Rationale and design of the Clinical and Histologic Analysis of Mesenchymal Stromal Cells in AmPutations (CHAMP) trial investigating the therapeutic mechanism of mesenchymal stromal cells in the treatment of critical limb ischemia

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ABSTRACT

Objective: Currently, there are no accepted nonsurgical therapies that improve the delivery of blood-derived nutrients to patients with critical limb ischemia. Here, we describe the ongoing phase 1/2 Clinical and Histologic Analysis of Mesenchymal Stromal Cells in AmPutations (CHAMP) trial, which will provide crucial evidence of the safety profile of mesenchymal stromal cells (MSCs) and explore their therapeutic mechanisms in the setting of critical limb ischemia requiring below-knee amputation (BKA).

Methods: In the CHAMP and the parallel marrowCHAMP trials (hereafter grouped together as CHAMP), a total of 32 extremities with rest pain or tissue loss requiring BKA will be enrolled to receive intramuscular injections of allogeneic MSCs (CHAMP; n = 16) or autogenous concentrated bone marrow aspirate (marrowCHAMP; n = 16) along the distribution of the BKA myocutaneous flap and proximal tibialis anterior. After treatment, subjects are randomized to BKA at four time points after injection (days 3, 7, 14, and 21). At the time of amputation, skeletal muscle is collected at 2-cm increments from the tibialis injection site and used to determine proangiogenic cytokine description, MSC retention, quantification of proangiogenic hematopoietic progenitor cells, and histologic description. Clinical limb perfusion before and after treatment will be quantified using transcutaneous oximetry, toe-brachial index, ankle-brachial index, and indocyanine angiography. Additional clinical end points include all-cause mortality, need for amputation revision, and gangrene incidence during the 6-month post-treatment follow-up.

Results: Enrollment is under way, with 10 patients treated per protocol thus far. We anticipate full conclusion of follow-up within the next 24 months.

Conclusions: CHAMP will be pivotal in characterizing the safety, efficacy, and, most important, therapeutic mechanism of allogeneic MSCs and autogenous concentrated bone marrow aspirate in ischemic skeletal muscle. (J Vasc Surg 2018;68:176-81.)

Critical limb ischemia (CLI) refers to a pathologic state in which blood flow to the leg is insufficient to deliver the resting metabolic needs of skeletal muscle. Unfortunately, those who are not candidates for surgical revascularization, the "gold standard" therapy, are at a higher risk of amputation secondary to refractory pain or persistent infection.[1] Cell-based therapies represent a potential treatment for patients without a suitable revascularization option, but the mechanism of action by which this occurs has yet to be clearly defined. Intramuscular (IM) injection of concentrated bone marrow aspirate (cBMA), containing $CD34^+$ proangiogenic hematopoietic progenitor cells (PHCs) and mesenchymal stromal cells (MSCs), has shown promise in small case series and large randomized controlled trials alike.[2-6] However, the source of cBMA must be autogenous, given a robust host rejection response, and therefore it is limited by the comorbidities of the treated patient. MSCs, as an isolated treatment product, potentially represent a more efficacious therapy as they do not constitutively express major histocompatibility complex (MHC) class II molecules and largely escape host immune recognition, allowing the use of healthy allogeneic donors for diseased hosts.[7]

METHODS

Synopsis. Clinical and Histologic Analysis of Mesenchymal Stromal Cells in AmPutations (CHAMP) is a

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Table I. Inclusion and exclusion criteria for the Clinical and Histologic Analysis of Mesenchymal Stromal Cells in AmPutations (CHAMP) trial

Inclusion	Exclusion
Age between 40 and 80 years	Evidence of infection • Suppurative gangrene: purulence emanating from gangrenous areas, with or without compression • Spreading cellulitis: proximal progression of erythema from wound within 24 hours • Systemic manifestations of infection: WBC count >11,500 cells (with antibiotic therapy >24 hours), increasing oxygen requirements or pulmonary failure, pressor support, change in creatinine concentration >50% from baseline
Requires BKA, as determined by an independent vascular surgeon, for rest pain, nonsuppurative gangrene, or tissue loss	Ulceration or gangrene due to venous insufficiency or lymphedema
Resting ABI ≤0.55 or TBI ≤0.40	Patent superficial femoral artery as determined by duplex ultrasound, angiography (including CT), or MRI within 6 months before enrollment
$TcPO_2$ at the calf ≤40 mm Hg	Patients who are pregnant, planning to become pregnant in the next 12 months, or lactating
Tissue loss distal to the malleoli	Hospitalization for congestive heart failure exacerbation within the last 1 month before enrollment
BKA can be safely performed up to 30 days after screening	Acute coronary syndrome in the last 1 month before enrollment
Women of childbearing potential must be willing to use one form of birth control for the duration of the study. Female participants must undergo a blood or urine pregnancy test at screening.	HIV positive, active HBV or HCV
	History of cancer within the last 5 years, except basal cell skin carcinoma
	Any bleeding diathesis, defined as an INR ≥2.0 (off anticoagulation therapy) or history of platelet count <70,000 or hemophilia
	Any condition requiring immunosuppressant medications
	Presence of any clinical condition that in the opinion of the PI or the sponsor makes the patient not suitable to participate in the trial

ABI, Ankle-brachial index; *BKA,* below-knee amputation; *CT,* computed tomography; *HBV,* hepatitis B virus; *HCV,* hepatitis C virus; *HIV,* human immunodeficiency virus; *INR,* international normalized ratio; *MRI,* magnetic resonance imaging; *PI,* principal investigator; *TBI,* toe-brachial index; *$TcPO_2$,* transcutaneous oximetry; *WBC,* white blood cell count.

single-center, phase 1/2, open-label clinical trial that will enroll 32 total CLI patients requiring semielective below-knee amputation (BKA) within 30 days for rest pain or dry gangrene. The Indiana University Institutional Review Board (No. 1510579216A015) reviewed and approved the trial protocol, which was designed in concordance with the latest iteration of the Declaration of Helsinki.[8] After informed consent is obtained, participants receive IM injections of autogenous cBMA or allogeneic MSCs along the distal thigh, the gastrocnemius, and the proximal aspect of the tibialis anterior before randomization to BKA and tissue harvest at four time points within 21 days.

cBMA and MSC preparation. The protocol for harvest, concentration, and injection of cBMA has been described by our group in detail elsewhere.[3] In short, a large-bore needle is inserted into the superior iliac crests under local anesthesia, and approximately 330 mL of bone marrow is aspirated. The MarrowStim PAD Kit (Zimmer Biomet, Warsaw, Ind) is used to create 33 mL of cBMA from this dilute suspension through centrifugation. Small aliquots (~1 mL) of the treatment product are injected into the muscle beds of the BKA myocutaneous flap and proximal tibialis anterior at set points. Subjects enrolled into the MSC cohort are typed for human leukocyte antigen A2 (HLA-A2) before selection of appropriately mismatched MSCs to allow ease of identification between host and donor at the time of tissue harvest through fluorescent in situ hybridization. HLA-$A2^-$ subjects will receive HLA-$A2^+$ donor cells, and HLA-$A2^+$ patients will receive gender-mismatched donor cells.

All MSCs in the treatment product are isolated from young, healthy donors at the Center for Advanced Cellular Therapeutics (University of Louisville). Mononuclear cells from the aspirate are cultured in 175-cm^2 flasks. Adherent cells are isolated, proliferated, and collected at passage two to ensure homogeneity of

Table II. Follow-up protocol for the Clinical and Histologic Analysis of Mesenchymal Stromal Cells in AmPutations (CHAMP) trial

Evaluation	Baseline	MSC injection	BKA (day varies)	Day 3	Day 14	Day 45	Week 12	Week 18	Week 24
Informed consent	X								
Medical history	X								
Physical examination	X	X	X	X	X	X	X	X	X
Vital signs	X	X	X	X	X	X	X	X	X
Medication list	X	X		X	X	X	X	X	X
Serum pregnancy test	X								
Adverse event evaluation	X	X	X	X	X	X	X	X	X
Infectious disease laboratory workup	X								
General laboratory workup	X		X			X	X	X	X
12-Lead ECG	X		X			X	X	X	X
Lower extremity arterial duplex ultrasound	X								
Randomization: time to BKA	X								

BKA, Below-knee amputation; *ECG*, electrocardiography; *MSC*, mesenchymal stromal cell.
The following tests are performed at baseline: baseline blood tests (complete blood count, complete metabolic panel); infectious disease panel; medical history and physical examination; medication history; pregnancy test; 12-lead electrocardiography. Additional noninvasive clinical testing of the ischemic limb includes ankle-brachial index, toe-brachial index, transcutaneous oximetry, and SPY Elite angiography with intravenous administration of indocyanine.

treatment product. MSC phenotype is confirmed by fluorescence-activated cell sorting observation of CD73, CD90, and CD105 coexpression.[9]

Inclusion and exclusion, randomization, and treatment. All patients with CLI between the ages of 40 and 80 years, without clinical evidence of infection, requiring BKA within 30 days are eligible for inclusion into the cBMA or MSC cohorts (Table I). Screening protocols, treatment timing, and follow-up schedule are detailed in Table II. Patency of lower extremity arteries is not routinely performed as part of the CHAMP screening protocol.

Computer-assisted, nonstratified randomization is performed to assign patients to BKA and tissue harvest at days 3, 7, 14, or 21 after injection (Fig 1). Cells are administered into 25 different sites within the gastrocnemius and distal thigh (Fig 2) muscle beds that compose the BKA myocutaneous stump. An additional injection point at a fixed point from the tibial tuberosity is performed in the most proximal aspect of the tibialis anterior for downstream mechanistic analysis. Tissue from the untouched soleus of the deep posterior compartment serves as an internal control.

Tissue harvest. Before BKA on the assigned day, the patient is subjected to a clinical limb perfusion evaluation with ankle-brachial index, toe-brachial index, transcutaneous oximetry, and indocyanine angiography for comparison to baseline studies. Intraoperatively, at least four muscle samples from the tibialis anterior in 2-cm increments distal to the cellular injection point are collected for downstream applications. Amputation proceeds in study patients in the standard fashion for our institution, consisting of the anterior rotation of a posterior compartment myocutaneous flap.

End points and follow-up. Through a review of adverse events by clinic visits during 6 months after the procedure, we plan to test the hypothesis that autogenous cBMA and allogeneic MSCs do not result in significant cardiovascular, respiratory, or infectious complications (Table II). Peripheral blood is also collected from the patients at days 3, 14, 45, 90, 135, and 180 and compared with baseline test results to determine changes in peripheral cytokine signaling, microRNA expression, and proangiogenic and inflammatory mononuclear phenotypes. Tissue collected at time of amputation will be subject to multiplex arrays, immunohistochemistry, fluorescence-activated cell sorting, and fluorescent in situ hybridization. In this way, we can characterize histologic appearance of muscle, local proangiogenic cytokine expression, inflammatory cell infiltrates, donor MSC retention, and the clinically important measure of capillary density at various time points after treatment. The plan for statistical analysis of data points isolated is detailed in the Supplementary Methods (online only).

Additional clinical investigations will be made into the clinical efficacy of MSCs and cBMA in promoting freedom from gangrene, above-knee amputation conversion, and death after BKA. Treatment groups will be compared with patients who qualified for CHAMP but declined participation.

Current status. Recruitment for CHAMP is ongoing, and the first 10 patients have been treated per protocol.

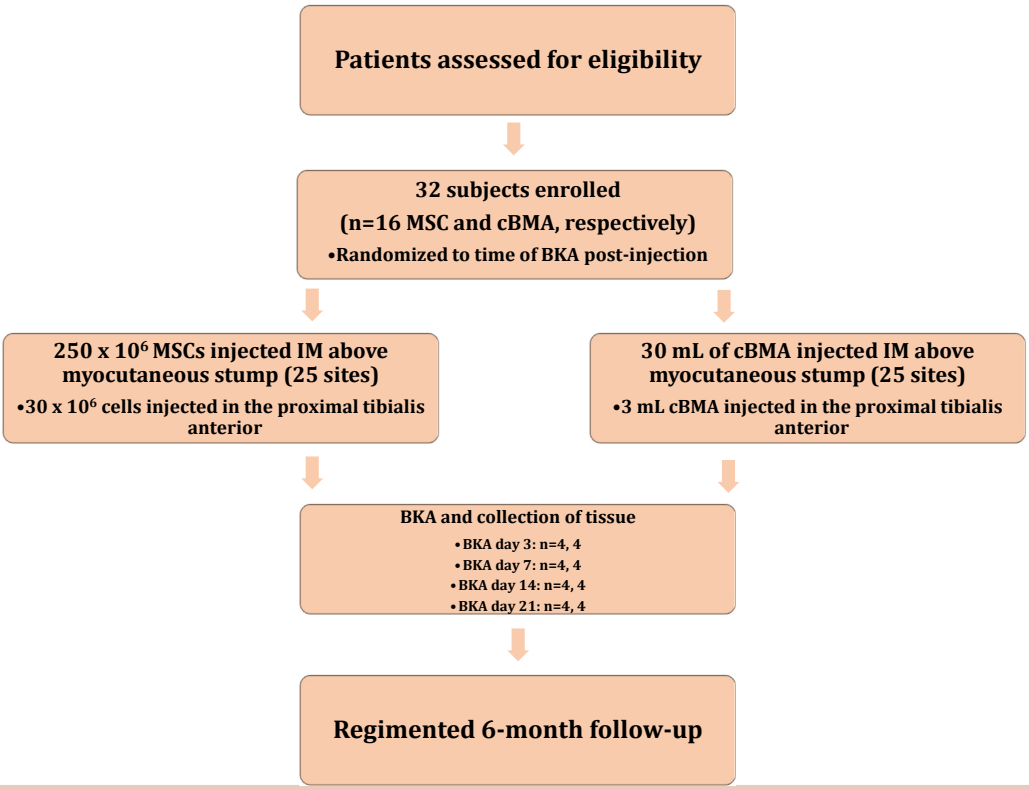

Fig 1. Trial design of Clinical and Histologic Analysis of Mesenchymal Stromal Cells in AmPutations (CHAMP). After screening, patients are enrolled in either the concentrated bone marrow aspirate (*cBMA*) or mesenchymal stromal cell (*MSC*) arm of CHAMP. Subjects are randomized to one of four time points within 21 days of cell therapy administration. At that time, below-knee amputation (*BKA*) is performed and tissue is harvested. All patients are observed for 6 months after amputation. *IM,* Intramuscularly.

Anticipated conclusion of recruitment, treatment, and follow-up will be within 24 months.

DISCUSSION

When amputation is required, below-knee creation of a myocutaneous stump is preferred as knee preservation decreases energy expenditure for prosthesis-assisted ambulation.[10,11] However, contemporary proximal conversion rates secondary to stump ischemia are reported at 20%, creating massive morbidity for the patient and increasing the overall health care burden.[11-13] Therefore, the effort to reduce BKA failure represents an important area of research for all patients with CLI.

CHAMP will be decisive in characterizing the biologic activity of allogeneic MSCs in human tissue. The idea for this study matured secondary to completion of our previous open-label phase 1 trial investigating the utility of IM injections of cBMA containing MSCs into 30 limbs with nonrevascularizable CLI. We found that these patients exhibited 1-year and 5-year amputation free survival rates of 86% and 74%, respectively, which is comparable to the CLI population that receives surgical revascularization.[6] In addition, we noted improvements in ankle-brachial index, transcutaneous oximetry, and rest pain. Results from that preliminary study allowed us to initiate a multicenter randomized controlled phase 2 study (MarrowStim PAD Kit for the Treatment of Critical Limb Ischemia in Subjects with Severe Peripheral Arterial Disease [MOBILE] trial), which demonstrated a statistically significant ability of cBMA to prevent major

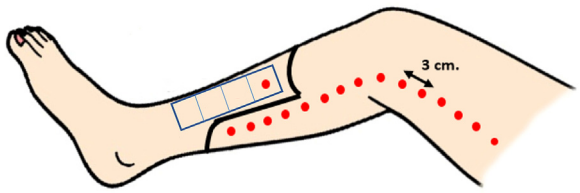

Fig 2. Simplified treatment plan of the Clinical and Histologic Analysis of Mesenchymal Stromal Cells in AmPutations (CHAMP) trial. A total of 280 million allogeneic mesenchymal stromal cells (MSCs; 33 mL of concentrated bone marrow aspirate [cBMA]) are injected into the muscle beds of patients undergoing below-knee amputation (BKA; *dots*); 250 million MSCs (30 mL of cBMA) are injected proximal to the myocutaneous flap along the gastrocnemius and distal thigh muscle beds. One additional site in the proximal tibialis anterior is injected with 30 million MSCs (3 mL of cBMA). Tissue harvest sites of the tibialis anterior at time of BKA are denoted (*rectangle*) along the length of the muscle bed in 2-cm increments. Untouched soleus muscle will serve as an internal tissue control and is harvested concurrently.

amputation in nonrevascularizable CLI patients with the caveat that they are not diabetic and do not have Rutherford class 5 disease. The number needed to treat to prevent one amputation was merely six patients.[3]

Although these studies reported promising results, several glaring limitations have prevented the advancement and adoption of cell-based therapy as a widespread treatment modality. First, isolation of autologous cells requires a harvest procedure under anesthesia, placing CLI patients, often of advanced age and severe cardiovascular comorbidities, at risk for additional complications. Second, autologous stem cells from patients with cardiovascular disease have shown limited neovascularization ability in experimental stroke and heart disease models compared with healthy controls.[14-16]

MSCs, on the other hand, can be sourced from healthy allogeneic donors and injected into diseased limbs because of reduced constitutive MHC class II molecule expression, bypassing the limitations of cell-based therapies.[7] This readily available, unique subset of cells has demonstrated the ability to induce angiogenesis, to decrease muscle fiber apoptosis, and to stimulate re-epithelialization of wound beds.[17-19] Our preliminary unpublished in vitro experiments indicated that MSCs secrete increased quantities of the proangiogenic cytokines vascular endothelial growth factor, hepatocyte growth factor, and angiopoietin 1 in response to hypoxic conditions. However, the current overall understanding of the bioactivity of this subset of stem cells arises entirely from in vitro or animal studies, which are limited by poor generalizability and genomic discordance in pathologic conditions.[17,20,21]

The lack of clarity regarding MSC bioactivity in ischemic human tissue continues to be a problem despite multiple clinical trials.[22-24] Initially, MSCs were thought to engraft, differentiate, and replace damaged tissue. However, subsequent work has established low levels of engraftment (<0.1%) of donor cells.[20] Current opinion reflects a mechanism of crosstalk between MSCs and injured cells to limit tissue destruction and to enhance repair. Specifically, this phenomenon may occur secondary to secretion of bioactive proteins that act in a paracrine or autocrine fashion, upregulation of genes that inhibit excessive inflammatory and immune reactions, and transfer of vesicular components that contain mitochondria and microRNA (exosomes).[21]

The MSC dosage selected in CHAMP was based on mouse models (by body surface area) and previous clinical trials, notably Percutaneous Stem Cell Injection Delivery Effects on Neomyogenesis (POSEIDON), in which MSCs were directly injected into the myocardium.[22] Therefore, it is entirely possible that we may not observe efficacy secondary to inadequate dosing. In addition, the primary goal of this phase 1/2 trial is to assess safety, and therefore it is not powered for clinical efficacy end points.

To describe the bioactivity of allogeneic MSCs in ischemic muscle, we plan to define donor MSC retention over time, host immune responses, recruitment of PHCs, changes in capillary density, and variation of skeletal muscle fiber morphology by histology. Based on previous animal models, we anticipate that the donor population will be significantly depleted by day 7 as MSCs begin to upregulate the expression of MHC class II antigen.[21] A peak infiltration of PHCs in the more perfused regions of the anterior tibialis should occur concurrently to peak cytokine concentration around this time. Capillary formation should continue to expand secondary to PHC recruitment until the last time point at 21 days.

CHAMP is designed to deliver critical evidence of the safety profile of IM injection of MSCs in CLI. Tissues harvested from the participants receiving both cBMA and MSCs will provide data that may elucidate the MSC therapeutic mechanism. Comparison to concurrent non-CHAMP risk factor-matched BKA patients at the same institution will provide controls to assess improvements in wound complications. As the next iteration after the conclusion of CHAMP, we hope to proceed with a larger, randomized controlled trial with escalating MSC dosages powered to provide outcomes data for real-world use.

CONCLUSIONS

Because CHAMP aims to harvest human tissue after IM injection of MSCs and cBMA at time points relevant to tissue reparation and cell survival, it will have the unique ability to provide a vital description of the in vivo biologic activity of MSCs and cBMA in ischemic limbs undergoing BKA.

AUTHOR CONTRIBUTIONS

Conception and design: SW, LG, ND, RM, AF, MM
Analysis and interpretation: SW, LG, ND, RM, AF, MM
Data collection: SW, LG, ND, RM, AF, MM
Writing the article: SW, ND, MM
Critical revision of the article: SW, LG, ND, RM, AF, MM
Final approval of the article: SW, LG, ND, RM, AF, MM
Statistical analysis: SW, MM
Obtained funding: MM
Overall responsibility: SW

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APPENDIX (online only).

Supplementary Methods

Safety and efficacy. Adverse events are divided into serious adverse events and major adverse cardiac events and categorized by systems (cardiovascular, respiratory, or infectious). Proportions of patients suffering adverse events will be compared with a historical control cohort by the Wilson score interval method. Rates of our composite end points, death at 6 months, conversion to above-knee amputation, and incidence of stump gangrene, will be compared with a historical control cohort with continuous confidence intervals at the 95% level. The critical levels for the multiplicity adjustment will be determined by simple Monte Carlo simulation.[1]

Mesenchymal stromal cell retention and activity. To estimate half-life of mesenchymal stromal cells, quantity over time will be fit to an exponential decay curve using a residual pseudolikelihood procedure.[2] Continuous confidence intervals at the 95% level will be calculated at all four time points for the $CD34^{+}CD133^{+}$ proangiogenic hematopoietic progenitor cells, capillary density, proangiogenic cytokines, and muscle fiber morphology. The correlation between capillary density (CD31 count) and tissue perfusion (indocyanine angiography) at all time points will be estimated by Spearman rank coefficient.[3]

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