Autologous Bone Marrow Mononuclear Cell Implantation and Its Impact on the Outcome of Patients With Critical Limb Ischemia
— Results of a Randomized, Double-Blind, Placebo-Controlled Trial —

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Background: Cell therapy is a therapeutic option for patients presenting with nonrevascularizable critical limb ischemia (CLI). However, there is a lack of firm evidence on its efficacy because of the paucity of randomized controlled trials.

Methods and Results: The BALI trial was a multicenter, randomized, controlled, double-blind clinical trial that included 38 patients. For all of them, 500 mL of bone marrow were collected for preparation of a BM-MNC product that was implanted in patients assigned to active treatment. For the placebo group, a placebo cell-free product was implanted. Within 6 months after inclusion, major amputations had to be performed in 5 of the 19 placebo-treated patients and in 3 of the 17 BM-MNC-treated patients. According to a classical logistic regression analysis there was no significant difference. However, when using the jackknife analysis, 6 months after inclusion BM-MNC implantation was associated with a lower risk of major amputation (odds ratio (OR): 0.55; 95% confidence interval (CI): 0.52–0.58; P<0.0001) and of occurrence of any event (major or minor amputation, or revascularization) (OR: 0.30; 95% CI: 0.29–0.31; P<0.0001). The secondary endpoints (i.e., pain, ulcers, TcPO2, and ankle-brachial index value) were not statistically different between groups.

Conclusions: Our results suggested that cell therapy reduced the risk of major amputation in patients presenting with nonrevascularizable CLI.

Key Words: Bone marrow cells; Cell transplantation; Limb salvage; Peripheral vascular diseases

Critical limb ischemia (CLI) is the end stage of peripheral arterial obstructive disease. In 25–40% of the cases, revascularization is not feasible or shows very low success rates. These “no-option” patients have a poor prognosis in terms of major amputation risk and mortality.1 Cell therapy provides a new therapeutic approach for such patients. In 2002, Tateishi-Yuyama et al were the first to report on the efficacy of intramuscular implantation of autologous bone marrow mononuclear cells (BM-MNC) in patients presenting with CLI.2 Subsequently, several studies have suggested the benefit of BM-MNC therapy.3–19 However, most of those studies included a limited number of patients in non-controlled, non-blinded trials. This led us to develop a randomized controlled trial (RCT) to evaluate the efficacy of intramuscular implantation of BM-MNC in CLI. The Bone marrow Autograft in Limb Ischemia (BALI) study was initiated in 2009 and it was decided to use unfractionated BM-MNC, which have been extensively and safely used in published trials. Furthermore, the procedure for BM-MNC
preparation is easily standardized in a multicenter trial. The second major point was to decide on the route of administration of BM-MNCs. It was decided to use intra-muscular administration rather than the intra-arterial route, because this method of administration is simple to handle. Finally, the procedure planned a single BM-MNC administration as initially described by Tateishi-Yuyama et al.2

Methods

Trial Design
The BALI trial was an investigator-initiated, multicenter, randomized, controlled, double-blind clinical trial conducted in 7 academic centers in France. Included patients were randomly assigned in a 1:1 plan to receive either BM-MNC or placebo. Randomization was performed by the local investigator in charge of the patient, through a dedicated website, with stratification by center. The randomization was transmitted to the local cell therapy unit. The steering committee, the investigators, the observers, and the patients were unaware of the treatment allocation. An independent Data and Safety Monitoring Board (DSMB) was regularly informed on the inclusion rate and the safety data.

The study protocol was approved by the French National Agency for Medicines and Health Products Safety and by an Institutional Review Board. Written informed consent was given by all patients. The trial was registered on www. clinicaltrials.gov (identifier NCT00904501).

Patients
Patients were eligible for the trial if they were ≥18 years of age, and presented with atherosclerosis-related CLI as previously defined with no sign of improvement after previous appropriate medical treatment including wound care and drug therapy. Enrolment was proposed by the local investigator in agreement with an independent vascular surgeon. Both ensured that surgery or percutaneous revascularization had previously failed or was considered impossible because of the poor quality of the run-off vessels. Exclusion criteria were: Buerger’s disease, active infection, uncontrolled diabetes mellitus, history of neoplasm or malignancy, contraindication for general anesthesia, dialysis, prothrombin time <50%, myocardial or brain infarction within 3 months, any medical condition contraindicating the modification of anticoagulation, unexplained hematological abnormality, human immunodeficiency virus, hepatitis B or C virus infection, or any concomitant disease associated with a life expectancy of <1 year.

Procedure
For all included patients, 500 mL of bone marrow were collected from the posterior iliac crests under general anesthesia. The procedure of BM-MNC separation depended on the equipment at each center: 8 centers (having included a total of 27 patients) used a blood-cell separator (Cobe Spectra, version 4, Bone Marrow Processing Program, Gambro BCT, Lakewood, CO, USA); 2 centers used a blood-cell separator requiring a Ficoll density-gradient for isolation of the BM-MNC (Cobe 2991, Gambro BCT). Whatever the procedure, a 40-mL BM-MNC product was obtained from which 10 mL were used for cell product characterization. For patients randomly assigned to receive BM-MNC, the cells were implanted within 3h of their preparation. For the placebo group, a “cell-like” product was prepared (30mL saline with 4mL autologous peripheral blood); collected BM-MNCs were cryopreserved. The assigned product (i.e., BM-MNC or placebo) was provided blindly to the clinical staff and was implanted through 30 intramuscular injections of 1 mL; the injections into the ischemic leg used 26G needles and were 1–1.5 cm deep, spaced 1 cm apart. At 1 h before the implantation, patients received 10 mg morphine subcutaneously. Whenever required, vitamin K antagonists were switched to heparin, which was discontinued 12 h before the bone marrow harvest. Antiplatelet intake was not allowed within 5 days of bone marrow aspiration except aspirin (at the dosage of 75mg/day). Previous anticoagulation or antiplatelet agents were resumed 6h after cell implantation.

Outcome
Follow-up visits were performed on post-procedure days 1, 3, 15 and 28, then monthly for 6 months, and at 12 months. Each visit included a clinical evaluation and the measurement of the ankle-brachial index (ABI) and transcutaneous pressure of oxygen (TcPO2). Pain was evaluated using a visual analog scale. TcPO2 was measured while supine.

The primary endpoint was major amputation or death between inclusion date to the 6-month follow-up. Major amputation was defined as an amputation through or above the ankle joint. All deaths, whatever the cause, were considered as an event. Secondary endpoints were defined by their evolution within the first 6 months follow-up: pain, ulcers, ABI, and TcPO2, as well as the occurrence within 6 months of any event including minor or major amputation, revascularization or death. The occurrence of major amputation or any other event at 12 months after the randomization date was analyzed.

Cell Product Characterization
Cell product characterization methods have been described. Hematocrit and nucleated cells count were determined using an automated counter; the differential was determined by optical microscopy. CD34(+) cells were measured by flow cytometry as previously described.

Statistical Analysis
When the BALI trial was initiated, taking the published data and our own experience into account, we estimated the 6-month risk of major amputation or death in patients with nonrevascularizable CLI as 35%. From a preliminary feasibility and safety study, we hypothesized that this risk would fall to 10% after cell therapy. It was therefore calculated that 48 participants in each treatment group would provide 80% power at a 5% significance level to detect a 25% absolute difference in the occurrence of the primary outcome.

However, during the data monitoring, which was done without unblinding of the trial participants, the occurrence of death or major amputation, defining the primary outcome, turned out to be much lower in both groups than initially estimated. The inclusion rate was also lower than expected. Considering time and financial constraints, the DSMB advised the trial’s steering committee to stop the study after the inclusion of 38 patients. The inclusions actually ceased on January 2013.

A modified intention-to-treat (ITT) analysis was performed. Quantitative variables are presented as median and interquartile range (IQR), qualitative variable as number and percentage. For the primary outcome (major
MNC isolation resulted in the destruction of the cells collected from a 76-year-old woman who had been allocated to the placebo group. Therefore, the modified ITT analysis excluded these 2 patients and the analysis was conducted on 19 patients in the placebo group and 17 in the BM-MNC group. The baseline patients’ characteristics are presented in Table 1. All patients presented with severe symptoms and high cardiovascular risk. There was no difference between the 2 groups especially in terms of pain and presence of ulcers. No vasodilator agent or nerve block was proposed after enrolment in the trial.

**Cell Product Characteristics**

Median and IQR of hematocrit, implanted platelets, and nucleated cells were respectively 0.03 [0.01–0.06], 14.9 \( \times 10^9 \) [10.1–22.4] and 1.6 \( \times 10^9 \) [0.9–1.7]. Median and IQR of implanted mononuclear cells and CD34(+) cells were, respectively, 1.3 \( \times 10^9 \) [0.9–1.5] and 33.5 \( \times 10^6 \) [24.2–55.5]. No significant variation in these cell product characteristics was observed according to the isolation procedure of BM-MNC.

**Efficacy Outcome**

The outcome of the 36 analyzed patients is reported in Figure 1. No deaths occurred within 12 months after inclusion. After implantation of either BM-MNC or placebo, some patients suffered from continuing uncontrolled pain that justified amputation. In the hope of avoid this issue, a revascularization procedure was performed despite the high risk of failure that had contraindicated this procedure at the inclusion. This was the case on days 25, 112 and 162 after implantation in 3 patients of the placebo group, and at days 76, 126 and 337 after implantation in 3 patients of the BM-MNC group.

Results of the statistical analysis are presented in Table 2. Within 6 months of inclusion, a major amputation at 6 months) odd ratios (ORs) with 95% confidence intervals (CIs) were calculated by logistic regression. The initial tests planned for comparisons between groups were the Chi-squared test or Fisher’s exact test for categorical variables, and the Mann-Whitney test for continuous variables, whenever appropriate. However, to face the problem of small sample size, at the time of the amendment, before unblinding the study, the jackknife method was chosen to calculate ORs and 95% CIs. This method is a resampling technic especially useful to reduce variance and bias estimations in small-sized studies. It consists of systematically leaving out each observation from a dataset and calculating the estimate and then finding the average of these calculations. The average thus obtained is more accurate and reduces the gap between the 95% CI limits. The Wald test was used to estimate P-values related to the jackknife method. The same analyses were performed for the secondary outcomes (all events at 6 months, major amputation and all events at 12 months). Ulcers, TcPO\(_2\), ABI and pain at 1 and 6 months were compared with baseline values at the inclusion period by McNemar test with Yates correction or Wilcoxon signed-rank test for the 2 treatment groups, whenever appropriate. All analyses were two-sided and performed using SAS version 8.2 (SAS Institute, Inc., Cary, NC, USA) and R version 3.1.2 (R Foundation for Statistical Computing, Vienna, Austria).

| Table 1. Demographic and Clinical Characteristics of Patients Included in the BALI Study |
|----------------------------------------|----------------------------------------|------------------|
|                                       | Placebo (n=20)                         | BM-MNC (n=18)    |
| Age, years**                          | 65 [56–71]                            | 72 [57–76]      |
| Male sex*                             | 18 (90)                               | 13 (72)         |
| Smoker                                |                                       |                  |
| Current*                              | 3 (15)                                | 7 (39)          |
| Past*                                 | 15 (75)                               | 8 (44)          |
| Diabetes mellitus*                    | 7 (35)                                | 10 (55)         |
| Hypertension*                         | 11 (55)                               | 13 (72)         |
| Cardioprotective drugs use            |                                       |                  |
| Antiplatelet therapy*                 | 14 (70)                               | 10 (55)         |
| Statins*                              | 18 (90)                               | 12 (66)         |
| ACEI*                                 | 16 (80)                               | 14 (77)         |
| Disease characteristics               |                                       |                  |
| Pain scale**                          | 60 [30–70]                            | 55 [40–85]      |
| Presence of ulcers*                   | 13 (65)                               | 12 (67)         |
| Rutherford class 4*                   | 7 (35)                                | 6 (33)          |
| Rutherford class 5*                   | 13 (65)                               | 12 (67)         |
| TcPO\(_2\), mmHg**                    | 4 [1–32]                              | 5 [2–26]        |
| ABI**                                 | 0.4 [0.2–0.6]                         | 0.4 [0.1–0.7]   |

\*n (%); **median and interquartile range. ABI, ankle-brachial index; ACEI, angiotensin-converting enzyme inhibitor; BALI, bone marrow autograft in limb ischemia; BM-MNC, [autologous] bone marrow mononuclear cells; TcPO\(_2\), transcutaneous pressure of oxygen.

Results

**Patients’ Characteristics**

From March 2009 to August 2012, 7 centers included 38 patients; 20 patients were allocated to the placebo group and 18 to the BM-MNC group. A 77-year-old woman who had been allocated to the BM-MNC group developed arrhythmia after randomization, so bone marrow harvest could not be performed. A technical problem during BM-MNC isolation resulted in the destruction of the cells collected from a 76-year-old woman who had been allocated to the placebo group. Therefore, the modified ITT analysis excluded these 2 patients and the analysis was conducted on 19 patients in the placebo group and 17 in the BM-MNC group. The baseline patients’ characteristics are presented in Table 1. All patients presented with severe symptoms and high cardiovascular risk. There was no difference between the 2 groups especially in terms of pain and presence of ulcers. No vasodilator agent or nerve block was proposed after enrolment in the trial.
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6 months for BM-MNC-treated patients (*Table 2*). Within 6 months of inclusion, a decrease in rest pain was observed, which was significant for placebo-treated patients (*P*=0.002) and of borderline significance for BM-MNC-treated patients (*P*=0.05); the degree of improvement was not significantly different between the 2 groups (*Figure 2*). An increase in TCPO2 was observed in both placebo-treated patients (*P=0.02*) and BM-MNC-treated patients (*P=0.02*), without any difference between groups (*Figure 2*). No significant variation was observed in any group concerning the frequency of ulcers and the ABI values (*Figure 2*).

In both groups, no major amputations occurred between 6 and 12 months. At 12 months after inclusion, there was no difference between groups according to the classical logistic regression test, but the jackknife method showed a significantly lower risk after BM-MNC implantation (*Table 2*).
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Events (i.e., minor or major amputations and revascularization). Because a revascularization procedure was performed in some patients after either BM-MNC or placebo treatment, the statistical analysis was completed by considering these patients as “amputated”. Using the jackknife method, a significantly lower risk was observed for BM-MNC-treated patients.

An improvement in pain and TcPO$_2$ was observed within 6 months of treatment; however, none was statistically different between groups. The frequency of ulcers and the ABI values were unchanged in both groups. However, the interpretation of these results has to take into account the large dispersion of values for these parameters and the low number of patients. Indeed, when an amputation or revascularization was performed, which was more frequent in the placebo group, these parameters were not evaluated.

The patients included in the BALI trial were followed for 12 months after randomization. No major amputations were performed between 6 and 12 months. Thus, the beneficial effect of BM-MNC implantation, which was observed at 6 months, was not offset by delayed amputation.

A major concern when analyzing clinical trials is the choice of statistical analyses. In the BALI trial, considering the number of included patients, before unblinding the

Figure 2. Evolution of clinical symptoms and hemodynamical parameters within the first 6 months after randomization of subjects in the Bone marrow Autograft in Limb Ischemia (BALI) study. (A,C,D) Median, interquartile ranges and extreme values are presented. (B) Columns indicate the percentage of patients presenting with ulcers. Total numbers of evaluable patients were: 34 at day 15, 31 at day 30, 29 at day 60, 25 at day 120, 24 at day 150, and 20 at day 180. ABI, ankle-brachial index; TcPO$_2$, transcutaneous pressure of oxygen.

Tolerance and Safety

No deaths or other serious adverse events that could be related to the procedure were reported. No systemic side-effects were reported. Pain commonly occurred during implantation whatever the product, and was easily controlled.

Discussion

To date, definite evidence on the efficacy of cell therapy in CLI has been lacking, probably because only a few RCTs have been conducted. This led us to conduct the BALI trial, which is a multicenter, double-blind, placebo-controlled RCT. Unfortunately, the trial had to be closed after the enrolment of 38 patients. Under these conditions, according to classical logistic regression analysis, cell therapy did not significantly influence the patient’s outcome. However, using the jackknife method, which was developed to tackle the problems of small-sizes studies, a significant decrease in the risk of major amputation was observed 6 months after BM-MNC implantation. It has to be noted that the ORs were the same whatever the method used, but the jackknife method enabled a reduction in their variability and made the range of 95% CI limits more precise. Similar results were observed when considering all
trial, we decided to use the jackknife method. This well-recognized method allows reduction of the bias of the estimator and its variability. In the present study, it has to be noted that, if calculated by Fisher’s exact test, the ORs would have been the same as determined by the jackknife method. The reduction in variability made the range of 95% CI limits more precise and less biased.

To our knowledge, there are 4 published RCTs that have evaluated the efficacy of unfractionated BM-MNC in CLI patients. Barc et al reported a trial including 29 patients randomized to receive either conventional therapy or cell therapy; no statistical difference in terms of efficacy was observed because of the lack of power of the study. The PROVASA trial was the first phase II, multicenter, double-blind, randomized-start trial: cell therapy was associated with significantly improved ulcer healing and reduced pain within 3 months, but BM-MNC treatment did not significantly increase ABI and the amputation rate did not differ between groups. Li et al reported a single blinded study: a significant improvement in rest pain, skin ulcers and ABI was observed 6 months after cell therapy, but there was no significant difference in the major amputation rate between the 2 groups. More recently, the results of the JUVENTAS trial were reported. The objective of that double-blind, placebo-controlled trial was to evaluate the effect of repetitive intra-arterial infusion of BM-MNC: no significant difference was observed in terms of major amputation at 6 months, but an improvement in rest pain, ABI, and TaPO2 was reported, although without any significant difference between the 2 groups. Thus, although previous trials have shown some beneficial effects of BM-MNC implantation, none could demonstrate any effect on the major amputation rate. However, a comparison of those studies and our trial is difficult because the strategies for cell therapy were different in several aspects, including cell product preparation, administration route and cell dose. Indeed, it has been documented that the cell isolation procedure has a major effect on the functional activity of BM-MNC when used as therapy of acute myocardial infarction. Red blood cell contamination may impair the effectiveness of implanted cells. In the BALI trial the hematocrit of the final cell product was very low. A dose-response relationship between the number of administered BM-MNC has been suggested. It should be noted that in the BALI trial, the number of implanted cells (i.e., \(12 \times 10^6\)) was larger than in the PROVASA and JUVENTAS studies. Trials also differ regarding the administration route of the cell product. Although there is no clear evidence suggesting that one route would bring a higher efficacy than another, we favored intramuscular injections, assuming that this route would be associated with a stronger paracrine effect than the intra-arterial route.

Meta-analyses of cell therapy clinical trials in CLI have included published studies regardless the type of cells used (BM-MNC, bone marrow-derived mesenchymal stromal cells, peripheral blood-derived mononuclear cells). These meta-analyses support the idea that cell therapy has beneficial effects in terms of pain relief, quality of life, wound healing, ABI, TaPO2 and amputation rates. However, 2 meta-analyses concluded on the nonstatistical beneficial effect on major amputation rates when the analysis considered placebo-controlled RCTs only. The meta-analysis published by Teraa et al has been recently updated with additional placebo-controlled trials. The ABI, TaPO2 and pain score were significantly improved after cell therapy. Surprisingly, no significant difference was observed in terms of the major amputation rate. Again, this meta-analysis did not select trials according to the cell therapy procedure or the type of cells. The best cell type to use is a critical question. BM-MNC have the advantage of containing a significant number of endothelial progenitor cells, which play a major role in angiogenesis. However, several studies favor an indirect effect of implanted cells via cytokines or growth factor production. Such a paracrine effect may be caused by mature cells that are also present in BM-MNC, such as monocytes, platelets, and lymphocytes. Moreover, mesenchymal stromal cells are worthwhile considering because (1) they produce many proangiogenic factors, (2) they have the ability to differentiate into endothelial cells, and (3) their immunomodulatory properties may allow their use not only in the autologous setting but also in the allogeneic situation.

A major issue in clinical trials aimed at establishing the benefit of cell therapy in CLI is estimating the number of patients to include to demonstrate efficacy when the major amputation rate is considered as the primary outcome. The amputation rate for CLI patients who are not candidates for revascularization is commonly estimated at approximately 40%. However, recent data suggest that this rate is now much lower, especially in the setting of the specialized centers in which clinical trials are performed. Indeed, in these centers, patients enrolled in clinical trials are previously treated according to the standard of care. In the BALI trial the 6-month amputation rate was 26% in the placebo group. Based on this low rate of amputation, large cohorts of patients would have been required to establish significance if using conventional statistical methods. Finally, the risk of major amputation is highly variable among the option CLI patients, depending on disease severity and comorbidities. Considering this heterogeneity of patients and the variability of their outcomes, a choice for further evaluation of cell therapy in CLI could be to assess efficacy in relevant categories of patients. In line with this purpose, Benoit et al suggested restricting inclusion to “Rutherford five” patients for whom the expected amputation rate is the highest. In contrast, others have reported that patients with advanced CLI may not benefit from cell therapy. This suggests that cell therapy should be evaluated at earlier stages of CLI and as an adjuvant therapy to surgical or endovascular procedures.

**Study Limitations**

(1) Patients were enrolled by local teams without any validation by a multicenter steering committee. Therefore, heterogeneity of the surgical assessment of the patient’s status cannot be excluded. (2) The low number of included patients precluded the use of conventional statistical methods. We therefore used the well-recognized jackknife method, which is a resampling technique especially useful for small-sample studies. However, results should be confirmed by larger RCTs.

**Conclusions**

The results of the BALI trial suggested that intramuscular implantation of BM-MNC reduced the risk of major amputation in patients with CLI. These results were observed thanks to the use of a statistical analysis adapted
for small-sized clinical studies.

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