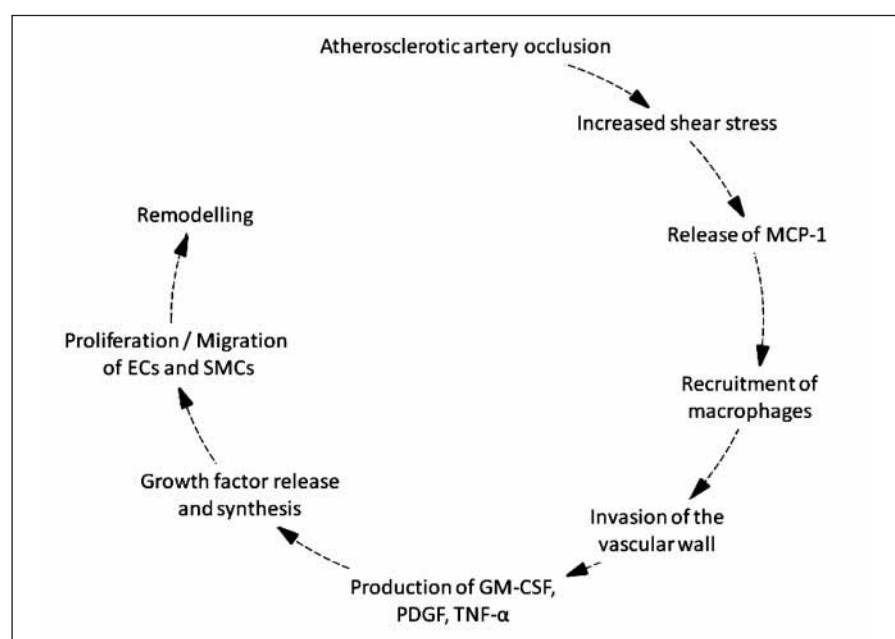


Figure 2: Schema of possible events in the arteriogenic process. EC, endothelial cell; SMC, smooth muscle cell; MCP-1, monocyte chemoattractant protein-1; GM-CSF, granulocyte-macrophage colony-stimulating factor; PDGF, platelet derived growth factor; TNF- α , tumour necrosis factor- α .

originate from the monocyte-macrophage lineage and are likely to be identical with the BM-derived monocytic cells active in the perivascular collateral artery space (22–25).

Two important points are to be remembered with respect to arteriogenesis: 1) arteriogenesis is driven by shear-stress, not by hypoxia, and therefore occurs proximal and parallel to the occluded artery, and 2) recruitment of monocytic BM cells is the central event of arteriogenesis. However, as outlined before, this adaptive repair mechanism fails to work adequately in a large number of patients, resulting in advanced peripheral ischaemia and, ultimately, in limb loss. However, regenerative capacity observed in young animals might not be comparable to that of humans, where it is much lower possibly due to widespread vascular disease (26). It is intriguing that the same risk factors for advanced ischaemia due to insufficient collateralisation – diabetes, smoking, hyperlipidaemia and advanced age – are also risk factors for a lower number of circulating, monocytic EPC. This observation strengthens the important role of BM-derived monocytes in PAD repair (27–32) yet again. Therefore, the rationale of cell therapy in PAD is to imitate and boost the physiological repair processes, using large numbers of functionally active autologous precursor cells to ultimately induce arteriogenesis.

Animal models of cell therapy in limb ischaemia

A large number of animal experiments in mice and rats as well as in larger animals have demonstrated the feasibility and efficacy of cell therapy in restoring blood flow to the ischaemic limb. These experiments were able to show that the number of circulating EPCs increase in response to ischaemia (33, 34), that these cells can be

found incorporated into capillaries and interstitial arteries in models of limb ischaemia (34, 35), and that they may act in a paracrine manner by secreting angiogenic growth factors and cytokines (36). Therapeutic relevance was demonstrated by the finding (37) that *ex vivo*-expanded human EPCs transplanted into athymic nude mice with hind limb ischaemia showed a recovery of blood flow, an enhanced collateral density, and a 60% limb salvage (7% controls). A short summary of experimental animal studies is given in ► Table 1, and an excellent review has been published recently (38).

In these studies hind limb ischaemia was induced in models of acute, but not chronic ischaemia by unilateral ligation or coagulation of the common femoral artery, this restriction being one of their major limitations. Such being the case, there is an urgent need for specific animal models with true degenerative arteriosclerotic disease, this being the most frequent etiology of human PAD. Furthermore, heterogeneous cell sources and cell preparations were used in the described studies, whereas most researchers BM-derived mononuclear cells or purified fractions thereof generally injected intravenously. Encouraging results of preclinical studies have rapidly led to several small clinical trials in which BM-derived mononuclear cells were also administered to patients with limb ischaemia caused by atherosclerotic PAD. In the following, we will review the available evidence for the use of cell therapy for patients with limb ischaemia.

Clinical results of autologous cell therapy

TACT (Therapeutic Angiogenesis using Cell Transplantation) was the first large report on the use of BM-derived mononuclear cells in the treatment of limb ischaemia (39). The protocol consisted of

an open pilot study and a randomised controlled confirmatory part.

In the pilot study, patients recruited to receive BM transplantation had chronic conditions of critical limb ischaemia, including rest pain, non-healing ulcers, or both due to PAD which was not amenable to revascularisation. Twenty-five patients with unilateral limb ischaemia were treated with BM-derived mononuclear cells (BMMNC; $1.6 \pm 0.6 \times 10^9$ cells), injected at 40 points into the gastrocnemius of the more ischaemic limb (ankle-brachial index [ABI] < 0.6). As a control treatment, saline solution was injected into the contralateral, less ischaemic leg (ABI > 0.6). The procedure was apparently safe and improved the ABI, transcutaneous oxygen pressure (TcPO₂) and rest pain, and the pain free walking distance at four and 24 weeks.

In the randomised, controlled part 22 patients with bilateral leg ischaemia were recruited and randomly treated with either BMMNC (active treatment) in one leg, or with unstimulated peripheral blood mononuclear cells (PBMNC) into the other leg as a control. ABI and TcPO₂ improved four weeks after cell treatment. ABI improved in legs injected with BMMNC in 13 out of 20 patients. Improvements were observed for TcPO₂ to a similar extent. Pain at rest in legs treated with BMMNCs was abrogated in 16 out of 20 patients, and pain-free walking time also improved.

PBMNC injection had no beneficial effect on outcome parameters proving that 1) PBMNC do not induce relevant angiogenesis / collateralisation, and 2) that needle punctures as well do not induce collateralisation. The authors of TACT suggest that the success of BMMNC transplantation is due to the approx. 500-times

Table 1: Overview of animal experiments (hind limb ischaemia) using cell therapy. Hind limb ischaemia was induced by unilateral ligation or coagulation of the common femoral artery in animals. hEPCs, human endothelial progenitor cells; PBMNC, mobilised peripheral blood mononuclear cells; PMNs, polymorphonuclear leukocytes; BMCs, bone marrow cells; EPCs, en-

dothelial progenitor cells; MSCs, mesenchymal stem-cell like cells; MNCs, mononuclear cells; ADSCs, adipose tissue-derived cells; hADSCs, human adipose tissue-derived cells; OECs, outgrowth endothelial cells; - increase, - decrease.

Reference	Cell type	Animal model	Outcome
1997 Asahara (21)	hEPCs	mouse	blood flow recovery ↑ capillary density ↑
2002 Iba (110)	PBMNC, PMNs	rat	collateral vessel formation ↑
2004 Ziegelhoeffer (18)	BMCs	mouse	collateral growth ↑ cell incorporation into perivascular space
2004 Kinnaird (111)	MSCs	mouse	collateral flow ↑, recovery limb function ↑ incidence of auto-amputation, muscle atrophy ↓
2004 Kinnaird (112)	MSCs	mouse	cell incorporation into perivascular space b-FGF-levels ↑, VEGF-levels ↑
2004 Niagara (113)	Skeletal myoblasts	rabbit	neovascularisation ↑
2005 Napoli (114)	BMCs	mouse	blood flow recovery ↑ capillary density ↑
2005 Takagi (115)	BMCs	rat	neovascularisation ↑
2005 Iwase (116)	MSCs, MNCs	rat	blood perfusion ↑
2005 Nakagami (117)	ADSCs	mouse	angiogenic score ↑
2005 Yoon (118)	EPCs, OECs	mouse	neovascularisation ↑
2006 Aicher (119)	EPCs	rat	recruitment and homing of EPCs ↑
2006 Awad (120)	EPCs	mouse	healing and vascular growth ↑
2006 Kobayashi (121)	BMCs	rabbit	angiographic score ↑, capillary density ↑, TcPO ₂ ↑ skin ulcer ↓
2006 Li (122)	PBMNC	mouse (nude)	capillary density ↑, limb loss ↓ cell incorporation into perivascular space
2006 Kim (123)	MSCs	mouse	sustainment of hind limbs ↑
2006 Moon (124)	hADSCs	mouse	muscle injury ↑, vascular density ↑
2006 Sica (125)	BMCs	mouse	blood flow ↑, capillary density ↑ interstitial fibrosis ↑
2007 Jeon (126)	BMCs	mouse	density of microvessels ↑ b-FGF-levels ↑, VEGF-levels ↑
2008 Zhang (127)	BMCs	mouse	blood flow ↑, capillary density ↑ Laser Doppler flow ↑

higher number of CD34+ haematopoietic stem cells as well as to the higher number of immature, precursor cells in the BM as compared to those in the peripheral blood stream.

The publication of TACT and the first works on cardiac stem cell therapy (40) raised considerable general interest, and lead to the use of stem cell / BM cell therapy in peripheral ischaemia in a number of different countries. The main features of these studies are shown in ► Table 2. As in animal studies, several cell isolation methods and modes of application were employed. Furthermore, the degree of ischaemia varied throughout the groups, ranging from Rutherford grade 2 / Fontaine IIa to severe CLI (Rutherford 6 / Fontaine IV) (41). Some studies were also hampered by the small number of study subjects, lack of a control groups and by differing outcome parameters.

Despite these limitations, the outcome of BM-derived cell therapy on perfusion parameters (ABI, TcPO₂) and clinical course (wound healing, walking distance) is remarkably consistent and positive throughout the different reports. Pooled results show that autologous cell therapy can induce an increase in ABI values between 0.1 and 0.2 points, and a TcPO₂ increase between 10 and 20 mmHg O₂. Depending on baseline values, walking distance can improve about 100 to 200 meters. In addition, no serious side-effects were reported.

Critical appraisal of methods

While results of clinical trials with BM-derived cell therapy in limb ischaemia are promising, a number of methodological questions still remain unsolved. In the following we will give an overview discussing questions still open from clinical trials.

Role of cell type

Implementation of cell therapies in cardiovascular disease was initially driven by the theoretical concept that BM-derived endothelial progenitor cells could incorporate into the damaged vessel endothelium. Alternatively, *de novo* vessel formation by association of haemangioblasts / EPCs (vasculogenesis) was discussed as a possible mechanism of cell therapy in ischaemic diseases. However, since the first description of EPCs by Asahara in 1997 (17), more than 800 scientific articles (42) have been published about putative EPCs, yet no definite description of this elusive cell type has been found so far (43). Also, vasculogenesis as a true formation of new blood vessels has never been convincingly shown in adult humans. As a consequence, the role of "EPCs" in human angiogenesis in the setting of peripheral vascular obstruction remains

Table 2: Studies on bone marrow-derived mononuclear cells. BMMNC, bone marrow-derived mononuclear cells; PAD, peripheral artery disease; DM, diabetes mellitus; TAO, thromboangiitis obliterans; CLI, critical limb ischaemia; ABI, ankle-brachial-index; TcPO₂, transcutaneous partial pressure of

oxygen; Amp., amputation (major or minor); +/-, overall result positive (+) or negative (-) or equivocal (+/-); ↑ increased; ↓ decreased; study level: 1a, double-blind; 1b, randomised, non-blinded controlled trial; 2, controlled trial; 3, cohort study/historical controls; 4, patient series/uncontrolled trial

	Study level	# Subjects	ABI	TcPO ₂	Pain	Amp.	+/-
2002 Tateishi-Yuyama (39)	1b	45, PAD, DM	↑	↑	↓	↓	+
2002 Esato (128)	4	8, PAD, TAO	↑	--	↓	↓	+
2004 Saigawa (129)	4	8, PAD, DM	↑	↑	↓	↓	+
2004 Higashi (130)	4	8, PAD	↑	↑	↓	↓	+
2004 Miyamoto (131)	4	12, PAD, CLI	↑	--	↓	--	+
2005 Nizankowski (132)	4	10, TAO, CLI	↑	↑	↓	↓	+
2006 Durdu (133)	1b	28, TAO	↑	↑	↓	↓	+
2006 Bartsch (134)	4	10, PAD, CLI	↑	↑	--	--	+
2006 Miyamoto (108)	4	8, TAO, CLI	--	--	↓	--	+
2007 Kajiiguchi (135)	4	7, CLI, TAO	→	(↑)	↓	?	+/-
2007 Huang (136)	2	74, PAD, DM	↑	↑	↓	--	+
2007 Hernandez (137)	4	12, PAD, DM	↑	↑	↓	↓	+
2008 Gu (138)	4	16, PAD/CLI	↑	↑	↓	↓	+
2008 Chochola (139)	4	28, CLI, PAD	↑	↑	↓	↓	+
2008 Wester (140)		8, CLI	--	--	↓	↓	+
2008 Van Tongeren (141)	4	27, PAD	↑	↑	↓	?	+
2008 De Vriese (142)	4	16, PAD	→	↑	↓	?	+/-
2009 Amann (143)	4	51, CLI	↑	↑	↓	↓	+
2009 Prohazka (56)	4	37, CLI, DM	↑	↑	↓	--	+

doubtful (44), and the translation of a truly “EPC” based endothelial repair into the clinic has not been achieved so far.

The well substantiated concept of arteriogenesis strengthens the importance of several different BM cell types, all sharing a monocytic phenotype, however. They migrate to the perivascular space of nascent collaterals and induce collateral artery growth by the release of angiogenic growth factors. It seems that cell therapies in PAD based on the application of whole BM monocytic cells or on whole stimulated PBMNCs are more successful than methods which use subfractionated cell preparations, e.g. CD 133+ (45) or highly purified CD 34+ cells from peripheral blood after granulocyte-colony stimulating factor (G-CSF) mobilisation only (46).

It must be kept in mind that all of the above mentioned studies used autologous BM or autologous peripheral blood cells. Therapies with allogeneous cells from another donor or pooled from several donors as in a placental cell concentrate are solely in animal or phase I trials with no publications on their effect in humans so far. A variety of allogeneous and autologous tissues have been suggested as alternative cell sources, such as dental pulp (47), adipose tissue (48), endometrial cells (49), placental cells (50) umbilical cord cells (51). Clinical results are pending, however.

Methods of cell separation

For practical purposes, cell therapy has to be applicable in a straightforward manner, and to be effective. This sounds self-evident; however, the sometimes complex requirements for cell isolation procedures have been obstacles for the wider application of cell therapy in PAD. It is necessary to isolate and concentrate monocytic precursor cells; the application of raw, untreated bone marrow does not have any effect on perfusion.

Between 100–800 ml of bone marrow blood were extracted in the majority of studies in Table 2, and the mononuclear cell fraction was enriched by different separation techniques: 1) Ficoll™ density gradient system centrifugation and variations thereof (52–55), 2) blood centrifugation and plasmapheresis systems (i.e. COBE® Spectra, Gambro, Sweden; CS 3000®-Plus, Baxter Healthcare, USA) (39) or 3) with a point-of-care, bedside centrifugation system (SmartPrep®, Harvest Technologies, USA) (56, 57). A total BMMNC number of between 1.5 and 10×10^9 cells was obtained using these techniques.

Both Ficoll™ and blood separator techniques require a good clinical practice (GCP)-certified blood handling facility, usually either a specialised transfusion service or a dedicated haematology unit, and both are very labor-intensive. If cultivation steps for the expansion of cells are used, an additional sterile cell biology laboratory is necessary (58, 59). Both Ficoll™ and separator techniques are tightly regulated EU-wide, and need special permission by the respective authorities.

To overcome these difficulties, a single-step bed-side, closed isolation system has been developed recently, independent of specialised hospital subservices and without legal hurdles. When using bed-side marrow collection and isolation, total therapy time

was shortened from 8–10 hours to 1 hour. In addition, this system seems to be considerably cheaper than separator or Ficoll™-based techniques (56, 57) and its arteriogenic potency at least equivalent to, or even higher than that of the BMMNC cell concentrate in an animal model (60). With simpler techniques for cell isolation, cell therapy may gain impetus in non-university hospitals as well, where the majority of PAD patients are currently treated. Further subfractionation or *ex vivo* expansion of cells does not improve results achieved with unselected BMMNC or PBMNC (61, 62); a finding also observed in the cardiac stem cell study TOPCARE-AMI (63).

Having ample experience with both the Ficoll™ density gradient system and the bed-side isolation method, we firmly believe that cell therapy will be more practicable with simpler techniques. BM seems to be the cell source of choice, similar to cardiac applications, because withdrawal of BM (usually between 100–250 ml) is fast (< 10 minutes), does not require general anesthesia but sedation only, and yields reproducible cell numbers. In contrast, PBMNC collection requires expensive G-CSF injections over five consecutive days and plasmapheresis for several hours.

Hernandez et al. (64) clinically compared Ficoll™-isolated and blood separator isolated cell preparations, and found identical positive clinical effects with both separation methods. In CAD, Seeger et al. (65) compared the Lymphoprep™ isolation method and the Ficoll™ method *in vitro* and *in vivo*. Like Ficoll™, Lymphoprep cell isolation is a polysaccharide based density gradient centrifugation method. This comparison was triggered by the ASTA-MI trial (66) which did not show an improvement of cardiac function after intra-myocardial injection of BMMNC isolated with the Lymphoprep™ method. In contrast, the TOPCARE-AMI (63) and REPAIR-AMI (67) trials showed that Ficoll™ isolated BMMNC did improve cardiac function. It was concluded by Seeger et al. that different diluents (saline vs. heparin plasma), as well as different buffer solutions and incubation media were responsible for the reduced (about one third) BMMNC number and for the reduced function of the Lymphoprep™ isolated cells (65).

Dosage: Is the number of therapeutically applied cells important?

The number of injected concentrated mononuclear cells (MNCs) in PAD has been as low as 0.1×10^9 MNCs and as high as 50×10^9 , with positive effects on perfusion reported even when low cell numbers were used (68). 1.6×10^9 MNCs were obtained in the TACT (39) and in the Higashi study (69), whereas Durdu et al. retrieved a 50-fold increase of that number of MNCs (70). The amount of BM harvested varied between 80 ml and 1,000 ml. Most groups aimed at MNC numbers around those used in the TACT study (1.6×10^9) (39). No direct comparisons of the degree of positive effects are available between different cell doses and the only study trying to establish a correlation between clinical response and cell number had only eight participants (71). MYSTAR is the only published clinical trial with a positive correlation between the

number of injected stem cells and the rate of improvement of cardiac perfusion in clinical cardiac stem cell therapy. It demonstrated that the only predictor for a reduction in infarct size was the number of intra-myocardially injected cells (72).

The large difference in cell numbers obtained may also be due to different cell counting methods. Using an automated cell counter might have included non-monocytic leukocytes (e.g. granulocytes) in the monocyte count. From our own experience 100 ml of fresh bone marrow aspirate contain (decreasing with age) about 0.5×10^9 to 3×10^9 MNCs. With isolation losses around 20–40%, the amount of transplantable BMMNC is between $0.3 - 2 \times 10^9$ per 100 ml BM.

Indirect evidence points to the importance of the monocytic cell number. It is intriguing that the young patients with TAO treated with cell therapy showed a much better response rate in terms of wound healing, abrogation of rest pain and increase in walking distance than patients with arteriosclerosis obliterans who are older, have several co-morbidities and also a 50% lower BMMNC count than TAO patients. However, no definite proof for the importance of cell numbers exists so far, and larger trials will be required directly addressing this dosage issue.

The percentage of implanted CD34+ haematopoietic stem cells is usually between 0.6% and 2.4% of total implanted MNC. Again, there is no proven correlation between CD34+ count and therapeutic response so far. Because CD34-negative cell preparations have an angiogenic effect similar to CD34+ cell concentrates (68), being CD34+ is probably not mandatory for cells used for arteriogenesis. Its measurement seems to be more of a quality control during the concentration process.

Colony-stimulating factors

The relatively large amount of BM needed to obtain the necessary number of cells has led a number of groups to use growth factor mobilised BM cells extracted from peripheral blood. This can be accomplished if growth factors like G-CSF or granulocyte-macrophage colony stimulating factor (GM-CSF) are given for several days. The BM then releases precursor and stem cells into the peripheral circulation (73) where they can be harvested by leukapheresis. This technique has long been used for donor stem cell collection in haematological disease (74, 75).

In vitro, G-CSF mobilised PBMNC have less angiogenic activity than BMMNC. Blocking monocyte apoptosis by GM-CSF could increase monocytic arteriogenic activity (73) as a potential advantage of growth factor application (76). However, subsequent clinical studies have not confirmed the suspected arteriogenic potency of GM-CSF alone (77). G-CSF is now used because of its good potential of BM cell release.

Several studies with growth factor liberated PBMNC were performed with results very similar to those of BMMNC trials (► Table 3). Among these, Huang et al. studied the effect of G-CSF-mobilised PBMNC (3×10^9 cells) in 28 diabetic patients with CLI in a randomised controlled design (78). The control group received

conventional wound care and both groups were supplemented with an intravenous injection of prostaglandin E_1 . The patients received G-CSF for a total of five days before PBMNC were collected from the peripheral circulation. Huang reported improvements in pain-free walking distance, healing of diabetic foot ulcers, ABI, and angiographic scores.

However, the vascular regenerative potential of G-CSF stimulated cells may be lower than that of BMMNC (79). Direct head-to-head comparison of different cell preparations are rare: the only study in this context, to our knowledge is that of Huang et al., in which 72 patients treated with G-CSF stimulated PBMNC were directly compared with 75 patients treated with BMMNC. Similar results in perfusion increase and clinical endpoints were demonstrated in this comparative study (62), but the degree of ischaemia remains unclear, since asymptomatic patients (Fontaine I) appear to have been included. The TOPCARE-AMI (80) study examined the effects of intracoronary infusion of BMMNC and *ex vivo*-expanded PB-EPC on contractile function and coronary flow reserve of the heart after acute myocardial infarction. No differences were found among the two cell populations.

How should cell therapy be applied?

In mouse and rat animal models of limb ischaemia, BMNC or PBMNC were given intravenously (Table 1) whereas MNC were injected into the myocardium in rabbits and pigs, (81). Unfortunately no studies exist on modes of application in larger animals with peripheral ischaemia.

Intramuscular (i.m.) and intraarterial (i.a.) injection or a combination of both has yielded promising results in human PAD. The rationale behind i.m. injection is to create a depot of cells with paracrine activity in the ischaemic area. The fate of MNC injected into peripheral muscle is unknown, however, and cell retention rates vary between 0.44 % and 10% after four days. Survival times up to 14 days were reported (82, 83). I.m. injection into the gastrocnemius muscle along a symmetric grid with a fixed number of injections (between 20 and 60) was the preferred application in most human trials (Tables 2 and 3), except for the recent pilot Bone marrow outcomes trial 1 (BONMOT-1) (57) and the follow-up placebo-controlled double blind study (BONMOT -CLI) (84). In these trials, injections were placed along the occluded native arteries because the density of preformed collaterals is highest in parallel to the axial arteries, and collateral growth occurs preferably at this location (13). In BONMOT 1 and 2, the number of injections was increased corresponding to the length of the arterial occlusion, from 40 injections for infra-popliteal disease only, to 60 injections if femoral, popliteal and infra-popliteal disease was present. No direct comparisons between different i.m. injection sites and numbers exist, however.

In the case of i.a. application, blood flow guides the injected cells to the border zone region of maximum ischaemia (85–87). Nutrient and oxygen supply is thought to be optimal there, but the degree of uptake of cells from the circulation is unknown (88).

Table 3: Mobilised peripheral blood mononuclear cells (PBMNC) – trials in peripheral artery disease. PAD, peripheral artery disease; PBMNC, peripheral blood mononuclear cells; DM, diabetes mellitus; TAO, thromboangiitis obliterans; CLI, critical limb ischaemia; ABI, ankle-brachial index; TcPO₂, negative transcutaneous partial pressure of oxygen; Amp., am-

putation (major or minor); +/- overall result positive (+) or negative (-) or equivocal (+/-); study level: 1a double-blind; 1b, randomised non-blinded controlled trial; 2, controlled trial; 3, cohort study/historical controls; 4, patient series/uncontrolled trial.

	Study level	# Subjects	ABI	TcPO ₂	Pain	Amp.	+/-
2004 Huang (144)	4	5, PAD	→	↑	↓	?	+
2005 Kawamura (145)	4	30, PAD, CLI	↑	↑	↓	↓	+
2005 Lenk (146)	4	7, CLI	↑	↑	↓	?	+
2005 Huang (147)	2	28, CLI, DM	↑	↑	↓	↓	+
2005 Ishida (148)	4	6, TAO	↑	↑	↓	?	+
2006 Kawamura (149)	4	75, CLI	↑	↑	↓	↓	+
2007 Huang (136)	2	76, PAD	↑	↑	↓	?	+

Bartsch et al. (89) report treatment of 13 patients with Fontaine IIa/b claudication with a combined i.m. / i.a. BMMNC application, and observe improvements in walking distance and ABI. Van Tongeren (90) compared i.m. and combined. i.a. + i.m. injection in a total of 27 patients and conclude that both i.m. and combined i.m.+ i.a. delivery of autologous BMC are safe and improve walking distance in a considerable proportion of patients with severe PAD not able to take part in conventional treatment.

Taken together, there are no head-to-head comparisons of different administration routes of cell therapy in patients with CLI. A study in rat hind limb ischaemia reports that both intraarterial and intramuscular administration provides similar angiogenic results, however (85).

Stimulation of impaired cell function

Albeit patients with atherosclerotic PAD have the highest need for endogenous induction of arteriogenesis, their EPCs and BMMNCs have been shown to have a compromised capacity to regenerate blood flow. These patients have a reduced number of circulating BM-derived cells, and the function of the latter is reduced (91–93).

A variety of drugs have been described possessing a proliferative effect on EPCs, including statins (94–97), angiotensin receptor blockers, vardenafil, puerarin, peroxisome proliferator-activated receptor (PPAR) γ agonists, estrogens, iloprost, and erythropoietin (98–102). In addition, pretreatment of cells with nitric oxide synthase enhancer and hypoxic preconditioning have been shown to improve the effect of autologous stem cell therapy in models of hind limb ischaemia (103, 104). Finally, VEGF gene transfer into EPCs was shown to enhance proliferation, adhesion, and incorporation into endothelial cell layers *in vitro* (105) and in animal models (105–107).

Taken together these data indicate that there is a potential for the improvement of repair function of autologous stem cells harvested from patients with severe atherosclerotic disease, diabetes and

chronic ischaemic heart disease. However, the role of stimulation or co-treatment of these cells has not been well defined so far.

Tolerability / safety considerations

Long-term concerns about unwanted side effects of cell therapy were originally raised by Miyamoto (108) following an uncontrolled and not-blinded pilot trial, testing the short-term clinical benefits of BM mononuclear cell transplantation in eight patients with CLI / TAO. At four weeks, improvement in pain was observed in all 11 treated limbs, with complete relief in four (36%). Pain scale (visual analogous scale) score decreased from 5.1 ± 0.7 to 1.5 ± 1.3 . An improvement in skin ulcers was observed in the eight limbs with an ischaemic ulcer, with complete healing in seven (88%). During the follow-up, clinical events occurred in four of the eight patients, however. The first patient suffered sudden death at 20 months after transplantation at 30 years of age. The second patient with an incomplete healing of a skin ulcer showed worsening of the lesion at four months. The third patient showed worsening of rest pain at eight months. The last patient developed a symptomless arteriovenous shunt in the calf at seven months, which had spontaneously regressed by one year.

These data are in strong disagreement with trials that have observed patients after autologous BMMNC transplantation for up to 3.2 years (57, 109). The TACT late study (109) was designed to assess the long-term safety and clinical outcomes of cell therapy by investigating the mortality and leg amputation-free interval as primary endpoints. The median follow-up time for surviving patients was 25 months, and three-year overall survival 80% of patients with atherosclerotic PAD (11 out of 74 patients died). This number increased to 100% in the 41 patients with TAO. Three-year amputation-free rate was 60% in PAD and 91% in patients with TAO. TACT late also reported no cases of unwanted neovascularisation, no increase of the expected mortality and no unwanted neovascularisation. BONMOT-1 (57) included 51 patients with impending major amputation due to severe CLI for BM cell transplantation

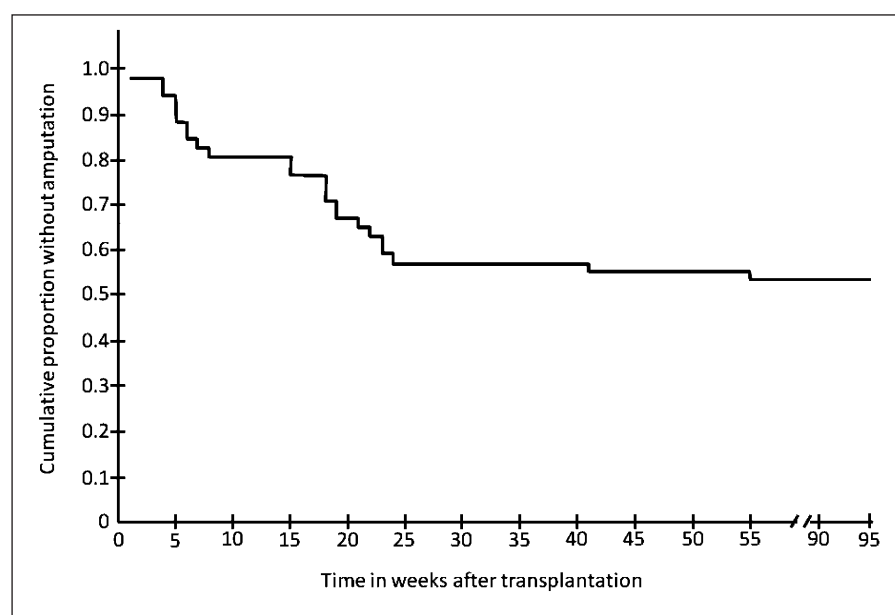


Figure 3: Cumulative proportion without amputation in BONMOT-1 (57). Amputation-free survival after bone marrow cell transplantation from the BONMOT-1 pilot study (reproduced with permission from [57]). Most amputations occurred between one and 24 weeks.

into the ischaemic leg with a 3.2-year follow-up. Limb salvage was 59% at six months and 53% at last follow-up (► Fig. 3). Most important from a clinical perspective, patients with limb salvage improved from a mean Rutherford category of 4.9 at baseline to 3.3 at six months. Three severe periprocedural adverse events occurred (two cases of anaemia after 500 ml BM aspiration, one case of large bowel puncture) and resolved without sequelae, but no unexpected long-term adverse events occurred.

Weighing the evidence from Miyamoto's report of late unwanted side-effects in eight patients against data from two long-

term studies with more than 100 patients, BM cell transplantation is a safe procedure with minimal short- and long-term side effects.

Ongoing randomised controlled trials

A number of currently ongoing placebo-controlled trials will further define the role of autologous BMMNC therapy (► Table 4). These include the BONMOT-CLI (NCT00434616), the JU-

Table 4: Ongoing randomised controlled cell therapy trials in limb ischaemia (as of November 12th 2009). BMAC, autologous bone marrow aspirate concentrate; DF, diabetic foot; CLI, critical limb ischaemia; VRC, vascular repair cells; G-CSF, granulocyte-colony stimulating factor; PBMNCs, mobilised peripheral blood mononuclear cells; BMMNCs, bone marrow-derived mononuclear cells; BMC, bone marrow cells.

Acronym	clinical-trials.gov	Sponsor/Location	Indication	Comparison	Phase	Completion
ACT34-CLI	NCT00616980	Baxter	CLI	CD34+ vs. placebo	I/II	10/2009
RESTORE-CLI	NCT00468000	Aastrom Biosciences/US	CLI	In vitro expanded autologous bone marrow cells) vs. placebo	II	06/2011
None	NCT00498069	Harvest Technologies/US	CLI	Autologous BMMNC vs. placebo	II	01/2014
JUVENTAS	NCT00371371	UMC Utrecht/NL	CLI	BMMNC vs. placebo	I/II	12/2010
ABC	NCT00539266	Leiden University Medical Centre/NL	CLI	Autologous BMMNC vs. placebo	II/III	09/2010
BONMOT-2	NCT00434616	Franziskus Krankenhaus Berlin/Germany	CLI	Autologous BMC concentrate vs. placebo	II/III	03/2010
None	NCT00595257	Harvest Technologies/India	CLI	i.m. Injection vs. i.m. injection and i.a. infusion of autologous BMMNC	I/II	
None	NCT00922389	Beike Biotech/India	CLI / DF	G-CSF mobilized PBMNC vs. standard therapy	I/II	01/2011
None	NCT00955669	Military Hospital Chongqing/China	CLI / DF	Cultured mesenchymal stem cells vs. placebo	II/III	09/2010
SCRIPT-CLI	NCT00913900	University of Wisconsin/ Madison, USA	CLI	Autologous CD133+ cells vs. placebo	I	05/2012

VENTAS trial (www.juventas-trial.nl; NCT00371371) and the “Feasibility Study of the Safety and Activity of Autologous Bone Marrow Aspirate Concentrate (BMAC) for the Treatment of Critical Limb Ischaemia Due to Peripheral Arterial Occlusive Disease” (NCT00498069).

The BONE Marrow Outcome Trial in Critical Limb Ischaemia (BONMOT-CLI) (84) evaluates the therapeutic value of BM cell-induced angiogenesis and arteriogenesis in severe, limb-threatening ischaemia. It is an investigator-initiated, double-blinded, 1:1 randomised, placebo-controlled multi-centre study at four sites in Germany. Ninety patients with no option for revascularisation or after failed revascularisation will be included and randomised either to a concentrate of autologous BM cells processed with the Harvest Smartprep2 BMAC system, or to a sham BM aspiration and placebo injection. The combined primary endpoint is major amputation or persisting CLI (no clinical or perfusion improvement) after three months. A cross-over option for placebo patients to BMMNC treatment after three months is implemented. Secondary endpoints are death, changes in perfusion (ABI, TcPO₂), quality of life (EQ5D), walking distance, minor amputations, wound healing, collateral density and cancer incidence. Post-study follow-up is up to two years.

JUVENTAS (Rejuvenating endothelial progenitor cells via transcutaneous intra-arterial supplementation) is a randomised, double-blind, placebo-controlled trial in the Netherlands. The clinical effects of repeated intra-arterial infusion of BMMNC will be investigated in 110 – 160 patients with CLI. Functional characteristics of the BMMNC obtained from CLI patients will also be studied and BMMNC dysfunction related to clinical outcome. A total volume of 100 ml bone marrow will be aspirated from the iliac crest in patients both from the active and the comparator arm. Patients in the active arm will receive repeated intra-arterial infusion of autologous stored BMMNC into the common femoral artery. The comparator arm will receive repeated i.a. infusion of placebo solution. The primary outcome measure is the rate of major amputation after six months. Secondary endpoints include minor amputation, number and extent of leg ulcers, resolution of rest pain, improvement of the ABI, improvement of TcPO₂, changes in quality of life (SF-36) and changes in clinical status.

The “Feasibility Study of the Safety and Activity of Autologous Bone Marrow Aspirate Concentrate (BMAC) for the Treatment of Critical Limb Ischaemia Due to Peripheral Arterial Occlusive Disease” is a randomised, double blind controlled multicenter trial in the US to assess the safety and activity of autologous bone marrow aspirate concentrate for the treatment of CLI due to peripheral arterial disease. It will include 48 patients, randomised in a 2:1 manner to BMMNC or placebo, respectively. The study will make use of the Harvest Smartprep2 BMAC System. Patients in the active arm will receive injection of autologous BM concentrate into ischaemic tissues of the lower extremity. Patients of the control arm will receive an injection of placebo into ischaemic tissues of the lower extremity. Outcomes are amputation-free survival, pain, pain free / maximal walking distances, TcPO₂, ABI, TBI, quality of life (SF-36).

In the other studies listed in Table 4, different cell preparations with and without cell culture are used; no further information except the NIH/NCT form is available, however. Therefore, these trials are solely listed and not further discussed.

Conclusions

Exploring new treatment strategies in patients with PAD is of utmost importance due to the high risk of major leg amputations and subsequent mortality if surgical or endovascular revascularisation has failed or is not possible. Autologous cell therapy using either BMMNC or PBMNC is a promising new treatment option for these patients, and clinical trials are very consistent in reporting clinical benefits including improvements of ABI, TcPO₂, reduction of pain and reduced need for amputation. However, there is still a need for large randomised, placebo-controlled, double-blind studies to provide a definitive role for this treatment option. Ongoing clinical trials like BONMOT-CLI (84), JUVENTAS and NCT00498069 are directed towards this goal.

As an increasing number of clinical trial evidence will support routine use of stem cell therapy, more practical aspects of cell therapy will gain importance. In this context, a single-step, bed-side, closed isolation system without the need for specialised hospital subservices and without legal hurdles is especially attractive. The total procedure is substantially shortened to one hour and is considerably less expensive than Ficoll™-based techniques. This may also provide non-university hospitals the option for to use stem cell therapy for their PAD patients.

A number of open issues remain to be resolved to that end, including optimal cell type, isolation method, cell dosage, role of colony-stimulating factors, administration route, and the supportive stimulation of cells with reduced functioning due to advanced PAD.

Taken together, autologous stem cell therapy for ischaemic peripheral disease seems to be a promising tool for the treatment of severe limb ischaemia. Preliminary evidence points at its safety, feasibility and effectiveness for several important endpoints. Several large endpoint studies are underway to further consolidate this evidence.

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