From the New England Society for Vascular Surgery

Early results and lessons learned from a multicenter, randomized, double-blind trial of bone marrow aspirate concentrate in critical limb ischemia

Mark D. Iafrati, MD,[a] John W. Hallett, MD,[b] George Geils, MD,[b] Gregory Pearl, MD,[c]
Alan Lumsden, MD,[d] Eric Peden, MD,[d] Dennis Bandyk, MD,[e] K. S. Vijayaraghava, MD,[f]
R. Radhakrishnan, MD,[f] Enrico Ascher, MD,[g] Anil Hingorani, MD,[g] and Sean Roddy, MD,[h] *Boston, Mass; Charleston, SC; Dallas and Houston, Tex; Tampa, Fla; Chennai, India; and Brooklyn and Albany, NY*

Objectives: Despite advances in endovascular therapies, critical limb ischemia (CLI) continues to be associated with high morbidity and mortality. Patients without direct revascularization options have the worst outcomes. We sought to explore the feasibility of conducting a definitive trial of a bone marrow-derived cellular therapy for CLI in this "no option" population.
Methods: A pilot, multicenter, prospective, randomized, double-blind, placebo-controlled trial for "no option" CLI patients was performed. The therapy consisted of bone marrow aspirate concentrate (BMAC), prepared using a point of service centrifugation technique and injected percutaneously in 40 injections to the affected limb. Patients were randomized to BMAC or sham injections (dilute blood). We are reporting the 12-week data.
Results: Forty-eight patients were enrolled. The mean age was 69.5 years (range, 42-93 years). Males predominated (68%). Diabetes was present in 50%. Tissue loss (Rutherford 5) was present in 30 patients (62.5%), and 18 (37.5%) had rest pain without tissue loss (Rutherford 4). Patients were deemed unsuitable for conventional revascularization based on multiple prior failed revascularization efforts (24 [50%]), poor distal targets (43 [89.6%]), and medical risk (six [12.5%]). Thirty-four patients were treated with BMAC and 14 with sham injections. There were no adverse events attributed to the injections. Renal function was not affected. Effective blinding was confirmed; blinding index of 61% to 85%. Subjective and objective outcome measures were effectively obtained with the exception of treadmill walking times, which could only be obtained at baseline and follow-up in 15 of 48 subjects. This pilot study was not powered to demonstrate statistical significance but did demonstrate favorable trends for BMAC versus control in major amputations (17.6% vs 28.6%), improved pain (44% vs 25%), improved ankle brachial index (ABI; 32.4% vs 7.1%), improved Rutherford classification (35.3% vs 14.3%), and quality-of-life scoring better for BMAC in six of eight domains.
Conclusions: In this multicenter, randomized, double-blind, placebo-controlled trial of autologous bone marrow cell therapy for CLI, the therapy was well tolerated without significant adverse events. The BMAC group demonstrated trends toward improvement in amputation, pain, quality of life, Rutherford classification, and ABI when compared with controls. This pilot allowed us to identify several areas for improvement for future trials and CLI studies. These recommendations include elimination of treadmill testing, stratification by Rutherford class, and more liberal inclusion of patients with renal insufficiency. Our strongest recommendation is that CLI studies that include Rutherford 4 patients should incorporate a composite endpoint reflecting pain and quality of life. (J Vasc Surg 2011;54:1650-8.)

Critical limb ischemia (CLI), has a 1-year mortality of approximately 25%, with major limb amputations afflicting another 30%.[1] Surgical and endovascular recanalization improves perfusion, pain, quality of life, and limb preserva-

From the Division of Vascular Surgery, Tufts Medical Center, Boston[a];
Roper S. Francis Medical Center, Charleston[b]; Division of Vascular Surgery, Baylor Heart and Vascular Hospital, Dallas[c]; Division of Vascular Surgery, Methodist Hospital, Houston[d]; Division of Vascular Surgery, University of South Florida, Tampa[e]; Division of Vascular Surgery, Sri Rmachandra Medical College and Research Institute, Chennai[f]; Division of Vascular Surgery, Maimonides Medical Center, Brooklyn[g]; and Division of Vascular Surgery, Vascular Group, Albany.[h]

tion in the majority of patients. However, 25% to 40% of CLI patients are not candidates for revascularization.[1-3]

In 1997, Asahara identified a class of bone marrow-derived, circulating endothelial progenitor cells that contribute to angiogenesis in ischemic tissue,[4] while Tateishi-Yuyama was the first to report the use of autologous bone marrow-derived cells (BMDCs) to treat CLI in a human trial. They used patients with bilateral ischemia as their own controls and compared bone marrow-derived mononuclear cells (BMDMC) versus saline or peripherally derived cells and showed improvement in objective and subjective measures of perfusion.[5] Since then, numerous studies have reported the safety and feasibility of autologous BMDMCs in CLI. The majority of these were case series,[6-27] with few randomized, controlled trials.[5,28-33] While there have been a small number of blinded studies in the cardiac literature,[34-37] Lu and colleagues provided the first well-controlled and blinded study in diabetic patients with CLI and ulcers. This study of 41 patients found bone marrow mesenchymal stem cells to be superior to mononuclear cells or normal saline in improving perfusion and ulcer healing.[33] Initial studies of cellular thera-

pies utilized time- and resource-intensive Ficoll density gradient separation techniques to isolate the target cells, with or without subsequent cell expansion, whereas several recent reports, including this study utilized automated point of service centrifugation techniques.[6,17,32]

Robust trials for cellular therapy in CLI are complicated by severe comorbidities, challenges of blinding, inclusion criteria, and choice of outcome measures. In order to access these trial-related issues as well as the safety of cellular therapy for CLI, we undertook a Food and Drug Administration (FDA)-cleared pilot study.

METHODS

Patients. Patients had chronic CLI, including rest pain (Rutherford class 4 [R4]) or mild-to-moderate tissue loss (Rutherford class 5 [R5]) and were not candidates for surgical or endovascular revascularization. Patients were determined to be poor candidates for conventional therapy based on anatomic (target vessel, conduit, failed bypasses, long or calcified occlusions) or physiological considerations, attested to by two vascular surgeons. Hemodynamic parameters included one of the following: ankle pressure <50 mm Hg or ABI <0.4; toe pressure <40 mm Hg or toe brachial index (TBI) <0.4; or tissue oxygen concentration ($TcPO_2$) < 20 mm Hg on room air. Patients with noncompressible ankle brachial indices (ABIs) were evaluated based on TBI or $TcPO_2$.

Exclusion criteria included: severe tissue loss (Rutherford class 6); creatinine ≥2.0 mg/dL or dialysis; ABI = 0; uncorrected iliac disease in the index limb; active infection; active malignancy; major cardiovascular procedure or myocardial infarction within 3 months; stroke within 6 months; bone marrow or hematologic disorders; uncontrolled diabetes (HgbA1C ≥ 10%); or hyperbaric oxygen therapy within 30 days.

Subjects underwent cancer (prostate, cervical, breast, lung, colon) and ophthalmologic screenings. The institutional review boards of all participating sites approved the study.

METHODS

The study procedure involved aspiration and processing of bone marrow and injection of BMAC or sham injectate into the study limb. Procedures were carried out under conscious sedation. Local anesthesia was utilized for the bone marrow harvest. For patients randomized to cell therapy, 120 mL of marrow was withdrawn from each iliac crest. For control patients, the iliac crest was punctured bilaterally and 2 mL withdrawn to maintain blinding. Marrow aspirate was processed using the SmartPReP2 Bone Marrow Aspirate Concentrate system (Harvest Technologies, Plymouth, Mass). This point-of-care system consists of an automated centrifuge that concentrates marrow by a gradient centrifugation method. The processing was carried out in the operating room (14 minutes). Aliquots (2 mL) of BMA and BMAC were collected and later analyzed for cell counts. The concentrate (40 mL) was loaded into syringes for injection into the index limb. Ten milliliters of peripheral blood was withdrawn from all patients at the time of intravenous placement; for control subjects this was

diluted 3:1 and presented in a syringe for intramuscular injection. Medial and or anterior linear injection patterns on the lower leg were selected based on preoperative imaging to replicate a "biologic bypass." Under ultrasound guidance, 40 1-mL intramuscular injections, spaced 1 to 2 cm apart, 5 to 10 mm from the popliteal, tibial, and/or pedal arteries, extended distally to the area of tissue loss or arterial reconstitution.

Randomization and blinding. Patients were randomized 2:1, investigational treatment or placebo using block stratification by investigational site, diabetes, and renal function (creatinine clearance ≤40 mg/dL). Forty-eight patients were enrolled: 34 cell therapy, 14 placebo. This study was FDA approved as a pilot and not powered for statistical significance. Study group assignment was revealed, to the individual performing the bone marrow aspiration, in the operating room after prepping and draping. Bilateral iliac punctures were performed. Treatment patients had 240 mL marrow aspirated versus 2 mL in controls. Following centrifugation of the aspirate (treatment group) or sham operation of the centrifuge (control), the unblinded physician and study coordinator left the procedure room and the blinded vascular surgeon and coordinator entered. The surgeon was presented with four syringes for injection without knowing their contents. The sham injectate has color and consistence indistinguishable from BMAC, and, like BMAC, will clot if inadvertently dripped in the field. Effectiveness of blinding was assessed by querying the patients and clinicians after the procedure and at the conclusion of the study. Although an interim analysis was performed at 3 months per protocol, blinding will be maintained during the 5-year follow-up.

Follow-up and outcome measurements. Patients were evaluated at 1, 4, 8, and 12 weeks postprocedure. Follow-up for 5 years is planned. Evaluation included subjective and objective measures of clinical, hemodynamic, and functional outcomes. Clinical outcomes included amputation, Rutherford classification, visual analog pain scale, and walking distance. Functional outcome was evaluated using the Rand-36 questionnaire. Hemodynamic outcome was evaluated by ABI and $TcPO_2$. Laboratory monitoring of hematology and blood chemistries was performed. Retinal examination was performed by an ophthalmologist at baseline and 3 months in diabetics.

RESULTS

Patient baseline characteristics are presented in Table I. All randomized patients received the prescribed treatment and presented for 1-week follow-up. Four subjects missed their 12-week visit but were available for subsequent evaluation, and thus their survival and amputation were included although hemodynamic and questionnaire data were missing. Missing data were handled using the last observation carried forward (LOCF) method.

Procedure

Marrow aspiration, processing, and injections were accomplished in the operating room at a single visit. Patients

Table I. Baseline characteristics of enrolled patients

	Bone marrow aspirate concentrate	*Control*	*Total*
Patients	34	14	48
Age (years)	72.5 (42-93)	65.7 (52-85)	69.5 (42-93)
Male	23 (67.6%)	9 (64.3%)	32 (66.7%)
Race			
Asian	5 (14.7%)	1 (7.1%)	6 (12.5%)
Black/African American	4 (11.8%)	6 (42.9%)	10 (20.8%)
Caucasian	25 (73.5%)	7 (50.0%)	32 (66.7%)
Tobacco use			
Never used	9 (26.5%)	6 (42.9%)	15 (31.3%)
Previous user	19 (55.9%)	8 (57.1%)	27 (56.3%)
Current user	6 (17.6%)	0 (0%)	6 (12.5%)
Diabetes mellitus			
Type 1 diabetes	1 (2.9%)	1 (7.1%)	2 (4.2%)
Type 2 diabetes	17 (50.0%)	5 (35.7%)	22 (45.8%)
Renal insufficiency (Cr clearance <40 mg/dL)	3 (8.8%)	1 (7.1%)	4 (8.3%)
Cardiac disease			
Angina	4 (11.8%)	3 (21.4%)	7 (14.6%)
Myocardial infarction	11 (32.4%)	2 (14.3%)	13 (27.1%)
Congestive heart failure	7 (20.6%)	4 (28.6%)	11 (22.9%)
Coronary revascularization			
Coronary artery bypass	11 (32.4%)	2 (14.3%)	13 (27.1%)
Coronary angioplasty	5 (14.7%)	6 (42.9%)	11 (22.9%)
Rutherford class			
Rutherford 4	11 (32.4%)	7 (50.0%)	18 (37.5%)
Rutherford 5	23 (67.6%)	7 (50.0%)	30 (62.5%)
Ankle brachial index	0.46 (0.13-1.23)	0.40 (0.17-0.74)	0.44 (0.13-1.23)
Previous amputation (contralateral)			
Major amputation	7 (20.6%)	1 (7.1%)	8 (16.7%)
Minor amputation	6 (17.6%)	2 (14.3%)	8 (16.7%)
Previous lower extremity revascularization			
Surgical bypass	23 (67.6%)	8 (57.1%)	31 (64.6%)
Angioplasty	8 (23.5%)	7 (50.0%)	15 (31.2%)
Stent	5 (14.7%)	6 (42.9%)	11 (22.9%)
Atherectomy	2 (5.9%)	4 (28.6%)	6 (12.5%)
Reason for no option status			
Failed revascularization	18 (52.9%)	6 (42.9%)	24 (50.0%)
Anatomic poor candidate	31 (91.2%)	12 (85.7%)	43 (89.6%)
Medical high risk	5 (14.7%)	1 (7.1%)	6 (12.5%)
Medication			
Cholesterol lowering agent	24 (70.6%)	11 (78.6%)	35 (72.9%)
Beta blocker	15 (44.1%)	5 (35.7%)	20 (41.7%)
Antiplatelet agent	28 (82.3%)	14 (100%)	42 (87.5%)

tolerated the procedure well, transient deep sedation was utilized to facilitate the injections, and none required intubation. There were no complications on the day of treatment. The mean number of nucleated cells injected per patient was 3.23×10^9 cells (range, 0.88-7.44×10^9). Cell counts did not correlate with age, diabetes, or outcome but did correlate with Hct.

Safety and adverse events

There were no deaths or severe unexpected adverse events during this 3-month reporting period. Bone marrow aspiration was well tolerated with no complications. Hematocrit decreased by 2.6% in the BMAC group (Table II), and no patient was transfused. Injections were well tolerated with no infections, ulceration, or persistent pain. One control patient experienced edema of the index limb following injection of placebo, without venous thrombosis. Muscle injury due to the intramuscular injections (rhabdo-

myolysis) was not observed clinically or chemically (creatine phosphokinase [CPK]). The mean CPK for the cell therapy group decreased by 31% 1 week after treatment and increased by 7% in the controls. Renal function remained stable in both groups (Table II).

No patient demonstrated inappropriate angiogenesis. Ophthalmologic examinations at screening demonstrated baseline proliferative retinopathy in four patients, but there were no cases of worsened or new retinopathy. There were no clinically evident cases of new or recurrent malignancy.

Efficacy and outcomes

Amputations. There were 10 major amputations during the 3-month period (Table III). All major amputations occurred in patients who were Rutherford 5 at screening. Among patients who were R5 at baseline, major amputations occurred in 26.1% of BMAC versus 57.1% of controls

Table II. Periprocedure laboratory values: Mean (range)

	Bone marrow aspirate concentrate	*Control*
Hematocrit (%)		
Screening	35.9 (27.7-42.7)	36.2 (28.7-42.9)
Day 7	33.4 (24.5-42.0)	36.0 (30.0-44.0)
Mean change	−2.6 (−7.0-2.6)	−0.1 (−5.8-4.5)
Creatine phosphokinase (u/L)		
Screening	103.8 (11.0-490.0)	99.0 (37.0-234.0)
Day 7	71.3 (10.0-281.0)	107.0 (5.2-237.0)
Mean change	−33.9 (−430.0-77.0)	+5.5 (−50.0-64.0)
Serum creatinine (mg/dL)		
Screening	1.1 (0.6-2.0)	1.0 (0.7-1.2)
Day 7	1.1 (0.5-1.8)	1.1 (0.7-1.5)
Mean change	0.1 (−0.1-0.4)	0.1 (−0.1-0.4)

Table III. Major amputations (3 month): Bone marrow aspirate concentrate versus control

	Amputations	*Total*	*% Amputations*	*P value*[a]
All patients	10	48	20.8%	
Bone marrow aspirate concentrate	6	34	17.6%	0.448
Control	4	14	28.6%	

Major amputations are above- or below-knee amputations.
[a]*P* calculated using Fisher's exact test, two-tailed.

Table IV. Change in Rutherford classification

	Improved	*Not improved*	*% Improved*	*P value*[a]
Bone marrow aspirate concentrate	12	22	35.3%	.18
Control	2	12	14.3%	
R4	10	8	55.6%	.003
R5	4	26	13.3%	

R4, Ischemic rest pain; *R5*, limited tissue loss.
[a]*P* values calculated using Fisher's exact test, two-tailed.

Table V. Outcome measures (pain and composite)

	Bone marrow aspirate concentrate	*Control*	P
Pain			.54
Improved	11/25 (44.0%)	2/8 (25.0%)	
Stable	12/25 (48.0%)	6/8 (75.0%)	
Worsened	2/25 (8.0%)	0/8 (0%)	
Composite endpoint			.11
Success	17/34 (50%)	3/14 (21.4%)	
Failure	17/34 (50%)	11/14 (78.6%)	

Pain is self-reported using a 100-mm visual analog scale.
Changes at 12 weeks compared to baseline of >30 mm are reported as "improved" or "worsened."
Changes of <30 mm are "stable."
Composite outcome: to be a "success," a subject must: A, be alive; B, be without a major amputation on the index limb; C, have not worsened in Rutherford classification or visual analog pain scale; and D, have improved in either Rutherford classification or visual analog pain scale. Subjects not meeting all of the criteria were classified as failures.

(P = .18). Minor amputations were performed in seven patients (four BMAC, three controls). Three minor amputations were subsequently revised to major amputations (one BMAC, two controls).

Rutherford class. All patients were Rutherford 4 or 5 at screening. Improvement in Rutherford score required complete wound healing (R5 → R4) or resolution of rest pain (R4 → R3, 2, 1), whereas the development of rest pain or irretrievable tissue loss (R6) represented a worsening of the score. All major amputations were recorded as worsened Rutherford score (Table IV).

Pain. Pain was assessed using a 100-mm VAS. An absolute change of 30 mm on the VAS from baseline was required to denote either improvement or worsening. Table V reports these data for patients without major amputation.

Walking distance. We endeavored to evaluate walking distance by treadmill testing. However, because of pain or associated comorbidities, many did not complete or even attempt the test. At screening, 21 patients did not complete the baseline test, and of those who did, another six were unable to complete follow-up.

Quality of life. Quality of life was measured using the Rand-36 questionnaire at baseline and 3 months. We calculated the change in individual patients' scores (Table VI). While BMAC patients demonstrated improvements compared with controls in six of the eight domains, only the Physical Function domain approached statistical significance (P = .06).

ABI and TcPO$_2$. TcPO$_2$ at the transmetatarsal level trended toward improvement in BMAC (12 increasing to 25 mm Hg) versus controls (15 increasing to 17 mm Hg; P = NS). Only 28 of 48 patients had evaluable baseline and

Table VI. Quality of life (Rand-36)

	Bone marrow aspirate concentrate	Control	P[a]
General health	2.46	−2.2	.45
Physical function	4.58	−15.3	.06
Role physical	10.77	−2	.24
Role emotional	6.73	15	.51
Vitality	4.5	0.4	.44
Mental health	5.12	9.2	.47
Social function	6.35	−4.3	.15
Bodily pain	8.96	1.6	.35

Rand-36 instrument utilized with permission.
Data are the means for changes in individuals who completed the questionnaire at baseline and 12-week follow-up.
[a]P calculated from two-sample Wilcoxon (nonparametric) test.

Table VII. Change in ankle-brachial index

	Improved	Not improved	Total	% Improved	P[a]
Bone marrow aspirate concentrate	11	23	34	32.4%	.08
Control	1	13	14	7.1%	

Increases in ankle-brachial index that are ≥0.1 are considered "improved." Patients who increased by less than 0.1, worsened, had major amputations, or did not have follow-up studies were considered "not improved."
[a]P value calculated using Fisher's exact test, two-tailed.

12-week ABI recordings as a result of amputation, missed testing, or noncompressibility. Analysis of the evaluable paired data demonstrated an improvement in mean ABI for BMAC of 0.12 (n = 22), whereas controls decreased −0.17 (n = 6; P = .13). A priori, we defined a significant change in ABI as a change ≥0.1. Patients were "improved" if their ABI increased by ≥0.1, while they were "not improved" if the ABI increased by <0.1, they had a major amputation, or did not have evaluable data. Noncompressible ABIs (>1.1) were excluded. TBIs were requested when the ABI > 1.1; however, small numbers of these cases preclude analysis (Table VII).

Blinding

Patients and investigators (except for the bone marrow aspirating team) were blinded. To evaluate blinding, patients and investigators were asked, on the day of treatment, to identify which treatment arm they thought the patient was assigned. The blinding index equals percent incorrect guesses + percent undecided guesses.[38] Blinding indexes >50% suggest successful blinding. The blinding index for subjects and investigators in this study ranged from 61.5% to 84.6%, indicating successful blinding (Table VIII).

DISCUSSION

Role of cell therapy. A recent study examining tissue in patients treated with bone marrow-derived cells has demonstrated growth of small blood vessels in humans with

Table VIII. Assessment of blinding

	Actual treatment	
Subject's guess	Bone marrow aspirate concentrate (n = 32)	Control (n = 13)
Bone marrow aspirate concentrate	9 (28.1%)	4 (30.8%)
Placebo	0 (0%)	2 (15.4%)
Undecided	23 (71.9%)	7 (53.8%)
Blinding index	71.9%	84.6%
Investigator's guess		
Bone marrow aspirate concentrate	8 (25.0%)	4 (30.8%)
Placebo	4 (12.5%)	5 (38.5%)
Undecided	20 (62.5%)	4 (30.8%)
Blinding index	75.0%	61.5%

Patients and investigators were asked prior to discharge on the day of treatment to identify which treatment arm they thought the patient was assigned.
Blinding index equals (percent [incorrect] + percent [undecided]).
When blinding exceeds 50%, subjects have been successfully blinded.[40]

CLI.[39] Our pilot study supports the potential of bone marrow-derived cells as a regenerative medical intervention for CLI.

Since Tateishi-Yuyama et al published their work describing the first use of cell therapy in CLI in 2002, reports have included over 1000 treated patients. However, the majority of these studies are case series. While early work on cell therapy for CLI has demonstrated its feasibility, it will not be accepted as a proven therapy until it has succeeded in robust, randomized, and blinded studies.

The point-of-care system (Harvest SmartPReP2), which processed cell concentrate in this study, has been used in other clinical trials for both cardiac[40,41] and vascular[6,17,32] indications. The system demonstrated several advantages: the centrifuge did not require a separate laboratory technician for operation; processing was done within 15 minutes; and samples did not leave the procedure room. Furthermore, the system was capable of achieving mean cell counts of 3.23×10^9 from 240 mL of marrow aspirate, numbers that are comparable to 1.6×10^9 achieved by the Tateishi-Yuyama (Ficoll) protocol that requires 500 mL aspirate. The smaller volume of marrow leads to shorter marrow harvesting times, less anesthesia risk, and less anemia.

This randomized, double-blinded controlled, pilot study of cell therapy in CLI, although not powered to prove efficacy, showed cell therapy to be feasible and safe in no-option patients. Furthermore, we were able to establish the utility of the study design as well as areas for improvement.

Study design. This study included rest pain (R4) and tissue loss (R5) patients. Although the designation of rest pain is based on subjective complaints, the accompanying hemodynamic inclusion criteria assured the severity of vascular disease at baseline. While limb salvage with a limited amputation of toes or forefoot was an acceptable outcome goal, we excluded patients with extensive mid foot or hind

foot necrosis (R6) for whom major amputation was unavoidable.

Important outcome measures for any study of CLI include amputation, pain, and quality of life. While amputation is objective and VAS is well established, both measures could be influenced by knowledge of treatment group assignment. For instance, a patient or his or her physician who believes that an active treatment was administered might tolerate severe pain or stable tissue loss for a longer period of time before committing to amputation. Likewise, self-reporting of pain or quality of life could be influenced. Therefore, blinding the investigator and patient is key. In this study, randomization was managed centrally ensuring strict adherence to protocol. Blinding was maintained by several processes: (1) separating the unblinded physician and research coordinator, who performed the bone marrow harvesting, from the vascular surgeon and coordinator, who carried out injections and subsequent care; (2) performing marrow punctures on both treatment and control patients; (3) operating the centrifuge for both groups of patients. Placebo injections were diluted peripheral blood, which closely simulated BMAC. Assessment of both patient and investigator demonstrated successful blinding. The blinding procedures presented here provide a robust approach that may be incorporated in future cell therapy trials in CLI.

Safety. Despite a high reported baseline amputation and death rate in this population, there were no deaths and no unanticipated major adverse events in either the treatment or control groups. There were no instances of clinically relevant anemia, rhabdomyolysis, or kidney injury associated with therapy.

Proliferative retinopathy is a serious issue for diabetics, and it is appropriate to ask whether a therapy designed to promote neovascularization might worsen this condition. To date, there has been no indication that cellular therapies worsen retinopathy.[21] Rather, some have postulated that cellular therapy may mitigate retinopathy.[42,43] Similarly, there is speculation that BMDCs may contribute to cancer development by supporting tumor angiogenesis.[44,45] However, Wickersheim showed that bone marrow-derived endothelial progenitor cells do not contribute to tumor endothelium in either primary or metastatic tumors.[46] A review of the literature demonstrates only a single study in which the cancer rate in stem cell patients was higher than expected.[13] However, in that series, all patients received granulocyte-colony stimulating factors (G-CSF), which may have played a role in the unexpectedly high cancer rate. In our pilot, no exogenous growth factors were administered, and no malignancies were diagnosed.

Efficacy and outcome measures. Given the historically high risk of death and amputation among patients with unreconstructable CLI, the use of amputation-free survival (AFS) as an objective primary outcome measure has merit. There is, however, a subjective component to deciding when an amputation is necessary, and maintenance of blinding is vital to avoid such bias. Variations in surgeons' threshold for amputation was addressed by requiring a

second opinion for amputations, while regional or hospital differences were mitigated by including the site as a stratification variable. The pilot study was not powered to show statistical significance. However, nearly every outcome measure favored the BMAC group over the controls: major amputations (17.6% vs 28.6%), improved pain (44% vs 25%), improved ABIs (32.4% vs 7.1%), improved Rutherford classification (35.3% vs 14.3%), and quality-of-life scoring better for BMAC in six of eight domains. Using these pilot data as assumptions in a power calculation, we estimate that a pivotal study with similar criteria, using AFS as a primary outcome measure, would require at least 210 patients to achieve a power of 0.8 for $P < .05$.

Composite outcome. Limb preservation and survival (AFS) are clearly the sine qua non of a successful CLI intervention. However, AFS fails to consider pain, limited mobility, and other factors that impact quality of life. Although the risk of amputation or death in CLI patients with the tissue loss is daunting in the near term, patients with rest pain have a more indolent course wherein quality of life is important. In this pilot, there were no amputations in R4 patients in the first 12 weeks. Thus, for studies which include R4 and R5 patients, we believe that AFS is not an appropriate primary stand-alone outcome measure. Therefore, we constructed a composite outcome which accounts for the goals of therapy that patients and physicians value. In this proposed metric, to be classified as a success, a subject must: (1) be alive; (2) without a major amputation on the index limb; and (3) have improved in either Rutherford classification or VAS pain scale, while not deteriorating in either. Subjects meeting all of these criteria were classified as successes. By this measure, success was achieved in 17 of 34 (50%) BMAC versus three of 14 (21.4%) control patients ($P = .11$). In this pilot study, the composite results are similar to the AFS; however, it is notable that most subjects were R5 at enrollment. If future studies enrolled a higher proportion of R4 patients, we believe that AFS would perform poorly. Therefore, a composite outcome measure such as the one proposed should be validated in future studies, as it reflects our therapeutic goals.

Rutherford classification. The Rutherford classification system distinguishes rest pain from tissue loss; however, clinical trials often group CLI patients together. Yet tissue loss is biologically different from rest pain. Recently, researchers have begun distinguishing differences in outcomes within the CLI population. In a study of 2240 patients with peripheral arterial disease, Taylor et al found that outcomes following revascularization varied with degree of disease at presentation. They state, "For every outcome measure, patients with claudication significantly outperformed those with ischemic rest pain, and patients with rest pain significantly outperformed those with ischemic tissue loss."[47] Valid data in CLI studies are therefore predicated on an equal distribution of disease severity between treatment groups.

Treadmill testing. Patients were scheduled to undergo treadmill testing at screening and follow-up. Unfortunately, 27 of 48 patients did not complete the treadmill

test at screening or at follow-up because of amputation, wounds, or unsteadiness. For the pivotal trial, we will eliminate the treadmill test and replace it by a simple question of whether or not the patient is ambulatory (with or without assistance) or nonambulatory. This questionnaire approach has been supported by several recent publications.[48-50]

Creatinine cut-off. Chronic renal insufficiency is a risk factor for death and amputation in CLI.[51-53] Although this population may have the most to gain from innovative therapies given their poor response to conventional modalities, the risk of failure is onerous, and therefore, dialysis patients are not appropriate for initial testing of biological therapies in CLI. However, since nearly 25% of patients with chronic renal insufficiency also have peripheral arterial disease,[54] requiring normal renal function in clinical trials would not reflect the general target population. Furthermore, patients with more severe renal insufficiency are less likely to be candidates for surgical or endovascular therapy. This pilot study required a creatinine <2.0, which resulted in the exclusion of many otherwise suitable candidates. Since we did not demonstrate any adverse effect on renal function nor change in outcome, we support a more liberal threshold for future studies, which will better reflect the CLI population as a whole.

Potential weaknesses of the study. In this trial, we did not aim for a particular cell count. However, analysis of our BMAC demonstrated cell yields equivalent or superior to those achieved in prior studies. The eight-fold range in cell counts in this protocol suggests that future refinements in technique might include individualizing the aspirate volume based on preoperative indicators of cellular yield such as Hct or C-reactive protein or the use of repeat treatments if yields are low. This protocol embraces a point-of-care technique for marrow processing, which is fast and easy. By not requiring dedicated laboratory facilities or personnel, this protocol may be feasible for a wide range of clinicians and patients.

CONCLUSION

In this multicenter, randomized, double-blind controlled trial of autologous bone marrow-derived cell therapy for CLI, the therapy was well tolerated without significant adverse events associated with the procedure during 3-month follow-up. The cell therapy group demonstrated trends toward improvement in amputation rate, pain, quality of life, Rutherford classification, TcPO$_2$, and ABI when compared with controls. This pilot identified several areas for improvement in future trials. Recommendations include elimination of treadmill testing, stratification by Rutherford class, and more liberal creatinine cut off. Our strongest recommendation is that CLI studies that include Rutherford 4 patients, stratify according to disease severity, and incorporate a composite endpoint reflecting pain and quality of life.

REFERENCES

1. 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). J Vasc Surg 2007;45 Suppl S:S5-67.
2. Fadini GP, Agostini C, Avogaro A. Autologous stem cell therapy for peripheral arterial disease meta-analysis and systematic review of the literature. Atherosclerosis 2010;209:10-7.
3. Sprengers RW, Moll FL, Verhaar MC. Stem cell therapy in PAD. Eur J Vasc Endovasc Surg 2010;39 Suppl 1:S38-43.
4. Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, et al. Isolation of putative progenitor endothelial cells for angiogenesis. Science 1997;275:964-7.
5. Tateishi-Yuyama E, Matsubara H, Murohara T, Ikeda U, Shintani S, Masaki H, et al; Therapeutic Angiogenesis using Cell Transplantation (TACT) Study Investigators. 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.
6. Amann B, Luedemann C, Ratei R, Schmidt-Lucke JA. Autologous bone marrow cell transplantation increases leg perfusion and reduces amputations in patients with advanced critical limb ischemia due to peripheral artery disease. Cell Transplant 2009;18:371-80.
7. Burt RK, Testori A, Oyama Y, Rodriguez HE, Yaung K, Villa M, et al. Autologous peripheral blood; CD133+ cell implantation for limb salvage in patients with critical limb ischemia. Bone Marrow Transplant 2010;45:111-6.
8. Cobellis G, Silvestroni A, Lillo S, Sica G, Botti C, Maione C, et al. Long-term effects of repeated autologous transplantation of bone marrow cells in patients affected by peripheral arterial disease. Bone Marrow Transplant 2008;42:667-72.
9. De Vriese AS, Billiet J, Van Droogenbroeck J, Ghekiere J, De Letter JA. Autologous transplantation of bone marrow mononuclear cells for limb ischemia in a Caucasian population with atherosclerosis obliterans. J Intern Med 2008;263:395-403.
10. 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;44:732-9.
11. Gu YQ, Zhang J, Guo LR, Qi LX, Zhang SW, Xu J, et al. Transplantation of autologous bone marrow mononuclear cells for patients with lower limb ischemia. Chin Med J 2008;121:963-67.
12. Hernández P, Cortina L, Artaza H, Pol N, Lam RM, Dorticós E, et al. Autologous bone-marrow mononuclear cell implantation in patients with severe lower limb ischaemia: a comparison of using blood cell separator and Ficoll density gradient centrifugation. Atherosclerosis 2007;194:e52-6.
13. Horie T, Onodera R, Akamastu M, Ichikawa Y, Hoshino J, Kaneko E, et al; Japan Study Group of Peripheral Vascular Regeneration Cell Therapy (JPRCT). Long-term clinical outcomes for patients with lower limb ischemia implanted with G-CSF-mobilized autologous peripheral blood mononuclear cells. Atherosclerosis 2010;208:461-6.

14. Kajiguchi M, Kondo T, Izawa H, Kobayashi M, Yamamoto K, Shintani S, et al. Safety and efficacy of autologous progenitor cell transplantation for therapeutic angiogenesis in patients with critical limb ischemia. Circ J 2007;71:196-201.

15. Kawamoto A, Katayama M, Handa N, Kinoshita M, Takano H, Horii M, et al. Intramuscular transplantation of G-CSF-mobilized; CD34(+) cells in patients with critical limb ischemia: a phase I/IIa, multicenter, single-blinded, dose-escalation clinical trial. Stem Cells 2009;27:2857-64.

16. Kawamura A, Horie T, Tsuda I, Abe Y, Yamada M, Egawa H, et al. Clinical study of therapeutic angiogenesis by autologous peripheral blood stem cell (PBSC) transplantation in 92 patients with critically ischemic limbs. J Artif Organs 2006;9:226-33.

17. Kolvenbach R, Kreissig C, Cagiannos C, Afifi R, Schmaltz E. Intraoperative adjunctive stem cell treatment in patients with critical limb ischemia using a novel point-of-care device. Ann Vasc Surg 2010;24: 367-72.

18. Koshikawa M, Shimodaira S, Yoshioka T, Kasai H, Watanabe N, Wada Y, et al. Therapeutic angiogenesis by bone marrow implantation for critical hand ischemia in patients with peripheral arterial disease: a pilot study. Curr Med Res Opin 2006;22:793-8.

19. Lara-Hernandez R, Lozano-Vilardell P, Blanes P, Torreguitart-Mirada N, Galmés A, Besalduch J. Safety and efficacy of therapeutic angiogenesis as a novel treatment in patients with critical limb ischemia. Ann Vasc Surg 2010;24:287-94.

20. Lenk K, Adams V, Lurz P, Erbs S, Linke A, Gielen S, et al. Therapeutical potential of blood-derived progenitor cells in patients with peripheral arterial occlusive disease and critical limb ischaemia. Eur Heart J 2005; 26:1903-9.

21. Matoba S, Tatsumi T, Murohara T, Imaizumi T, Katsuda Y, Ito M, et al; TACT Follow-up Study Investigators. 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.

22. Miyamoto K, Nishigami K, Nagaya N, Akutsu K, Chiku M, Kamei M, et al. Unblinded pilot study of autologous transplantation of bone marrow mononuclear cells in patients with thromboangiitis obliterans. Circulation 2006;114:2679-84.

23. Moriya J, Minamino T, Tateno K, Shimizu N, Kuwabara Y, Sato Y, et al. Long-term outcome of therapeutic neovascularization using peripheral blood mononuclear cells for limb ischemia. Circ Cardiovasc Interv 2009;2:245-54.

24. Motukuru V, Suresh KR, Vivekanand V, Raj S, Girija KR. Therapeutic angiogenesis in Buerger's disease (thromboangiitis obliterans) patients with critical limb ischemia by autologous transplantation of bone marrow mononuclear cells. J Vasc Surg 2008;48:53S-60S; Discussion 60S.

25. Saigawa T, Kato K, Ozawa T, Toba K, Makiyama Y, Minagawa S, et al. Clinical application of bone marrow implantation in patients with arteriosclerosis obliterans, and the association between efficacy and the number of implanted bone marrow cells. Circ J 2004;68:1189-93.

26. Saito Y, Sasaki K, Katsuda Y, Murohara T, Takeshita Y, Okazaki T, et al. Effect of autologous bone-marrow cell transplantation on ischemic ulcer in patients with Buerger's disease. Circ J 2007;71:1187-92.

27. Wester T, Jørgensen JJ, Stranden E, Sandbaek G, Tjønnfjord G, Bay D, et al. Treatment with autologous bone marrow mononuclear cells in patients with critical lower limb ischaemia. A pilot study. Scand J Surg 2008;97:56-62.

28. Arai M, Misao Y, Nagai H, Kawasaki M, Nagashima K, Suzuki K, et al. Granulocyte colony-stimulating factor: a noninvasive regeneration therapy for treating atherosclerotic peripheral artery disease. Circ J 2006; 70:1093-8.

29. Bartsch T, Brehm M, Zeus T, Kögler G, Wernet P, Strauer BE. Transplantation of autologous mononuclear bone marrow stem cells in patients with peripheral arterial disease (the TAM-PAD study). Clin Res Cardiol 2007;96:891-9.

30. 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;28:2155-60.

31. 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. Thromb Haemost 2007;98:1335-42.

32. Procházka V, Gumulec J, Jalùvka F, Salounová D, Jonszta T, Czerný D, et al. Cell therapy, a new standard in management of chronic critical limb ischemia and foot ulcer. Cell Transplant 2010;19:1413-24.

33. Lu D, Chen B, Liang Z, Jiang Y, Li S, Xu J, 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.

34. Assmus B, Rolf A, Erbs S, Elsässer A, Haberbosch W, Hambrecht R, et al; REPAIR-AMI Investigators. Clinical outcome 2 years after intracoronary administration of bone marrow-derived progenitor cells in acute myocardial infarction. Circ Heart Fail 2010;3:89-96.

35. Ge J, Li Y, Qian J, Wang Q, Niu Y, Fan B, et al. Efficacy of emergent transcatheter transplantation of stem cells for treatment of acute myocardial infarction (TCT-STAMI). Heart 2006;92:1764-7.

36. Janssens S, Dubois C, Bogaert J, Theunissen K, Deroose C, Desmet W, et al. Autologous bone marrow-derived stem-cell transfer in patients with ST-segment elevation myocardial infarction: double-blind, randomised controlled trial. Lancet 2006;367:113-21.

37. Wöhrle J, Merkle N, Mailänder V, Nusser T, Schauwecker P, von Scheidt F, et al. Results of intracoronary stem cell therapy after acute myocardial infarction. Am J Cardiol 2010;105:804-12.

38. James KE, Bloch DA, Lee KK, Kraemer HC, Fuller RK. An index for assessing blindness in a multi-centre clinical trial: disulfiram for alcohol cessation–a VA cooperative study. Stat Med 1996;15:1421-34.

39. Duong Van Huyen JP, Smadja DM, Bruneval P, Gaussem P, et al. Bone marrow-derived mononuclear cell therapy induces distal angiogenesis after local injection in critical leg ischemia. Mod Pathol 2008;21:837-46.

40. Reyes G, Allen KB, Alvarez P, Alegre A, Aguado B, Olivera M, et al. Mid term results after bone marrow laser revascularization for treating refractory angina. BMC Cadiovsc Disord 2009;10:42.

41. Turan RG, Bozdag-Turan I, Ortak J, Akin I, Kische S, Schneider H, et al. Improvement of cardiac function by intracoronary freshly isolated bone marrow cells transplantation in patients with acute myocardial infarction. Circ J 2011;75:683-91.

42. Friedlander M, Dorrell MI, Ritter MR, Marchetti V, Moreno SK, El-Kalay M, et al. Progenitor cells and retinal angiogenesis. Angiogenesis 2007;10:89-101.

43. Jonas JB, Witzens-Harig M, Arseniev L, Ho AD. Intravitreal autologous bone marrow-derived mononuclear cell transplantation: a feasibility report. Acta Ophthalmol 2008;86:225-6.

44. Li Calzi S, Neu MB, Shaw LC, Kielczewski JL, Moldovan NI, Grant MB. EPCs and pathological angiogenesis: when good cells go bad. Microvasc Res 2010;79:207-16.

45. Lyden D, Hattori K, Dias S, Costa C, Blaikie P, Butros L, et al. Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. Nat Med 2001; 7:1194-201.

46. Wickersheim A, Kerber M, de Miguel LS, Plate KH, Machein MR. Endothelial progenitor cells do not contribute to tumor endothelium in primary and metastatic tumors. Int J Cancer 2009;125:1771-7.

47. Taylor SM, Cull DL, Kalbaugh CA, Senter HF, Langan EM 3rd, Carsten CG 3rd, et al. Comparison of interventional outcomes according to preoperative indication: a single center analysis of 2,240 limb revascularizations. J Am Coll Surg 2009;208:770-8; Discussion:8-80.

48. Crawford RS, Cambria RP, Abularrage CJ, Conrad MF, Lancaster RT, Watkins MT, et al. Preoperative functional status predicts perioperative outcomes after infrainguinal bypass surgery. J Vasc Surg 2010;51:351-8; Discussion:8-9.

49. Flu HC, Lardenoye JH, Veen EJ, Van Berge Henegouwen DP, Hamming JF. Functional status as a prognostic factor for primary revascularization for critical limb ischemia. J Vasc Surg 2010;51:360-71 e1.

50. Taylor SM, Kalbaugh CA, Blackhurst DW, Cass AL, Trent EA, Langan EM 3rd, et al. Determinants of functional outcome after revascularization for critical limb ischemia: an analysis of 1000 consecutive vascular interventions. J Vasc Surg 2006;44:747-55; Discussion:55-6.

51. Owens CD, Ho KJ, Kim S, Schanzer A, Lin J, Matros E, et al. Refinement of survival prediction in patients undergoing lower extremity bypass surgery: stratification by chronic kidney disease classification. J Vasc Surg 2007;45:944-52.

52. Schanzer A, Mega J, Meadows J, Samson RH, Bandyk DF, Conte MS. Risk stratification in critical limb ischemia: derivation and validation of a model to predict amputation-free survival using multicenter surgical outcomes data. J Vasc Surg 2008;48:1464-71.

53. Arvela E, Söderström M, Albäck A, Aho PS, Tikkanen I, Lepäntalo M. Estimated glomerular filtration rate (eGFR) as a predictor of outcome after infrainguinal bypass in patients with critical limb ischemia. Eur J Vasc Endovasc Surg 2008;36:77-83.

54. O'Hare AM, Glidden DV, Fox CS, Hsu CY. High prevalence of peripheral arterial disease in persons with renal insufficiency: results from the National Health and Nutrition Examination Survey 1999-2000. Circulation 2004;109:320-3.

Access to *Journal of Vascular Surgery Online* is reserved for print subscribers!

Full-text access to *Journal of Vascular Surgery Online* is available for all print subscribers. To activate your individual online subscription, please visit *Journal of Vascular Surgery Online,* point your browser to ***http://www.jvascsurg.org,*** follow the prompts to **activate your online access**, and follow the instructions. To activate your account, you will need your subscriber account number, which you can find on your mailing label (*note:* the number of digits in your subscriber account number varies from 6 to 10). See the example below in which the subscriber account number has been circled:

Sample mailing label

This is your subscription account number →

```
************************3-DIGIT 001
                                    SJ P1
FEB00 J024 C: 1 (1234567-89) U 05/00 Q: 1
J. H. DOE, MD
531 MAIN ST
CENTER CITY, NY 10001-001
```

Personal subscriptions to *Journal of Vascular Surgery Online* are for individual use only and may not be transferred. Use of *Journal of Vascular Surgery Online* is subject to agreement to the terms and conditions as indicated online.