Intraarterial Administration of Bone Marrow Mononuclear Cells in Patients With Critical Limb Ischemia

A Randomized-Start, Placebo-Controlled Pilot Trial (PROVASA)

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Background—Critical limb ischemia due to peripheral arterial occlusive disease is associated with a severely increased morbidity and mortality. There is no effective pharmacological therapy available. Injection of autologous bone marrow-derived mononuclear cells (BM-MNC) is a promising therapeutic option in patients with critical limb ischemia, but double-blind, randomized trials are lacking.

Methods and Results—Forty patients with critical limb ischemia were included in a multicenter, phase II, double-blind, randomized-start trial to receive either intraarterial administration of BM-MNC or placebo followed by active treatment with BM-MNC (open label) after 3 months. Intraarterial administration of BM-MNC did not significantly increase ankle-brachial index and, thus, the trial missed its primary end point. However, cell therapy was associated with significantly improved ulcer healing (ulcer area, 3.2 ± 4.7 cm² to 1.89 ± 3.5 cm² [P=0.014] versus placebo, 2.92 ± 3.5 cm² to 2.89 ± 4.1 cm² [P=0.5]) and reduced rest pain (5.2 ± 1.8 to 2.2 ± 1.3 [P=0.009]) versus placebo, 4.5 ± 2.4 to 3.9 ± 2.6 [P=0.3]) within 3 months. Limb salvage and amputation-free survival rates did not differ between the groups.

Repeated BM-MNC administration and higher BM-MNC numbers and functionality were the only independent predictors of improved ulcer healing. Ulcer healing induced by repeated BM-MNC administration significantly correlated with limb salvage (r=0.8; P<0.001).

Conclusions—Intraarterial administration of BM-MNC is safe and feasible and accelerates wound healing in patients without extensive gangrene and impending amputation. These exploratory findings of this pilot trial need to be confirmed in a larger randomized trial in patients with critical limb ischemia and stable ulcers.

Clinical Trial Registration—URL: http://www.clinicaltrials.gov. Unique identifier: NCT00282646.

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Key Words: stem cells ■ peripheral vascular disease ■ angiogenesis

Critical limb ischemia, defined as rest pain or tissue necrosis with ulceration or gangrene, attributable to peripheral arterial occlusive disease (PAOD) not only has a major impact on quality of life, but also is associated with a dramatically increased mortality.1 Despite recent advances in interventional or surgical techniques, prognosis and amputation-free survival remains poor because a large number of patients with critical limb ischemia are not candidates for such revascularization procedures.1,2 At present, no effective pharmacological therapy is available.3

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Recent experimental studies indicated that autologous bone marrow-derived mononuclear cells (BM-MNC) as well as selected CD34+, CD133+, or CXCR4+ cells or cultured peripheral blood-derived proangiogenic cells may be a promising therapeutic option in patients with critical limb ischemia.4 Specifically, the first landmark trial, the Therapeutic Angiogenesis by Cell Transplantation (TACT) study,5 dem-

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onstrated a significant improvement of ankle-brachial index (ABI), rest pain, and transcutaneous oxygen pressure (TCO₂) after intramuscular injection of autologous BM-MNC. However, despite a number of additional smaller, nonrandomized trials showing promising results of cell transplantation in patients with PAOD or thrombangiitis obliterans (TAO)⁶–¹¹ (for review, see Sprengers et al⁴), definitive proof is still not available because of the lack of double-blinded controls. In addition, although the majority of trials have tested a potential effect of intramuscular injection of cells, no data are available that assessed the effects of intraarterial administration of BM-MNC. Therefore, we designed the Intraarterial Progenitor Cell Transplantation of Bone Marrow Mononuclear Cells for Induction of Neovascularization in Patients With Peripheral Arterial Occlusive Disease (PROVASA) trial in order to test the hypothesis that intraarterial administration of BM-MNC is associated with improved limb perfusion, resulting in an increase of ABI with reduction of ischemic rest pain and improved healing of tissue necrosis as clinically relevant secondary end points.

**Methods**

**Patients and Study Design**

The PROVASA trial is a multicenter phase II trial with a double-blind randomized-start design. At baseline, in a double-blind fashion, 40 patients were randomly assigned (1:1) to receive BM-MNC treatment or placebo and were followed for 3 months (randomized start). At 3 months, all patients received active treatment with BM-MNC and were followed for another 3 months (open-label). Thus, at the end of 3 months, all patients who had received placebo switched to active treatment (BM-MNC, crossover), and patients who had received active treatment initially received a repetitive treatment at 3-month intervals (initial verum group). A prespecified analysis was performed after the randomized-start phase as well as at the end of 6 months follow-up, where the 3-month effects of a single treatment with BM-MNC (crossover from the initial placebo group) could be compared with potential effects of a repetitive treatment at 3-month intervals (initial verum group). For patients with ulcers (Rutherford class 5) and evidence of delayed wound healing after 6 months, the PROVASA trial was extended by an amendment in order to allow for additional serial treatments with up to 3 more intraarterial applications of BM-MNC (verum). Timing and indication for continuation of BM-MNC therapy was decided according to the estimation of the treating physician in case of delayed wound healing. An interval of at least 3 months was required between serial treatments. The study outline is shown in Figure 1.

The study was approved by the review boards of the local ethics committees in Frankfurt, Hamburg, and Bern, Germany, and by the national authority, the Paul-Ehrlich-Institut, Langen, Germany. Independent on-site monitoring was available at each study site. The study was conducted in accordance with the Declaration of Helsinki.

**Inclusion Criteria**

Patients aged 18 to 80 years with ischemic rest pain (Rutherford class 4) or nonhealing ulcers (Rutherford class 5 or 6) due to PAOD and...
TAO who were not candidates for interventional or surgical revascularization or who failed to respond to interventional or surgical procedures were eligible for inclusion in the study. To be able to document a cell effect, technically successful interventional or surgical procedures (defined as patent artery after percutaneous transluminal angioplasty [PTA] or patent bypass graft) must have been performed at least 3 months before inclusion. Patients had either infrainguinal vessel occlusions or chronic femoropopliteal occlusions. Written informed consent was obtained from all patients before enrollment.

Exclusion Criteria
Technically successful interventional or surgical procedures <3 months before screening, a history of infectious diseases (HIV, active hepatitis) or evidence for chronic inflammatory diseases (eg, Crohn disease, rheumatoid arthritis), a history of malignancies without complete remission (<5 years), a history of stroke or myocardial infarction <3 months before screening, or advanced renal insufficiency (creatinine >2.0 mg/dL at the time of treatment) were the exclusion criteria.

End Points
The primary end point was the change in ABI of the treated leg at 3 and 6 months based on the findings of the TACT study. Secondary end points were complete healing of all ulcers or a reduction of ulcer area (cumulative ulcer size in square centimeters), amputation-free survival (major amputation above the ankle) and overall survival, and freedom from rest pain or reduction of rest pain (by at least 1 point). Wound healing was assessed by documentation using digital cameras and standardized measurement (Visitrak) to quantify total ulcer area (square centimeters). Rest pain or severity of pain was assessed on a visual analog scale ranging from 0 to 10 points, with 0 indicating the best (complete relief of pain without analgesics) and 10, the worst pain.

TCO2 was measured with the TCM3 (Radiometer; Copenhagen, Denmark) at 2 prespecified sites of the lower limb (dorsal surface of the foot and surface of the lower calf) at 2 centers (Frankfurt, Bern) and is presented as a subgroup analysis. Quantitative analyses for ulcer area and pain scale are presented for patients with complete serial measurements at all time points (baseline, month 3, and month 6). Physicians or study nurses who measured end points (ABI, ulcer size, pain scale, TCO2) were blinded to the treatment.

BM-MNC Isolation
Processing of bone marrow aspirates and cell isolation procedures were identical to the protocols used in the REPAIR-AMI (Reinfusion of Enriched Progenitor Cells and Infarct Remodeling in Acute Myocardial Infarction) trial.12,13 In brief, 50 mL of bone marrow was aspirated from the iliac crest into heparin-treated syringes with the use of local anesthesia. The bone marrow aspirate, together with 20 mL of venous blood used to produce the patient’s own serum, was shipped at room temperature to the central cell-processing laboratory at the Red Cross Blood Donation Center in Frankfurt, where patients were randomly assigned to receive BM-MNC or placebo in the randomized-start phase. The randomization was performed for the entire study cohort at the cell-isolation center by a simple random allocation. There was no blocking at each center.

BM-MNC were isolated and enriched using density gradient centrifugation. After several washing steps, cells were resuspended in X-VIVO 10 medium (a serum-free medium containing pharmaceutical-grade human components (Cambrex) supplemented with 2 mL of the patient’s own serum. Cell analysis was performed from the final cell preparation. Placebo control was prepared by mixing X-VIVO 10 medium with autologous serum. All cell processing and labeling of the product were performed according to good clinical practice guidelines. The method of cell isolation has been validated extensively in Frankfurt,13 and cell viability is proven until at least 24 hours. Cell viability was >98% in all cases, and neither viability nor functional capacity of cells was compromised by shipping to external centers. Our preclinical experiments and previous clinical trials using BM-MNC in patients with ischemic heart disease provided the basis for the PROVASA trial.12,14–19

Cell Characterization
The cell suspension consisted of a heterogeneous cell population that included hematopoietic, mesenchymal, and other progenitor cells as well as mononuclear cells. The total number of mononuclear cells was determined using a differential counter (XT-1800R; Sysmex). Cells were further characterized by flow cytometry using CD34+/CD45-/CD133+ antibodies. Viability was assessed using trypan blue staining. Functional parameters of the final cell product, such as basal or stromal-derived factor 1-induced migration, were assessed in a Boyden chamber as previously described.18 Colony forming unit (CFU) capacity assays were performed using Methocult H4534 Classic without erythropoietin (Stemcell Technologies), which supports growth of granulocyte, macrophage, and granulocyte-macrophage CFUs. For quantification, all 3 types of colonies were detected.

Intraarterial Catheter-Based Application of BM-MNC
After arterial puncture, all patients received at least 5000 U of heparin. Angiographies were performed by way of crossover sheath. Cells or placebo solutions were administered by hand injection into the distal superficial femoral artery in patients with infrainguinal disease or into the deep femoral artery in patients with additional chronic femoropopliteal occlusions. Low-pressure balloon occlusions of the superficial femoral artery (SFA) for 1×5 minutes were used in 20 patients with faster distal runoff (eg, patients with TAO with small vessel disease of the foot) but not in patients with slow distal runoff in the lower limb by way of collaterals (n=20).

Data and Statistical Analysis
Sample size calculation for the primary end point was based on the findings in the TACT study5: assuming a mean increase in ABI of 0.13 (SD, 0.1) after BM-MNC treatment and no increase after placebo, a sample size of 15 patients per group was considered necessary to document a significant effect with a statistical power of 90% (2-sided α=0.05). Taking patient dropout and death or major amputation within 6 months into consideration led to a sample size of 20 patients per treatment group.

Data are expressed as counts and percentages for discrete variables and as mean±SD for continuous variables or median values with interquartile ranges (IQRs) or 95% CIs. Categorical variables were compared by means of the Fisher exact test. Continuous variables were compared by nonparametric methods (Mann-Whitney U test). Paired comparisons were performed by Wilcoxon test. Multivariate analysis was performed using the Cox regression analysis on SPSS version 15.0 software, including fixed covariates. Statistical significance was assumed to be 2-sided at P<0.05. Long-term clinical outcome and associations with wound healing or amputation were compared by Kaplan-Meier survival curves, and the corresponding P value was obtained from the log-rank test. Overall survival and amputation-free survival were counted as the duration before amputation or death, and the patients still alive were marked as censored at the date of last follow-up.

Results
Patients were enrolled from October 2005 to January 2009 at 3 centers. Mean follow-up was 30.2 months (median, 28 months; range, 6 to 57 months). All surviving patients have an ongoing follow-up >1 year. The patient characteristics are listed in Table 1. There were no clinically relevant differences between the study groups. Before inclusion into the study, the rate of previous interventional or surgical procedures was higher in the placebo group. However, according to the design of the study, these procedures were performed >3 months (mostly >6 months) before screening in all cases and had failed to induce wound healing. Therefore, the long

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The table shows the characteristics of patients in the BM-MNC and placebo initial treatment groups.

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>BM-MNC Initial Treatment (n=19)</th>
<th>Placebo Initial Treatment (n=21)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>64.4±15</td>
<td>64.5±16</td>
<td>0.97</td>
</tr>
<tr>
<td>Male sex</td>
<td>16 (84)</td>
<td>13 (62)</td>
<td>0.31</td>
</tr>
<tr>
<td>History of smoking</td>
<td>9 (47)</td>
<td>11 (52)</td>
<td>1.0</td>
</tr>
<tr>
<td>Current smoker</td>
<td>4 (21)</td>
<td>1 (4.8)</td>
<td>0.17</td>
</tr>
<tr>
<td>Hypertension</td>
<td>14 (74)</td>
<td>14 (67)</td>
<td>0.74</td>
</tr>
<tr>
<td>Diabetes</td>
<td>10 (53)</td>
<td>10 (48)</td>
<td>1.0</td>
</tr>
<tr>
<td>IDDM</td>
<td>9 (47)</td>
<td>6 (29)</td>
<td>0.33</td>
</tr>
<tr>
<td>Hyperlipidemia</td>
<td>15 (79)</td>
<td>16 (76)</td>
<td>1.0</td>
</tr>
<tr>
<td>Kidney dysfunction (creatinine &gt;1.4 mg/dL)</td>
<td>7 (37)</td>
<td>6 (29)</td>
<td>0.74</td>
</tr>
<tr>
<td>History of myocardial infarction</td>
<td>6 (32)</td>
<td>4 (19)</td>
<td>0.47</td>
</tr>
<tr>
<td>Heart failure EF ≥35%</td>
<td>3 (16)</td>
<td>1 (4.8)</td>
<td>0.33</td>
</tr>
<tr>
<td>Chronic venous insufficiency</td>
<td>2 (11)</td>
<td>3 (14)</td>
<td>1.0</td>
</tr>
<tr>
<td>PAOD-specific details</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infrapopliteal Disease</td>
<td>13 (68)</td>
<td>14 (67)</td>
<td>1.0</td>
</tr>
<tr>
<td>Femoropopliteal occlusions</td>
<td>6 (32)</td>
<td>7 (33)</td>
<td>1.0</td>
</tr>
<tr>
<td>Buerger's disease (TAO)</td>
<td>3 (16)</td>
<td>5 (24)</td>
<td>0.69</td>
</tr>
<tr>
<td>Mediasclerosis</td>
<td>5 (26)</td>
<td>3 (14)</td>
<td>0.44</td>
</tr>
<tr>
<td>Fontaine class 3</td>
<td>4 (21)</td>
<td>6 (29)</td>
<td>0.72</td>
</tr>
<tr>
<td>Fontaine class 4</td>
<td>15 (79)</td>
<td>15 (71)</td>
<td>1.0</td>
</tr>
<tr>
<td>Rutherford class 4 (rest pain)</td>
<td>4 (21)</td>
<td>6 (29)</td>
<td>0.72</td>
</tr>
<tr>
<td>Rutherford class 5 (minor tissue loss)</td>
<td>12 (63)</td>
<td>14 (67)</td>
<td>1.0</td>
</tr>
<tr>
<td>Rutherford class 6 (gangrene)</td>
<td>3 (16)</td>
<td>1 (4.8)</td>
<td>0.33</td>
</tr>
<tr>
<td>Highly resistant bacteria (MRSA, ESBL, or Pseudomonas)</td>
<td>11 (58)</td>
<td>9 (43)</td>
<td>0.71</td>
</tr>
<tr>
<td>History of PTA &gt;3 mo</td>
<td>3 (16)</td>
<td>10 (48)</td>
<td>0.046</td>
</tr>
<tr>
<td>History of peripheral bypass operation</td>
<td>3 (16)</td>
<td>9 (43)</td>
<td>0.089</td>
</tr>
<tr>
<td>Successful &gt;3 mo</td>
<td></td>
<td>1 (4.8)</td>
<td></td>
</tr>
<tr>
<td>History of minor amputation</td>
<td>3 (16)</td>
<td>2 (10)</td>
<td>0.65</td>
</tr>
<tr>
<td>Second BM-MNC therapy at 3 mo</td>
<td>14 (74)</td>
<td>19 (90)</td>
<td>0.23</td>
</tr>
<tr>
<td>Extended protocol: patients with cell applications &gt;6 mo</td>
<td>6 (32)</td>
<td>6 (29)</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Data are presented as no. (%) or mean±SD. CAD indicates coronary artery disease; EF, ejection fraction; ESBL, extended-spectrum β-lactamase; IDDM, insulin-dependent diabetes mellitus; MRSA, methicillin-resistant Staphylococcus aureus.

run-in phase excluded a potential bias between intervention and surgery or potential cell effects.

Seventy-three bone marrow aspirations and catheter procedures (52 BM-MNC and 21 placebo applications) were performed until 6 months; 1 patient in the placebo group refused the second procedure at 3 months (and had no improvement of rest pain since). Fourteen additional BM-MNC administrations were performed between 6 and 18 months in 12 patients. There were no complications during a total of 87 bone marrow aspiration procedures. There were no cell-related adverse events observed in all intraarterial catheter-based BM-MNC applications. In 1 patient, a small thrombus developed after low-pressure balloon occlusion in the distal SFA within a preexisting stent. The thrombus could be aspirated, resulting in an uneventful clinical course. There was 1 puncture-related hematoma and 1 pseudoaneurysm in the groin, which healed without sequelae.

After 6 months, 12 patients with incomplete healing of ulcers qualified and gave separate informed consent for continuation of cell therapy in the extended protocol. Of these, 6 belonged to the initial verum group and 6 to the placebo group during the randomized-start phase of the trial. Eleven patients received 1 additional BM-MNC application; 1 patient obtained 3 repeated BM-MNC therapies >6 months. Cells are characterized in Table 2.

**ABI**

There were no significant differences in ABI changes between the BM-MNC and the placebo groups (Figure 2). During the double-blind, randomized-start phase of the study until 3 months, the median ABI slightly, but non-significantly, increased from 0.66 (IQR, 0.12 to 0.85) at baseline to 0.75 (IQR, 0.57 to 0.89; P=0.4) in the BM-MNC group and remained unchanged in the placebo group (baseline median, 0.64; IQR, 0.32 to 0.83; 3-month median, 0.66; IQR, 0.43 to 1.00; P=0.2). At 6 months, ABI had increased to 0.85 (IQR, 0.46 to 0.95; P=0.6 versus baseline) in the initial BM-MNC group receiving an additional BM-MNC administration at 3 months and to 0.70 (IQR, 0.43 to 1.08; P=0.1 versus baseline) in the initial placebo group receiving a single BM-MNC treatment at 3 months. Excluding patients with TAO and medial atherosclerosis, which frequently results in false-positive distal pressure recordings, did not alter the results. At baseline, the 8 patients with TAO had higher ABI values (median, 0.67; IQR, 0 to 0.7) compared to the 32 patients with PAOD (median, 0.33; IQR, 0.53 to 1.03; P=0.037 versus TAO).

**Death, Limb Salvage, and Amputation-Free Survival**

There were no differences in clinical outcome with respect to death and limb salvage during the randomized-start, placebo-controlled, double-blind phase I of the study. One patient in the verum group and none in the placebo group died during the first 3 months. At the end of phase II (6 months), an additional 5 deaths had occurred, 2 in the initial verum group and 3 in the initial placebo group (Figure 1, Table 3). All patients who died had atherosclerotic PAOD, whereas all patients with TAO survived. Cause of death was sudden cardiac death in 2, heart failure in 1, sepsis after amputation in 1, stroke in 1, and metastatic colon cancer in 1. Thus, overall cumulative survival was 86% at 6 months and 84% at 1 year. The patient with advanced colon cancer was discovered to have cancer 1 month after inclusion in the study, indicating that the cancer must have been present but yet unknown before cell therapy. No further tumors were observed during a mean follow-up of 30.2 months.

As summarized in Table 3, 4 patients (1 in the initial placebo group, 3 in the verum group) underwent amputation...
above the ankle during the randomized-start phase, and an additional 2 patients (1 in the placebo group and 1 in the initial verum group) underwent amputation above the ankle during the second phase of the study until 6 months. Importantly, all patients with Rutherford class 6 at inclusion into the study (3 in the verum group, 1 in the placebo group) underwent amputation above the ankle within 3 months during the randomized-start phase, regardless of treatment allocation. Two patients with advanced Rutherford class 5 initially randomized to placebo or verum during the start phase of the trial. Clinical outcome dependent on the initial Rutherford class is shown in Table 4.

Ulcer Healing
At inclusion into the randomized-start phase, 30 patients had nonhealing ulcers (15 in the BM-MNC group, 15 in the placebo group). As illustrated in Figure 3A, for patients with serial measurements at all time points (excluding patients with amputations), ulcer area significantly (P=0.014) decreased in the BM-MNC group during the randomized-start phase of the study, whereas no changes in ulcer area were observed in the placebo group up to 3 months follow-up. In both groups, 3 patients had complete ulcer healing after the double-blind, randomized-start phase. In the initial placebo group that switched to BM-MNC at 3 months, ulcer area was significantly decreased at 6 months, whereas in the initial BM-MNC group receiving a second BM-MNC administration at 3 months, ulcer area continued to decrease until 6-month follow-up (Figure 3A). To demonstrate the effect of a single BM-MNC treatment, ulcer area data of the BM-MNC patients of the randomized-start phase was combined with ulcer area data of the initial placebo group patients during phase II after switching to BM-MNC treatment at 3 months and revealed that within 3 months after a single BM-MNC administration, ulcer area was significantly (P=0.003) reduced from 3.21±4.5 cm² to 1.75±3.2 cm² (n=19), whereas ulcer area remained constant 3 months after placebo administration (2.92±3.5 cm² to 2.89±4.1 cm²; P=0.5) (n=12) (Figure 3B). Overall, the median relative change in ulcer area was

Table 3. Major Adverse Events

<table>
<thead>
<tr>
<th>Event</th>
<th>0–3 Months Initial Treatment</th>
<th>4–6 Months</th>
<th>7–12 Months</th>
<th>&gt;12 Months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Major amputation</td>
<td>3</td>
<td>1*</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Death</td>
<td>1</td>
<td>5*</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Cancer (4 weeks after)</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
</tbody>
</table>

Data are presented as counts.

*One patient with major amputation and death <6 months.

Figure 2. ABI during the randomized-start phase and until 6 months follow-up (open-label phase). ABI values are shown as median and IQR for 26 patients with complete serial measurements at all time points. BL indicates baseline; M2, month 2; M3, month 3; M4, month 4; M5, month 5; M6, month 6. *P=0.6 at 3 months BM-MNC versus placebo and P=0.7 BM-MNC versus placebo after switching to BM-MNC.
including 12 patients with a third or fourth BM-MNC treatment, 10 had completely healing wounds, 1 underwent amputation, and 1 had a small residual ulcer at last visit, further supporting that serial treatments are necessary for a clinical response.

We divided the ulcer area data into 2 groups according to the median of 2.3 cm², resulting in 14 with an ulcer area < 2.3 cm² versus 16 with larger wounds ≥ 2.3 cm². Among the 30 patients with ulcers, 12 (85%) of 14 had healing wounds in the group with smaller ulcers versus only 8 (50%) of 16 in the group with larger ulcers (P = 0.038). The absolute reduction in ulcer area after 2 BM-MNC treatments was higher than after 1 BM-MNC treatment and significantly higher versus placebo (Figure 3C).

During the entire study observation period, there was complete ulcer healing in 20 (66.6%) of the 30 patients with ulcers at baseline. Ulcer healing occurred at a mean time of 10.9 months after inclusion in the study. Fourteen patients had healing ulcers after ≥ 2 BM-MNC treatments; 3 had healing ulcers after 1 BMC application, whereas 3 small ulcers healed after placebo. Repeated BM-MNC administration (≥ 2) was significantly correlated with complete ulcer healing (log-rank P = 0.017 for ≥ 2 BM-MNC therapies versus 1-time BM-MNC). Interestingly, 1 patient’s ulcers healed twice after BM-MNC treatments (after the first and again after the third cell application) while a novel ulcer intermittently occurred at 8 months due to progression of atherosclerosis requiring additional intervention of a newly occluded SFA. Taken together, these findings support the concept that serial treatments are frequently necessary to stimulate ulcer healing in patients with critical ischemia.

### Determinants of Ulcer Healing and Limb Salvage

Several factors influenced ulcer healing (30 patients with ulcers at baseline). Younger age, better ejection fraction, smaller ulcer size, better renal function (creatinine < 1.4 mg/dL), a higher number of administered BM-MNC, and repeated BM-MNC administration positively correlated with ulcer healing. To disclose a potential association between the number and functionality of the administered BM-MNC and ulcer healing, we compared total BM-MNC number and their migratory capacity (marker of cell retention) as well as their CFU capacity (marker for intrinsic progenitor cell activity) in patients with healing versus nonhealing ulcers. Importantly, the 20 patients with healing ulcers received significantly greater numbers of total BM-MNC (178 ± 113 × 10⁶ versus 87 ± 29 × 10⁶ BM-MNC; P = 0.003) as well as of CD34⁺/CD45⁺ BM-MNC (3.57 ± 1.7 × 10⁶ versus 1.78 ± 1.7 × 10⁶; P = 0.033). Likewise, the migratory capacity (87 ± 46 cell counts versus 51 ± 28 cell counts; P = 0.016) as well as the CFU capacity (27.7 ± 14.6 versus 18.2 ± 9.4; P = 0.048) of the administered cells were significantly better in patients with subsequently healing ulcers (Table 5).

Most importantly, on multivariate analysis (Table 6), including all relevant clinical parameters that individually correlated with ulcer healing, repeated BM-MNC administr-
Figure 3. A. Ulcer area during the randomized-start phase and until 6 months (open label) presented as mean ± SE in patients with serial measurements. B, Serial measurements of ulcer area in the placebo group until 3 months compared with the effect after 1 single BM-MNC treatment. Ulcer area data of the BM-MNC patients were combined for the randomized-start phase with ulcer area data of the initial placebo group patients after crossover to BM-MNC treatment (data are presented as mean ± SD). C, Absolute decrease in ulcer size according to treatment groups (data are presented as mean ± SE).
tion as well as the number and functionality of administered 
BM-MNC were significant independent predictors for complete 
ulcer healing. Ulcer healing induced by repeated BM-MNC 
administration significantly correlated with limb salvage 
\(r=0.8; P<0.001\). Importantly, all 20 patients with healing 
wounds experienced subsequent limb salvage.

In addition, we measured cell surface markers of the 
BM-MNC by fluorescence-activated cell sorter analysis. As 
seen in Table 5, the number of absolute CD45\(^+\)/CD34\(^+\) cells 
were significantly higher in 20 patients with healing wounds 
versus nonresponders \(n=10\). Likewise, the absolute number 
of CD45\(^+\)/CD133\(^+\) was higher, although the difference did 
not achieve statistical significance (see Table 5). In contrast, 
the number of BM-MNC expressing the endothelial cell 
marker kinase insert domain receptor (KDR) did not differ 
between responders and nonresponders. These data suggest 
that the number of hematopoietic CD34\(^+\) cells have the major 
impact on the functional improvement.

Finally, to identify clinical responders to therapy defined by 
either partial or complete healing of ulcers or freedom from rest 
pain or reduction in pain medication consumption, potential 
determinants were analyzed in all 40 patients. Twenty-six (65\%) 
of 40 patients had evidence for either healing ulcers or less rest 
pain during follow-up. All 8 (100\%) patients with TAO were 
responders, whereas 18 (56\%) of 32 patients with atherosclerotic 
PAOD clinically improved by BM-MNC treatments \(P=0.02\) 
TAO versus PAOD). Patients with gangrene (Rutherford class 
6) did not respond at all. On multivariate analysis, repeated 
BM-MNC administration as well as the number and functionality 
of administered BM-MNC were the only independently 
associated predictors of clinical improvement.

**Angiographies**

Angiographic follow-up at 6 months in the first 10 patients 
did not show any significant differences in angiographically 
visible collateral formation on gross inspection. Therefore,
routine control angiography at 6 months was abandoned unless it was necessary for a repeat BM-MNC application.

**Discussion**

To our knowledge, the present study is the first randomized, double-blind, placebo-controlled multicenter phase II trial to assess potential effects of intraarterial BM-MNC administration in patients with critical limb ischemia due to PAOD. The results of the double-blind, placebo-controlled randomized-start phase suggest that BM-MNC administration does not alter ABI, but accelerates ulcer healing and pain reduction in patients with stable ulcers or rest pain within 3 months. However, critically ill patients with impending amputation due to Rutherford 6 did not derive any benefit from BM-MNC administration. Additionally, the unique design of the trial with a randomized-start phase followed by a switch of the placebo patients to BM-MNC administration and repetitive BM-MNC administration in the initial verum group at 3 months enabled us to address a number of clinically critical questions with respect to the safety and potential efficacy of BM-MNC administration in patients with critical limb ischemia but stable ulcers or rest pain. Successful ulcer healing associated with improved limb salvage requires repeated BM-MNC administration as well as functionally competent BM-MNC in sufficient numbers. Moreover, in line with previous results, patients with TAO appeared to demonstrate improved responsiveness to BM-MNC administration compared with patients with atherosclerotic PAOD. However, these exploratory findings in the present pilot trial need to be confirmed in a larger randomized trial in patients with critical limb ischemia and stable ulcers.

In retrospect, the choice of change in ABI as the primary end point of the double-blind, randomized-start phase represents the major drawback of the present trial. Although ABI reflects distal perfusion pressures in patients with PAOD, changes in ABI values do not correlate well with ulcer healing and limb salvage, the clinically most relevant therapeutic goals in the treatment of patients with critical limb ischemia. Moreover, in patients with TAO, who responded most favorably to BM-MNC administration, ABI values are spuriously high and do not reflect the degree of distal ischemia and tissue necrosis. Changes in ABI values do not predict clinical outcome or are suitable for comprehensively

![Figure 5. TCO2 during the randomized-start phase and until 6 months (open label) in 16 patients with serial measurements. Data are presented as mean±SE.](image)

**Table 6. Multivariate Analysis: Association With Complete Wound Healing**

<table>
<thead>
<tr>
<th>Factor</th>
<th>Exp (B) Hazard</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at baseline</td>
<td>0.68</td>
<td>1.009</td>
<td>0.96–1.05</td>
</tr>
<tr>
<td>Ulcer size at baseline</td>
<td>0.2</td>
<td>0.83</td>
<td>0.62–1.1</td>
</tr>
<tr>
<td>EF</td>
<td>0.16</td>
<td>1.07</td>
<td>0.97–1.18</td>
</tr>
<tr>
<td>Creatinine &lt;1.4 mg/dL</td>
<td>0.34</td>
<td>2.91</td>
<td>0.32–26.3</td>
</tr>
<tr>
<td>Cell no.</td>
<td>0.001</td>
<td>1.03</td>
<td>1.01–1.04</td>
</tr>
<tr>
<td>Repeated BM-MNC (≥2 applications)</td>
<td>0.005</td>
<td>0.116</td>
<td>0.025–0.53</td>
</tr>
<tr>
<td>Cell function (migration)</td>
<td>0.036</td>
<td>1.025</td>
<td>1.002–1.05</td>
</tr>
<tr>
<td>Colony forming units</td>
<td>0.36</td>
<td>1.027</td>
<td>0.97–1.087</td>
</tr>
</tbody>
</table>

Multivariate analysis obtained with Cox regression analysis. Creatinine values, repeated BM-MNC applications were categorized.
assessing potential beneficial therapeutic effects on ulcer healing and rest pain in patients with critical limb ischemia. Therefore, it is not surprising that the beneficial effects of BM-MNC treatment on ulcer healing and rest pain were observed despite no effect on the primary end point of change in ABI during the placebo-controlled, double-blind randomized-start phase of the present study. Moreover, because there is substantial uncertainty about the best end point to measure therapeutic benefit in critical limb ischemia, the presentation of composite end points instead of choosing 1 primary end point and several secondary end points would be an adequate option in designing future trials, according to the outcome of this study.

Previous uncontrolled trials using intramuscular injection of BM-MNC or blood-derived mononuclear cells reported 6-month limb salvage rates of 71% (TACT) and 59% (Bone Marrow Outcome Trial). Likewise, a recent randomized controlled gene therapy trial using a plasmid-encoding fibroblast growth factor reported limb salvage rates of 75% at 1 year, whereas ulcer healing did not differ from placebo treatment at 6 months. One-year limb salvage rate (84%) and amputation-free survival (73%) in the present study are consistent with these trials. However, it should be kept in mind that the severity of the underlying disease accompanied by advanced necrosis or infections will affect the necessity for major amputations. Indeed, all patients with extensive gangrene with impending amputation (Rutherford class 6) at inclusion in the study had to undergo amputation above the ankle already during the initial 3-month randomized-start phase, regardless of treatment allocation. Despite these seemingly disappointing observations in patients with stable ulcers at inclusion, complete ulcer healing occurred at a mean time of 10.9 months after BM-MNC administration, although significant improvements in ulcer size and rest pain had occurred within 3 months after BM-MNC administration. Thus, assessing potential clinical benefits of cell therapy in patients with critical limb ischemia will require follow-up periods of at least 18 months.

The identification of cell number and cell functionality as independent predictors of subsequent ulcer healing and clinical improvement is an intriguing finding of the present study because it indeed may indicate a cause-and-effect relationship. Similar observations have been reported in applying BM-MNC to patients with acute myocardial infarction and chronic heart failure. Although we cannot fully exclude that higher numbers and better functionality of the BM-MNC retrieved from bone marrow aspirates simply reflect less severely ill patients with a better natural ulcer healing capacity, the pivotal role of preserving cell functionality to enhance blood flow recovery is firmly established in animal models of limb ischemia. Thus, future studies using cell enhancement strategies and retrieving larger amounts of bone marrow aspirates to increase the number of BM-MNC to be administered are necessary not only to finally document a cause-and-effect relationship for BM-MNC therapy in patients with critical limb ischemia, but also to improve therapeutic efficacy in patients with severely impaired BM-MNC functionality.

Given the long-standing chronic disease process leading to critical limb ischemia in PAOD, it is not surprising that ulcer healing appears to be significantly promoted by repeated BM-MNC administration. The excellent procedural safety profile of bone marrow harvest and intraarterial BM-MNC administration documented in the present study provides the necessary framework for a repeated treatment strategy that can be easily implemented into clinical guidance of patients with critical limb ischemia.

Regarding mechanistic aspects of a cell effect, angiographic follow-up at 6 months in the first 10 patients did not show any significant differences in angiographically visible collateral formation. We speculate on the basis of a body of evidence from animal models that neovascularization most likely involves vessels of the microvasculature that were smaller than the angiographic resolution. Indeed, using an inducible suicide approach to eliminate previously administered BM-MNC in an experimental model of myocardial infarction, we could demonstrate that the beneficial effects of BM-MNC administration are paralleled by increased capillary and arteriolar vessel density.

Limitations

Although the design of the present study with a randomized-start phase during the first 3 months followed by a switch to active treatment in the initial placebo group or repetitive active treatment in the initial verum group during the second phase from 3 to 6 months provided for the unique opportunity to address a potentially beneficial effect of repeated BM-MNC administration, it precluded the comparison of active treatment with placebo administration throughout the entire study period. However, we believed it to be unethical to extend the placebo phase >3 months in these patients with critical limb ischemia. In addition, the delayed or randomized-start design as used in the PROVASA trial is one way of evaluating disease-modifying treatments, and there has been regulatory support for this trial design, as previously discussed in an editorial. Moreover, if there had been no measurable effect of active treatment on ulcer size or pain within 3 months after BM-MNC administration, it would be highly unlikely that such treatment may be capable of modifying the disease process in these critically ill patients. Finally, the repetitive treatment group could be used to evaluate potential additive disease-modifying effects of repeated BM-MNC administration in this chronically progressing disease.

The number of patients included into the trial is small. The present study was designed as a pilot trial, and the sample size was based on the number of patients included in the TACT trial. Thus, the inclusion of 40 patients (19 BM-MNC, 21 placebo) is essentially comparable to the TACT trial with regard to sample size. Nevertheless, during the placebo-controlled crossover phase of the study, a total of 73 procedures (52 BM-MNC administrations, 21 placebo treatments) were performed until 6 months followed by an additional 14 BM-MNC administrations during the extended study period. Thus, in combination with the mean follow-up time of 30.2 months, the size of the study population appears to be...
sufficiently large to derive clinically relevant conclusions with respect to the safety of intraarterial BM-MNC administration and its effects in patients with stable ulcers and rest pain.

The enrollment phase of the study lasted 3.5 years. However, screening processes were frequently delayed because the inclusion criteria required at least a 3-month interval after PTA or bypass procedures before cell therapy in potential candidates in order to achieve stable baseline conditions and to be able to document a cell effect without conflicting bias by other interventions.

The present study did not address the question of whether intraarterial cell administration is superior to intramuscular injections. Given that experimental studies demonstrated that the administered BM-MNC needs to persist for at least 3 weeks to improve cardiac function associated with increased capillary and arteriolar vessel density,26 we used the intraarterial route of application based on the assumption that intraarterially applied cells will only home to tissue with preserved nutrient blood supply.

Finally, we cannot exclude that selected cell populations might be superior to the heterogeneous BM-MNC used in the present study. Indeed, a recently reported, but not yet published study by Losordo and colleagues (2010) demonstrated that intramuscular injection of granulocyte colony-stimulating factor-mobilized peripheral blood-derived CD34+ cells was associated with a significantly reduced amputation rate in patients with critical limb ischemia.

Conclusion

In patients with critical limb ischemia, intraarterial administration of BM-MNC does not increase ABI but promotes ulcer healing and reduces rest pain. Successful ulcer healing associated with improved limb salvage requires repeated administration of functionally competent BM-MNC. However, critically ill patients with extensive gangrene and impending amputation (Rutherford class 6) did not derive any benefit, whereas patients with TAO in general responded very well. Thus, large-scale randomized trials are warranted to assess the clinical effect of repeated BM-MNC administration in patients with critical limb ischemia and stable ulcers and rest pain.

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Disclosures

Drs Zeiher and Dimmeler are cofounders and advisors to 2cure GmbH. Drs Walter, Krankenberg, Balzer, Kalka, Baumgartner, Schlüter, Tomn, Seeger, and Lindhoff-Last have no disclosures to report.

References


**CLINICAL PERSPECTIVE**

Injection of autologous bone marrow-derived mononuclear cells (BM-MNC) is a promising therapeutic option in patients with critical limb ischemia, but double-blind, randomized trials are lacking. The present study is the first randomized, placebo controlled trial showing that intraarterial BM-MNC administration accelerates wound healing and induces pain reduction until 3 months in patients with critical limb ischemia with stable ulcers but not in patients with extensive gangrene. Ulcer healing induced by repeated BM-MNC administration significantly correlated with limb salvage. Successful ulcer healing required repeated applications of functionally competent BM-MNC. These exploratory findings of this pilot trial need to be confirmed in a larger randomized trial in patients with critical limb ischemia and stable ulcers.
Intraarterial Administration of Bone Marrow Mononuclear Cells in Patients With Critical Limb Ischemia: A Randomized-Start, Placebo-Controlled Pilot Trial (PROVASA)

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