

## Phase I trial: the use of autologous cultured adipose-derived stromal stem cells to treat patients with non-revascularizable critical limb ischemia

ALESSANDRA BURA<sup>1,2</sup>, VALERIE PLANAT-BENARD<sup>3,4,5,6</sup>, PHILIPPE BOURIN<sup>4,7</sup>, JEAN-SEBASTIEN SILVESTRE<sup>8</sup>, FABIAN GROSS<sup>9,10</sup>, JEAN-LOUIS GROLLEAU<sup>2,11</sup>, BERTRAND SAINT-LEBESE<sup>13</sup>, JULIE-ANNE PEYRAFITTE<sup>3,4,5,6</sup>, SANDRINE FLEURY<sup>3,4,5,6</sup>, MELANIE GADELORGE<sup>3,4,5,6</sup>, MARION TAURAND<sup>3,4,5,6</sup>, SOPHIE DUPUIS-CORONAS<sup>3,4,5,6</sup>, BERTRAND LEOBON<sup>2,12</sup> & LOUIS CASTEILLA<sup>3,4,5,6</sup>

<sup>1</sup>Service de médecine vasculaire, Pôle cardiovasculaire et métabolique, Centre hospitalo-universitaire de Toulouse, Toulouse, France, <sup>2</sup>Université de Toulouse III, Paul Sabatier, Toulouse, France, <sup>3</sup>CNRS, Université Toulouse III, UPS UMR5273 STROMAlab, Toulouse, France, <sup>4</sup>EFS (Etablissement Français du Sang), STROMAlab, Toulouse, France, <sup>5</sup>Inserm U1031 STROMAlab, Toulouse, France, <sup>6</sup>Université Toulouse III, UPS UMR5273 STROMAlab, Toulouse, France, <sup>7</sup>CSA21, 7 chemin des silos, Toulouse, France, <sup>8</sup>INSERM UMRS 970, Paris Cardiovascular Research Center, Université Paris Descartes, Sorbonne Paris cité, Paris, France, <sup>9</sup>Centre d'Investigation Clinique de Biothérapies de Toulouse, Toulouse cedex 9, France, <sup>10</sup>Direction de la Recherche Médicale et Innovation (DRMI) du CHU de Toulouse, Toulouse cedex 9, France, <sup>11</sup>Service de chirurgie plastique, reconstructrice et esthétique, Centre hospitalo-universitaire de Toulouse, Toulouse, France, <sup>12</sup>Service de chirurgie cardiovasculaire, Pôle cardiovasculaire et métabolique, Centre hospitalo-universitaire de Toulouse, Toulouse, France, and <sup>13</sup>Service de chirurgie vasculaire, Pôle cardiovasculaire et métabolique, Centre hospitalo-universitaire de Toulouse, Toulouse, France

### Abstract

**Background aims.** Non-revascularizable critical limb ischemia (CLI) is the most severe stage of peripheral arterial disease, with no therapeutic option. Extensive preclinical studies have demonstrated that adipose-derived stroma cell (ASC) transplantation strongly improves revascularization and tissue perfusion in ischemic limbs. This study, named ACellDREAM, is the first phase I trial to evaluate the feasibility and safety of intramuscular injections of autologous ASC in non-revascularizable CLI patients. **Methods.** Seven patients were consecutively enrolled, on the basis of the following criteria: (i) lower-limb rest pain or ulcer; (ii) ankle systolic oxygen pressure <50 or 70 mm Hg for non-diabetic and diabetic patients, respectively, or first-toe systolic oxygen pressure <30 mm Hg or 50 mm Hg for non-diabetic and diabetic patients, respectively; (iii) not suitable for revascularization. ASCs from abdominal fat were grown for 2 weeks and were then characterized. **Results.** More than 200 million cells were obtained, with almost total homogeneity and no karyotype abnormality. The expressions of stemness markers Oct4 and Nanog were very low, whereas expression of telomerase was undetectable in human ASCs compared with human embryonic stem cells. ASCs (10<sup>8</sup>) were then intramuscularly injected into the ischemic leg of patients, with no complication, as judged by an independent committee. Trans-cutaneous oxygen pressure tended to increase in most patients. Ulcer evolution and wound healing showed improvement. **Conclusions.** These data demonstrate the feasibility and safety of autologous ASC transplantation in patients with objectively proven CLI not suitable for revascularization. The improved wound healing also supports a putative functional efficiency.

**Key Words:** adipose tissue, ASC, cell therapy, CLI, mesenchymal stromal cells

### Introduction

Critical limb ischemia (CLI), defined by the presence of rest pain or skin ulcer, is the most severe stage of peripheral arterial disease (PAD) and is a crucial health care issue, associated with high rates of mortality and amputation. Despite the currently available

treatments to restore the blood flow in the limbs (surgical bypass or endovascular procedures), many CLI patients will die or undergo major amputation within 5 years. Furthermore, one third of affected patients are not candidates for bypass surgery, leaving

them with no other option than amputation, which subsequently worsens their quality of life.

Therapeutic angiogenesis, either as recombinant protein, gene transfer or stem cell therapy delivered to the ischemic muscle, has demonstrated exciting results and constitutes a promising approach for the treatment of CLI patients who are unsuitable candidates for revascularization (1,2). In 2002, Tateishi-Yuyama *et al.* (3) were the first to publish a pilot randomized, controlled trial to test autologous non-cultured bone marrow-derived mononuclear cells (BMMNCs), which were intra-muscularly re-implanted in CLI patients. They reported a significant increase in transcutaneous oxygen pressure (TcPO<sub>2</sub>) and pain-free walking time. Since then, and in the context of ischemia, several cell types have been tested and reviewed for their therapeutic potential. Most of these are derived from bone marrow or peripheral blood (4,5). Recent meta-analysis indicates that patients with PAD, and particularly those with CLI, have improvement in symptoms when treated with a cell therapy providing acceptable tolerability (6), although the efficacy must be definitively proven in a definitive, double-blinded, placebo-controlled clinical trial.

Different cell preparations have been used and can be proposed. The first ones were derived from bone marrow. The use of BMMNCs displays the advantage of preparing the therapeutic cell product at bench side. Unfortunately, this is counterbalanced by the need for large amounts of bone marrow aspirate with systemic or epidural anesthesia and the great heterogeneity of the therapeutic product that cannot be controlled in the time of intervention. MSCs may overcome these limits because they can be expanded, characterized as a homogeneous population and display pleiotropic regenerative effects, mainly through their marked paracrine activity. One of the limits of this technique is the poor frequency of these cells in BM aspirates; this requires harvesting large amounts of BM under general anesthesia and/or expansion of such cells, with more time needed to obtain enough cells, and increased risk associated with cell proliferation. In this view, the efficiency of BM-MSc-based therapy was tested in diabetic patients with CLI not suitable for revascularization (2,4). Besides such sources, white adipose tissue (WAT) is a seemingly unexpected and very promising alternative source of therapeutic cells: It is unexpected because it plays a main role in metabolic disease most often related to PAD but promising because it can be easily sampled under the skin and under local anesthesia only and hosts a large pool of mesenchymal stromal cells, referred to as ASC (adipose-derived stroma/stem cells) (7), which display striking therapeutic features. The use of adipose

tissue as the source of therapeutic cells can further improve the outcome of cell therapy for at least two reasons. First, because the frequency of these cells is much greater than that of MSCs, the volume of harvested tissue can be low and harvested under local anesthesia only, and the yield of expansion can be limited to decrease the delay between the tissue biopsy and cell injection and to eliminate any risk of cell transformation during the culture process (7). Second, ASCs display a very strong angiogenic potential. As far back as 2004, we first showed that these cells express the stem/progenitor cell marker CD34 and display strong angiogenic properties *in vitro* as well as *in vivo* in an experimental model of CLI (8). From this seminal study, concomitant and increasingly numerous reports confirmed our initial findings and proposed different but complementary mechanisms involved in the regenerative capacities of ASCs (9–13). Overall, their angiogenic properties rely on combinatory effects of direct participation in new vessel formation and extensive angiogenic paracrine activities involving several secreted molecules (14). Although ASCs share many features with BM-MSCs, they display a stronger angiogenic and regenerative potential when compared in an identical experimental setting of a CLI model to BM-MSc administration (15,16). Particularly, these differences could be attributed to a stronger paracrine activity of ASCs compared with BM-MSCs (15–17). Altogether, these studies suggest that the same benefit could be achieved more efficiently with the use of a lower number of ASCs than BM-MSCs and/or significantly lower putative risks associated with the expansion process, although the risks of chromosomal aberration are very limited in adult MSCs and more related to a senescence process than to a cancer process (18). Furthermore, we recently published the lack of toxicity even after ASC infusion (19).

On the basis of these concordant and convincing pre-clinical results in animals, we designed a phase I clinical trial ACeLLDREAM (for Adipose derived Cell Driven Research for Efficient Angiogenic Medicine) to test the safety and feasibility of cell therapy with autologous ASCs in “no option” CLI patients.

## Methods

### *Enrollment criteria*

Patients were enrolled in this study on the basis of the following enrollment criteria: (i) existence of rest pains of ischemic origin, which requires analgesic treatment, and/or non-healing ischemic ulcers for at least 2 weeks; (ii) for non-diabetic patients, ankle systolic oxygen pressure <50 mm Hg or first-toe systolic oxygen pressure <30 mm Hg; for diabetic

patients, ankle systolic oxygen pressure <70 mm Hg or first-toe systolic oxygen pressure <50 mm Hg; (iii) patients who were not suitable candidates for vascular or endovascular surgery, after a multidisciplinary surgical and medical decision. Exclusion criteria were (i) age <40 years, (ii) life expectancy <6 months, (iii) recent (<3 months) myocardial infarction or stroke, cachexia, proliferative diabetic retinopathy, history of cancer, severe wound infection or sepsis, (iv) treatments with prostaglandin analogs or arterial vasodilators. No sex-based or ethnic-based differences were present.

A written informed consent form was obtained from all the patients. The ethics committee of the participating university medical center approved the protocol. The clinical trial was registered with the French regulatory agency (TC194) and on the Clinical Trials website (NCT01211028, URL: <http://clinicaltrials.gov/show/NCT01211028>) and approved by the ethics committee (CPP SOOM 1 No. 1–05–23).

#### *Staging of the procedure*

Adipose tissue was harvested through the use of liposuction from subcutaneous abdominal adipose tissue in the operating room under local anesthesia. Approximately 30 g (or 60 mL of lipoaspirate containing anesthetic fluid) of adipose tissue and anesthetic fluid was removed by suction with the use of 10-mL syringes and centrifuged for 1 min at 400g. The upper (oil and mature adipocytes) and lower (blood and fluid) phases were then discarded. Syringes were then transferred to the of the Cell Therapy Unit of Etablissement Français du Sang Pyrénées-Méditerranée. All disposables containing patient materials were labeled with the unique patient identifiers.

#### *Cell processing and culture*

The stroma vascular fraction (SVF) was obtained by means of collagenase digestion. Briefly, aliquots of 10 g of adipose tissue were mixed with 34 mL of the collagenase solution (NB6; Coger, Paris, France) and incubated at 37°C for 45 min. Enzymatic digestion was stopped by the addition of complete culture medium (CCM) containing minimum essential medium (MacoPharma, Tourcoing, France), human platelet growth factor–enriched plasma, 10 µg/mL ciprofloxacin and 1 U/mL heparin. After homogenization, the digested suspension was passed through sterile 100-µm filters. The cells were centrifuged at room temperature for 10 min at 600g. The supernatant was discarded and the SVF was resuspended in 20 mL of CCM. An aliquot of the SVF was removed for the quality controls: cell count, viability, phenotyping (CD34, CD45 and CD14) and sterility. The SVF was regarded as not

compliant if cell viability was <70%, if the percentage of the cells' CD45<sup>+</sup> or CD14<sup>+</sup> was >80% or if that of the CD34<sup>+</sup> cells was <5% or non-sterile (assessed by aerobic and anaerobic hemoculture for 10 d).

The cells from the SVF were then seeded in a 1270-cm<sup>2</sup> CellStack culture chamber (MacoPharma) at a density of  $4 \times 10^3$  cells/cm<sup>2</sup> in CCM, with the use of a seeding kit (MacoPharma), at 37°C in an atmosphere saturated with moisture and 5% CO<sub>2</sub>. After an initial 24-h incubation, the non-adherent cells were removed. The adherent cells were washed once with Dulbecco's phosphate-buffered saline (PBS), and CCM medium was added for 7 d. The medium was completely replaced at day 4 and day 6 of culture with the use of medium exchange kits (MacoPharma). The primary culture (P0) was regarded as non-compliant if viability was <80% or the percentage of the CD45<sup>+</sup> or CD14<sup>+</sup> cells was >20% or that of the CD34<sup>+</sup> cells was <1% or if hTert was detected by quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR) or non-sterile.

At day 8, the cells were harvested with the use of a detachment kit (MacoPharma) according to the following protocol: after aspiration of the medium and washing with Dulbecco's PBS, 50 mL of irradiated trypsin solution was added for 5 min at room temperature. After the inhibition of trypsin activity by the addition of CCM, the cells were collected in a transfer bag (MacoPharma). An aliquot of the cell suspension was aseptically removed for cell count, viability, phenotyping (CD34, CD45 and CD14), measures of messenger RNA (mRNA) contents by qRT-PCR and assessment of sterility. The cells were seeded in a 1270-cm<sup>2</sup> Cell-Stack culture chambers at a density of  $2 \times 10^3$  cells/cm<sup>2</sup> and incubated for 6 d. The CCM was completely replaced at day 11 and day 13. At day 14, the cells were harvested according to the same procedure as described above. The cell suspension was placed in a transfer bag (MacoPharma) and washed with Dulbecco's PBS. The ASCs were then resuspended in a solution containing 3.6% human albumin provided by Laboratoire français du Fractionnement et des Biotechnologies (Courta-boeuf, France) and a poly-ionic solution containing glucose. An aliquot of the ASC suspension was aseptically removed for cell count, and its quality was evaluated as described above. There is no other step, no freezing and no storage during cell culture.

SVF cells ( $2 \times 10^5$  cells treated for 5 min at room temperature with a lysis buffer [Vitalyse; BioE, Saclay, France] to remove red blood cells) or ASCs ( $2 \times 10^5$  cells) were stained with saturating amounts of monoclonal antibodies conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE) and their respective isotype controls for 30 min in the dark at 4°C in PBS/0.5% human albumin and 0.1% sodium azide. After washing, the labeled cells were

Table I. Specific oligonucleotide probes used in the study.

Genes	Forward	Reverse
<i>Oct4</i>	ACATCAAAGCTCTGCAGAAAGAACT	CTGAATACCTTCCCAAATAGAACCC
<i>Nanog</i>	CAGCTGTGTGTACTIONCAATGATAGATTT	CAACTGGCCGAAGAATAGCAATGGTG
<i>hTERT</i>	GGAGCAAGTTGCAAAGCATTG	TCCCACGACGTAGTCCATGTT
$\beta$ -Actin	CCTGGCACCCAGCACAAT	GCCGATCCACACGGAGTACT
Catalase	TTCCAGATGGACATCGCCAC	AAGTCTCGCCGCATCTTCAA
<i>HGF</i>	CAGAGGGACAAAGGAAAAGAA	GCAAGTGAATGGAAGTCCTTTA
<i>HIF-1<math>\alpha</math></i>	TCCAGTTACGTTCCCTTCGATCA	TTTGAGGACTTGCCTTTCA
<i>IDO-1</i>	GCCTGATCTCATAGAGTCTGGC	TGCATCCCAGAACTAGACGTGC
<i>IGF</i>	CTCTTCAGTTCGTGTGTGGAG	CAGCCTCCTTAGATCACAGCT
<i>IL-1-RA</i>	ATGGAGGGAAGATGTGCCTGTC	GTCCTGCTTTCTGTTCTCGCTC
<i>IL-6</i>	TCCACAAGCGCCTTCGGTCC	GCAGGGAAGGCAGCAGGCAA
<i>MMP-9</i>	CGCACGACGTCTTCCAGTACCG	TCACGCGCCAGTAGAAGCGG
<i>PUM-1</i>	AGTGGGGGACTAGGCGTTAG	GTTTTTCATCACTGTCTGCATCC
<i>SOD-1</i>	GGTGTGGCCGATGTGTCTAT	CCTTTGCCCAAGTCATCTGC
<i>SOD-2</i>	CTGGACAAACCTCAGCCCTAA	AACCTGAGCCTTGGACACCAA
<i>VEGF</i>	CTCTACCTCCACCATGCCAAG	AGACATCCATGAACCTTCACTTC
<i>TIMP-1</i>	GGGCTTCACCAAGACCTACA	TGCAGGGGATGGATAAACA

analyzed by flow cytometry (EPICS XL-MCL flow cytometer; Beckman-Coulter, Nyon, Switzerland). FITC anti-CD14, FITC anti-CD45, PE anti-CD34, PE anti-CD73, PE anti-CD90 and immunoglobulin (Ig)G1 PE were from BD Pharmingen (Le Pont de Claix, France) (respectively, 555397, 555482, 555822, 550257, 555596 and 559320); IgG1 FITC was from Immunotech (Marseille, France) (A07795).

qRT-PCR was performed according to European pharmacopeia 2.6.21. Briefly, cells were lysed and RNA was recovered with the use of a specific kit (Rneasy; Qiagen, Courtaboeuf, France). The specific oligonucleotide probes used in the study are shown in Table I.

Quantitative measurement was performed during the amplification process with the use of SYBR green heparin labeled oligonucleotides (Roche, Paris, France). The data are expressed as  $2^{-\Delta Ct}$  with actin gene expression used as control ( $\Delta Ct = Ct_{\text{gene}}$  minus  $Ct_{\text{actin}}$ ).

#### Clonogenic assay

Cells were plated at low density (16 cells/cm<sup>2</sup>). After 2 weeks, cells were rinsed with PBS, fixed in methanol and stained with 6% Giemsa. Colonies containing >50 colony-forming unit fibroblasts (CFU-Fs) were counted. CFU-F frequency was calculated by dividing the colony number by the number of seeded cells.

#### Quantification of secreted cytokines

ASCs were cultured with clinical protocol followed, as described above. After the last change of the medium, cells were cultured for 48 h and supernatants were collected and frozen at -80°C for enzyme-linked immunosorbent assays. Interleukin (IL)-6, IL-8,

vascular endothelial growth factor (VEGF) (Peprotech, Neuilly sur Seine, France), hepatocyte growth factor (HGF) and insulin-like growth factor (IGF) (Eurocell Diagnostics, Chartes de Bretagne, France) were assessed in these supernatants according to the manufacturer's instructions. Cytokine concentrations were expressed as ng/mL for  $1 \times 10^6$  cells in culture.

#### Calculation of population doubling

The population doubling was calculated as the ratio between the total number of cells harvested at the end of the culture by the number of CFU-F.

#### Release criteria

After culture, cell count and cell viability had to be >90%. The percentage of CD45<sup>+</sup> or CD14<sup>+</sup> cells and a percentage of CD90<sup>+</sup> or CD73<sup>+</sup> had to be lower and higher than 2% and 90%, respectively. Finally, no expression of hTert at the end of P0 could be detected. Because of the time required for performing the karyotype, the karyotype was performed but not included in release criteria. They were evaluated by G-banding cytogenetic according to INSR standards. Microbiological tests (aerobic and non-aerobic) were performed with the use of BacT Alert (BioMerieux, Craponne, France) (European Pharmacopeia Monograph 2.6.27) up to 12 d.

#### Cell transplantation

Two weeks after liposuction, 10<sup>8</sup> fresh cultured cells were implanted by means of intramuscular injection into the internal and external gastrocnemius and anterior compartment of the ischemic leg. Approximately 0.5 mL of cell suspension was used in

each injection site (15 sites for each muscle with the use of a standard grid), with 26-gauge needle. Total injection volume was approximately 23 mL.

#### *Assessment of outcomes*

The primary end points were feasibility and safety. Feasibility was defined as the ability of the expansion procedure to yield the target numbers of cells ( $10^8$ ) within 2–3 weeks. Safety referred to the lack of local or general adverse events related to biopsy sampling or cell transplantation. Local adverse effects were defined as inflammation or necrosis or thrombotic events at the sites of injection. To evaluate the safety, general adverse effects were clinically assessed and completed by measurements of C-reactive protein (CRP) and creatine kinase (CK), CK-myocardial band (MB) myoglobin until 30 and 90 d after transplantation, respectively, to estimate putative inflammatory syndrome and rhabdomyolysis. All serious adverse events were collected during follow-up and evaluated by an independent reviewing committee.

The secondary end point was efficacy, which was assessed by clinical and hemodynamic criteria of lower-limb ischemia. For clinical criteria, we evaluated pain and ulcer healing. Pain was self-assessed by the patient with the use of a visual analog scale (EVA) and the consumption of analgesic molecules.

Improved ulcer wound healing was assessed by means of standardized measurement of the largest diameter of the ulcer as performed by two independent technicians. Objective criteria of limb ischemia were assessed by the ankle or toe systolic pressure and by TcPO<sub>2</sub> and the ankle-brachial index (ABI) measurements (laser Doppler PeriFlux System 500, Perimed, France).

Safety and efficacy parameters were collected at day 1 and day 3 and at months 1, 3 and 6 after the injection procedure.

#### *Statistical analysis*

All values are expressed as mean  $\pm$  standard deviation. Comparisons of mRNA contents were assessed by means of the Mann-Whitney *U* test. TcPO<sub>2</sub> values at the end point of the trial were compared with values before cell transplantation by means of the Wilcoxon nonparametric paired test. A value of  $P < 0.05$  was considered statistically significant.

## **Results**

#### *Patient characteristics*

Sixty consecutive patients with CLI and non-suitable for revascularization were screened as recommended

by international guidelines (20), and 39 of them were rejected from enrollment in the study (Figure 1). Among the 21 patients enrolled, 10 did not undergo WAT sampling (six of the patients required urgent amputation, one presented with proliferative retinopathy, one displayed foot ischemia without arteriopathy of the leg and one did not have enough WAT). In addition, four patients were not transplanted with cultured ASC because two WAT samples displayed bacterial contamination and two required amputation quickly before liposuction. Finally, seven patients were enrolled and underwent the entire procedure. Patient characteristics and medical history are summarized in Table II. All patients were smokers and had undergone angioplasty or bypass surgery, and all of them except for one thromboangitis obliterans (TAO) patient displayed the most severe state of PAD: ankle TcPO<sub>2</sub>  $< 30$  mm Hg (normal value  $> 50$  mm Hg) and a Rutherford PAD ultimate grade of III-6.

It is noteworthy that among the patients, three were diabetic and one of them was morbidly obese (body mass index [BMI]  $> 40$ ). Patient 5, who did not have the minimal ankle TcPO<sub>2</sub>, had TAO disease characterized by non-atherosclerotic segmental inflammatory disease of small- and medium-sized arteries of the distal extremities (21). These features lead to a very difficult-to-perform percutaneous transluminal angioplasty and a bypass operation.

#### *Cell characteristics*

After liposuction, two fat samples were contaminated and the corresponding patients were withdrawn from this study, as mentioned above. However, the tissues were digested and the cells were processed similarly to the other batches. No additional contamination was detected after the culture process, suggesting that the aspirate sample was contaminated by intra-tissue abscess. Considering all ASC preparations, the mean viable cell yield ( $85.7\% \pm 3.2\%$  of viable cells in SVF) was  $445,600 \pm 100,600$  mono-nucleated cells per gram of WAT. The percentages for CD14-, CD34- and CD45-positive cells to detect macrophages, hematopoietic and endothelial/ASC cells, respectively, were  $6.8\% \pm 1.2\%$ ,  $27.3\% \pm 3.6\%$  and  $32.4\% \pm 5.6\%$ , respectively.

After isolation, cells were grown for 2 weeks before intra-muscle injection. During the time course of culture, as expected, the number of CD45-, CD14- and CD34-positive cells largely decreased to reach approximately 1% (Figure 2A), whereas 99% of the cultured ASC expressed mesenchymal stromal/stem cell markers ( $99.9\% \pm 0.4\%$  and  $99.4\% \pm 0.5\%$  of cultured cells positive for CD73 and CD90, respectively). During the culture process, the number of CFU-F did not

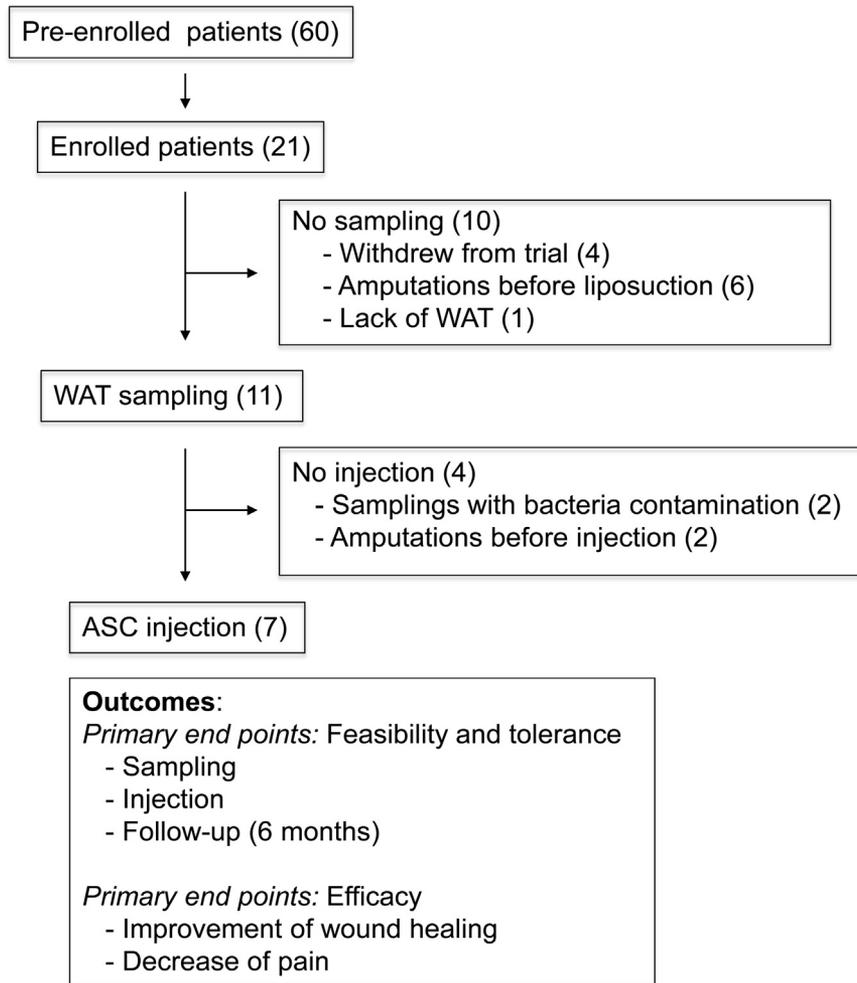


Figure 1. The number of patients at the different steps is indicated in brackets. Sixty patients were successively pre-enrolled. After further clinical examination, one third were definitively enrolled and finally seven were treated with ASC injections.

significantly increase (Figure 2B), whereas the number of population doublings significantly increased with the passage to reach  $4.7 \pm 0.1$  (Figure 2C). For all preparations,  $200$  to  $250 \times 10^6$  ASCs ( $230.0 \pm 9.8$ ) were harvested at the end of the process, except in patient 6, who displayed the highest BMI at 40.5 ( $14.7 \times 10^6$  ASCs were obtained for this patient). At the end of the culture process, we assessed Oct4, Nanog and Telomerase gene expression that were defined by French regulatory agency as safety controls to exclude any contaminating pluripotent or

cancer cells in the culture (22–24). As expected, the data clearly showed that the expressions of these pluripotency markers were significantly lower than their expressions in gold-standard pluripotency cells, for example, human embryonic stem cells (Figure 2D). The karyotype of each cell preparation was performed and showed no abnormality in any patient (data not shown). To complete the characterization of cells, we assessed the expression of several genes involved in redox metabolism, paracrine activity and matrix remodeling in ASCs from

Table II. Characteristics of patients enrolled in ACellDREAM and transplanted with autologous ASC.

Case	Age in years/sex	BMI	Risk factors	PAD origin	Previous surgical treatment for PAD	Rutherford grade
1	58/M	28.7	Smoking, hypertension	Atheromatous	Bypass (2)	III-6
2	81/M	28.3	Smoking, diabetes, hypertension	Atheromatous	Bypass (2)	III-6
3	46/M	29.3	Smoking, diabetes, hypertension	Atheromatous	Bypass, angioplasty, stent	III-6
4	60/M	19.5	Smoking	Atheromatous	Bypass (3) and thrombectomy	III-6
5	36/M	21.7	Smoking	TAO	None	III-6
6	50/F	40.5	Smoking, diabetes, hypertension	Atheromatous	Angioplasty	III-6
7	58/M	20	Smoking	Atheromatous	Bypass (4)	III-6

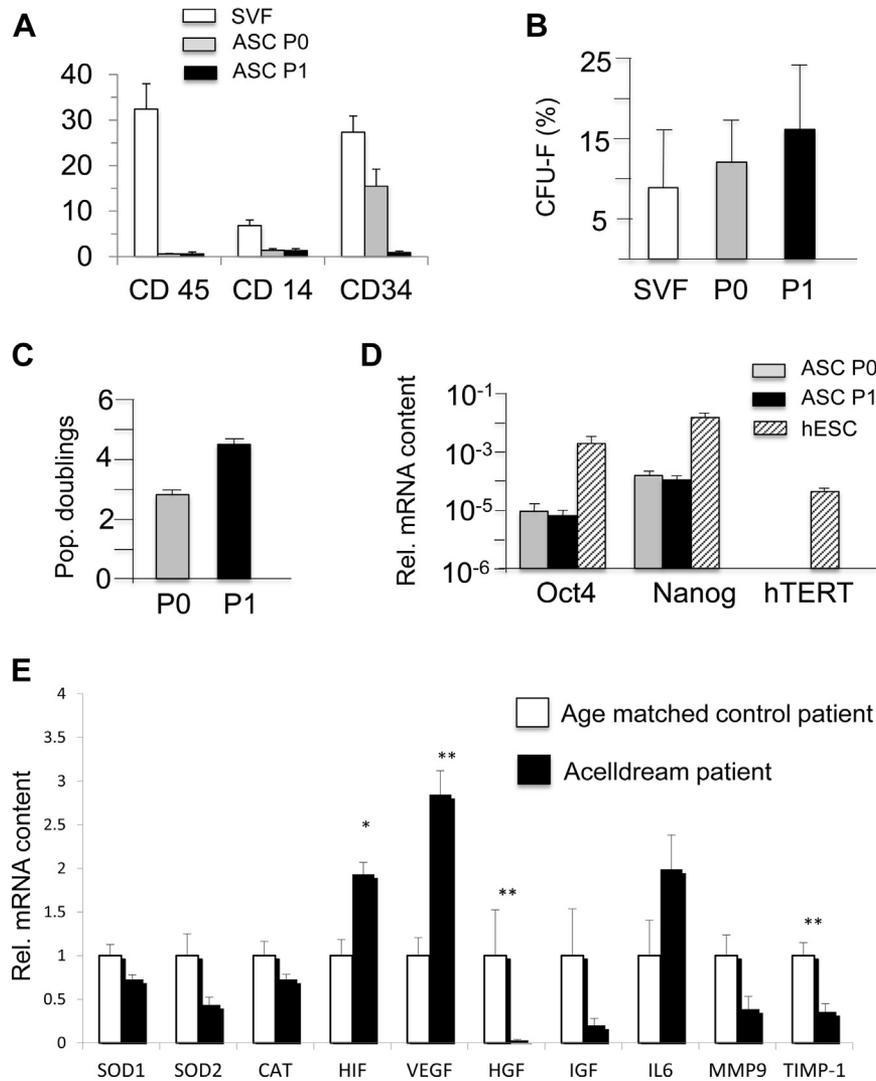


Figure 2. Characteristics of transplanted ASCs. (A) Antigenic profiles of cells were assessed by means of FACS analysis just after their isolation from liposuction (SVF) and after passages of adherent cells (P0 and P1). (B) Clonogenic potential assessed by CFU-F activity expressed in percentage of plating cells (%) and (C) population doubling were estimated for AC at P0 and P1. (D) The expressions of pluripotency genes (*Oct4*, *Nanog* and *hTERT*) used as safety control and genes involved in redox metabolism and paracrine activity (E) were estimated by means of qRT-PCR in ASCs after P0 and P1 compared with human embryonic stem cell line HD129. Data are expressed as  $2^{-dCt}$  with actin gene expression used as control ( $dCt = CT_{\text{gene}} - \text{minus } CT_{\text{actin}}$ ). All values corresponds to mean  $\pm$  standard error of the mean of values for all patients; \* $P < 0.05$ , \*\* $P < 0.01$ .

non revascularizable CLI patients compared with the control age-matched patients (Figure 2E). Control patients were  $59 \pm 2.8$  years old and had a BMI of  $25 \pm 0.8$ . Both values were not statistically significant from those of ACellDREAM patients. The profile showed large changes, although only some of them were statistically significant. HIF-1 $\alpha$  and VEGF mRNA contents were significantly upregulated, whereas the mRNA contents of mRNA coding for HGF, matrix metalloproteinase (MMP)-9 and TIMP-1 were significantly downregulated.

We then quantified for five patients the secretion of IL-6, IL-8, HGF, VEGF and IGF in the supernatants before (P0) and at the end of the first

passage (P1) (Table III). The contents of all the cytokines and growth factors were easily detectable and were the highest at the end of P0. After passage, the secretions were strongly downregulated except for HGF, for which significant levels were always and easily detected. It is noteworthy that the amounts for all secreted proteins at P0 were the lowest for patient 4.

#### Injection and follow-up

Subcutaneous abdominal WAT liposuction under local anesthesia was well tolerated in all patients, with no side effects. ASC transplantations were well

Table III. Paracrine activity of ACellDREAM ASC (mean  $\pm$  standard error of the mean).

Patient	IL-6		IL-8		VEGF		HGF		IGF-I	
	P0	P1	P0	P1	P0	P1	P0	P1	P0	P1
1	8.08 $\pm$ 0.02	0.032 $\pm$ 0.001	3.761 $\pm$ 0.086	0.004 $\pm$ 0.02	4.630 $\pm$ 0.01	0.003 $\pm$ 0.001	4.635 $\pm$ 0.03	0.169 $\pm$ 0.03	4.752 $\pm$ 0.27	0.025 $\pm$ 0.03
2	0.119 $\pm$ 0.023	0.025 $\pm$ 0.02	0.003 $\pm$ 0.005	0.001 $\pm$ 0.02	0.037 $\pm$ 0.001	0.007 $\pm$ 0.001	1.000 $\pm$ 0.006	0.77 $\pm$ 0.004	0.172 $\pm$ 0.17	0.007 $\pm$ 0.01
4	0.109 $\pm$ 0.014	0.011 $\pm$ 0.001	0.004 $\pm$ 0.001	0.001 $\pm$ 0.02	0.036 $\pm$ 0.003	0.006 $\pm$ 0.001	0.398 $\pm$ 0.003	0.33 $\pm$ 0.002	0.019 $\pm$ 0.02	0.002 $\pm$ 0.001
6	0.022 $\pm$ 0.003	0.009 $\pm$ 0.001	0.004 $\pm$ 0.001	0.001 $\pm$ 0.02	0.010 $\pm$ 0.001	0.006 $\pm$ 0.001	1.093 $\pm$ 0.21	0.135 $\pm$ 0.03	0.048 $\pm$ 0.05	0.005 $\pm$ 0.001
7	ND	0.042 $\pm$ 0.001	ND	0.000	ND	0.029 $\pm$ 0.006	ND	0.251 $\pm$ 0.03	ND	0.012 $\pm$ 0.001

IL-6, IL-8, VEGF, HGF and IGF cytokines and growth factor contents of ASC supernatants were assessed by means of enzyme-linked immunosorbent assay, as described in the Methods section, and expressed in ng/mL/10<sup>6</sup> cells. The media conditioned for 48 h were sampled just before (P0) and at the end of the first passage (P1) at the time of cell transplantation.

tolerated except in one patient who had pain during the procedure, requiring the use of a mixture of oxygen and nitrous oxide. ASC transplantation induced no local inflammatory reactions or complications such as muscle edema, bleeding and rejection, allergic reactions, infection or signs of tumor development. No increases in laboratory markers of inflammatory syndrome and rhabdomyolysis (CRP, CK, CK-MB and myoglobin) were observed (data not shown) except in patient 2, who displayed a slight increase in the biochemical parameters of inflammation before the procedure. In this patient, CRP and white blood cells were elevated before the procedure, suggesting the rise of an infection, and indeed continued to increase after the procedure. He received amputation at day 30 because of a foot infection, as indicated by the circulating inflammatory indexes.

At 24 weeks after transplantation, rest pain in the ASC-treated limbs was slightly improved, as measured by EVA scale (Table IV), even if the consumption of analgesic molecules did not change significantly (data not shown). As expected from the natural history of critical limb ischemia, three patients underwent major amputation at 30, 90 and 150 d after ASC injection (patients 2, 7 and 4, respectively), but these amputations were not considered adverse events as expected for patients at the most severe stage of the disease, as judged by the independent review committee of the participating university medical center. Except for patient 2, because of his rapid amputation, ankle systolic TcPO<sub>2</sub> and ABI were recorded to assess limb ischemia (Figure 3 and Table IV). For all non-TAO patients except for patient 4, the difference of ankle TcPO<sub>2</sub> values between ASC transplantation and their respective end points showed a significant increase (from +8 for patient 1 up to 12 for patient 7;  $P = 0.046$ ), whereas ABI tended to decrease. For TAO patients, the increase in TcPO<sub>2</sub> was the highest (+24), and it was the single one for which ABI was improved (from 0.61–0.81).

Patient 1 displayed striking results, with no ulcers at 6 months and a large decrease in CLI severity (Rutherford grade: from III-6 to II). For non-amputated patients, the Rutherford grade was also improved but to a lesser extent.

Interestingly, and except for patient 2, who was given amputation at day 30, the number of lesions decreased (Table IV). For other patients, and except for patient 4, who was given amputation at day 150, the whole ulcer surface measurement was largely decreased (Figure 4). When the initial wound surface was <250 mm<sup>2</sup> (Figures 4A and 5), the decrease occurred within the first half of follow-up (90 d). In patient 6, with a large overall surface, the improvement occurred more progressively (Figure 4B).

Table IV. Main results for all enrolled patients after the day of ASC transplantation (EVA).

Case	Rutherford grade		Rest pain (EVA)		TcPO <sub>2</sub> (mm Hg)		ABI		No. of ulcers		
	Day 0	Day 180	Day 0	Day 180	Day 0	Changes (end point, day 0)	Day 0	Day 180	Day 0	End point	Amputation
1	III-6	II	6	0	26	+8	0.46	0.36	3	0	None
2	III-6	NA	6	NA	0	NA	0.3	NA	2	NA	Major, day 30
3	III-6	III-5	8	3	15	+9	0.65	0.56	4	2	None
4	III-6	NA	4	NA	8	-5	0.46	NA	6	4	Major, day 150
5	III-6	III-5	7	4	4	+24	0.61	0.81	1	1	None
6	III-6	III-5	0	0	30	+10	0.31	0.25	2	1	None
7	III-6	NA	7	NA	5	+12	0.37	0.18	1	0	Major, day 90

Values are indicated at day 0 (day of ASC transplantation) and end point corresponding either to the day of amputation or 180 d after ASC transplantation. Rest pain and TcPO<sub>2</sub> were assessed by analogic scale and laser Doppler analysis, respectively.

No adverse event associated with ASC treatment was reported, as judged by the independent review committee for this trial in the participating university medical centers.

**Discussion**

ACellDREAM is the first clinical trial to use expanded ASCs to treat patients with CLI objectively proven by hemodynamic and oxygen perfusion parameters and not suitable for revascularization. As previously mentioned, cell-based therapy appears to be a promising alternative for PAD. The administration of the first source of cells, for example, BMMNC that have been tested in this context, has been shown to improve the clinical parameters of patients with CLI (3). In a 2-year follow-up, a significant improvement in leg pain, ulcer size and pain-free walking distance were also described, whereas ABI and TcPO<sub>2</sub> were unaffected (25). From this seminal trial, several trials have been conducted with different bone marrow-derived cells. In a very recent meta-analysis including all types of bone

marrow-derived cell populations, Teraa *et al.* (26) concluded that there is a true and significant benefit of bone marrow-derived cell therapy, although the benefit on major amputation, which is the key criteria, is not significant when only placebo-controlled trials are considered. A single study reported the comparison between the BM-MNCs that form a rather heterogeneous population containing a large and uncontrolled proportion of hematopoietic cells and the BM-MSC that corresponds to expanded and selected mesenchymal stromal cells (4). In this study, Lu *et al.* (4) reported that MSCs are more potent than are BM-MNC. This advantage must be associated with the possibility, because of the time of culture, of efficiently controlling the cell product before its release. Unfortunately, the benefits of the uses of this last type of cells are counter-balanced by the time required for their expansion. In this context, adipose-derived stromal

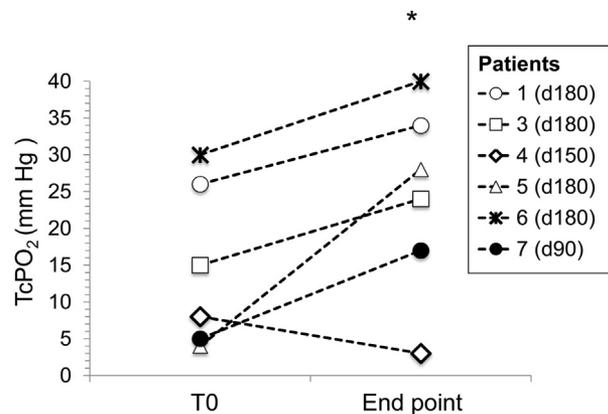


Figure 3. Individual TcPO<sub>2</sub> evolution after ASC transplantation. The TcPO<sub>2</sub> was assessed by means of laser Doppler measurements. Number in brackets corresponds to the time (in days, from the day of ASC transplantation) of the measure (\*P < 0.05).

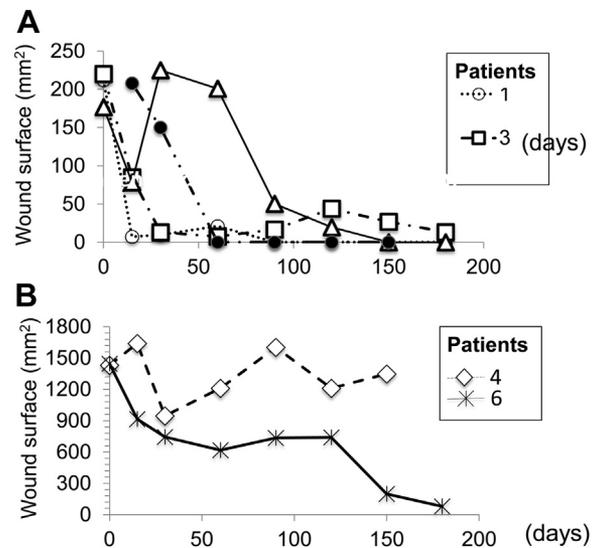


Figure 4. Evolution of wound healing after ASC transplantation. For the different patients, the effects on wound healing were assessed by the evolution of ulcer areas (mm<sup>2</sup>) measured by two independent technicians as described in the Methods section and after ASC transplantation (day 0). The triangle and the black dot correspond to patients 5 and 7, respectively.



Figure 5. Images of wound ulcers. Three examples of ulcer wound improvement from the day of ASC injection (left panel, day 0) to the end point of the follow-up (right panels, day 180).

cells represent a promising alternative for two main reasons. First, they display a greater angiogenic potential compared with that of MSCs (14). These effects are mediated in part by their differentiation toward endothelial-like cells and by their paracrine activities. Second, a greater number of mesenchymal cells can be obtained in a shorter time from adipose tissue compared with bone marrow because the frequency of ASCs per volume unit of tissue is higher than those of BM-MSCs. It is the case in this study in which, in only 2 weeks,  $>10^8$  ASCs from 30 mL of initial liposuction are obtained for all patients, including one obese patient and two diabetic patients. This also indicates that a slight improvement of the culture process and/or a slight increase in the volume of liposuctions could additionally reduce the delay before injections. The cell culture procedure also induces a strong homogenization of the cell population as revealed by the antigenic

profile and the expression of classic markers of MSCs (ie, CD73 and CD90). The decreases in the number of CD45-positive hematopoietic cells and CD14-positive macrophage cells confirm the lack of contamination by cells from hematopoietic lineages and the efficiency of selection by adhesion. The decrease in the percentage of CD34-positive cells is expected and previously attributed to ASC proliferation (27). This is consistent with the profile defined in a statement paper by Bourin *et al.* (28). Notably, the number of cell doublings is low and is associated with the absence of hTert expression and pluripotency/tumorigenic markers, as expected (22–24,29). No karyotype abnormality is reported, confirming the safety of our procedure. It is also noteworthy that we carefully avoided the use of any animal products or purified growth factors during cell processing. The gene expression analysis shows that unlike the culture process, changes can be observed.

The strong induction of *VEGF* gene expression associated with a concomitant increase in *HIF-1 $\alpha$*  gene expression appears to be consistent with the ischemic situation for the patient, although the site of liposuction is far from the limb and no clear change is observed for the genes involved in redox metabolism. Such a profile would be prone to induction of angiogenesis (30), but this gene expression profile is not necessarily correlated with a high secretion of VEGF *in vitro*. The significant decrease in HGF, MMP-9 and TIMP-1 mRNA contents clearly suggests that ASCs derived from CLI patients could be defective in paracrine and remodeling tissue activity (31) independent of an aging effect. This is consistent with the very low level of secreted cytokines and growth factors at the time of cell transplantation. Among the secreted proteins, HGF secretion is relatively maintained after passage and could participate to the effects (9). It is noteworthy that ASCs from patient 4 display the lowest paracrine activity at the end of passage 0 associated with no improvement in TcPO<sub>2</sub> and wound healing. In contrast, paracrine activities of ASCs from patients 1 and 7 are the highest but are not associated with better improvement. This clearly demonstrates that more experiments with more patients are needed to define a control of efficacy.

Several trials with ASCs are ongoing, but few of these are already published. The most advanced clinical study concerns a phase III trial for the treatment of patients with Crohn's disease and fistula (32). A very recent report also tested autologous ASC therapy in the treatment of patients with TAO (32). In this study, the definition of CLI was not supported by objective evaluation of hemodynamic parameters. Furthermore, clinical presentation of CLI was less severe than that in our study. Concerning the culture process, they used fetal bovine serum. This study also reveals a decrease in pain-free walking time and revealed an improvement in tissue perfusion, although there is no change in the CLI grade at 6 months after ASC transplantation (33). One clear weakness of our current report is the small number of patients, which is explained by our choice of strict and documented enrollment/exclusion criteria. Nevertheless, the 6-month amputation rate was 42.8%, which is comparable to the 40% published in the literature by the Inter-Society Consensus (TASC) II guidelines (34). In the RESTORE-CLI trial, major amputations occurred in 19% of expanded BM-derived cell-treated patients only, compared with 43% in saline-treated control subjects (4). Although comparisons among trials are very difficult to make, and only blinded trials with rigorous, simultaneous placebo controls can be used to evaluate efficacy, a careful examination of the data reveals that the improvement occurs mostly in patients who already have an amputation. In patients who

underwent their first major amputation during the trial, as with patients enrolled in our trial, the rate of major amputations is very low and the difference between both groups is not significant (4).

On the basis of the low number of patients and the design of the present trial, the efficiency of ASC therapy cannot be objectively evaluated, and, for these reasons, our investigations were limited to the key clinical, robust and routine parameters. Instead of these limitations and except for ABI, our results appear to be consistent with those of the RESTORE-CLI trial, in which all measured parameters (ulcer healing rate, painless walking time, ABI and TcPO<sub>2</sub>) were improved in cell-treated patients (Figures 3 and 4). Additionally, in our study, all CLI grades improved and TcPO<sub>2</sub> tended to significantly increase, even if most of them did not increase >30 mm Hg, which is the critical threshold for the definition of CLI. Furthermore, the number of ulcers decreased and their healing improved. Similar results are also observed in ASC-treated TAO patients (33). It is noteworthy that in our trial, the best results on ischemia were obtained in TAO patients. Regardless of these considerations, it appears that our protocol is safe for treating non-revascularizable CLI patients. This suggests that ASC transplantation could represent an alternative for no-option CLI, and, on the basis of these data, its efficacy now must be statistically tested in a larger phase II trial. The difficulties in enrolling patients were related to the severe set of inclusion/exclusion criteria, particularly to the criteria "non-suitable for revascularization," because revascularization surgery is always possible even when its chances of success are negligible. On the basis of the rapid evolution of the disease, such a decision induces a deleterious delay in enrollment. The design of the trial must be refined for further investigations.

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## References

- Benoit E, O’Donnell TF Jr, Kitsios GD, Iafrazi MD. Improved amputation-free survival in unreconstructable critical limb ischemia and its implications for clinical trial design and quality measurement. *J Vasc Surg.* 2012;55:781–9.
- Powell RJ. Update on clinical trials evaluating the effect of biologic therapy in patients with critical limb ischemia. *J Vasc Surg.* 2012;56:264–6.
- Tateishi-Yuyama E, Matsubara H, Murohara T, Ikeda U, Shintani S, Masaki H, et al. Therapeutic angiogenesis for patients with limb ischaemia by autologous transplantation of bone-marrow cells: a pilot study and a randomised controlled trial. *Lancet.* 2002;360:427–35.
- Lu D, Chen B, Liang Z, Deng W, Jiang Y, Li S, et al. Comparison of bone marrow mesenchymal stem cells with bone marrow-derived mononuclear cells for treatment of diabetic critical limb ischemia and foot ulcer: a double-blind, randomized, controlled trial. *Diabetes Res Clin Pract.* 2011;92:26–36.
- Losordo DW, Dimmeler S. Therapeutic angiogenesis and vasculogenesis for ischemic disease, part ii: cell-based therapies. *Circulation.* 2004;109:2692–7.
- De Haro J, Acin F, Lopez-Quintana A, Florez A, Martinez-Aguilar E, Varela C. Meta-analysis of randomized, controlled clinical trials in angiogenesis: gene and cell therapy in peripheral arterial disease. *Heart Vessels.* 2009;24:321–8.
- Gimble JM, Katz AJ, Bunnell BA. Adipose-derived stem cells for regenerative medicine. *Circ Res.* 2007;100:1249–60.
- Planat-Benard V, Silvestre JS, Cousin B, Andre M, Nibbelink M, Tamarat R, et al. Plasticity of human adipose lineage cells toward endothelial cells: physiological and therapeutic perspectives. *Circulation.* 2004;109:656–63.
- Rehman J, Traktuev D, Li J, Merfeld-Clauss S, Temm-Grove CJ, Bovenkerk JE, et al. Secretion of angiogenic and antiapoptotic factors by human adipose stromal cells. *Circulation.* 2004;109:1292–8.
- Kondo K, Shintani S, Shibata R, Murakami H, Murakami R, Imaizumi M, et al. Implantation of adipose-derived regenerative cells enhances ischemia-induced angiogenesis. *Arterioscler Thromb Vasc Biol.* 2009;29:61–6.
- Moon MH, Kim SY, Kim YJ, Kim SJ, Lee JB, Bae YC, et al. Human adipose tissue-derived mesenchymal stem cells improve postnatal neovascularization in a mouse model of hindlimb ischemia. *Cell Physiol Biochem.* 2006;17:279–90.
- Nakagami H, Maeda K, Morishita R, Iguchi S, Nishikawa T, Takami Y, et al. Novel autologous cell therapy in ischemic limb disease through growth factor secretion by cultured adipose tissue-derived stromal cells. *Arterioscler Thromb Vasc Biol.* 2005;25:2542–7.
- Cao Y, Sun Z, Liao L, Meng Y, Han Q, Zhao RC. Human adipose tissue-derived stem cells differentiate into endothelial cells in vitro and improve postnatal neovascularization in vivo. *Biochem Biophys Res Commun.* 2005;332:370–9.
- Casteilla L, Planat-Benard V, Cousin B, Laharrague P, Bourin P. Vascular and endothelial regeneration. *Curr Stem Cell Res Ther.* 2010;5:141–4.
- Kim Y, Kim H, Cho H, Bae Y, Suh K, Jung J. Direct comparison of human mesenchymal stem cells derived from adipose tissues and bone marrow in mediating neovascularization in response to vascular ischemia. *Cell Physiol Biochem.* 2007;20:867–76.
- Ikegame Y, Yamashita K, Hayashi S, Mizuno H, Tawada M, You F, et al. Comparison of mesenchymal stem cells from adipose tissue and bone marrow for ischemic stroke therapy. *Cytotherapy.* 2011;13:675–85.
- Nakanishi C, Nagaya N, Ohnishi S, Yamahara K, Takabatake S, Konno T, et al. Gene and protein expression analysis of mesenchymal stem cells derived from rat adipose tissue and bone marrow. *Circ J.* 2011;75:2260–8.
- Sensebe L, Tarte K, Galipeau J, Krampera M, Martin I, Phinney DG, et al. Limited acquisition of chromosomal aberrations in human adult mesenchymal stromal cells. *Cell Stem Cell.* 2012;10:9–10.
- Toupet K, Maumus M, Peyrafitte JA, Bourin P, van Lent P, Ferreira R, et al. Long-term detection of human adipose derived mesenchymal stem cells after intra-articular injection. *Arthritis Rheum.* 2013;65:1786–94.
- Dormandy JA, Rutherford RB. Management of peripheral arterial disease (pad): TASC working group: Transatlantic inter-society consensus (TASC). *J Vasc Surg.* 2000;31:S1–296.
- Dargon PT, Landry GJ. Buerger’s disease. *Ann Vasc Surg.* 2012;26:871–80.
- Hiyama E, Hiyama K. Clinical utility of telomerase in cancer. *Oncogene.* 2002;21:643–9.
- Li C, Yan Y, Ji W, Bao L, Qian H, Chen L, et al. Oct4 positively regulates survivin expression to promote cancer cell proliferation and leads to poor prognosis in esophageal squamous cell carcinoma. *PLoS One.* 2012;7:e49693.
- Dai X, Ge J, Wang X, Qian X, Zhang C, Li X. Oct4 regulates epithelial-mesenchymal transition and its knockdown inhibits colorectal cancer cell migration and invasion. *Oncol Rep.* 2013;29:155–60.
- Matoba S, Tatsumi T, Murohara T, Imaizumi T, Katsuda Y, Ito M, et al. Long-term clinical outcome after intramuscular implantation of bone marrow mononuclear cells (therapeutic angiogenesis by cell transplantation [tact] trial) in patients with chronic limb ischemia. *Am Heart J.* 2008;156:1010–8.
- Teraa M, Sprengers RW, van der Graaf Y, Peters CE, Moll FL, Verhaar MC. Autologous bone marrow-derived cell therapy in patients with critical limb ischemia: a meta-analysis of randomized controlled clinical trials. *Ann Surg.* 2013;258:922–9.

27. Maumus M, Peyrafitte JA, D'Angelo R, Fournier-Wirth C, Bouloumie A, Casteilla L, et al. Native human adipose stromal cells: localization, morphology and phenotype. *Int J Obes (Lond)*. 2011;35:1141–53.
28. Bourin P, Bunnell BA, Casteilla L, Dominici M, Katz A, March K, et al. Stromal cells from adipose tissue-derived stromal vascular fraction and culture expanded adipose tissue-derived stromal/stem cells: a joint statement of the International Federation for Adipose Therapeutics and Science (IFATS) and the International Society for Cellular Therapy (ISCT). *Cytotherapy*. 2013;15:641–8.
29. Glinsky GV. “Stemness” genomics law governs clinical behavior of human cancer: implications for decision making in disease management. *J Clin Oncol*. 2008;26:2846–53.
30. Sadat S, Gehmert S, Song YH, Yen Y, Bai X, Gaiser S, et al. The cardioprotective effect of mesenchymal stem cells is mediated by IGF-I and VEGF. *Biochem Biophys Res Commun*. 2007;363:674–9.
31. Cai L, Johnstone BH, Cook TG, Liang Z, Traktuev D, Cornetta K, et al. Suppression of hepatocyte growth factor production impairs the ability of adipose-derived stem cells to promote ischemic tissue revascularization. *Stem Cells*. 2007;25:3234–43.
32. Herreros MD, Garcia-Arranz M, Guadalajara H, De-La-Quintana P, Garcia-Olmo D. Autologous expanded adipose-derived stem cells for the treatment of complex cryptoglandular perianal fistulas: a phase III randomized clinical trial (FATT 1: Fistula Advanced Therapy Trial 1) and long-term evaluation. *Dis Colon Rectum*. 2012;55:762–72.
33. Lee HC, An SG, Lee HW, Park JS, Cha KS, Hong TJ, et al. Safety and effect of adipose tissue-derived stem cell implantation in patients with critical limb ischemia. *Circ J*. 2012;76:1750–60.
34. Norgren L, Hiatt WR, Dormandy JA, Nehler MR, Harris KA, Fowkes FG, et al. Inter-society consensus for the management of peripheral arterial disease (TASC II). *Eur J Vasc Endovasc Surg*. 2007;33(Suppl 1):S1–75.