Therapeutic Efficacy of Autologous Non-Mobilized Enriched Circulating Endothelial Progenitors in Patients With Critical Limb Ischemia — The SCELTA Trial

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Background: The therapeutic efficacy of bone marrow mononuclear cells (BM-MNC) autotransplantation in critical limb ischemia (CLI) has been reported. Variable proportions of circulating monocytes express low levels of CD34 (CD14+CD34low cells) and behave in vitro as endothelial progenitor cells (EPCs). The aim of the present randomized clinical trial was to compare the safety and therapeutic effects of enriched circulating EPCs (ECEPCs) with BM-MNC administration.

Methods and Results: ECEPCs (obtained from non-mobilized peripheral blood by immunomagnetic selection of CD14+ and CD34+ cells) or BM-MNC were injected into the gastrocnemius of the affected limb in 23 and 17 patients, respectively. After a mean of 25.2±18.6-month follow-up, both groups showed significant and progressive improvement in muscle perfusion (primary endpoint), rest pain, consumption of analgesics, pain-free walking distance, wound healing, quality of life, ankle-brachial index, toe-brachial index, and transcutaneous PO2. In ECEPC-treated patients, there was a positive correlation between injected CD14+CD34low cell counts and the increase in muscle perfusion. The safety profile was comparable between the ECEPC and BM-MNC treatment arms. In both groups, the number of deaths and major amputations was lower compared with eligible untreated patients and historical reference patients.

Conclusions: This study supports previous trials showing the efficacy of BM-MNC autotransplantation in CLI patients and demonstrates comparable therapeutic efficacy between BM-MNC and ECEPCs.

Key Words: Angiogenesis; Endothelial cells; Limb ischemia; Stem cell; Transplantation and therapy

Peripheral arterial disease (PAD) is due primarily to an atherosclerotic obstruction of the arteries of the lower limbs. Disease prevalence ranges between 3% and 10%, and increases up to 20% in patients over 70 years of age.1,2 When the obstruction becomes critical, a typical cluster of signs and symptoms occurs, such as ischemic leg pain at rest and an incident ulcerative or gangrenous lesion.
of the affected extremity. Only 1–2% of the all PAD patients over the 50 years of age progresses to critical limb ischemia (CLI). The fast and spontaneous deterioration occurring in most CLI patients unavoidably leads to amputation, unless a surgical or procedural intervention can restore arterial flow. Therefore, a timely attempt to achieve effective arterial revascularization should always be made. Nonetheless, quite often many patients are not eligible for any surgical or percutaneous intervention. In patients progressing to CLI, the development of an effective net of collateral vessels is often lacking, especially in diabetic patients, affecting the fate of the ischemic leg. This compensatory mechanism consists of microcirculatory neovascularization and may be accomplished by circulating endothelial progenitor cells (EPCs) and vascular progenitor cells. Over the past 2 decades, stimulation of angiogenesis has been attempted using gene transfer of growth or hypoxia-inducible factors, with some indication of efficacy but also with important limitations due primarily to poor yield of gene transfer. Another approach has been the use of bone marrow (BM) stem cells (SC) that may differentiate towards endothelium or also stimulate resident endothelial cells through the release of growth factors. After the pioneering study of Tateishi-Yuyama et al., several reports confirmed the clinical efficacy of the intramuscular transplantation of autologous BM mononuclear cells (BM-MNC) or of circulating CD34+SC mobilized by granulocyte colony stimulating factor (G-CSF) or granulocyte-macrophage colony stimulating factor (GM-CSF). In a previous study, we demonstrated the existence of a subpopulation of circulating CD14+ cells in healthy subjects that exhibited low expression of CD34 (CD14+CD34low), being the major source of circulating EPCs. We assumed that these cells may represent an accessible and convenient source of EPC obtained without mobilization. To verify this possibility, we evaluated the number of these cells in the peripheral blood (PB) of healthy subjects at different ages, and in patients with chronic vascular diseases. We then developed a cell-enrichment system based on the immunomagnetic isolation of both CD14+ (including CD14+CD34low) and CD34+ circulating cells, defined "enriched circulating (EC) EPCs" (patent no. FI2004A0000238). We then designed a single-center randomized unblinded clinical trial, the Stem Cell Emergency Life Threatening Arteriopathy (SCELTA) study, with the aim of comparing the safety and efficacy of intramuscular transplant of autologous ECEPCs vs. BM-MNCs in the limbs of CLI patients. The final analysis performed on 40 patients (17 treated with BM-MNCs and 23 treated with ECEPCs) demonstrated similar safety and efficacy of the 2 treatments, as revealed by clinical, functional, and imaging evaluation.

**Methods**

**Study Design**

The SCELTA study was a single-center randomized unblinded clinical trial with the aim of assessing the safety and therapeutic efficacy of an intramuscular injection of autologous ECEPCs in 30 CLI patients compared with 30 CLI patients injected with BM-MNCs. Eligible subjects were selected among men and women aged >40 years with persistent rest pain requiring systemic analgesic treatment during the last 15 days and/or the presence of trophic lesions amenable to occluding arteriopathy, and only if an ankle-brachial index (ABI) <0.40 (ankle systolic pressure <50–70 mmHg), a toe-brachial index (TBI) <0.40 (toe systolic pressure <30–50 mmHg), and transcutaneous (TC) PO2 <30 mmHg could be demonstrated. Patients were enrolled in the study only if, according to vascular Duplex and computed tomography angiography (CTA) or arteriography findings, intravascular or surgical revascularization was not feasible or when the patient refused to undergo surgical treatment, and after written informed consent had been obtained. The final analysis performed on 40 patients (17 treated with BM-MNCs and 23 treated with ECEPCs) demonstrated similar safety and efficacy of the 2 treatments, as revealed by clinical, functional, and imaging evaluation.
Results of the SCELTA Randomized Clinical Trial

Table 1. Baseline Characteristics of Patients Treated With Enriched Circulating ECEPC or BM-MNC

|                        | ECEPCs | BM-MNC | P value |
|------------------------|--------|--------|---------|
| No. PAD patients       | 23     | 17     | NS      |
| Age (years)            | 71.2±9.4 | 68.7±10.8 | NS   |
| Sex (M/F)              | 20/3   | 13/4   | NS      |
| Trophic lesions        | 16 (69) | 14 (82) | NS      |
| Previous endovascular therapy | 16 (70) | 11 (64) | NS      |
| Previous bypass surgery | 17 (74) | 8 (47)  | NS      |

Rutherford class
- C4: 7 (30)
- C5: 8 (35)
- C6: 8 (35)

Hypertension
- 17 (74)

Diabetes mellitus
- 12 (52)

Dyslipidemia
- 16 (69)

Past smoker
- 18 (78)

Therapy with statins
- 18 (78)

Ejection fraction (%)
- 56±6.5
- 58.1±6.8

Creatinine (mg/dL)
- 0.95±0.3
- 0.86±0.2

Hemoglobin (g/dL)
- 13.1±1.5
- 13.5±1.6

Previous CAD (<6 months)
- 15 (65)

Previous TIA or stroke
- 4 (17)

Infected lesions
- 10 (43)

Unless indicated otherwise, data are given as the mean±SD or as n (%). BM-MNC, bone marrow mononuclear cells; CAD, coronary artery disease; ECEPC, endothelial progenitor cells; TIA, transient ischemic attack.

Results

Circulating CD14+CD34low Cell Counts in Healthy Subjects and Patients With Chronic Vascular Disorders

Before starting the SCELTA trial, the relative recovery of CD14+CD34low cells using immunomagnetic isolation from healthy subjects at different ages and patients with chronic vascular disorders was calculated. The number of CD14+CD34low cells was variable, independent of age or the presence of PAD (Figure S1). The absolute number of CD14+CD34low and CD34+ cells recoverable from leukapheresis after immunomagnetic cell isolation was also calculated in donors from 3 different blood banks. The number of CD14+CD34low cells in patients ranged from 3.0x10^6 to 840x10^6, which is much higher than the number of classic CD34+ SC, which ranged from 0.56x10^6 to 11x10^6 (Table S1).

Enrolled Patients and Dropouts

Patient enrolment started in December 2009 and the evaluation ended on October 2015; the trial was stopped before 60 patients were enrolled in the study (30 in each of the BM-MNC and ECEPC groups) because of time limits and for the achievement of a primary endpoint. The SCELTA trial design is shown in Figure 1. By October 2015, 44 of the 126 patients screened were found to be eligible for the study and had therefore been enrolled and randomized. An additional 18 patients were considered eligible for inclusion, but they were not randomized for different reasons, including late refusal to undergo treatment, the presence of monoclonal gammopathy of undetermined significance (MGUS; 8 patients), rapid mind deterioration (1 patient), and the absence of accessible venous sources (1 patient). Forty of 44 enrolled patients were treated according to the inclusion and exclusion criteria (4 patients dropped out). Follow-up visits were performed at 30, 150, and 360 days. According to treatment randomization, 23 patients received intramuscular injections of ECEPCs and 17 received intramuscular injections of BM-MNCs. Baseline clinical, functional, and biochemical data are summarized in Table 1.

Details of the patients who dropped out of the study are given in Results S1.

Historical Reference Group

A placebo control group was not envisaged in the study design. Instead, in order to obtain a comparable sample of untreated CLI patients, outcome data (at 1 year) were collected for 40 patients in a historical clinical reference group (HCRG) with similar disease severity (Rutherford classification), matched for age, sex, and comorbidities, whose medical records clinical and instrumental data were
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After injection of contrast medium (1 patient). The post-procedural AEs and their possible relationship to the entire procedure are reported in Table S3.

AEs Occurring >5 Days After Procedures

AEs that occurred more than 5 days after procedure included the death of 1 patient randomized to ECEPC 5 months after treatment due to myocardial infarction and major amputations in 7 patients (5 in the ECEPC-treated group and 2 in the BM-MNC-treated group; P=NS). Major amputations were required exclusively in patients showing progressive extension of local infection, untreatable pain, and/or preseptic conditions (just before or soon after treatment; 18 patients). No amputations were required in patients in whom there was no infection (0/22 patients; P<0.001; Table S3). Minor amputations were required in 7 patients (2 in the BM-MNC-treated group, 5 in the ECEPC-treated

Figure 2. Clinical improvements in enriched circulating endothelial progenitor cell (ECEPC)- and bone marrow mononuclear cells (BM-MNC)-treated patients. (A) Changes in rest pain, as measured using a visual analogue scale (VAS), and (B) pain-free walking distance from enrollment to Day 360. P values are reported in the tables next to each graph. (C) Quality of life (QoL) in patients with critical limb ischemia, as evaluated using the ST-22 questionnaire, after ECEPC or BM-MNC treatment. The questionnaire was divided into 4 sections (A, B, C, and D), with each question scored between 1 (extremely good health) and 5 (extremely compromised health); lower scores indicate a better QoL. P values for the comparison of scores on Day 0 and Day 150 are shown above the columns. Data are the mean±SEM.

Retrieved from medical records (see Methods S1). The HCRG cohort also included CLI patients who were not eligible for surgical or interventional revascularization procedures (Table S2).
Rest Pain

The mean pain intensity score showed no differences between patients randomized to treatment with ECEPCs or BM-MNCs at any follow-up visit. Pain scores, as evaluated using a visual analog scale (VAS), were significantly lower than at Time 0 at all follow-up times in both groups; in addition, pain scores were significantly reduced between the 1st and the 3rd follow-up visits in the ECEPC arm (Figure 2A).

Consumption of Analgesic Drugs

No difference was found in mean analgesic drug consumption between patients randomized to treatment with ECEPCs or BM-MNCs at any time point (data not shown).

Pain-Free Walking Distance

There was no difference between the ECEPC and BM-MNC groups with regard to PFWD at Time 0 and at subsequent follow-up visits, but mean PFWD increased gradually and significantly in both groups and had been all planned at the beginning of the study due to persisting trophic or gangrenous lesions. No amputations were required in the remaining 18 (of 23; 78.3%) patients in the ECEPC group and the remaining 15 (of 17; 88.2%) patients in the BM-MNC group (P=NS). During the follow-up period, 3 of 17 patients (17.6%) in the BM-MNC-treated group developed new trophic lesions, compared with 2 of 23 patients (8.7%) in the ECEPC-treated group (P=NS). Of these patients, 3 (1 in the BM-MNC group, 2 in the EPEPC group) subsequently underwent major amputation.

Comparison of Clinical Results Between the ECEPC and BM-MNC Groups

Clinical comparisons were made between the ECEPC and BM-MNC groups for 5 parameters: rest pain, analgesic drug consumption, pain-free walking distance (PFWD), trophic limb lesions (TLL), and quality of life (QoL).

Rest Pain

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Pain-Free Walking Distance

There was no difference between the ECEPC and BM-MNC groups with regard to PFWD at Time 0 and at subsequent follow-up visits, but mean PFWD increased gradually and significantly in both
groups after treatment (Figure 2B).

**Wound Evaluation** The number of patients with TLL before treatment was not significantly different in the ECEPC compared to the BM-MNC group: 16/23 (69.6%) vs 14/17 (82.4%) respectively (P=NS). In both groups, the number of patients with TLL decreased significantly after treatment (6/17 [35%] and 6/15 [40%] in the ECEPC and BM-MNC groups, respectively; P<0.05 and P<0.01, respectively). In order to evaluate the evolution of single trophic lesions, as assessed by Wagner grades, those lesions with a grade ≤2 were checked before and after cell treatment, and there was a tendency for an improvement in the grades of the lesions in both groups. There was difference between the 2 groups (Table S4).

**QoL** The mean QoL scores did not differ between the 2 groups before treatment, and there was a marked increase in QoL scores (to the same extent) in the 2 groups at the second follow-up examination (Figure 2C).

**Comparison of Instrumental Vascular Tests Between the ECEPC and BM-MNC Groups**

**ABI and TBI** Mean ABI and TBI values were comparable in all patients before treatment, and they improved significantly (to a similar extent) in both treatment groups at each follow-up visit compared with baseline. No differences were found after treatment between the BM-MNC and ECEPC groups (Figure 3A).

**TC PO2** Mean TC PO2 values were similarly low in both groups before treatment, and increased significantly at each follow-up examination, without any difference between the BM-MNC and ECEPC groups (Figure 3C).

**Muscle Perfusion** Muscle perfusion, as measured by contrast-enhanced ultrasound sonography (CEUS) at the upper calf and forefoot of the ischemic limb, was similar in the 2 groups. There was a gradual, significant increase in muscle perfusion at both the upper calf and forefoot seen in all follow-up examinations in both groups (Figure 4). Since we believe muscle represents the most direct functional test to define recovery of limb vascularization, we chose this parameter as a primary endpoint in the present study. As expected, there was a significant correlation between muscle perfusion and other vascular parameters, particularly TC PO2 (Figure S2).

**Anatomical Vessel Assessment** CTA was used to characterize the anatomy of arteries within the limbs at the time of screening or enrolment examination, at the 1st and 3rd follow-up visits. No differences in collateral circulation were found between the BM-MNC and ECEPC groups before and after treatment, whereas a significant increase in microcirculation was noted at the 3rd compared with the 1st follow-up visit (P<0.05 in the BM-MNC group; P<0.01 in ECEPC group; Figure 5A). Figure 5B shows 1 representative case from the ECEPC-treated group.

**Correlations Between the Number of CD14+CD34low Cells Inoculated and Improvement in Muscle Perfusion** Because the injected ECEPC suspension also included high numbers of CD14+CD34- cells and low numbers of CD34+ SC in addition to the CD14+CD34low cells, the total number of circulating or BM-derived cells was counted, as was the
Figure 5. Changes in limb vessels in patients treated with ECEPC or BM-MNC. (A) Changes in vessels and the collateral circulation were evaluated by computed tomography angiography and scored as described in Methods S1 as follows: 0, worsening; 1, unchanged; 2, slight improvement; 3, clear improvement. (B) Radiograms of 1 patient before (Left) and after (Right) ECEPC treatment. The arrows indicate the new vessels found 12 months after ECEPC treatment (Right) compared with the baseline image (Left). Abbreviations as in Figure 2.

Figure 6. Correlation between the number of cells inoculated and changes in blood perfusion of the ischemic limb after treatment of patients with ECEPC or BM-MNC. The variation in blood perfusion (change in TTP [ΔTTP]) was measured at the calf level, comparing Time 0 with Day 150. The values obtained were correlated with the absolute number of total cells inoculated, or the number of CD14^+CD34^low or CD14^-34^+, HSC obtained from peripheral blood leukapheresis or bone marrow. R² values are reported in each graph, but P values are reported only when significant. WBC, white blood cells; HSC, hematopoietic stem cell. Other abbreviations as in Figures 1, 2.
number of each of these three 3 cell subpopulations, in order to investigate their possible relationship with improvements in muscle perfusion. As shown in Figure 6, the number of CD14+CD34− SC cells from PB was positively correlated with calf time to peak (TTP), whereas there was no correlation between TTP and total leukocytes or CD34+ SC. No appreciable correlation was found between muscle perfusion and BM-MNC. Similar data were obtained for forefoot TTP (data not shown).

Clinical Outcomes in Treated Patients and the HCRG
The outcomes observed in the treated patients from both groups (present study) and the HCRG are reported in Table 2. The 1-year death rate (all-cause mortality) was significantly higher in the HCRG than in the treated patient group, with 18/40 (45%) and 1/40 (2.5%) of HCRG and treated patients dying, respectively (P < 0.0001). Interestingly, sepsis was the cause of death in 9 of 18 deaths (50%) in the HCRG, whereas acute myocardial infarction was the cause of death for the only treated patient who died. The number of major amputations was higher in the HCRG than treated patient group (12/40 [30%] vs. 7/40 [17.5%], respectively) although the difference did not reach statistical significance. However, the proportion of patients who died after a major amputation was higher in HCRG than treated patient group (7/12 [58.3%] vs. 0/7 [0%], respectively; P < 0.001).

With regard to clinical parameters, the proportion of survivors in the non-amputated group with healed trophic lesions was higher for treated patients than the HCRG (10/28 [37.7%] vs. 3/30 [10%], respectively). The PFWD for 53% of treated patients was >200 m, whereas none of the HCRG patients had a PFWD during the 1-year follow-up period. During the follow-up period, 2 patients worsened compared to enrollment (6%), 14 were stable (43.7%), and 17 improved (51.5%) in the treated group, compared with 2 (11.7%), 15 (88.2%), and 0 (0%) in the HCRG patients, respectively. In the HCRG, analgesic drug consumption, evaluated in survivors, decreased significantly in the treated patients compared with the HCRG (13/39 [33.3%] vs. 22/22 [9%], respectively; P < 0.001; Table 2). These results are in keeping with other studies in the field.⁷⁻¹⁵,⁴⁵,⁴⁷

Table 2. Comparison Between All Treated Patients: Both ECEPC and BM-MNC (Present Study) and the HCRG

| Group          | No. patients | Follow-up duration (months) | No. deaths | Major amputations | Improvements in PFWD | Drug consumption | Trophic lesions |
|----------------|--------------|-----------------------------|------------|-------------------|----------------------|-----------------|-----------------|
|                |              |                             |            |                   |                      |                 |                 |
| Patients       |              |                             |            |                   |                      |                 |                 |
| Treated        | 40           | 12                          | 0          | 0                 | 1                    | 0/7             | 17/32 (53)      |
| Untreated      | 40           | 12                          | 4          | 5                 | 2                    | 7               | 0/17            |
| HCRG           |              |                             | 0          | 1                 | 1                    | 0               | 2/22 (9)        |
|                |              |                             |            |                   |                      |                 | 3/30 (10)       |
| P value        | NS           | NS                          | <0.001     | <0.001            | <0.001               | NS              | <0.001          |

Unless indicated otherwise, data are given as n (%) or as the number of patients in each group. +Amp, with amputation; −Amp, without amputation; HCRG, historical clinical reference group; PFWD, pain-free walking distance.

Discussion
Several studies have shown that autologous or even allogeneic implantation of BM-MNCs promotes therapeutic angiogenesis in patients with CLI, although some review of the existing literature dose not fully support this correlative hypothesis.⁸⁻¹²,¹⁶,¹⁷,²⁷⁻⁴² Alternative techniques have also been attempted, such as implantation of autologous circulating CD34+ SC, following G-CSF mobilization, with evidence of some positive results.⁹⁻¹¹,¹⁴,¹⁵ However, SC mobilization may be failing in approximately one-third of subjects,³⁷⁻⁴² and it virtually does not happen in diabetic patients.³³,³⁴

Some years ago, we demonstrated the existence of a subpopulation of CD14+ cells exhibiting low CD34 expression (CD14+CD34low) that appeared to be the major source of circulating EPCs.¹⁷ The proportion of these cells in the PB varies individually, ranging from 10% to 70% of total CD14+ cells. The relative amount of circulating CD14+CD34low cells was not dependent on age and was unaffected in chronic vascular diseases.

To demonstrate the therapeutic efficacy of ECEPCs in CLI patients, we designed a single-center randomized clinical trial (SCELTA) in which 2 groups of patients received intramuscular leg injections of autologous ECEPCs or BM-MNCs.

At present, the mechanisms regulating SC homing, tissue incorporation, survival, and differentiation are partially known,³⁵⁻³⁶ and studies have not reported any significant differences between intra-arterial or intramuscular injections.³⁷⁻³⁹ Thus, we chose to use the multiple-site (n=40) gastrocnemius injection method because it results in a cellular depot in the ischemic tissue.³⁸

In the present study, 23 patients were treated with ECEPCs and 17 were treated with BM-MNCs, and the absolute number of viable injected ECEPCs and BM-MNCs was comparable to that reported in other studies using the same source of cells and route of administration.⁸⁻¹²,⁴¹,⁴² However, it should be noted that the number of recovered CD34+ SC was in the order of 10⁶ cells, whereas none of the HCRG patients had a PFWD during the 1-year follow-up period. During the follow-up period, 2 patients worsened compared to enrollment (6%), 14 were stable (43.7%), and 17 improved (51.5%) in the treated group, compared with 2 (11.7%), 15 (88.2%), and 0 (0%) in the HCRG patients, respectively. In the HCRG, analgesic drug consumption, evaluated in survivors, decreased significantly in the treated patients compared with the HCRG (13/39 [33.3%] vs. 22/22 [9%], respectively; P < 0.001; Table 2). These results are in keeping with other studies in the field.⁷⁻¹⁵,⁴⁵,⁴⁷
patients treated; however, treatment with autologous PB MNCs after G-CSF mobilization has been demonstrated to be safe by previous studies.\textsuperscript{10,11,14,15}

A placebo control group was not envisaged in the present study because many studies have already demonstrated the efficacy of BM-MNC implantation;\textsuperscript{14,27–30,43} these data were recently confirmed by Pignon et al,\textsuperscript{47} who demonstrated the efficacy of BM-derived MNCs in reducing the frequency of major amputations in CLI patients in a randomized double-blind placebo-controlled trial. Thus, in the present study we considered the BM-MNC arm as the control condition, in accordance with the decision of the Ethics Committee. We also planned an internal control represented by the longitudinal evaluation of each patient before the injection procedure: (1) screening time (3–4 months before the procedure); (2) enrollment time (28 days before the procedures); and (3) procedure time (24 h before the injection). The data clearly show that in this preprocedural period, all parameters evaluated were stable if not worsening. In some cases the worsening was statistically significant due to the typical clinical instability of CLI patients. Moreover, we took advantage of an HCRG comprising patients (matched for number, disease severity, therapy regimen, and general characteristics) who had previously been evaluated for CLI in our institutions (Careggi University Hospital and San Giovanni di Dio Hospital). This close similarity in patient characteristics and the standard clinical management can overcome, at least in part, the well-known limitations in interpreting the results after comparison with a retrospective reference group rather than with a standard prospective control group. Indeed, the patients in the HCRG represent the classic history of CLI patients in our institutions in the absence of cell therapy. In the HCRG, a major incidence of deaths, major amputations, and a worsening of ulcer scores was observed over a 12-month follow-up compared with patients in both treatment arms in the present study, indicating that both cell regimens are useful in CLI. Similarly, there was a significantly higher incidence of deaths and major amputations in the cohort of screened eligible patients that did not undergo treatment compared with treated subjects (data not shown). It is also of note that major amputations in treated patients were required exclusively for patients with extensive local infections and/or presepticemic conditions before treatment.

The trial was stopped after the enrolment of 40 patients because the statistical improvement of the single primary endpoint (i.e., muscle perfusion) was largely obtained before 60 patients had been enrolled in the study. The trial was not powered for treatment efficacy; nevertheless, all the clinical parameters evaluated (i.e., rest pain, analgesic drug consumption, PFWD, wound healing and QoL), improved significantly in both treatment arms at the earliest follow-up visit (30 days after treatment), with additional progressive improvements at the 2nd (150 days) and 3rd (360 days) follow-up examinations. The favorable clinical effects were consistent with the objective instrumental results of different vascular and metabolic tests (ABI, TBI, TC PO\textsubscript{2}, TTP). CTA revealed data favoring an improvement in macrovessel and collateral vessel representation at the 3rd follow-up visit, being an expression of neovascularogenesis, which is a late event. These data suggest that injection of EPCs induces a widening of the microcirculatory net and a run-off microvascular bed that can be considered prerequisites for the later development of larger proximal vessels.

All the clinical improvements in the treated patients persisted even beyond 360 days (3rd follow-up visit) and some patients in both groups are still in good conditions up to 6 years after treatment. Moreover, none of the patients surviving at the 1-year follow-up examination died or underwent major amputations in the following period, with a mean (±SD) follow-up for the entire group of 25.2±18.6 months. The question of whether vascular regeneration induced by EPCs is due to their differentiation into endothelial cells remains to be answered. SC-tracing studies in animal models have shown that injected cells are retained for only a short period in the limb,\textsuperscript{48} and that incorporation into the vascular bed does not contribute to the observed proangiogenic effects.\textsuperscript{49,44} SCs could act through a paracrine effect, either directly on the local endothelium\textsuperscript{48} or indirectly through the recruitment of angiogenic monocytes.\textsuperscript{44}

**Conclusions**

The results of the present trial do not only confirm the results of several previous studies on the therapeutic efficacy of autologous transplantation of BM-MNCs in CLI,\textsuperscript{8,16,47} but they also provide evidence of the efficacy of an additional procedure, based on the injection of a circulating cell population consisting of monocytes (containing the CD14\textsuperscript{+}CD34\textsuperscript{low} population) and of CD34\textsuperscript{+} cells, in absence of G-CSF mobilization. These cells, particularly the CD14\textsuperscript{+}CD34\textsuperscript{low} population, have been shown to be prone towards differentiation, at least in vitro, into endothelial cells.\textsuperscript{17} Thus, the results of the present study provide strong support for the “no superiority” of non-mobilized ECEPCs vs. BM-MNCs treatment, and encourage the use of an ECEPC method as a possible source of EPCs for therapeutic purposes as an alternative to BM.

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**Supplementary Files**

Supplementary File 1

Supplementary Methods

**Supplementary Results**

**Figure S1.** Recoverable CD14+CD34low cells from the blood of critical limb ischemia (CLI) patients and healthy donors.

**Figure S2.** Correlation between muscle perfusion, shown as time to peak (TTP) at the level of the calf and transcutaneous (TC) PO2 on Days 30, 150, and 360 of follow-up.

**Table S1.** Number of cells injected

**Table S2.** Baseline characteristics of the treated with both ECEPC and BM-MNC patients and HCRG

**Table S3.** Adverse events (AEs)

**Table S4.** Evaluation of the evolution of TLL

Please find supplementary file(s); http://dx.doi.org/10.1253/circj.CJ-17-0720