Published in final edited form as:

Cytotherapy. 2014 December ; 16(12): 1720–1732. doi:10.1016/j.jcyt.2014.07.011.

Bilateral Administration of Autologous CD133+ Cells in Ambulatory Patients with Refractory Critical Limb Ischemia: Lessons Learned from a Pilot Randomized, Double blind, Placebo-controlled Trial

Amish N. Raval, MD¹, Eric Schmuck, PhD¹, Girma Tefera, MD², Cathlyn Leitzke, RN¹, Cassandra Vander Ark, RN¹, Derek Hei, PhD³, John M. Centanni, MS¹, Ranil de Silva, FRCP PhD⁴, Jill Koch, PhD¹, Richard Chappell, PhD⁵, and Peiman Hematti, MD⁶

¹Division of Cardiovascular Medicine, Department of Medicine, University of Wisconsin School of Medicine and Public Health, Madison, WI, USA

²Division of Vascular Surgery, Department of Surgery, University of Wisconsin School of Medicine and Public Health, Madison, WI, USA

³Waisman Biomanufacturing Facility, Madison, WI, USA

⁴National Heart and Lung Institute, Imperial College London and NIHR Cardiovascular Biomedical Research Unit, Royal Brompton and Harefield NHS Foundation Trust, United Kingdom

⁵Department of Biostatistics and Medical Informatics, University of Wisconsin School of Medicine and Public Health, Madison, WI, USA

⁶Division of Hematology/Oncology, Department of Medicine, University of Wisconsin School of Medicine and Public Health, Madison, WI, USA

Abstract

Introduction—CD133+ cells confer angiogenic potential and may be beneficial for the treatment of critical limb ischemia (CLI). However, patient selection, blinding methods and endpoints for

© 2014 International Society for Cellular Therapy. Elsevier Inc. All rights reserved.

Correspondence to: Amish N. Raval MD, FACC, FSCAI Associate Professor of Medicine Division of Cardiovascular Medicine University of Wisconsin School of Medicine and Public Health H4/568 Clinical Sciences Center 600 Highland Ave, Madison, WI 53792-3248, USA Office:(608) 263-6146 Fax:(608) 263-0405 anr@medicine.wisc.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Disclosure of Interest

Project support was provided in part by:

- i. National Heart, Lung, and Blood Institute, National Institutes of Health, Department of Health and Human Services under contract numbers HHSN268201000010C Production Assistance for Cellular Therapies (PACT). Partial financial support was provided for leukapheresis and CD133+ cell selection.
- ii. National Institutes of Health grant UL1TR000427 to the University of Wisconsin Institute of Clinical Translational Research from National Center for Advancing Translational Sciences (NCATS). Partial project support was provided in the form of data management system and statistics support.
- iii. Miltenyi Biotec Inc. provided CD133+ reagent kits and partial financial support for the trial.

clinical trials is challenging. We hypothesized that bilateral intramuscular administration of cytokine mobilized CD133+ cells in ambulatory patients with refractory CLI would be feasible and safe.

Methods—In this double-blind, randomized, sham-controlled trial, subjects received subcutaneous injections of granulocyte colony stimulating factor (10 mcg/kg/d) for 5 days, followed by leukapheresis, and intramuscular administration of 50-400 million sorted CD133+ cells delivered into both legs. Control subjects received normal saline injections, sham leukapheresis and intramuscular injection of placebo buffered solution. Subjects were followed for 1 year. An aliquot of CD133+ cells was collected from each subject to test for genes associated with cell senescence.

Results—70 subjects were screened, of whom 10 were eligible. Subject enrollment was suspended due to a high rate of mobilization failure in subjects randomized to treatment. Of 10 subjects enrolled (7 randomized to treatment, 3 randomized to control), there were no differences in serious adverse events at 12 months and blinding was preserved. There were non-significant trends toward improved amputation free survival, 6 minute walk distance, walking impairment questionnaire and quality of life in subjects randomized to treatment. Successful CD133+ mobilizers expressed fewer senescence associated genes compared to poor mobilizers.

Conclusion—Bilateral administration of autologous CD133+ cell in ambulatory CLI subjects was safe and blinding was preserved. However, poor mobilization efficiency combined with high CD133+ senescence suggests futility in this approach.

Keywords

Stem cell; Critical limb ischemia; Peripheral artery disease; Angiogenesis

Introduction

Critical limb ischemia (CLI) is defined by ischemic rest pain, ulcers and/or gangrene and is usually caused by atherosclerosis in lower extremity arteries. CLI is associated with high mortality, amputation, permanent disability and increased healthcare costs(1, 2). Surgical and/or catheter-based revascularization can be performed to preserve the limb and restore ambulatory function. However, approximately 25-40% of patients are not candidates for revascularization due to poor distal targets, lack of suitable autogenous conduits, significant co-morbidities or failure of prior revascularization attempts(3). Over time, increased emphasis on cardiovascular risk reduction through lifestyle modification and pharmacotherapy has improved outcomes in patients with CLI. However, there still remains a significant unmet need for sustained and effective treatments(4, 5). For example, in a recent therapeutic gene transfer trial involving patients with refractory CLI, placebo-treated subjects suffered a one year combined mortality and major amputation rate of 33%(6).

Cell-based therapy may offer a novel approach to induce neovascularization, improve lower extremity perfusion and prevent tissue loss in patients with refractory CLI. Cells that express the surface glycoprotein CD133+ have been characterized as immature bone marrow derived progenitor cells with high proliferative, vasculogenic and regenerative capacity *in vitro* and *in vivo*(7-10). These findings suggest that local administration of CD133+ cells may induce

vasculogenesis, improve limb perfusion, prevent tissue loss and restore ambulatory function in patients with refractory CLI.

Several small, randomized trials have demonstrated that local administration of selected and unselected autologous cells of bone marrow origin for the treatment of CLI is safe, though the reported benefits were variable(11-25). A meta-analysis of twelve, randomized, autologous bone marrow derived cell therapy for CLI trials suggested beneficial effects of cell treatment when compared to placebo (n=5 trials) or standard of care (n=7 trials)(26). However, when the placebo-controlled trials were analyzed separately, limb salvage rates were not improved.

Study design is of critical importance for CLI cell therapy trials. For example, amputation is widely considered to be a major hard clinical endpoint. However, in clinical practice, a combination of medical and social factors influences the decision to proceed with amputation. Typically, this is a shared decision made by both the patient and their surgeon. Therefore, *double-blind* treatment allocation is necessary for CLI cell therapy trials to minimize treatment suspicion bias and post-randomization drop-outs. However, blinding methods can become complex when multi-step autologous cell mobilization and/or harvest procedures are required. For example, Losordo et al. tested intramuscular delivery of autologous CD34+ progenitor cells obtained from granulocyte colony stimulating factor (G-CSF) mobilized leukapheresis products in subjects with refractory CLI(24). G-CSF is associated with side-effects such as malaise, myalgias, and bone and therefore, these investigators chose an approach where both the active treatment and the control group received G-CSF to preserve blinding. However, a major limitation of this approach was that the active therapy group was not compared against standard of care.

Regulatory agencies and investigators to date have focused on time to amputation as the major defining endpoint in CLI trials cell therapy trials. However, amputation as an endpoint may not be able to detect moderate improvements in limb perfusion, especially in trials designed to administer only single dose cell therapy. Alternatively, walking distance is a clinically relevant, reproducible, continuous functional parameter that is measurable with or without an artificial limb prosthesis(27). Furthermore, most trials have tested cell administration into a single pre-defined “index” limb. However, patients with unilateral CLI typically also have significant contra-lateral peripheral artery disease(4). Revascularization of one limb commonly reveals functionally limiting symptoms in the contra-lateral limb, which may lead to an overall negative effect limitation in walking. Therefore, bilateral limb administration of cells may be required to demonstrate improvement in walking distance. Finally, the optimal cell dose continues to remain poorly defined for CLI trials, despite over a decade of experience. The usual approach in CLI cell therapy trials is to administer pre-specified fixed cell doses. This can be a problem for minimally manipulated autologous cell therapy trials where the chosen dose must be sufficiently low to be applied to the majority of enrolled subjects given the variability in the harvested yield. With this approach, partitioning of the harvested product is required and the non-administered cells are discarded. Furthermore, this approach does not account for the inter-subject variability in the potency of mobilized cells. An alternative and potentially simpler approach is to administer the entire

harvested cell product into the patient as an overall treatment strategy; however, little is known about the feasibility and safety of this approach.

To address these issues, we designed a randomized, double-blind, placebo/ sham controlled pilot trial to test the feasibility and safety of a treatment strategy that would require bilateral injection of total mobilized dose of autologous CD133+ cells into both lower extremities of *ambulatory* subjects with refractory CLI.

Methods

Trial Design

The Stem Cell Revascularization for Patients with Critical Limb Ischemia (SCRIPT-CLI) trial was an investigator-initiated, randomized-controlled, double blind, clinical trial conducted at two academic medical centers in the United States. U.S. FDA Investigational New Drug (IND) and local institutional review board approvals were obtained to perform the trial. An independent data safety and monitoring board (DSMB) monitored the trial conduct, recruitment and adverse events. Subjects were screened and randomized 2:1 to receive autologous CD133+ cells obtained from peripheral blood or control.

Major Inclusion and Exclusion Criteria

Subjects were eligible to participate if aged 18 years or over and had CLI defined as: i) atherosclerosis-induced ischemic rest pain with or without slow or non-healing ulcer in at least 1 limb; ii) stable symptoms for at least 30 days; iii) unsuitable for surgical/catheter based revascularization based on anatomical considerations or significant co-morbidities; and iv) at least one level obstructive peripheral artery disease in the contra-lateral limb. Eligible patients were also required to fulfill any of the following vascular function inclusion criteria: ankle-brachial Index (ABI) <0.6 or absolute ankle pressure of <60mmHg or toe pressure <40mmHg or pulse volume recording (PVR) that was flat or barely pulsatile in both limbs. Angiographic documentation of obstructive atherosclerosis of at least 1 major artery in both lower extremities within one year of screening was also required. Subjects needed to have been established on guideline recommended medical therapy to ensure optimized control of blood pressure, cholesterol, glycemic status as well as at least one antiplatelet agent. Subjects with non-atherosclerotic disorders such as vasculitis, history of chronic immunosuppression, active malignancy, prior organ transplant, and those with glomerular filtration rate <20 mL/min were excluded.

Study Treatment

Subjects randomized to active treatment received granulocyte colony stimulating factor (G-CSF, Neupogen, Amgen, Thousand Oaks, CA) 10 µg/kg subcutaneously daily for 5 days, followed by leukapheresis (COBE Spectra Leukapheresis System, Terumo, BCT, Lakewood, CO) on the final day. The resulting leukapheresis product was stored overnight at 4 degrees Celsius. The following day, the leukapheresis product underwent closed-system selection of CD133+ cells using the CliniMACS CD133 reagent system (Miltenyi Biotec Inc, Auburn, CA). Selected CD133+ cells underwent viability, purity and sterility testing. The entire enriched CD133+ cell product was suspended in phosphate buffered saline(PBS)/

ethylene-diamine-tetraacetic acid(EDTA)/0.5% Human Serum Albumin (HSA), brought to a final volume of 10mL and distributed into injection syringes for intramuscular administration.

Purity and viability studies were also performed on mobilized leukapheresis products obtained from healthy normal volunteers (AllCells, Alameda, CA). The dose of G-CSF for normal volunteers was 5 mcg/kg subcutaneous for 4 days. Specific release criteria for the trial were: CD133+ purity 70% CD133+ based on flow cytometry, non-CD133+ cell impurities <30%, viability of CD133+ 70% based on 7-AminoActinomycin D (7AAD) assay, hematocrit 5% as measured with an automated hematology analyzer and Gram stain negative for bacteria. Subjects randomized to the control group received a sham mobilization procedure with 0.9% normal saline (instead of G-CSF) subcutaneous injections for 5 days, sham leukapheresis, followed by intramuscular injection of cell suspension buffer PBS/EDTA buffer/0.5% Human Serum Albumin.

CD133+ Dose

The safe and tolerable dose of autologous CD133+ cells necessary to achieve a measurable effect in humans with refractory CLI was unknown prior to the initiation of our trial. Our goal was to test a *therapeutic strategy* of injecting the entire enriched CD133+ product. The U.S. Food and Drug Administration (FDA) mandated a pre-specified lower and upper dose threshold to allow the trial to proceed. To comply, a lower dose limit of 50×10^6 CD133+ cells and upper dose limit of 400×10^6 cells was considered acceptable and was based on estimates of G-CSF mononuclear cell mobilization efficiency in the literature at that time. If less than 50×10^6 CD133+ cells were selected, subjects were considered to have mobilization failure and would, by protocol, receive intramuscular injection of placebo. Although the primary analysis plan was intention to treat, a secondary analysis of subjects with mobilization failure would also be performed. If greater than 400×10^6 CD133+ cells were selected, then subjects were administered with only 400×10^6 CD133+ cells.

Injection Procedure

An injection map of the leg's major muscle groups was used to guide injection locations (Figure 1). Using sterile technique, 10 intra-muscular injections of CD133+ cells or placebo (0.4mL volume per injection) were administered into both lower extremities for a total of 20 injections per subject. Subjects were placed prone for posterior compartment injections and supine for anterior compartment injections. Injections were performed using a 25G needle with an injection depth that varied between 1 to 5 cm. Injection depth was estimated by the physician. The injection target was to be approximately in the center of the muscle bundle. Each injection was administered over 30 seconds. CD133+ cell to 25G needle bench-top biocompatibility validation studies were performed prior to trial initiation, confirming preserved cell viability and number with each injection. Conscious sedation with supplemental oxygen was administered as needed. Heart rate, blood pressure and oxygen saturation were monitored throughout the injection procedure and for one hour following injections.

Blinding Strategy

Investigators were divided into blinded and unblinded teams. The blinded team consisted of physician investigators and study coordinators who participated in subject screening, placement of the 11.5F Mahurkar dialysis catheter (Covidien, Dublin, Ireland) into the right internal jugular vein, intramuscular injections of study agent, and clinical follow-up. The unblinded team consisted of a hematologist, pharmacy, leukapheresis nurses and lab personnel who performed CD133+ selection and flow cytometry product characterization for subjects randomized to active therapy, and sham leukapheresis, preparation of placebo syringes for those subjects randomized to placebo control. All subjects were positioned in an infusion center bed, with drapes positioned to blind subjects to the intravenous lines and leukapheresis equipment. To maintain a credible blind, unattached lines were intermittently manipulated for those subjects receiving sham leukapheresis. Questions such as “how are you feeling?”, “any numbness or tingling?” were asked by nurse personnel. Duration of sham leukapheresis procedure varied between 3 and 4 hours at the discretion of the leukapheresis nurse and un-blinded hematologist. Subjects were surveyed following the leukapheresis and sham leukapheresis procedure to determine if the blinding effort was successful.

Endpoints

The goals of this pilot study were i) to demonstrate the feasibility and safety of intramuscular injection of autologous CD133+ cells in ambulatory subjects with refractory, no-option CLI and ii) to demonstrate the feasibility of the trial design and blinding strategy which might inform the design and conduct of future, larger, multi-center trials. Clinical endpoint assessments were performed daily during mobilization, leukapheresis and treatment phases and then at 1, 2, 4, 12, 24 and 52 weeks following treatment. Vascular endpoints consisted of rest ABI, toe-pressure, segmental PVR, TcPO₂. Functional endpoints consisted of peak walking time on treadmill using the Gardner Treadmill protocol, 6-minute walk test, and 2-minute exercise ABI. Medical Outcomes Health Status Survey SF-36, Walking Impairment Questionnaire, Visual pain scale, and adequacy of study blinding questionnaire were recorded.

CD133+ Senescence

In each subject, an aliquot of mobilized and selected CD133+ cells was cryogenically preserved for subsequent senescence testing. Control cells were obtained from three healthy male donors between 21-22 years of age (AllCells, Alameda CA; frozen mobilized peripheral blood CD133+ cells, mPb024-1). Cryopreserved CD133+ cells were rapidly thawed in a 37°C water bath, washed in phosphate buffered saline and total RNA isolated (Qiagen, Germantown MD; RNeasy Mini Kit). RNA concentrations were determined using a NanoDrop ND-1000 spectrophotometer. First-strand cDNA synthesis was performed using 80ng of total RNA using an RT² First Strand Kit (Qiagen), then run on the “Human Cellular Senescence” RT² Profiler PCR array (PAHS-050Z, Qiagen) which contains 84 genes of interest that were normalized to the average of 4 housekeeping genes: B2M, HPRT1, RPLO, GADPH. All kits were used according to manufacturer's instructions and analysis was performed using open source software available at www.pcrdataanalysis.sabiosciences.com.

Statistical Methods

Continuous variables were summarized using means and standard deviations, and comparisons made using Student t-test. Categorical outcomes were summarized as frequencies and percentages and compared using Fischer's Exact test.

Results

Seventy subjects were screened of which 60 were considered screen failures (Figure 2). Of the 10 randomized subjects, the first was treated on October 7, 2009. After the 10th subject, the independent data safety monitoring board (DSMB) recommended suspending new enrollment into the trial (August 8, 2012) due to the high rate of mobilization failure. At the point that enrollment was suspended, three subjects were randomized to control, and 7 subjects were randomized to active treatment. All randomized subjects were followed for 1 year and there was no loss to follow-up. Of the active treatment group, 4 of 7 subjects failed to achieve the CD133+ low dose threshold following mobilization. Per protocol, these subjects received intramuscular injection of placebo. The remaining 3 of 7 subjects randomized to active treatment actually received CD133+ cell injections. Baseline characteristics of enrolled subjects are listed in Table 1. Mobilization efficiency and CD133+ purity among subjects randomized to active treatment are listed in Table 2. CD133+ selection was not performed on subject 6 due to extremely poor mobilization and futility. Blinding assessment questionnaires from 6 subjects indicated they were 100% unsure of treatment allocation, 2 subjects identified their treatment allocation incorrectly (1 in treatment group and 1 in control group) and 2 subjects identified their treatment allocation correctly (both in the treatment group).

Safety Endpoints

At 6 months, there was no difference in serious adverse events (SAE's) per subject between those subjects randomized to treatment and subjects randomized to control, (0.7/subject versus 1.3/subject, $p=0.5$). There was no difference in the number of SAE's per subject across actual treatment cohorts (0.7/subject, 0.8/subject, 1.3/subject; CD133+ treatment, Mobilization failure, Control groups respectively). Notably, GCSF mobilization was not associated with any cardiac-associated adverse events, including any rise in cardiac enzymes during the mobilization period.

Efficacy Endpoints

At 12 months, there was no difference in overall survival, freedom from amputation, or freedom from hospitalization in subjects randomized to CD133+ treatment compared to those assigned to control (Table3). There was a trend towards improved amputation free survival (86% versus 33%, treatment versus control, $p=0.2$), but this also did not reach statistical significance. Furthermore, there were no trends to benefit when events were assessed by actual treatment, instead of intention to treat.

Histopathology was performed in one subject who required below knee amputation 22 days following CD133+ active treatment injections. The CD133+ injection map was provided to the pathologist and surgeon. Hematoxylin and eosin staining revealed no evidence of

endothelial proliferation or tumor formation in tissue that would approximate below knee injection locations. The CD133+ selection antibody contains an iron moiety that does not dissociate unlike other antibodies used to select cells using the magnetic bead separation technique (i.e. CD34+ selection antibody). Prussian blue staining on these specimens revealed no evidence of the CD133+ iron anti-body used for cell selection (Miltenyi Biotec Inc.). Sampling error cannot be ruled out despite provision of the injection map.

Change in six-minute walk distance between baseline and 12 months was not different between treatment and control subjects when analyzed by intention to treat (Figure 3). However, those who successfully mobilized and were treated with CD133+ cells were able to walk further, on average, compared to mobilization failure and control subjects. Similarly, Walking Impairment Questionnaire scores were not different between those randomized to treatment versus control; however, those who received CD133+ had improved scores in distance, speed and stairs scores (Figure 4). Improved SF36 categories were generally observed among those who actually received CD133+ cells compared to other groups (Figure 5). In contrast, no such trends were observed with treadmill peak walking time, transcutaneous tissue oxygenation of either the index limb or non-index limb, or ABI of either the index or non-index limb.

CD133+ Senescence

Compared to healthy donor CD133+ cells (n=3), CD133+ cell obtained from subjects randomized to the treatment arm (n=7) had 2 fold or greater expression in several genes that are associated with cell senescence: mitogen-activated protein kinase 3 (MAP2K3)(28), transforming growth factor β 1/1 (TGF- β 1/1)(29), plasminogen activator urokinase (PLAU) (30), Cyclin E1 (CCNE1)(31), E3 ubiquitin-protein ligase (MDM2)(32) and Insulin like growth factor 1 (IGF1)(33) (Figure 6A). Comparison was made between successful and unsuccessful mobilizers in those subjects randomized to the treatment group (Figure 6B). A trend toward 2-fold or greater expression of 4 of 5 genes that are associated with cellular senescence was observed in the mobilization group [Insulin Like Growth Factor Binding Protein 5 (IGFBP5)(34), Calreticulin CALR(35), v-ets avian erythroblastosis virus E26 oncogene homolog 2 (ETS2)(36), growth arrest and DNA damage-inducible alpha (GADD45A)(37-39)]. The lone *down regulated* gene, super oxide dismutase 2 (SOD2), encodes an enzyme to clear free radicals and is considered to be an anti-senescence gene(40, 41). Although the sample size is small, these results suggest that patients with CLI who mobilize poorly with GCSF may have heightened cellular senescence gene expression compared to those who successfully mobilized.

Discussion

The SCRIPT-CLI trial was designed as a pilot study to test the feasibility and safety of a novel therapeutic strategy involving bilateral lower extremity injection of all mobilized autologous CD133+ cells in ambulatory subjects with refractory CLI. Subject enrollment was suspended early due to an unexpected failure to mobilize sufficient CD133+ cells to the required low dose threshold and increased CD133+ expression of senescence markers. The novel blinding approach was successful and no major safety concerns were identified.

However, feasibility of the approach could not be demonstrated given that there was a high failure to mobilize sufficient cells to generate the protocol defined lowest dose limit of CD133+ cells. The investigators considered lowering the lowest dose threshold even further, but the conclusion was that this approach would be unlikely to generate a positive outcome in future, appropriately powered, efficacy trials given the low CD133+ yield and increased senescence observed in our pilot study.

Subjects who failed to mobilize CD133+ cells may represent a CLI sub-population with a worse prognosis compared to those who mobilized CD133+ well, although post-hoc analysis demonstrated no clear differences between successful and failed G-CSF mobilizers for safety events, major clinical events, six-minute walk distance, walking impairment questionnaire, and SF 36 quality of life. Gene expression analysis identified up-regulation of several senescence associated genes linked to subjects who mobilize CD133+ cells poorly (Figure 6B and Table 4); however, these data can only be viewed as hypothesis generating given the small sample size.

Circulating endogenous progenitor cells that originate primarily from the bone marrow which are capable of vascular repair were first described by Asahara et al.(42) Exogenously administered G-CSF and granulocyte-macrophage colony stimulating factor (GM-CSF) are implicated in endothelial cell proliferation and neovascularization(43). However, clinical trials testing the therapeutic effects of these cytokines alone have not been successful for peripheral artery disease(44), stroke(45) and myocardial infarction(46-48). Alternatively, intramuscular administration of cytokine mobilized leukapheresis cell product has demonstrated some benefit in subjects with refractory CLI using unfractionated mononuclear cells(49-52), selected CD34+ cells(18, 24), and mesenchymal stem cells(53). Two small trials demonstrated that single-limb, intramuscular administration of autologous peripheral blood acquired CD133+ cells in subjects with CLI is feasible but efficacy could not be assessed as these trials were uncontrolled(54, 55). However, these favorable reports should be tempered by emerging reports of safety and futility concerns of local administration of G-CSF mobilized leukapheresis cell product in subjects with CLI. For example, Jonsson et al. terminated a trial testing intramuscular administration of peripheral blood mononuclear cells obtained following G-CSF mobilization due to safety concerns from excess peri-procedural complications and futility due to highly variable paracrine and metabolic responses in the treatment group(56).

Intramuscular injection of populations of bone marrow cells that include CD133+ cells has been associated with local endothelial proliferation and angiogenesis in CLI subjects where the target limb required amputation(57, 58). In SCRIPT-CLI, there was no evidence of endothelial proliferation or presence of CD133+ antibody in the one subject where histology of the amputated limb was performed 22 days following CD133+ cell injection. Potential explanations for this are either sampling error or perhaps reduced angiogenic potency when CD133+ are harvested via cytokine mobilization/leukapheresis versus direct bone marrow aspiration.

Autologous cell-based therapy for CLI faces a number of challenges. CLI subjects are older, carried a heavy burden of atherosclerosis and have multiple comorbidities. Risk factors such

as advanced age, obesity and diabetes are independently associated with reduced endothelial progenitor cell number and angiogenic potential(59-63). Teraa et al demonstrated that CLI patients have lower circulating CD133+ cell numbers, reduced paracrine function and mobilization capability due to an impairment of the MMP9 and SDF-1/CXCR4 axis(64). To our knowledge, our study is the first to demonstrate increased CD133+ senescence marker expression among subjects with CLI compared to healthy controls. The combination of reduced CD133+ cell yield and potentially altered function may prevent this cell type from being a viable therapeutic product for patients with CLI.

Translating our approach to a larger trial will be challenged with issues around leukapheresis, CD133+ selection, and injection. These are potentially costly procedures that would likely be restricted to highly specialized centers. There may be significant institutional variation in product yield and quality. Centralized cell processing has been suggested as an alternative. However, complexities of cell processing and logistics of transportation of cellular products in a timely fashion can be challenging and costs are likely to remain high. Furthermore, although sham leukapheresis was feasible in our trial, it may not be a viable approach across multiple institutions due to the extensive training and expertise that is required.

SCRIPT-CLI was designed as a pilot feasibility trial. Observed efficacy trends are hypothesis generating and potentially useful for sample size calculation for future larger scale efficacy trials. In SCRIPT-CLI, efficacy data was presented as total number of events within the follow-up period. Other methods to present efficacy data in placebo-controlled trials include time to event analysis and the global rank method (hierarchical efficacy analysis) described by Subherwal(65). Time to event analysis of wide ranging composite endpoints can be misleading if lesser clinical impact events such as ulcer healing occur before major events such as time to major amputation. Alternatively, the global rank method is a powerful analytic method that assigns consensus panel driven hierarchical priority to each of the efficacy endpoints. Categorical events such as death and amputation are well defined. There is less consensus agreement on what constitutes a clinically meaningful improvement in walking distance in CLI patients. Future CLI therapy trials should consider hierarchical efficacy analysis with walking distance as part of the composite endpoint.

There are several limitations to this pilot study. Enrollment into this study was slow due to the narrow inclusion criteria. This may be surmounted by increasing the number of trial sites; however, this is costly and funding is another major challenge for biologic therapy in peripheral artery disease(66). Although CD133+ angiogenic gene expression is of interest, only senescence gene expression was tested on residual CD133+ samples.

Screen failure rate was high due to the attempt to restrict the study to a subset of CLI patients who remain ambulatory. This approach enabled exploring walking parameters as highly clinically relevant and sensitive functional study endpoints, compared to amputation as an endpoint which may not be able to detect early or moderate signals of recovery in this early phase, single dose clinical trial. Interestingly, one subject who was randomized to treatment, mobilized well and received CD133+ injections, but suffered a below knee amputation 22 days later. The subject received an artificial limb prosthesis and had an

exceptional return to ambulatory function. Specifically, six minute walk distance improved from 180 meters at baseline to 358 meters at 1 year with the prosthesis. At face value, it would appear that intramuscular administration of autologous CD133+ cells offered no benefit to this patient. An alternative hypothesis is that CD133+ administration enabled stump healing as several CD133+ injections were above the knee. This may have permitted more rapid adoption of the limb prosthesis and improved contra-lateral limb flow so that ambulatory function was fully restored. This hypothesis would need to be tested in future trials.

Furthermore, the high screen failure rate suggests that this trial design is impractical for clinical trials with larger sample sizes that are more appropriate to test efficacy. G-CSF was administered only in treated subjects, unlike other cardiovascular disease trials where it was administered to both treatment and control subjects(24, 67). This pro-inflammatory cytokine is associated with side effects that are rarely severe, but are commonly mild and may be perceptible to patients, which could lead to unblinding. The elderly CLI subjects with numerous co-morbidities in SCRIPT-CLI were unable to accurately identify treatment assignment based on the results of the post treatment blinding questionnaire. The lack of perceptible G-CSF related side-effects appeared to correspond to low mobilization efficiency in this CLI population. However, blinding may be challenging in future trials using our mobilization approach, if the subject pool includes younger subjects with less co-morbidity and hence, potentially more perceptible G-CSF related side-effects.

Conclusion

The therapeutic strategy of bilateral lower extremity intramuscular injection of all mobilized autologous CD133+ cells isolated from GCSF mobilized apheresis products in subjects with refractory CLI was not feasible in this pilot study. The primary reason for this was failure to achieve the pre-specified minimum mobilized cell dose threshold. These data combined with the observed increased CD133+ expression of senescence associated genes suggest this therapeutic approach in this subject population is not likely to be successful.

Acknowledgments

Sincere thanks to the Janelle McManus CRN and other leukapheresis and cell selection lab staff at the University of Wisconsin Hospital and Clinics.

Abbreviations

7AAD	7-AminoActinomycin D assay
ABI	ankle brachial index
CLI	critical limb ischemia
DSMB	data safety monitoring board
EDTA	Ethylenediaminetetraacetic acid
FDA	Food and Drug Administration

G-CSF	granulocyte colony stimulating factor
G-MCSF	granulocyte-macrophage colony stimulating factor
HSA	human serum albumin
IND	Investigational New Drug
PVR	pulse volume recording
PBS	phosphate buffered saline

REFERENCES

1. Norgren L, Hiatt WR, Dormandy JA, Nehler MR, Harris KA, Fowkes FG, Bell K, Caporusso J, Durand-Zaleski I, Komori K, Lammer J, Liapis C, Novo S, Razavi M, Robbs J, Schaper N, Shigematsu H, Sapoval M, White C, White J, Clement D, Creager M, Jaff M, Mohler E 3rd, Rutherford RB, Sheehan P, Sillesen H, Rosenfield K. Inter-society consensus for the management of peripheral arterial disease (tasc ii). *Eur J Vasc Endovasc Surg.* 2007; 33(Suppl 1):S1–75. [PubMed: 17140820]
2. Hirsch AT, Haskal ZJ, Hertzner NR, Bakal CW, Creager MA, Halperin JL, Hiratzka LF, Murphy WR, Olin JW, Puschett JB, Rosenfield KA, Sacks D, Stanley JC, Taylor LM Jr, White CJ, White J, White RA, Antman EM, Smith SC Jr, Adams CD, Anderson JL, Faxon DP, Fuster V, Gibbons RJ, Hunt SA, Jacobs AK, Nishimura R, Ornato JP, Page RL, Riegel B. Acc/aha 2005 practice guidelines for the management of patients with peripheral arterial disease (lower extremity, renal, mesenteric, and abdominal aortic): A collaborative report from the american association for vascular surgery/society for vascular surgery, society for cardiovascular angiography and interventions, society for vascular medicine and biology, society of interventional radiology, and the acc/aha task force on practice guidelines (writing committee to develop guidelines for the management of patients with peripheral arterial disease): Endorsed by the american association of cardiovascular and pulmonary rehabilitation; national heart, lung, and blood institute; society for vascular nursing; transatlantic inter-society consensus; and vascular disease foundation. *Circulation.* 2006; 113:e463–654. [PubMed: 16549646]
3. Becker F, Robert-Ebadi H, Ricco JB, Setacci C, Cao P, de Donato G, Eckstein HH, De Rango P, Diehm N, Schmidli J, Teraa M, Moll FL, Dick F, Davies AH, Lepantalo M, Apelqvist J. Chapter i: Definitions, epidemiology, clinical presentation and prognosis. *European journal of vascular and endovascular surgery : the official journal of the European Society for Vascular Surgery.* 2011; 42(Suppl 2):S4–12.
4. de Vries SO, Donaldson MC, Hunink MG. Contralateral symptoms after unilateral intervention for peripheral occlusive disease. *J Vasc Surg.* 1998; 27:414–421. [PubMed: 9546226]
5. Rollins KE, Jackson D, Coughlin PA. Meta-analysis of contemporary short- and long-term mortality rates in patients diagnosed with critical leg ischaemia. *Br J Surg.* 2013; 100:1002–1008. [PubMed: 23649310]
6. Belch J, Hiatt WR, Baumgartner I, Driver IV, Nikol S, Norgren L, Van Belle E. Effect of fibroblast growth factor *nv1fgf* on amputation and death: A randomised placebo-controlled trial of gene therapy in critical limb ischaemia. *Lancet.* 2011; 377:1929–1937. [PubMed: 21621834]
7. Ma N, Ladilov Y, Moebius JM, Ong L, Piechaczek C, David A, Kaminski A, Choi YH, Li W, Egger D, Stamm C, Steinhoff G. Intramyocardial delivery of human *cd133+* cells in a scid mouse cryoinjury model: Bone marrow vs. Cord blood-derived cells. *Cardiovascular research.* 2006; 71:158–169. [PubMed: 16730684]
8. Gehling UM, Ergun S, Schumacher U, Wagener C, Pantel K, Otte M, Schuch G, Schafhausen P, Mende T, Kilic N, Kluge K, Schafer B, Hossfeld DK, Fiedler W. In vitro differentiation of endothelial cells from *ac133*-positive progenitor cells. *Blood.* 2000; 95:3106–3112. [PubMed: 10807776]

9. Peichev M, Naiyer AJ, Pereira D, Zhu Z, Lane WJ, Williams M, Oz MC, Hicklin DJ, Witte L, Moore MA, Rafii S. Expression of vegfr-2 and ac133 by circulating human cd34(+) cells identifies a population of functional endothelial precursors. *Blood*. 2000; 95:952–958. [PubMed: 10648408]
10. Friedrich EB, Walenta K, Scharlau J, Nickenig G, Werner N. Cd34-/cd133+/vegfr-2+ endothelial progenitor cell subpopulation with potent vasoregenerative capacities. *Circulation research*. 2006; 98:e20–25. [PubMed: 16439688]
11. Tateishi-Yuyama E, Matsubara H, Murohara T, Ikeda U, Shintani S, Masaki H, Amano K, Kishimoto Y, Yoshimoto K, Akashi H, Shimada K, Iwasaka T, Imaizumi T. Therapeutic angiogenesis for patients with limb ischaemia by autologous transplantation of bone-marrow cells: A pilot study and a randomised controlled trial. *Lancet*. 2002; 360:427–435. [PubMed: 12241713]
12. Esato K, Hamano K, Li TS, Furutani A, Seyama A, Takenaka H, Zempo N. Neovascularization induced by autologous bone marrow cell implantation in peripheral arterial disease. *Cell transplantation*. 2002; 11:747–752. [PubMed: 12588106]
13. Huang P, Li S, Han M, Xiao Z, Yang R, Han ZC. Autologous transplantation of granulocyte colony-stimulating factor-mobilized peripheral blood mononuclear cells improves critical limb ischemia in diabetes. *Diabetes Care*. 2005; 28:2155–2160. [PubMed: 16123483]
14. Miyamoto K, Nishigami K, Nagaya N, Akutsu K, Chiku M, Kamei M, Soma T, Miyata S, Higashi M, Tanaka R, Nakatani T, Nonogi H, Takeshita S. Unblinded pilot study of autologous transplantation of bone marrow mononuclear cells in patients with thromboangiitis obliterans. *Circulation*. 2006; 114:2679–2684. [PubMed: 17145986]
15. Durdu S, Akar AR, Arat M, Sancak T, Eren NT, Ozyurda U. Autologous bone-marrow mononuclear cell implantation for patients with rutherford grade ii-iii thromboangiitis obliterans. *J Vasc Surg*. 2006; 44:732–739. [PubMed: 16926085]
16. Arai M, Misao Y, Nagai H, Kawasaki M, Nagashima K, Suzuki K, Tsuchiya K, Otsuka S, Uno Y, Takemura G, Nishigaki K, Minatoguchi S, Fujiwara H. Granulocyte colony-stimulating factor: A noninvasive regeneration therapy for treating atherosclerotic peripheral artery disease. *Circulation journal : official journal of the Japanese Circulation Society*. 2006; 70:1093–1098. [PubMed: 16936417]
17. Huang PP, Yang XF, Li SZ, Wen JC, Zhang Y, Han ZC. Randomised comparison of g-csf-mobilized peripheral blood mononuclear cells versus bone marrow-mononuclear cells for the treatment of patients with lower limb arteriosclerosis obliterans. *Thrombosis and haemostasis*. 2007; 98:1335–1342. [PubMed: 18064333]
18. Kawamoto A, Katayama M, Handa N, Kinoshita M, Takano H, Horii M, Sadamoto K, Yokoyama A, Yamanaka T, Onodera R, Kuroda A, Baba R, Kaneko Y, Tsukie T, Kurimoto Y, Okada Y, Kihara Y, Morioka S, Fukushima M, Asahara T. Intramuscular transplantation of g-csf-mobilized cd34(+) cells in patients with critical limb ischemia: A phase i/ia, multicenter, single-blinded, dose-escalation clinical trial. *Stem Cells*. 2009; 27:2857–2864. [PubMed: 19711453]
19. Prochazka V, Gumulec J, Jaluvka F, Salounova D, Jonszta T, Czerny D, Krajca J, Urbanec R, Klement P, Martinek J, Klement GL. Cell therapy, a new standard in management of chronic critical limb ischemia and foot ulcer. *Cell transplantation*. 2010; 19:1413–1424. [PubMed: 20529449]
20. Murphy MP, Lawson JH, Rapp BM, Dalsing MC, Klein J, Wilson MG, Hutchins GD, March KL. Autologous bone marrow mononuclear cell therapy is safe and promotes amputation-free survival in patients with critical limb ischemia. *Journal of vascular surgery*. 2011; 53:1565–1574. e1561. [PubMed: 21514773]
21. Lu D, Chen B, Liang Z, Deng W, Jiang Y, Li S, Xu J, Wu Q, Zhang Z, Xie B, Chen S. 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. [PubMed: 21216483]
22. Walter DH, Krankenberg H, Balzer JO, Kalka C, Baumgartner I, Schluter M, Tonn T, Seeger F, Dimmeler S, Lindhoff-Last E, Zeiher AM. Intraarterial administration of bone marrow mononuclear cells in patients with critical limb ischemia: A randomized-start, placebo-controlled pilot trial (provasa). *Circulation. Cardiovascular interventions*. 2011; 4:26–37. [PubMed: 21205939]

23. Powell RJ, Marston WA, Berceli SA, Guzman R, Henry TD, Longcore AT, Stern TP, Watling S, Bartel RL. Cellular therapy with ixmyelocel-t to treat critical limb ischemia: The randomized, double-blind, placebo-controlled restore-cli trial. *Molecular therapy : the journal of the American Society of Gene Therapy*. 2012; 20:1280–1286. [PubMed: 22453769]
24. Losordo DW, Kibbe MR, Mendelsohn F, Marston W, Driver VR, Sharafuddin M, Teodorescu V, Wiechmann BN, Thompson C, Kraiss L, Carman T, Dohad S, Huang P, Junge CE, Story K, Weistroffer T, Thorne TM, Millay M, Runyon JP, Schainfeld R. A randomized, controlled pilot study of autologous cd34+ cell therapy for critical limb ischemia. *Circulation. Cardiovascular interventions*. 2012; 5:821–830. [PubMed: 23192920]
25. Benoit E, O'Donnell TF Jr, Iafrafi MD, Asher E, Bandyk DF, Hallett JW, Lumsden AB, Pearl GJ, Roddy SP, Vijayaraghavan K, Patel AN. The role of amputation as an outcome measure in cellular therapy for critical limb ischemia: Implications for clinical trial design. *Journal of translational medicine*. 2011; 9:165. [PubMed: 21951607]
26. 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. *Annals of surgery*. 2013
27. Montgomery PS, Gardner AW. The clinical utility of a six-minute walk test in peripheral arterial occlusive disease patients. *Journal of the American Geriatrics Society*. 1998; 46:706–711. [PubMed: 9625185]
28. Jia M, Souchelnytskyi N, Hellman U, O'Hare M, Jat PS, Souchelnytskyi S. Proteome profiling of immortalization-to-senescence transition of human breast epithelial cells identified map2k3 as a senescence-promoting protein which is downregulated in human breast cancer. *Proteomics Clin Appl*. 2010; 4:816–828. [PubMed: 21137025]
29. Shibanuma M, Mochizuki E, Maniwa R, Mashimo J, Nishiya N, Imai S, Takano T, Oshimura M, Nose K. Induction of senescence-like phenotypes by forced expression of hic-5, which encodes a novel lim motif protein, in immortalized human fibroblasts. *Molecular and cellular biology*. 1997; 17:1224–1235. [PubMed: 9032249]
30. West MD, Shay JW, Wright WE, Linskens MH. Altered expression of plasminogen activator and plasminogen activator inhibitor during cellular senescence. *Experimental gerontology*. 1996; 31:175–193. [PubMed: 8706787]
31. Dulic V, Drullinger LF, Lees E, Reed SI, Stein GH. Altered regulation of g1 cyclins in senescent human diploid fibroblasts: Accumulation of inactive cyclin ecdk2 and cyclin d1-cdk2 complexes. *Proceedings of the National Academy of Sciences of the United States of America*. 1993; 90:11034–11038. [PubMed: 8248208]
32. Mikawa T, Maruyama T, Okamoto K, Nakagama H, Lleonart ME, Tsusaka T, Hori K, Murakami I, Izumi T, Takaori-Kondo A, Yokode M, Peters G, Beach D, Kondoh H. Senescence-inducing stress promotes proteolysis of phosphoglycerate mutase via ubiquitin ligase mdm2. *The Journal of cell biology*. 2014; 204:729–745. [PubMed: 24567357]
33. Handayaningsih AE, Takahashi M, Fukuoka H, Iguchi G, Nishizawa H, Yamamoto M, Suda K, Takahashi Y. Igf-i enhances cellular senescence via the reactive oxygen species-p53 pathway. *Biochemical and biophysical research communications*. 2012; 425:478–484. [PubMed: 22877754]
34. Kim KS, Seu YB, Baek SH, Kim MJ, Kim KJ, Kim JH, Kim JR. Induction of cellular senescence by insulin-like growth factor binding protein-5 through a p53-dependent mechanism. *Molecular biology of the cell*. 2007; 18:4543–4552. [PubMed: 17804819]
35. Johnson RJ, Xiao G, Shanmugaratnam J, Fine RE. Calreticulin functions as a molecular chaperone for the beta-amyloid precursor protein. *Neurobiology of aging*. 2001; 22:387–395. [PubMed: 11378243]
36. Sheydina A, Volkova M, Jiang L, Juhasz O, Zhang J, Tae HJ, Perino MG, Wang M, Zhu Y, Lakatta EG, Boheler KR. Linkage of cardiac gene expression profiles and ets2 with lifespan variability in rats. *Aging Cell*. 2012; 11:350–359. [PubMed: 22247964]
37. Chen Y, Ma X, Zhang M, Wang X, Wang C, Wang H, Guo P, Yuan W, Rudolph KL, Zhan Q, Ju Z. Gadd45a regulates hematopoietic stem cell stress responses in mice. *Blood*. 2014; 123:851–862. [PubMed: 24371210]

38. Jackson JG, Pereira-Smith OM. P53 is preferentially recruited to the promoters of growth arrest genes p21 and gadd45 during replicative senescence of normal human fibroblasts. *Cancer research*. 2006; 66:8356–8360. [PubMed: 16951143]
39. Lal A, Gorospe M. Egad, more forms of gene regulation: The gadd45a story. *Cell Cycle*. 2006; 5:1422–1425. [PubMed: 16775423]
40. Gualandi F, Morelli C, Pavan JV, Rimessi P, Sensi A, Bonfatti A, Gruppioni R, Possati L, Stanbridge EJ, Barbanti-Brodano G. Induction of senescence and control of tumorigenicity in bk virus transformed mouse cells by human chromosome 6. *Genes, chromosomes & cancer*. 1994; 10:77–84. [PubMed: 7520270]
41. De Benedictis G, Carotenuto L, Carrieri G, De Luca M, Falcone E, Rose G, Cavalcanti S, Corsonello F, Feraco E, Baggio G, Bertolini S, Mari D, Mattace R, Yashin AI, Bonafe M, Franceschi C. Gene/longevity association studies at four autosomal loci (ren, tho, parp, sod2). *Eur J Hum Genet*. 1998; 6:534–541. [PubMed: 9887369]
42. Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, Isner JM. Isolation of putative progenitor endothelial cells for angiogenesis. *Science*. 1997; 275:964–967. [PubMed: 9020076]
43. Bussolino F, Wang JM, Defilippi P, Turrini F, Sanavio F, Edgell CJ, Aglietta M, Arese P, Mantovani A. Granulocyte- and granulocyte-macrophage-colony stimulating factors induce human endothelial cells to migrate and proliferate. *Nature*. 1989; 337:471–473. [PubMed: 2464767]
44. Poole J, Mavromatis K, Binongo JN, Khan A, Li Q, Khayata M, Rocco E, Topel M, Zhang X, Brown C, Corriere MA, Murrow J, Sher S, Clement S, Ashraf K, Rashed A, Kabbany T, Neuman R, Morris A, Ali A, Hayek S, Oshinski J, Yoon YS, Waller EK, Quyyumi AA. Effect of progenitor cell mobilization with granulocyte-macrophage colony-stimulating factor in patients with peripheral artery disease: A randomized clinical trial. *JAMA : the journal of the American Medical Association*. 2013; 310:2631–2639.
45. Ringelstein EB, Thijs V, Norrving B, Chamorro A, Aichner F, Grond M, Saver J, Laage R, Schneider A, Rathgeb F, Vogt G, Charisse G, Fiebach JB, Schwab S, Schabitz WR, Kollmar R, Fisher M, Brozman M, Skoloudik D, Gruber F, Serena Leal J, Veltkamp R, Kohrmann M, Berrouschot J. Granulocyte colony-stimulating factor in patients with acute ischemic stroke: Results of the ax200 for ischemic stroke trial. *Stroke; a journal of cerebral circulation*. 2013; 44:2681–2687.
46. Zohnhofer D, Ott I, Mehilli J, Schomig K, Michalk F, Ibrahim T, Meisetschlager G, von Wedel J, Bollwein H, Seyfarth M, Dirschinger J, Schmitt C, Schwaiger M, Kastrati A, Schomig A. Stem cell mobilization by granulocyte colony-stimulating factor in patients with acute myocardial infarction: A randomized controlled trial. *JAMA*. 2006; 295:1003–1010. [PubMed: 16507801]
47. Ripa RS, Jorgensen E, Wang Y, Thune JJ, Nilsson JC, Sondergaard L, Johnsen HE, Kober L, Grande P, Kastrup J. Stem cell mobilization induced by subcutaneous granulocyte-colony stimulating factor to improve cardiac regeneration after acute st-elevation myocardial infarction: Result of the double-blind, randomized, placebo-controlled stem cells in myocardial infarction (stemmi) trial. *Circulation*. 2006; 113:1983–1992. [PubMed: 16531621]
48. Engelmann MG, Theiss HD, Hennig-Theiss C, Huber A, Wintersperger BJ, Werle-Ruedinger AE, Schoenberg SO, Steinbeck G, Franz WM. Autologous bone marrow stem cell mobilization induced by granulocyte colony-stimulating factor after subacute st-segment elevation myocardial infarction undergoing late revascularization: Final results from the g-csf-stemi (granulocyte colony-stimulating factor st-segment elevation myocardial infarction) trial. *J Am Coll Cardiol*. 2006; 48:1712–1721. [PubMed: 17045910]
49. Zafarghandi MR, Ravari H, Aghdami N, Namiri M, Moazzami K, Taghiabadi E, Fazel A, Pournasr B, Farrokhi A, Sharifian RA, Salimi J, Moini M, Baharvand H. Safety and efficacy of granulocyte-colony-stimulating factor administration following autologous intramuscular implantation of bone marrow mononuclear cells: A randomized controlled trial in patients with advanced lower limb ischemia. *Cytotherapy*. 2010; 12:783–791. [PubMed: 20078390]
50. Ozturk A, Kucukardali Y, Tangi F, Eriksi A, Uzun G, Bashekim C, Sen H, Terekeci H, Narin Y, Ozyurt M, Ozkan S, Sayan O, Rodop O, Nalbant S, Sildiroglu O, Yalniz FF, Senkal IV, Sabuncu H, Oktenli C. Therapeutical potential of autologous peripheral blood mononuclear cell

- transplantation in patients with type 2 diabetic critical limb ischemia. *J Diabetes Complications*. 2012; 26:29–33. [PubMed: 22240264]
51. Maksimov AV, Kiiasov AP, Plotnikov MV, Maianaika SD, Shamsutdinova II, Gazizov IM, Mavlikeev MO. [outcomes of using autologous peripheral-blood stem cells in patients with chronic lower arterial insufficiency]. *Angiol Sosud Khir*. 2011; 17:11–15. [PubMed: 21983456]
 52. Dubsky M, Jirkovska A, Bem R, Pagacova L, Fejfarova V, Varga M, Skibova J, Langkramer S, Sykova E. [treatment of critical limb ischemia and diabetic foot disease by the use of autologous stem cells]. *Vnitr Lek*. 2011; 57:451–455. [PubMed: 21695925]
 53. Mohammadzadeh L, Samedanifard SH, Keshavarzi A, Alimoghaddam K, Larijani B, Ghavamzadeh A, Ahmadi AS, Shojaeifard A, Ostadali MR, Sharifi AM, Amini MR, Mahmoudian A, Fakhraei H, Aalaa M, Mohajeri-Tehrani MR. Therapeutic outcomes of transplanting autologous granulocyte colony-stimulating factor-mobilised peripheral mononuclear cells in diabetic patients with critical limb ischaemia. *Experimental and clinical endocrinology & diabetes : official journal, German Society of Endocrinology [and] German Diabetes Association*. 2013; 121:48–53.
 54. Burt RK, Testori A, Oyama Y, Rodriguez HE, Yaung K, Villa M, Bucha JM, Milanetti F, Sheehan J, Rajamannan N, Pearce WH. Autologous peripheral blood cd133+ cell implantation for limb salvage in patients with critical limb ischemia. *Bone Marrow Transplant*. 45:111–116. [PubMed: 19448678]
 55. Canizo MC, Lozano F, Gonzalez-Porras JR, Barros M, Lopez-Holgado N, Briz E, Sanchez-Guijo FM. Peripheral endothelial progenitor cells (cd133 +) for therapeutic vasculogenesis in a patient with critical limb ischemia. One year follow-up. *Cytotherapy*. 2007; 9:99–102. [PubMed: 17354105]
 56. Jonsson TB, Larzon T, Arfvidsson B, Tidefelt U, Axelsson CG, Jurstrand M, Norgren L. Adverse events during treatment of critical limb ischemia with autologous peripheral blood mononuclear cell implant. *Int Angiol*. 2012; 31:77–84. [PubMed: 22330628]
 57. Pignon B, Sevestre MA, Chatelain D, Albertini JN, Sevestre H. Histological changes after implantation of autologous bone marrow mononuclear cells for chronic critical limb ischemia. *Bone marrow transplantation*. 2007; 39:647–648. [PubMed: 17384655]
 58. Duong Van Huyen JP, Smadja DM, Bruneval P, Gaussem P, Dal-Cortivo L, Julia P, Fiessinger JN, Cavazzana-Calvo M, Aiach M, Emmerich J. Bone marrow-derived mononuclear cell therapy induces distal angiogenesis after local injection in critical leg ischemia. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc*. 2008; 21:837–846.
 59. Hill JM, Zalos G, Halcox JP, Schenke WH, Waclawiw MA, Quyyumi AA, Finkel T. Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. *N Engl J Med*. 2003; 348:593–600. [PubMed: 12584367]
 60. Vasa M, Fichtlscherer S, Aicher A, Adler K, Urbich C, Martin H, Zeiher AM, Dimmeler S. Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. *Circ Res*. 2001; 89:E1–7. [PubMed: 11440984]
 61. Chen YL, Chang CL, Sun CK, Wu CJ, Tsai TH, Chung SY, Chua S, Yeh KH, Leu S, Sheu JJ, Lee FY, Yen CH, Yip HK. Impact of obesity control on circulating level of endothelial progenitor cells and angiogenesis in response to ischemic stimulation. *Journal of translational medicine*. 2012; 10:86. [PubMed: 22568992]
 62. Tepper OM, Galiano RD, Capla JM, Kalka C, Gagne PJ, Jacobowitz GR, Levine JP, Gurtner GC. Human endothelial progenitor cells from type ii diabetics exhibit impaired proliferation, adhesion, and incorporation into vascular structures. *Circulation*. 2002; 106:2781–2786. [PubMed: 12451003]
 63. Rauscher FM, Goldschmidt-Clermont PJ, Davis BH, Wang T, Gregg D, Ramaswami P, Pippen AM, Annex BH, Dong C, Taylor DA. Aging, progenitor cell exhaustion, and atherosclerosis. *Circulation*. 2003; 108:457–463. [PubMed: 12860902]
 64. Teraa M, Sprengers RW, Westerweel PE, Gremmels H, Goumans MJ, Teerlink T, Moll FL, Verhaar MC. Bone marrow alterations and lower endothelial progenitor cell numbers in critical limb ischemia patients. *PLoS One*. 2013; 8:e55592. [PubMed: 23383236]
 65. Subherwal S, Anstrom KJ, Jones WS, Felker MG, Misra S, Conte MS, Hiatt WR, Patel MR. Use of alternative methodologies for evaluation of composite end points in trials of therapies for critical limb ischemia. *American heart journal*. 2012; 164:277–284. [PubMed: 22980292]

66. Sherman W, Mazouz C, Deans R, Patel AN. Commercialization of trials for peripheral artery disease. *Cytotherapy*. 2011; 13:1157–1161. [PubMed: 21999371]
67. Losordo DW, Henry TD, Davidson C, Sup Lee J, Costa MA, Bass T, Mendelsohn F, Fortuin FD, Pepine CJ, Traverse JH, Amrani D, Ewenstein BM, Riedel N, Story K, Barker K, Povsic TJ, Harrington RA, Schatz RA. Intramyocardial, autologous cd34+ cell therapy for refractory angina. *Circ Res*. 2011; 109:428–436. [PubMed: 21737787]

Figure 1. Intramuscular Injection Protocol. Left panel: Schematic and actual injection locations mapped to 10 major muscle groups in the leg. Middle panel: Posterior injection locations. Right panel: Injection procedure.

Figure 2.

Trial design. G-CSF: granulocyte colony stimulating factor, IM: intramuscular IV: intravenous, GFR: glomerular filtration rate, ABI: ankle-brachial index.

Figure 3.
Baseline to 12 month change in 6 minute walk distance by intention to treat and actual treatment allocation in surviving subjects. (*paired, t-test)

Figure 4. Baseline to 12 month change in Walking Impairment Questionnaire (WIQ) Score by intention to treat and actual treatment allocation in surviving subjects. (*paired t-test)

Figure 5.
Baseline to 12 month change in SF36 categories by actual treatment allocation.

Figure 6.

Gene array assay for senescence associated genes. 6A. Compared to normal healthy donor CD133+ cells, CD133+ cell obtained from subjects randomized to the treatment arm (n=6) had 2 fold or greater expression in several senescence markers indicated above with horizontal p value cut off of 0.1. 6B. Subjects treated with CD133+ compared to mobilization failure subjects showed >2 fold upregulation in 4 of 5 genes expressing senescence markers and > 2 fold downregulation of 1 of 5 genes expressing an anti-senescence marker. Red vertical lines indicate 2-fold change in gene expression while the black horizontal line indicates a p value of 0.1. CLI: critical limb ischemia

Table 1

Baseline Characteristics

	Treatment (n=7)	Control (n=3)
Age mean	65	85
Male Gender n (%)	6 (86%)	2 (66%)
Presence of Ulcer n (%)	2 (29%)	1 (33%)
Presence of Diabetes n (%)	3 (43%)	1 (33%)
History of Tobacco abuse n(%)	6 (86%)	1 (33%)
Prior revascularization n (%)	3 (43%)	2 (66%)

Table 2

CD133+ yield, purity and viability. G-CSF: granulocyte colony stimulating factor, mcg: micrograms, HD: healthy donor

	Subject	#CD133+ Pre selection (10 ⁶)	#CD133+ Post selection (10 ⁶)	CD133+ Purity %	CD133+ Viability %
CD133+ Treated G-CSF 10mcg/kg/d × 5 days	1	437	99	81	100
	2	562	228	93.5	100
	3	177	80	92	98
Mobilized Untreated G-CSF 10mcg/kg/d × 5 days	4	33	18	63	90
	5	43	26	58	99
	6	23	n/a	n/a	n/a
	7	49	29	81	96
Healthy Donor G-CSF 5mcg/kg/d × 4 days	HD1	---	62.7	90	96
	HD2	---	138	95.2	98
	HD3	---	82.7	85.6	97

Table 3

Major clinical events at 12 months by intention to treat (A) and by actual treatment (B). G-CSF: granulocyte colony stimulating factor

A. Major Clinical Events By Intention to Treat at 12 months			
	Active Treatment (n=7)	Control (n=3)	p value
Major Amputation Free Survival	86%	33%	0.2
Overall Survival	100%	66%	0.3
Freedom from any amputation	57%	66%	1.0
Freedom from hospitalization	57%	0%	0.2

B. Major Clinical Events By Actual Treatment at 12 months			
	G-CSF + CD133 (n=3J)	G-CSF alone (n=4)	Control (n=3)
Major Amputation Free Survival	66%	75%	33%
Overall Survival	100%	100	66%
Freedom from any amputation	33%	75%	66%
Freedom from hospitalization	33%	75%	0%

Table 4

Per subject clinical events and senescence associated gene expression.

	Subject	Alive at 12 months	Amputation at 12 months	Ulcer Healing at 12 months	Change in walking distance (meters) compared to baseline at 6 months	CD133+ gene expression for senescence genes (>2 fold expression compared to healthy volunteers)
CD133+ Treated	1	Alive	Toe amputation	Ulcer at baseline– no healing	+85	Up regulated: CDKNA, COL1A1, GADD45A, IGF1, PLAUI, SERPINB2, THBS1, TWIST1 Down regulated: ERG 2, ID1, IFNG, SERPINE1
	2	Alive	Below Knee amputation	Ulcer at baseline– no healing	+175	Up regulated: ALB1, ALDH1A3, ATM, CALR, CCNB1, CCND1, CCNE1, CD44, CDC25C, CDK2, CDKN1A, CDKN1C, COL1A1, E2F3, ETS2, HRAS, IGF1, IGF1R, IGFBP3, IRF3,IRF7, MAP2K1, MAPSK3, MAPK14, MDM2, MORC3, NBN, NFKB1, PIK3CA, PIK3CA, PLAUI, PRKCD, RBL2, SIERT1, TERF2, TERT, TGFB1, TGFB1I1, THBS1, TP53BP1, VIM Down regulated: E12
	3	Alive	No Amputation	No Ulcer	–30	Up regulated: CCND1, CCNE1, CDC25C, CDKN1A, CDKN1C, COL1A1, COL3A1, ETS2, IF1, IGF1, MAP2K3, PLAUI, SERPINE1, TGFB1I1, THBS1, VIM Down regulated: IFNG, SERPINB2
Mobilization Failure	4	Alive	No Amputation	No Ulcer	+27	Up regulated: ALDH1A3, CDKN1A, CDKN1C, CDKN2B, CDKN2D, ID1, PIK3CA, PRKCD, SERPINB2, SOD2, SPARC, TGFB1, THBS1, TWIST Down regulated: CCNA2, COL3A1, PCNA, RBL1
	5	Alive	Toe amputation	Ulcer at baseline – no healing	0	Up regulated: CCNB1, CDKN1A, CDKN1C, COL1A1, IGF1, MAPSK3 PLAUI, PRKCD, TGFB1I1, THBS1, TWIST Down regulated: SERPINE1
	6	Alive	No Amputation	No Ulcer	–165	Up regulated: CCNB1, CDKN1A, CDKN1C, COL1A1, IGF1, MAP2K3, PLAUI, PRKCD, TGFB1I1, THBS1, TWIST Down regulated: SERPINE1
	7	Alive	No Amputation	No Ulcer	–62	Insufficient cells
Control	8	Death	No Amputation	No Ulcer	N/A (died)	N/A
	9	Alive	No Amputation	No Ulcer	–32	N/A

	Subject	Alive at 12 months	Amputation at 12 months	Ulcer Healing at 12 months	Change in walking distance (meters) compared to baseline at 6 months	CD133+ gene expression for senescence genes (>2 fold expression compared to healthy volunteers)
	10	Alive	Below knee amputation	Ulcer after enrollment – no healing	–50	N/A