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# Cell therapy in critical limb ischemia: A comprehensive analysis of two cell therapy products

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**Abstract**

*Background.* Cell therapy has been proposed as a salvage limb procedure in critical limb ischemia (CLI). In spite of the fact that clinical trials found some efficacy, the mechanism of action remains elusive. The objective of this study was to characterize two autologous cell therapy products (CTPs) obtained from patients with advanced peripheral arterial disease. *Methods.* Bone marrow (BM-CTPs) (n = 20) and CTPs obtained by non-mobilized cytapheresis (peripheral blood [PB]-CTPs) (n = 20) were compared. CTPs were characterized by their cell composition, by the quantification of endothelial progenitor cells (EPCs) and mesenchymal stromal cells (MSCs) and by transcriptomic profiling. The angiogenic profile and the 6-month outcome of CLI patients are described. *Results.* Patients presented inflammation syndrome and high levels of CXCL12, soluble stem cell factor and granulocyte colony-stimulating factor, whereas granulocyte macrophage colony-stimulating factor was low. Circulating CD34+ cells represented rare events. BM and PB-CTPs were heterogeneous. Mature cells and colony-forming unit–endothelial cells were in higher concentration in PB-CTPs, whereas CD34+ stem cells and EPCs were more abundant in BM-CTPs. MSCs were identified in both CTPs. Transcriptomic profiling revealed the strong angiogenic potential of BM-CTPs. Transcutaneous partial pressure of oxygen, C-reative protein and neutrophil content in CTPs are predictive of the clinical outcome at 6 months. *Discussion.* Transcriptomic allows an accurate characterization of CTPs. BM-CTPs have the richest content in terms of stem cells and transcriptome. The high content of mature cells in PB-CTPs means that they work via a paracrine mechanism. The clinical outcome indicates the deleterious influence the patients' status and the limits of an autologous approach. In this respect, MSCs may allow an allogenic strategy.

**Key Words:** *angiogenesis, cell therapy, critical limb ischemia, endothelial progenitor cells, mesenchymal stem cells, transcriptome*

## Introduction

The most common cause of chronic lower limb ischemia is atherosclerotic peripheral arterial disease (PAD). In the absence of control of cardiovascular risk factors, the disease will progress leading to critical limb ischemia (CLI) [1]. The annual incidence of CLI is estimated to be 500–1000 cases per million people in developed countries [1]. CLI may lead to limb loss or even death unless treated promptly. Up to 25% of patients are not eligible for standard revascularization procedures and are defined as "no-option CLI" (NO-CLI). For these patients, autologous cell therapy has been proposed. The Therapeutic Angiogenesis using Cell Transplantation (TACT) trial was the first large report on the use of bone marrow (BM)-derived mononuclear cells (MNCs) in the treatment of CLI [2]. Since 2002, 88 clinical trials have been published, and around 3000 patients have been treated with autologous transplantation. These studies were rarely randomized and generally included limited numbers of patients, which may explain that no definite conclusion about efficacy could be drawn. Recent meta-analyses were in favor of a beneficial effect of cell therapy on major amputation rates and amputation-free survival [3,4]. A major pitfall, when interpreting clinical trials including meta-analyses, is that many different cell therapy products (CTPs) were used. If the majority of CTPs were obtained from BM, peripheral blood (PB) with different protocols was also used (with or without granulocyte-colony stimulating factor [G-CSF]-mobilization and sub-fractionated cell preparations, e.g., CD34+ or CD133+ cells). If the safety

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of cell therapy can be considered as established [5], the mechanism of the neovascularization remains to be elucidated. In most clinical trials, the exact composition of the cellular product remains elusive and is obviously highly variable [6]. To improve our knowledge on the mechanism of action of CTPs, it is mandatory to characterize extensively each product. A clinical trial gave us the opportunity to perform an ancillary study that is presented here. The clinical trial (trial number NCT00533104) initiated in two French academic hospitals was designed as a two-arm, open labelled phase IIa trial, aimed at establishing the feasibility and safety of two different CTPs (BM- versus PB-CTPs). The originality of the clinical trial is related to the fact that PB-CTPs were collected without any G-CSF-mobilization. Forty patients presenting with CLI were included in this clinical trial and were implanted with BM- or PB-CTPs (20 in each group). We could demonstrate the safety and, particularly, the absence of thrombogenicity of both CTPs [7].

The ancillary study was designed as a comprehensive analysis of CTPs, using transcriptomic characterization in addition to the quantification of stem and progenitor cells. The objectives of the present study were: (i) to characterize the angiogenic potential of non-mobilized PB-CTPs in comparison with BM-CTPs, (ii) to evaluate the influence of the patient status on the characteristics of CTPs and (iii) to compare the CTPs' characteristics and patients' status with clinical outcome. The angiogenic profile of CLI patients was determined before cell therapy (baseline). Angiogenic potential of each CTP was evaluated by clonogenic assays (colony forming unit-endothelial cells, [CFU-EC] and CFU-fibroblast [CFU-F]) and by the quantification in flow cytometry (FC) of hematopoietic stem cells (HSCs) and endothelial progenitor cells (EPCs). Clonogenic assays, FC quantification and transcriptomics indicate that BM-CTPs possess a much higher angiogenic potential than PB-CTPs. The analysis of clinical response indicates that the patients' status at baseline is preponderant for their outcome at 6 months.

## Materials and methods

### *Patients, BM- and PB-cell products preparation*

The study protocol was approved by the Ethics Committee of Champagne Ardennes. The design of the clinical trial can be found on the site www.clinicaltrials.gov (trial number NCT00533104). Briefly, 40 patients presenting with unilateral CLI but not suitable candidates for non-surgical or surgical revascularization were included. Transcutaneous partial pressure of oxygen ($TcPO_2$) was measured using a TINA TCM4 monitor (Radiometer) in standardized conditions. CTPs were obtained by BM (n = 20) or cytapheresis (n = 20). For the preparation of BM-cell products, 500 mL of BM were collected under general anesthesia through multiple punctures of the posterior iliac crest. BM-CTPs were isolated using a blood-cell separator (Cobe Spectra, version 4, Bone Marrow Processing Program, Gambro BCT). PB-CTPs were collected by cytapheresis of one blood mass (5.1 ± 1.1 L) during 90 min on the same blood-cell separator (Cobe Spectra, version 6, auto-PBSC program) [8]. BM- and PB-CTPs were suspended in an autologous patient's plasma and were implanted once within 3 h after preparation, by 30 multiple intramuscular (IM) injections into the gastrocnemius of the ischemic leg (1 mL per injection). Follow-up visits were performed on post-procedure days 1, 2, 3, 14 and 28 and at months 3 and 6. The clinical outcome was assessed 6 months after CTP implantation.

### *Patient's blood parameters*

Quantitative determination of interleukin-6 (IL-6) and vascular endothelial growth factor (VEGF) in ethylenediaminetetraacetic acid (EDTA) plasma was performed by specific human antibodies using the Quantikine ELISA kit (R&D Systems Inc), according to the manufacturer's instructions. Chemokine, CXC motif, ligand 12 (CXCL12 or stromal-cell-derived factor, $SDF_{(1\alpha+1\beta)}$), soluble stem cell factor (sSCF or KIT Ligand), granulocyte macrophage-colony stimulating factor (GM-CSF) and granulocyte-colony stimulating factor (G-CSF) EDTA plasma levels were measured by Luminex multi-analyte profiling (xMAP) technology using Milliplex MAP kit human cytokine panel I or II (Millipore Corporation). C-reactive protein (CRP) serum levels were measured using standardized methods (COBAS, Roche Diagnostics). CD34+ cells analysis was performed according to the reference method [9] using phycoerythrin (PE)-conjugated CD34 (clone 581, epitope class III; Beckman Coulter) and fluorescein (FITC)-conjugated CD45 anti-human monoclonal antibodies, the 7-AAD viability dye (Beckman Coulter), PE-immunoglobulin (Ig)G1 and FITC-IgG1 (Beckman Coulter) as isotypic controls, and StatusFlowPro control as target values.

### *CTP parameters*

#### *Cell counts*

Cells counts were performed with an XE-2100™ Sysmex counter (Roche Diagnostics) in Reims Hospital, and a Gen's counter (Beckman Coulter) in Amiens Hospital. BM- or PB-cell products' characterization methods have been previously described [8].

#### *Characterization of mononuclear cells, stem and progenitor cells in FC*

A three-color FC analysis was performed as previously described to determine the proportion of CD2+