A very primitive population of hematopoietic cells has been isolated, originally from human cord blood, based on the presence of the cytosolic enzyme aldehyde dehydrogenase (ALDH), which is considered a marker for stem and progenitor cells (Lin- CD34+CD38-) (23). This sub-population of bone marrow progenitor cells is referred to as ALDH bright ($ALDH^{br}$) and represents about 1% of total bone marrow mononuclear cells. ALDH bright cells contain a variety of cell types thought to be needed for ischemic repair, including hematopoietic, endothelial, and mesenchymal progenitor cells. $ALDH^{br}$ cells express CD34 (57%) or CD133 (27%) markers. From the total population of $ALDH^{br}$ CD133+ cells, 78% were also CD34+, in contrast a very small percentage of $ALDH^{dim}$ cells were CD34+ (0.18%), CD133+ (0.21) or both (0.015%) (24-26). The gene expression of 69 of 84 known angiogenic factors expressed by human bone marrow derived $ALDH^{br}$ cells has been detected and compared to $ALDH^{dim}$ cells using quantitative real-time polymerase chain reaction (27). These include soluble cytokines such as IL8,TGFβ, VEGF and MDK and enzymes that generate soluble molecules with angiogenic signals for endothelial cells such as SPHK1, PTGS1. Some genes that are highly expressed by $ALDH^{dim}$ cells, INFG, TNF, IL6, compare to $ALDH^{br}$ cells are soluble inflammatory cytokines with anti-angiogenic activity.

In a preclinical study human $ALDH^{br}$ cells have been shown to cause neoangiogenesis and restore blood flow after intravenous infusion in an immunodeficient model of hind limb ischemia compared to $ALDH^{dim}$ cells (24). Compared to animals injected with placebo (PBS) or injected with unfractionated bone marrow mononuclear cells (50 million cells) or purified $ALDH^{dim}$ cells (0.5 million cells), mice transplanted with $ALDH^{br}$ cells (0.1 to 0.2 million cells) showed a greater recovery of perfusion and increased capillary density in ischemic limbs at 7 days after injection. $ALDH^{br}$ injected mice continued to have improved perfusion up to 3 weeks compared to the other groups. All these might explain why using a mixed unsorted BMMNCs population that includes a variety of cell types has the potential to inhibit the angiogenic activity of a pure $ALDH^{br}$ cell or CD34+ cell population (22, 24)

2.3 Background Clinical

The safety and efficacy of discrete stem cell populations, such as CD34+ or CD133+ cells (isolated on the basis of cell phenotype) and $ALDH^{br}$ (isolated on the basis of cell function) have been shown in clinical trials. (28-32).

Bartunek et al (28) enrolled 35 patients with acute myocardial infarction treated with stenting, 19 underwent intracoronary administration of CD133+ progenitor cells. At 4 months, left ventricular ejection fraction increased significantly in the treatment group from 45.0±2.6% to 52.1±3.5%, $P<0.05$), but only tended to increase in control patients (from 44.3±3.1% to 48.6±3.6%, P=NS). This was paralleled by a reduction in the perfusion defect in treatment group (from 28.0±4.1% to 22.5±4.1%, $P<0.05$) and no change in the control group (from 25.0±3.0% to 22.6±4.1%, P=NS).

Colombo et al (29) randomized 15 patients with large anterior STEMI, to receive CD133 cells isolated from either bone marrow (group A) or peripheral blood (group B), or to stay on drug therapy alone (group C). The cells were intracoronary injected within 10-14 days of STEMI. Infarct-related myocardial blood flow (MBF) was evaluated by NH positron emission tomography 2-5 days before cell administration and after 1 year. MBF increased in the infarct area from 0.419 (0.390-0.623) to 0.544 (0.371-0.729) mL/min per g in group A, decreased from 0.547 (0.505-0.683) to 0.295 (0.237-0.472) mL/min per g in group B and only slightly changed from 0.554 (0.413-0.662) to 0.491 (0.453-0.717) mL/min per g in group C (A vs. C: P = 0.023; B vs. C: P = 0.066). Left ventricular volume tended to increase more in groups B and C than in group A. These 2 studies demonstrate efficacy of the CD 133 subpopulation of BMMNC's in a clinical setting of acute myocardial infarction.

Losordo et al (31) studied the safety and efficacy of intramyocardial injections of autologous CD34+ cells in patients with refractory angina in a prospective, double-blind randomized, pilot study. They randomized 167 patients with refractory angina. Patients received 1 of 2 doses ($1x10^5$or $5x10^5$ cells/kg) of mobilized autologous CD34+ cells or an equal volume of diluent (placebo). Cells or placebo injections were distributed into 10 sites of ischemic, viable myocardium with a NOGA mapping injection catheter. The primary outcome of weekly angina frequency was significantly lower in the low-dose group than in placebo-treated patients at both 6 months (6.8±1.1 versus 10.9±1.2, P=0.020) and 12 months (6.3±1.2 versus 11.0±1.2, P=0.035); measurements in the high-dose group were also lower, but not significantly. Similarly, improvement in exercise tolerance was significantly greater in low-dose patients than in placebo-treated patients (6 months: 139±151 versus 69±122 seconds, P=0.014; 12 months: 140±171 versus 58±146 seconds, P=0.017) and greater, but not significantly, in the high-dose group. This study shows efficacy of the CD 34 subpopulation of BMMNC's in a clinical setting of refractory angina.

The CCTRN recently conducted a randomized trial evaluating the effect of BMMNCs in chronic ischemic heart failure (33). The study was a randomized, double-blind, placebo-controlled trial of symptomatic patients (New York Heart Association classification II-III or Canadian Cardiovascular Society classification II-IV) with a left ventricular ejection fraction of 45% or less, a perfusion defect by single-photon emission tomography (SPECT), and coronary artery disease not amenable to revascularization who were receiving maximal medical therapy at 5 National Heart, Lung, and Blood Institute-sponsored CCTRN clinical sites between April 29, 2009, and April 18, 2011. A total of 92 patients were randomized (2:1) to either receive NOGA-guided transendocardial injection of $100x10^6$ autologous BMMNCs or placebo. Co-primary end points assessed at 6 months: changes in LVESV by echocardiography, maximal oxygen consumption, and reversibility on SPECT. Changes in LVESV index (-0.9 mL/m^2 [95% CI, -6.1 to 4.3]; P=.73), maximal oxygen consumption (1.0 [95% CI, -0.42 to 2.34]; P=.17), and reversible defect (-1.2 [95% CI, -12.50 to 10.12]; P=.84) were not statistically significant. Authors concluded among patients with chronic ischemic heart failure, transendocardial injection of autologous BMMNCs compared with placebo did not improve LVESV, maximal oxygen consumption, or reversibility on SPECT. However exploratory analyses revealed that LVEF improved in the BMMNCs group compared with the placebo group by 2.7%. Interestingly, there was a correlation between improvement in LVEF and the percentage of CD34+ and CD133+ cells in BMMNC samples even when adjusted for covariates of age and therapy (R^2= 0.16 P=0.04). Even though the overall efficacy endpoints relating to the use of BMMNC's were negative these data suggest the potential for efficacy being driven by a bone marrow subpopulation.

In the setting of PAD, Kawamoto et al (30) conducted a phase I/IIa clinical trial of transplantation of autologous CD34+ cells, in no-option patients with atherosclerotic peripheral artery disease or Buerger's disease with critical limb ischemia (CLI). CD34+cells were isolated from the G-CSF-mobilized apheresis product using a magnetic cell sorting system. CD34+ cells (10^5/kg, n=6; 5 x 10^5/kg, n=8; or 10^6/kg, n=3) were injected intramuscularly into the leg with more severe ischemia. The primary endpoint was defined by an Efficacy Score, representing changes in the toe brachial pressure index, Wong-Baker FACES pain rating scale, and total walking distance at 3 months after treatment. The primary endpoint was positive, indicating improvement in limb ischemia in all patients, although no significant dose-response relationship was observed.

There have been two clinical phase I studies utilizing $ALDH^{br}$ cells. In one study $ALDH^{br}$ cells were injected transendocardially in the left ventricle in heart failure patients (FOCUS bright) (34).

Twenty patients were randomized to treatment (n=10) and control (n=10) groups and received 15 transendocardial injections of either autologous $ALDH^{br}$ cells or placebo respectively. In the periprocedural period (up to 1 month), no major adverse cardiovascular or cerebrovascular events

occurred in treatment patients, electromechanical mapping-related ventricular tachycardia (n=2) and fibrillation (n=1) occurred in control patients. Aldehyde dehydrogenase-bright-treated patients showed a significant decrease in left ventricular end-systolic volume at 6 months (P=.04) and a trend toward improved maximal oxygen consumption. The single photon emission computed tomography delta analysis showed improvement in reversibility on SPECT in cell-treated patients (P=.053).

More pertinent to the present study, is a phase I trial by Perin et al. evaluating the safety and feasibility of direct intramuscular injections of ALDH^{br} cells isolated from BMMNCs and BMMNCs in patients with CLI who were not eligible for percutaneous or surgical revascularization. This is the first clinical trial to evaluate the effect of a discrete bone marrow derived progenitor cell population and the unfractionated bone marrow. ALDH^{br} cells and BMMNCs were successfully administered to all patients. No therapy-related serious adverse events occurred. Patients treated with ALDH^{br} cells (n=11) showed improvements in Rutherford category from baseline to 12 weeks (mean, 4.09± 0.30 to 3.46±1.04; P=0.05) and a significant increase in ABI at 6 (mean, 0.22±0.19 to 0.30±0.24; P= 0.02) and 12 weeks (mean, 0.36±0.18; P=0.03) compared with baseline. Patients in BMMNC group (n=10) showed no significant improvements in Rutherford class at 6 or 12 weeks but did show improvement in ABI from baseline to 12 weeks (0.38±0.06 to 0.52±0.16; P=0.03) (13).

Taken together these clinical trials provide evidence that using discrete subpopulations of bone marrow derived progenitor cells is feasible and safe. There is a suggestion of beneficial effects and, when considered in the context of the preclinical trials, might be superior to total bone marrow mononuclear cells.

2.4 Background Conclusions

The prevalence of intermittent claudication is high in adults and while there are currently useful symptom-relieving therapies, there is an unmet need for new claudication therapies for individuals who do not respond to claudication pharmacotherapy, who may not be able to enroll in supervised exercise programs, or who do not have PAD anatomy favorable for revascularization. The identification of ALDH^{br} cells provides an attractive cell type that may have therapeutic benefit in this population. An initial clinical study has suggested safety in the application of this cell type in patients with PAD and further studies are warranted to assess the potential efficacy of this therapy. Furthermore, the identification of new imaging endpoints (in addition to traditional clinical endpoints) provides the Network the opportunity to perform groundbreaking work in the study of this promising cell type in patients with a common and morbid form of atherosclerotic disease. This cardiovascular scientific goal is within the scope of the CCTRN mission.

3.0 INVESTIGATIONAL PLAN

3.1 Research Questions

- Do autologous bone marrow-derived ALDH^{br} cells improve PWT in patients with intermittent claudication?
- Do autologous bone marrow-derived ALDH^{br} cells increase calf muscle blood flow in the ischemic limb of patients with PAD and claudication?
- Can novel MRI endpoints be utilized in a multicenter clinical trial context to quantify lower extremity tissue perfusion in a PAD patient population treated with ALDH^{br} cells?
- How do these imaging and perfusion endpoints inform PAD physiology compared with more traditional endpoints in a PAD patient population?

3.2 Study Design

This is a randomized, double-blind, placebo-controlled clinical trial designed to evaluate the effect of ALDH^{br} cells versus placebo in patients with PAD and intermittent claudication. Eighty patients will be randomized (1:1) to receive either autologous ALDH^{br} cells or placebo (vehicle). After enrollment, baseline imaging and study product injection, patients will be followed up at 1 week, 1 month, 3 months, and 6 months. The MRI efficacy endpoints will be assessed only at the 6 month visit, whereas the TMET endpoints will be assessed at 3 and 6 months. For the purpose of primary and secondary endpoint analysis and safety evaluations, we will utilize an "intention to treat" study population. The six month follow-up time point was selected based upon previous cell therapy trials in CLI patients as a reasonable and commonly used time frame to assess potential clinical benefit and risk (sections 2.1 and 2.3). A twelve month telephone contact will be made to conduct the PAQ and to assess current medications, as well as morbidity and mortality in trial participants.

3.3 Study Endpoints

3.3.1 Primary Endpoints

(Reflecting change from baseline to 6 months between groups)

- PWT
- Leg collateral artery anatomy (via contrast enhanced MR): number of new vessel developments
- Vascular flow (via phase-contrast MR): change in peak flow (mL/sec)
- Perfusion (via cuff-induced ischemia using perfusion MR): change in peak hyperemic flow (mL/sec)

3.3.2 Secondary Endpoints

(Reflecting change over time between groups)

- Pre-exercise ABI at 3 and 6 months
- Post-exercise ABI at 3 and 6 months
- COT at 3 and 6 months
- PWT at 3 months
- Assessment of the relationship between PWT and the three imaging based primary endpoints
- Walking Impairment Questionnaire (WIQ) at 1, 3, and 6 months
- Peripheral Artery Questionnaire (PAQ) at 1, 3, and 6 months

Brief descriptions of the MR assessments are included in Appendix A. Methods to be used are further described in the MRI Core Lab Procedure manual.

Treadmill testing with ECG monitoring will utilize the graded Gardner-Skinner protocol (see Appendix B) and will be used to measure the PWT and COT endpoints. A Treadmill Core Lab will provide training, monitoring, and equipment qualification for participating clinical sites.

Patients will be instructed to indicate the onset of any exercise-limiting symptoms; typical leg pain /claudication (to record claudication onset time), general fatigue, chest pain or shortness of breath. Patients will be asked to continue until they experience maximally tolerated claudication to record PWT. Exercise will also be terminated if the patient experiences limiting angina or chest pain, shortness of breath, or general fatigue. Immediately after exercise, arm and pedal pressures will be measured in the supine position. COT and PWT will be recorded as well as an ECG printout at both of those time points.

More detailed methods will be described in the Treadmill Core Lab Procedure manual.

3.3.3 Safety Evaluations

Adverse events will be assessed through the six month endpoint visit. The following will be documented in the adverse event reporting system.

- Local reactions to study product administration (pain, edema, rash, cellulitis, skin breaks, localized infectious processes, and peripheral nerve injury, hematoma, compartment syndrome, deep vein thrombosis,) detected during study follow-up.
- Any systemic reaction: fever, allergic reaction, anaphylaxis or any clinical untoward event that occurs within 30 days of study product administration.
- Abnormal laboratory results which are deemed clinically significant.

3.3.4 Biorepository Evaluations

Baseline bone marrow (BM) will be analyzed using flow cytometry markers and circulating colony-forming cell assays to answer the following questions:

- Does the frequency of circulating angiogenic cells (CD45dimCD34+) and type 2 monocytic cells (CD14lowCD16+) predict (a) delta hyperemic peak flow and (b) peak walking time?
- Does the number of CFC colony forming cells per/10^6 cells predict (a) delta hyperemic peak flow and (b) peak walking time?
- Does the percentage of viable B cells (CD19+) and monocytes (CD11b+) predict (a) delta hyperemic peak flow and (b) peak walking time?
- Does the number of immunomodulatory/stromal mesenchymal stromal cells (MSCs) (reflected by the frequency of CD45-CD34- MSCA-1+ CD271+ cells) predict (a) delta hyperemic peak flow and (b) peak walking time?
- Does the potency of immunomodulatory/stromal MSCs (as reflected by frequency of colony forming units (CFU-F) colonies/10^6 mononuclear cells present), predict (a) delta hyperemic peak flow and (b) peak walking time?

3.4. Sample Size Computations and Assumptions

Hypothesis testing for the primary endpoints will be carried out at the 0.05 level. All sample sizes are based on 1:1 active to placebo.

3.4.1 Primary Endpoint 1 – Peak Walking Time

The first primary endpoint of this trial is an improvement in PWT at 6 months. We expect PWT to increase in both the placebo and active patients, but anticipate a greater increase in the active arm.

The sample size is based on the execution of a general linear model,

$$E[\Delta y_i] = \beta_0 + \beta_1 w_i + \beta_2 x_i \qquad (1.1)$$

where the dependent variable, Δy_i is the change in PWT (follow-up – baseline). The predictor variables are the baseline measure of PWT, w_i (continuous) and the effect of therapy, x_i (dichotomous). The effect of therapy is based on a hypothesis test for the coefficient β_2

H_0:$\beta_2 = 0$ versus H_a:$\beta_2 \neq 0$.

Without loss of generality, we can standardize the baseline value such that its mean is zero for each of the cell and placebo groups. We can find the variance of the parameter estimate of b_2 of β_2, directly by writing (1.1) as E[y]=Xb. where X is the design matrix of dimension (k+1)n by 3 design matrix. Assuming ordinary (non-weighted) least square approach, the variance of b_2 is the entry in the third row, third column of $(\mathbf{X'X})^{-1}$, denoted as $(\mathbf{X'X})^{-1}_{(3,3)}$. Writing this symbolically, noting that $MSE = \frac{\left(1-R^2\right)SST}{n-3}$, we can write the sample size (k+1)n as a function as

$$N = (k+1)n = \frac{(k+1)\left[\frac{k+1}{k}\left(1-R^2\right)\sigma_\Delta^2\left[\frac{Z_{1-\left[\alpha/2\right]} - Z_\beta}{\beta_2}\right]^2 + 3\right]}{1-f}$$

where

- N = number of placebo patients + number of active group patients
- α = Type I error
- β = Type II error
- Z_c = the c^{th} percentile from the standard normal probability distribution
- B_2 = effect size (i.e., Difference between the change in the active group over time minus the change in the placebo group over time
- σ_Δ^2 = the variance in the change over time (incorporates the correlation over time). Pooled between the active and placebo groups.
- k = ratio of number of active group to placebo group patients
- f = expected proportion of patients anticipated to be lost to follow-up

The CLEVER study (35) provides the most useful information on which to base the sample size for PWT. In CLEVER, PWT increased by 1.2±2.6 (mean ± SD) minutes in the optimal medical care group, 5.8±4.6 in the supervised education group, and 3.7±4.9 in the stent revascularizaton group. The differences between these means provide a measure of the effect of therapy (i.e., the difference in the change over time between two groups). From this information, we may compute the required sample size from the formula above (assuming a 15% follow-up loss rate) (Table 1).

Table 1. Peak Walking Time
Type I error = 0.05; power = 90%; k = 1, followup losses = 15%

			Treatment Effect (β_2)				
			1.2	2	2.5	3	3.5
	Std Dev	2.50	220	84	56	41	32
R^2 = 0.01	of Diff	3.00	313	117	78	56	43
	(σ_Δ)	3.50	423	157	103	74	56
		4.00	551	203	132	94	71
			1.2	2	2.5	3	3.5
	Std Dev	2.50	211	80	54	40	31
R^2 = 0.05	of Diff	3.00	301	113	75	54	42
	(σ_Δ)	3.50	407	151	99	71	54
		4.00	529	195	127	91	68
			1.2	2	2.5	3	3.5
	Std Dev	2.50	200	77	52	38	30
R^2 = 0.1	of Diff	3.00	285	107	71	52	40
	(σ_Δ)	3.50	386	143	94	68	52
		4.00	502	185	121	86	65

A sample size of 75 provides 90% power to detect a 2.5 minute difference in improvement in PWT assuming a standard deviation of the difference of 3.

3.4.2 Primary MR Endpoint 2 - Leg Collateral Artery Anatomy

(Contrast enhanced MR)

The next primary endpoint is the assessment of change in the number of patent vessels over time. The average change in the number of patent vessels in the active group will be compared to the average change in same average in the placebo group. The analysis will be based on the Mann-Whitney U statistic for independent samples. Sample size computations are based on the asymptotic relative frequency (ARE) of the normal distribution to that to Mann Whitney U statistic. This is the asymptotic limit of the ratio of sample sizes. A conservative approach is to assume the worst case for the ARE for the Mann Whitney U statistic, which is 0.864 (36). The sample size is computed by computing that required by the normal distribution and dividing the sample size by 0.864, which provides adequate protection regardless of the underlying distribution. Type I error is 0.05 (two tailed) and we assume 90% power in this study. This produces the following sample sizes (active group plus placebo group, assuming a 15% follow-up loss rate) in Table 2 (37).

Table 2. Effect of Therapy on Change in the Number of Vessels
Type I error = 0.05; power = 90%; followup losses = 15%; ARE factor = 86.4%

		Treatment Effect (δ)				
		2	2.5	3	3.5	4
Std Dev	2.50	103	67	47	35	27
of Diff	3.00	147	95	67	50	39
(σ_Δ)	3.50	199	129	90	67	52
	4.00	260	167	117	86	67

A sample size of 67 provides 90% power to detect a mean difference of 3 in the change in the number of patent vessels between the groups with a standard deviation of the difference of 3.

3.4.3 Primary MR Endpoint 3 - Vascular Flow

(Phase Contrast MRA)

The third primary endpoint is vascular flow using phase contrast MRA. Our sample size assumption is based on Versluis (37) with the endpoint being peak flow (mL/s). The standard deviation in patients was 1.4. This translates to a standard deviation of the difference over six months as 1.08, assuming a correlation of 0.70. We anticipate a treatment effect of 1.5 mL/s. Assuming 90% power, and a two sided type I error rate of 0.05, the following table of trial sizes (active group plus placebo group, assuming a 15% follow-up loss rate) follows (Table 3).

Table 3. Vascular Flow Phase Contrast MRA
Type I error = 0.05; power = 90%; k = 1;followup losses = 15%

			Treatment Effect (β_2)				
			1	1.5	2	2.5	3
	Std Dev	1.00	56	29	19	15	12
R^2 = 0.01	of Diff	1.20	78	38	25	18	15
	(σ_Δ)	1.30	90	44	28	20	16
		1.40	103	50	31	22	18
			1	1.5	2	2.5	3
	Std Dev	1.00	54	28	19	15	12
R^2 = 0.05	of Diff	1.20	75	37	24	18	15
	(σ_Δ)	1.30	86	42	27	20	16
		1.40	99	48	30	22	17
			1	1.5	2	2.5	3
	Std Dev	1.00	52	27	18	14	12
R^2 = 0.1	of Diff	1.20	71	36	23	17	14
	(σ_Δ)	1.30	82	40	26	19	15
		1.40	94	46	29	21	17

A sample size of 75 provides 90% power with two sided type 1 error of 0.05, assuming $R^2 = 0.05$ to detect a difference of 1 with a standard deviation of the difference of 1.20in the change over time of peak flow (mL/s) of the active group to that of the placebo group

3.4.4 Primary MR Endpoint 4 – Perfusion

(Cuff-induced Ischemia using Perfusion MR)

The final primary endpoint is hyperemic fractional microvascular blood plasma volume by dynamic contrast enhanced (DCE) MRI. Our sample size assumption is based on Versluis (38) which in its review of DCE MRI, demonstrates standard deviations of 1.6% to 3.6%, reflected in the range of standard deviations in the following table. The SD of the difference is $2\sigma^2$ (1 - ρ) where ρ is the correlation coefficient. Correlation coefficients are not provided, so we assume over a six month time course that r = 0.50. Type I error is 0.05 (two tailed) and we assume 90% power in this study. Sample sizes are conservatively estimated between 1.00 and 2.00 percent (the change in percent in the active group minus the change in the placebo group) (Table 4).

Table 4. Hyperemic Fractional Microvascular Blood Plasma Volume by Dynamic Contrast MRI
Type I error = 0.05; power = 90%; k = 1;followup losses = 15%

			Treatment Effect (β_2)				
			1.00	1.25	1.50	1.75	2.00
	Std Dev	1.0	56	29	19	15	12
R^2 = 0.01	of Diff	2.0	203	94	56	38	29
	(σ_Δ)	2.5	313	143	84	56	41
		3.5	607	274	157	103	74
			1.00	1.50	2.00	2.50	3.00
	Std Dev	1.0	54	28	19	15	12
R^2 = 0.05	of Diff	2.0	195	91	54	37	28
	(σ_Δ)	2.5	301	138	80	54	40
		3.5	582	263	151	99	71
			1.00	1.50	2.00	2.50	3.00
	Std Dev	1.0	52	27	18	14	12
R^2 = 0.10	of Diff	2.0	185	86	52	36	27
	(σ_Δ)	2.5	285	131	77	52	38
		3.5	552	249	143	94	68

A sample size of 80 provides 90% power with two sided type 1 error of 0.05, assuming R^2 = 0.05. to detect an effect size of 2.0 with a standard deviation of the difference of 2.5.

Thus 80 patients (40 in the active group and 40 in the placebo group) will provide 90% power for each of these hypotheses to be carried out at the 0.05 level. The type I error will not be apportioned across these four primary endpoint analysis, but rather 0.05 will be applied equally to each of these four analyses. This permits PACE greater sensitivity to detect a signal of efficacy from any of these four endpoints, which is in keeping with our role to discover efficacy, and for ensuing larger clinical trials to build on these results for confirmation.

3.4.5 Sample Size Recommendation

Sample size of 80 subjects (40 active, 40 placebo group) provides sufficient sample size for the four primary endpoints.

3.5 Multiple Testing Issue

Type I error correction in the context of an early phase clinical trial that performs multiple tests can serve to protect the study interpretation against Type I error inflation. However, the use of corrections for multiple comparisons in “proof-of-concept-studies” is problematic. At this investigational level, tight control of the overall family-wise Type I error rate would increase the likelihood that the Investigators would miss a finding of potential importance by attributing it to chance simply because the p-value is large. The CCTRN Investigators have, in this protocol, attempted to balance the need to control the number of evaluations with the need to identify new effects by not incorporating a multiplicity correction.

4.0 SUBJECT SELECTION AND WITHDRAWAL CRITERIA

4.1 Introduction

Potential subjects will be males and females, 40 years of age and older, with PAD and intermittent claudication, identified and recruited from outpatient physicians’ offices, vascular specialty clinics, and referrals from primary care providers.

4.2 Inclusion Criteria

To be considered eligible to participate in this study, the patient must meet the following criteria:

1. Patients with atherosclerotic peripheral artery disease with classic claudication (exercise-induced pain, cramps, fatigue, or other equivalent discomfort involving large muscle groups of the leg(s) that is consistently relieved by rest) or atypical leg pain (exertional leg pain that does not begin at rest or does not resolve consistently with rest) as defined by the San Diego Claudication Questionnaire.

2. Age ≥40 years

3. Resting ankle-brachial index <0.90 or a resting toe-brachial index of <0.70 at baseline testing

4. Presence of significant stenosis or occlusion of infrainguinal arteries including the superficial femoral artery, popliteal artery and/or infrapopliteal arteries as determined by: Duplex ultrasound imaging (occlusion or focal doubling of peak systolic velocity of one or more affected segments) *OR* lower extremity CTA *OR* *lower extremity* MRA *OR* lower extremity catheter-based contrast arteriography. Each of these noninvasive and invasive anatomic assessments will identify patients with at least a 50% stenosis in the affected segment.

4.3 Exclusion Criteria

Patients will be excluded from the study if they have any of the following conditions:

1. Presence of any musculoskeletal disease, cardiac or pulmonary disease, or neurological disease that limits the patient's ability to walk to fulfill protocol requirements (claudication must be the consistent primary exercise limitation)

2. Inability to complete treadmill testing per protocol requirements.

3. Ability to walk for more than 12 minutes on the treadmill during treadmill testing.

4. Patients who identify both legs as equivocally symptomatic or alternate between symptomatic legs on the baseline treadmill tests.

5. Patients with critical limb ischemia (ischemic rest pain or ischemia-related non healing wounds or tissue loss (Rutherford categories 4, 5 or 6). See Appendix D.

6. Recent (<3 months) infrainguinal revascularization (surgery or endovascular revascularization) or revascularization planned during study period

7. Patients with a patent infrainguinal bypass graft in the index limb, with or without evidence of a hemodynamically significant stenosis or other defect (kinking, pseudoaneurysm, or fistula). Patients with an occluded infrainguinal bypass graft or a patent aortobifemoral or femoral-femoral bypass graft are NOT excluded.

8. Patients with >2+ lower extremity pitting edema

9. Patients with myelodysplastic syndrome (MDS)

10. Patients who are pregnant or lactating, planning to become pregnant in the next 12 months, or are unwilling to use acceptable forms of birth control during study participation

11. CHF hospitalization within the last 1 month prior to enrollment*

12. Acute coronary syndrome in the last 1 month prior to enrollment*

13. HIV positive, active HBV or HCV disease

14. History of cancer within the last 5 years, except basal cell skin carcinoma

15. Any bleeding diathesis defined as an INR ≥ 2.0 (off anticoagulation therapy) or history of platelet count less than 100,000 or hemophilia

16. Contraindication to MRI (including knee/tibial/fibular replacement hardware in the index leg) or known allergy to MR contrast media

17. Chronic kidney disease (eGFR <30 by MDRD or Mayo or Cockcroft-Gault formula)

18. Uncontrolled diabetes (HbA1C>8.5)

19. Planned change (initiate or terminate) to active involvement in a supervised exercise program (e.g., with a trainer, exercise protocol, and goals, such as in a PAD, cardiac or pulmonary rehabilitation program) during study participation.

20. Plans to change medical therapy during the duration of the study, (i.e. patients who use cilostazol should remain on a stable dose for four weeks prior to enrollment and should not change doses for the 6 months of the study duration.) As always, cilostazol can be discontinued if new heart failure or intolerance occurs during study participation.

21. Any condition requiring immunosuppressant medications (e.g., for treatment of organ transplants, psoriasis, Crohn's disease, alopecia areata).

22. History of inflammatory or progressively fibrotic conditions (e.g. rheumatoid arthritis, systemic lupus erythematosis, vasculitic disorders, idiopathic pulmonary fibrosis, retroperitoneal fibrosis).

23. Patients with any untreated stenosis > 70% of the distal aorta, common iliac, or external iliac arteries by CT, Angiography or MRI imaging will be excluded from enrollment (patients with previously successfully revascularized inflow stenoses may enroll in PACE). Subjects who were screen failures for a flow-limiting proximal lesion may be rescreened 3 months after successful angioplasty/stenting.

24. Inability to provide written informed consent due to cognitive or language barriers (interpreter permitted)

25. Concurrent enrollment in another clinical interventional investigative trial.

26. 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

As defined by the standard definitions of CHF and ACS by the American Heart Association

Please note: If at any time prior to the study procedure the randomized patient meets any of the above exclusion criterion, treatment will be postponed, or if the condition is not resolvable, the patient will be excluded from participation.

4.4 Prescreening (prior to consent)

Prescreening patients includes reviewing medical records for inclusion/exclusions prior to consent, as well as patients' imaging studies (duplex, angiography or MRI). **Note: Imaging studies are acceptable within 12 months prior to enrollment and PI will determine if individual patient needs to repeat any imaging modalities to use as screening.**

4.5 Consent

All participants enrolled in this clinical trial will be identified as having symptomatic claudication and will be referred from vascular clinics, outpatient care clinics, and other physician referrals or self-referrals generated by advertising. If identified in a clinic, potential participants will be approached by one of the investigators or research coordinators after discussion with the individual's primary physician. The information provided to the potential participant is included in the informed consent. The informed consent will provide information regarding standard alternative claudication therapies and will include information regarding possible risks of participation.

4.6 Baseline Screening

The baseline screening period extends from the date informed consent is signed until the day of randomization. The baseline screening window will not exceed 60 days prior to randomization. The following evaluations will be carried out at baseline:

- Baseline blood test (Section 6.2.6)
- hsCRP, lipid panel, and alkaline phosphatase (Section 6.2.6)
- Infectious disease panel (Section 6.2.6)
- Comprehensive medical and surgical history, vital signs and physical examination (Section 6.2.1)
- Current use of prescription and OTC medications within last 7 days (Section 6.2.1)
- Smoking history (Section 6.2.1)
- Pregnancy test (for women of childbearing potential; i.e. females who are neither surgically sterile nor at least two years postmenopausal) (Section 6.2.6)
- 12 lead ECG (Section 6.2.7)
- San Diego Claudication Questionnaire (Section 6.2.2)
- Walking Impairment Questionnaire (WIQ) (Section 6.2.3)
- Peripheral Artery Questionnaire (PAQ) (Section 6.2.4)
- Patient Expectation Questionnaire (PEQ) (Section 6.2.5)
- Pre-exercise ABI/TBI and post-exercise ABI (Section 6.3.1, Appendix B)
- Treadmill-based COT and PWT (Section 6.3.1, Appendix B)
- MRI imaging (CE-MRA, Phase-contrast, Perfusion) (Section 6.3.2, Appendix A)

Subjects may be excluded from the study if any of the above baseline testing is not completed or meets an exclusion criterion during the screening period.

4.7 Randomization

Randomization will be performed in accordance with written standard operating procedures by personnel that are not involved in the selection, screening, or enrollment of subjects and blinded as to clinical documentation. Randomization to treatment assignment will be conducted using a web access database created and maintained by the Data Coordinating Center (DCC). After the research coordinator verifies that baseline testing is complete and the inclusion and exclusion cri-

teria for the study have been satisfied, an unblinded member of the local stem cell laboratory will have secured access to the patient's treatment assignment. The patient will be randomized a minimum of 2 days before the scheduled date of the bone marrow harvest to arrange for the courier to pick up the bone marrow and alert the manufacturing lab. See Manual of Operations for details.

4.8 Stopping Early or Withdrawal from the Study

Patients that have revascularization, changes in medical therapy or develop a clinical event during the 6 month duration of the trial will still be followed, if feasible and included in intent to treat analysis. Patients that withdraw from the study before the 6 month endpoint is collected will be considered lost to follow-up and included in intent to treat analysis.

Screen Failure

Subjects are considered screen failures when they meet one of the following criteria after signing consent: Screening tests reveal that the subject is ineligible, OR the subject withdraws consent before being randomized.

Discontinued

Subjects are considered discontinued when they meet one or more of the following criteria: Subject withdraws consent after being randomized, OR subject is withdrawn after enrollment before randomization by investigator.

5.0 STUDY TREATMENTS

After baseline testing is complete and before 60 days have elapsed from the receipt of informed consent, all patients will be randomized to study treatment.

5.1 Procurement

The target amount for procurement is approximately 180 mL (±10mL) of bone marrow, however the minimum amount of bone marrow required to manufacture the study product is 50mL. The target amount will be collected from the posterior superior iliac spine of the patient using established, standard collection procedures by a trained physician or designated personnel. Conscious sedation or anesthesia will be used per local clinical site's institutional guidelines for bone marrow aspirations of this volume. Sterile technique will be followed to prevent contamination of the marrow collection and infection at the site of collection. The manufacturer will provide bone marrow shipping kits with the necessary materials to accurately collect, label, and transport the materials for processing. The bone marrow aspirate will be transferred in a sterile manner to a local stem cell laboratory. The details of the aspiration procedure are located in Appendix C. Patients on aspirin and/or Plavix (clopidogrel) at the time of consent should remain on aspirin and/or Plavix (clopidogrel) for the bone marrow aspiration procedure. Continuance or discontinuance of other medications at the time of bone marrow aspiration, (e.g. warfarin and heparins) are left to the discretion of the study physician.

5.2 Treatment Assignment

The study products (active and placebo) are each individually described below but shall thereafter be referred to throughout the protocol as "study product". In the very rare circumstance that less than 50mL can be procured from the harvest, the subject will continue to be followed (intent to treat analysis) but will not receive study product. Similarly, should manufacturing or transportation failures involving the processed study product occur, there will be no repeat bone marrow aspiration, the patient will remain randomized to their original treatment condition, and they will be followed per protocol as part of the intent to treat analysis.

5.2.1 Active Therapy Group

Product Testing: The final active treatment study product will have the following release testing completed before distribution to the clinical sites: phenotyping (including CD15, CD14, CD19, CD3, and CD235a(GlyA)), ALDH^{br} content, Gram Stain, Endotoxin, and Sterility testing. In addition to the phenotypic markers used for release testing, other markers will be assessed for informational purposes (CD45, CD34, CD133, EphB4, CD162, CD105, CD29 and CD49d). Final product (lot) release criteria listed below (Table 5):

Table 5. Study Product Release Specifications

Assay	Specification
Phenotyping	≤ 12% ALDHdim/CD3+ ≤ 15% ALDHdim/CD14+ ≤ 12% ALDHdim/CD19+ ≤ 15% ALDHdim/CD15+ ≤ 15% ALDHdim/CD235a+
ALDH^{br} content	Entire sample must meet ≥70% purity specification
Gram Stain	Negative
Endotoxin	≤33.3 Eu/mL
Sterility	Sterility results will be pending at time of release. Sites will be notified if a positive culture is identified or at the conclusion of the culture period for a negative result *

*The subject will keep a daily temperature log for 7 days to help determine the development of an infection before the test results are known. If the patient notes a fever, he/she will be requested to notify the investigator/study team.

Should the 14-day sterility culture testing produce a positive culture after the cell processing product has been administered to the patient, then the following steps will take place:

a) The manufacturer will conduct a laboratory investigation. The investigation and proposed corrections for a failure of lot release sterility will include: identification of the microorganism(s) and anti-microbial agent sensitivity testing; evaluation of the current procedures for collecting and processing the study product to determine the step at which contamination could be introduced; development of changes in procedures that will assist in preventing sterility lapses in the future; and implementation of appropriate testing to ensure that such changes to the procedures produce a sterile product. This information will be provided to the DCC (Sponsor).
b) The DCC will report the sterility failure, results of the investigation of the cause, and a corrective action plan to the FDA within 30 calendar days after the initial receipt of the positive culture test result.
c) The failure will also be reported as an “unanticipated problem” to the NHLBI, DSMB, and IRB.
d) The site principal investigator will be notified at once by the DCC that the specimen was positive.
e) The patient will remain in the study and be monitored for clinical signs of infection. If the patient experiences any serious and unexpected adverse drug experience that could be from administration of the sterility failure of the study product, an IND safety report will be filed by the DCC with the FDA within 15 calendar days of the receipt of the information.
f) Antibiotic prophylaxis will be considered.

Every effort will be made to protect the blinding of those involved in the study endpoint and safety event collection.

Dosing and Administration: ALD-301 is composed of ALDH^{br} cells isolated from autologous bone marrow of patients who have intermittent claudication. ALD-301 will be provided by the Center for Gene and Cell Therapy (CAGT) at Baylor College of Medicine (*Houston, Texas*) ALDH^{br} is supplied as a 10.5 mL clear liquid and packaged in a 12 mL fluoridated ethylene propylene (FEP) bag (American Fluoroseal) with a Luer-lock.

The entire volume of each bag is intended as a single dose. Product bag should be gently inverted several times to ensure uniform suspension before each syringe is filled. The needleless port on the product bag should be wiped with a new alcohol wipe each time the bag is accessed. Once the suspension is withdrawn into the syringes, it is ready for administration. Study product must be administered within 96 hours from the end of bone marrow harvest and cannot be left at room temperature for more than 4 hours. The number of cells contained in each dose is determined by the volume of bone marrow collected from the individual subject and the outcome of processing those cells. Based on previous experience of using ALDH^{br} cells in CLI and ischemic heart failure trials (13, 34), it is expected that about 150 mL of bone marrow will yield 1 to 4 x 10^6 ALDH^{br} cells and can be safely aspirated under conscious sedation.

Forty patients will receive the active study product. A total of 10 injections will be placed in the calf and thigh muscles of the index limb. After preparation of the target leg as described in Appendix E a total of 10 injection sites will be marked according to a standardized pattern (Appendix E). Eight of the injections will be placed in the calf and two injections will be placed in the posterior lower thigh. Injections will be performed using a 25 gauge needle. Injection sites will be at least 1 inch apart and be given at a depth to deliver the study product intramuscularly. Each injection will contain approximately 1mL of active study product and will be injected slowly (approximately 45 – 60 seconds). When each injection is completed the site will be covered with a small bandage after gentle pressure is applied.

Availability for Biorepository: For patients randomized to active study product, the CCTRN will have 30 ± 10 mL for scientific study of bone marrow cells in PAD patients. With appropriate consent, bone marrow mononuclear cells isolated from these samples will be used to evaluate the progenitor/stem cell numbers and function using cell-based potency assays and analysis of molecular markers. All surplus cells will be cryopreserved and saved in the CCTRN biorepository. (See section 6.4 for more detail)

The unblinded stem cell laboratory personnel at each site will conduct the following activities:

- Prepare, label, package, and deliver bone marrow sample and chain of custody form to courier as appropriate,
- Receive ALDH^{br} from courier or obtain placebo as appropriate, and store as per study procedures,
- Maintain ALDH^{br} chain of custody forms, temperature monitor logs, certificate of analysis forms and accountability logs (so that clinic staff does not have access),
- Keep completed chain of custody forms and downloaded temperature logs on site for study files and quality assurance purposes.

5.2.2 Placebo Group

Dosing and Administration: The placebo product is composed of phenol red-free CellGro® SCGM serum-free medium (CellGenix Technologie Transfer GmbH, Frieburg, Germany) supplemented with 1% HSA. The placebo is supplied as a 10.5 mL clear liquid packaged in a 12 mL FEP bag (American Fluroseal) with a Luer-lock. The placebo will be provided in advance by the manufacturer to the local cell processing labs to be kept on-site. The placebo is identical in appearance to the active study product. Forty patients will receive placebo (vehicle). Patients randomized to the placebo group will undergo the same baseline testing and bone-marrow aspiration procedure (180 ± 10 mL) to ensure blinding of the study team. Study product injection

will occur within 96 hours of bone marrow aspiration. An identical procedure as described in 5.2.1 will be followed to prepare, mark and inject placebo study product at 10 sites in the target leg.

Availability for Biorepository: If randomized to placebo, the CCTRN will have 180 ± 10 mL for scientific study of bone marrow cells in PAD patients. With appropriate consent, these samples will be analyzed with a focus on progenitor/stem cell activity as described in the previous section (5.2.1). All surplus cells will be cryopreserved at the CCTRN biorepository. (See section 6.4 for more detail)

5.3 Randomization and Blinding

Blinding of the physician giving the injections will be facilitated by the fact that all patients (placebo and active) will undergo bone marrow harvest and injection. All study products will be matched in color and consistency. The primary and secondary endpoints of the study will be determined by core laboratories whose personnel are blinded to therapy assignment. In addition, each of the clinical centers will take steps to ensure that endpoint and adverse event assessments are carried out in a blinded fashion.

Should unblinding occur, the site PI must contact the DCC PI directly to report the circumstances and discuss the circumstances of the unblinding. Any instance of unblinding will be documented in the study database and will be reported to the site's institutional review board (IRB) as required.

5.4 Study Product Requirements

5.4.1 Study Product Packaging and Labeling

Study product packaging and labeling will meet all local and federal requirements. Study product (active and placebo) label includes the product number, subject ID number, volume, expiration date and time, storage conditions, and Sponsor name and address. All products will be labeled for investigational use only and stipulated for autologous use. The 96 hour expiration will be calculated from the time the bone marrow harvest procedure concluded.

5.4.2 Study Product Storage Requirements

Study product must be administered within 96 hours of bone marrow harvest and must be stored at 2º to 18º C from the time of manufacture until the time of administration preparation. The study product should not be at room temperature for more than four hours. Products that exceed either of these expiration limits will not be used and the manufacturer should be notified immediately. Study product will be transported to the clinical trial site cell processing lab in a transport cooler that has been validated to maintain a temperature of 2º to 18º C. Once the product arrives at the lab, the data should be downloaded from the temperature logger and the transport temperature verified by cell processing lab personnel. If the logger indicates that the temperature was not maintained at 2º to 18º C during transport, manufacturer should be notified immediately.

5.4.3 Study Product Accountability

Upon receipt of study product from manufacturer, the cell processing lab personnel will open the shipment and verify shipment inventory, temperature, and condition of the study product by completing and signing the chain of custody form and forwarding the document and the temperature data files to the unblinded manufacturing personnel. The cell processing lab personnel will also compare subject ID number. Records shall be kept of all study supplies received, disposition, and any study products not used or left over. Study product accountability logs contain unblinded information and should be maintained by the cell processing lab staff only. The product accountability logs will record use of placebo as well.

Under no circumstances will any investigator(s) allow study product to be used for any purpose other than that specified by the protocol. The study product is an autologous product and is only to be used to treat the subject who supplied the bone marrow.

5.4.4 Study Product Handling and Disposal

Destruction of unused study product will proceed as directed by manufacturer or designee, with appropriate documentation completed and retained by site personnel. All records involving receipt, disposition, administration, return and/or destruction of the study product will be made available by the PI (or designee) to the Sponsor or designee upon request.

5.5 Concomitant Medications

Subjects will remain on medications used at the time of enrollment through completion of the 6 month trial. Medications will be assessed throughout the study to the 12 month telephone call.

6.0 CLINICAL AND LABORATORY EVALUATIONS

6.1 Schedule of Procedures

Procedures	**Baseline Screen-ing**	**Day -4 RZ**	**Day -2 BMA**	**Day 0 SPI**	**Wk 1 visit**	**Mo 1 visit**	**Mo 3 visit**	**Mo 6 visit**	**Mo 12[9] phone call**
Informed Consent	X								
Complete Medical History	X								
Physical Exam	X		X	X	X	X	X	X	
Vital Signs (Temp, BP, HR, Resp)	X		X	X[1]	X	X	X	X	
Concomitant Medications, including OTC	X		X	X	X	X	X	X	X
AE/SAE Evaluations	X		X	X	X	X	X	X	X
San Diego Claudication Questionnaire	X								
Patient Expectation Questionnaire	X							X	
Walking Impairment Questionnaire (WIQ)	X					X	X	X	
Peripheral Artery Questionnaire (PAQ)	X					X	X	X	X
Infectious Disease Labs	X[2]								
Laboratory evaluations	X[3]				X[3]	X[3]		X[3]	
12 Lead ECG	X		X[4]		X	X		X	
Pre-exercise ABI/TBI	X						X	X	

Peak walking time (PWT)	X[5]						X	X	
Claudication onset time (COT)	X[5]						X	X	
Post-exercise ABI	X						X	X	
Baseline eCRFs completed to allow for randomization		X							
MRI (primary end-point testing)	X							X	
Randomization		X							
Bone Marrow Aspiration (BMA)			X						
Peripheral blood draw			X[6]	X[6]	X	X		X	
Topical anesthetic cream to leg prior to injections				X					
Study Product Injections (SPI)				X[7]					
Injection Site Product Reaction Assessment				X	X				
Temperature Log				X[8]					

1. Pre-SPI, 30 min post-SPI, 60 min post-SPI
2. Infectious disease testing should be the bone marrow donor panel per local institutional guidelines, including HIV, Hep B (HBsAG, Anti-HBs, Anti-HBc), and Hep C (Anti-HCV)
3. CBC with diff., Glucose, Calcium, Sodium, Potassium, Chloride, CO2, BUN, Creatinine, eGFR, Magnesium, HbA1C, Total Bilirubin, Direct Bilirubin, SGPT (ALT), SGOT (AST), Albumin, Total Protein, PT, INR, Pregnancy (childbearing Females-baseline only), hsCRP, Lipid panel (total cholesterol, LDL, HDL, and TG), and alkaline phosphatase
4. Within 24 hrs prior to BMA
5. Baseline PWT and COT will be highest measured value from two baseline treadmill exercise tests as determined by treadmill core lab.
6. Day -2 draw to be collected after anesthesia but prior to harvest. Day 0 draw to be collected at 30 minutes post study product injection.
7. CCTRN cell processing labs receive study product from manufacturer within 48-72 hours from BM harvest.
8. Temperature log 2x/day x 7 days12 month contact is a telephone visit only.

6.2 Procedure Details

6.2.1 Patient History and Physical Exam

A complete patient history including medical, surgical, smoking, and medication review will be conducted at baseline. This history will include past use of claudication treatments (cilostazol, supervised exercise, and revascularization therapies).

A complete physical exam including vitals, height, and weight will be completed at baseline as well as a vascular exam. Similar physical exams will be conducted at each additional clinic visit during the study.

6.2.2 San Diego Claudication Questionnaire (SDCQ)

This questionnaire will assess patient symptoms in order to accurately assign each patient into their symptom class (e.g., classic claudication vs. atypical leg pain) and those with typical claudication pain. It will be administered by a research team member at baseline.

6.2.3 Walking Impairment Questionnaire (WIQ)

This questionnaire will assess the severity of the subjective walking impairment on distance, speed and stair climbing scales. It will be administered as a self-report at baseline, and at 1 month, 3 and 6 months.

6.2.4 Peripheral Artery Questionnaire (PAQ)

This questionnaire will assess subjective physical limitations, leg symptoms, social function, treatment satisfaction and quality of life. It will be administered as a self-report at baseline and at 1 month, 3 and 6 months. It will also be administered by a research team member at the 12 month telephone visit.

6.2.5 Patient Expectation Questionnaire (PEQ)

This questionnaire will assess patients' current levels of pain, fatigue, distress and interference due to their PAD and what they expect to gain from the study treatment. It will be administered as a self-report at baseline and 6 months.

6.2.6 Laboratory Testing

Pregnancy testing will be conducted at baseline on females of child bearing potential (i.e. females who are neither surgically sterile nor at least two years postmenopausal). Pregnancy test results must be negative to continue in the study. **If sexually active, females must be willing to use appropriate contraceptive measures for at least a month prior to treatment and while taking part in this study.** Medically acceptable contraceptives include: (1) surgical sterilization (such as tubal ligation or hysterectomy), 2) approved hormonal contraceptives (such as birth control pills, patches, implants or injections), (3) barrier methods (such as a condom or diaphragm) used with a spermicide, or (4) intrauterine device (IUD).

Baseline laboratory evaluations will include: CBC with differential, Glucose, Calcium, Sodium, Potassium, Chloride, CO2, BUN, Creatinine, eGFR, Magnesium, HbA1C, Total Bilirubin, Direct Bilirubin, SGPT (ALT), SGOT (AST), Albumin, Total Protein, PT, and INR. hsCRP and lipid panel will include: total cholesterol, LDL, HDL and TG. Alkaline phosphatase will also be included.

Infectious disease testing should be the bone marrow donor panel per local institutional guidelines, including HIV, Hep B (HBsAG, Anti-HBs, Anti-HBc), and Hep C (Anti-HCV); results must be known prior to randomization.

All laboratory evaluations will be repeated at the 1 week, 1 month, and 6 month visits except for the infectious disease tests.

With appropriate consent, 20 mL of peripheral blood will also be collected and transported to the CCTRN biorepository for scientific study at multiple time points. The first draw will occur on the day of the bone marrow aspiration (day -2) after sedation but prior to the start of the aspiration, on the day of study product injection (day 0) during the 30 minute injection site evaluation, and as part of the blood draw collections at the 1 week, 1 month, and 6 month visits (See Section 6.4 Collection of Biospecimens). Cells and plasma will be analyzed using flow cytometry markers, circulating colony-forming cell assays, and microRNA arrays.

6.2.7 Electrocardiogram (ECG)

A 12 lead ECG will be collected at baseline, again within 24hrs of the bone marrow aspiration, and at the 1 week, 1 month and 6 month visits.

6.3 Collection of Endpoints

For the purposes of endpoint collection, references to the index leg refer to: that leg which is most symptomatic for the patient, and will be the treated (injected) limb. The index leg will be determined through patient self-report at baseline physical/vascular exam, as well as by COT and PWT determined during treadmill testing.

6.3.1 Pre and Post-exercise ABI and Treadmill Claudication Measurements

(Methods detailed in Treadmill Core Lab Procedure Manual)

Pre-Exercise ABI/TBI:

Blood pressure and ankle-brachial index (ABI) for the symptomatic limb(s) will be obtained immediately prior to the treadmill tests at baseline, 3 months and 6 months. The arm and ankle blood pressures will be obtained with the patient supine. The patient should be supine for at least 10 minutes prior to the pre-exercise ABI. This may be done at the end of the supine rest period after participant familiarization with the treadmill, but prior to the actual treadmill test. If ABI is >1.3 or documented as unable to obtain due to noncompressible vessels at baseline, a TBI can be obtained by photoplethysmography (PPG). TBI must be <.0.70 for patient to qualify for study.

Treadmill Testing:

Participants will complete a treadmill familiarization session and three treadmill testing sessions (baseline, 3 months and 6 months) at 2 mph using a graded Gardner-Skinner treadmill protocol with ECG monitoring. The baseline session will consist of two treadmill exercise tests (TMET-1 and TMET-2) to determine PWT and COT. Each test must be conducted at least 72 hours apart from each other. Both TMET PWTs must be under the 12 minute exclusionary cut-off and the higher of the two will be used as the Baseline PWT. Participants that have a PWT ≥12 minutes on any baseline treadmill test will be excluded from the study.

The primary endpoint treadmill testing session will occur at the 6 month follow-up visit. The baseline, 3 month and 6 month tests should be completed at the same time of day within a four hour window. Participants should be instructed to fast for at least 2 hours prior to the treadmill test except for clear decaffeinated liquids and should refrain from smoking for at least 2 hours prior to the treadmill test. There should also be at least 2 hours between MRI testing and treadmill testing on any visit where both occur. Any sedatives given to the participant on the day of a treadmill test (for example, during the MRI) will need to clear the system before completing the treadmill test.

For each of these tests, the following information will be reported:

- Description of the participant's typical leg pain/claudication (to determine COT)
- COT (min/sec)

Onset of any exercise-limiting symptoms, including:

- PWT (min/sec)
- Most symptomatic leg (which will be the index leg)
- Treadmill grade when exercise terminated
- Stability of the ECG during exercise (absence of ST-segment changes and arrhythmias to document safety)
- Reason for stopping test (e.g., general fatigue, chest pain, shortness of breath, claudication pain)

Post-Exercise ABIs:
Once the participant stops walking, transfer them as quickly as reasonably possible to a stretcher and obtain systolic blood pressure (use either the dorsalis pedis or posterior tibial artery, whichever gave the HIGHER reading at rest if pre-exercise pressure was obtained by ABI) for the Index Leg, **WITHIN 2 MINUTES** after exercise is stopped. Since the pressures should be obtained within 2 minutes after completion of the treadmill test, it is important to have the stretcher or bed nearby and set up for the participant ahead of time. For some participants, the "immediate" ankle pressure in the index leg will be non-detectable due to absence of Doppler signals—this result should be entered as "zero" ("0"). If the ABI is zero in the Index Leg, continue to check pressure every 2 minutes for up to 10 minutes until pressure goes up to confirm it was zero post exercise. If pre-exercise TBI was used to qualify the participant, no post-exercise ABI or TBI will need to be collected.

More detailed methods are included in Appendix B – Gardner Skinner Treadmill Test Procedure.

6.3.2 MRI Testing

(Methods detailed in MRI Core Lab Procedure Manual)

The following MRI tests will be collected at baseline and repeated at 180 days post treatment (± 30 days):

- CE-MRA
- Phase-contrast
- Perfusion

MRI images/data should be submitted to the MRI Core Laboratory following enrollment and the 6 month visit.

6.4 Collection of Biospecimens

A central CCTRN biorepository will be utilized in this study. The biorepository will be included in the consent form and subjects will have the option of participating in the sample donation. Participation in the study does not equate with participation in donating to the biorepository; subjects can decline the biorepository donation and still participate in the overall trial.

The goal of this biorepository is three-fold: 1) to provide storage of critical biomaterials derived from patients enrolled in clinical protocols within the Cardiovascular Cell Therapy Research Network 2) to provide long-term integrity (up to 10 years) of these specimens and samples, and 3) to provide progenitor cell profiles and cytokine analyses of samples obtained during the clinical protocols undertaken by the CCTRN with an aim toward gaining insight into diagnostics of disease progression and prognostics of successful intervention. The biorepository will store these samples in cryovials for up to 10 years.

These stem cells will be used for research purposes only (not for profit), will be stored without personal identifying information, and will be shared with approved researchers who will conduct studies to improve the understanding of the effects of cell therapies. Cell samples will be destroyed after 10 years.

During the bone marrow aspiration procedure, 180mL (±10mL) bone marrow will be harvested from active and placebo patients. The bone marrow will be then transported to the local cell processing lab for packaging/shipping. 150mL bone marrow obtained from active patients will be

transported to manufacturer and the remaining 30 mL (±10mL) will be shipped (with appropriate consent) to the CCTRN biorepository. The whole harvest (180mL) from placebo patients (with appropriate consent) will be shipped to the CCTRN biorepository.

In the biorepository, samples will be processed for mononuclear cell isolation and then subjected to quantitative and qualitative analysis to evaluate cellular characteristics inherent to the individual enrolled in the trial. Cell potency will be assessed by assays designed to evaluate the content and function of progenitor cells based on colony-forming units (hematopoietic and mesenchymal) and expression of surface markers (CD34, CD133, CD271, MSCA-1) quantification, RNA and protein expression, growth kinetics and metabolic patterns.

The following variables will be used to explore the relationship between the cell phenotype and function (in bone marrow) and change in hyperemic peak flow and peak walking time (PWT) over time:

- Frequency of circulating angiogenic cells (CD45dimCD34+) and type 2 monocytic cells (CD14lowCD16+)
- Percentage of viable B cells (CD19+), and monocytes (CD11b+)
- Number of immunomodulatory/stromal MSCs (reflected by the frequency of CD45-CD34- MSCA-1+ CD271+ cells)
- Number of CFC colony forming cells per/10^6 cells
- Potency of immunomodulatory/stromal MSCs (as reflected by frequency of CFU-F colonies/10^6 mononuclear cells present)

Additionally, 20mL of peripheral blood will be withdrawn first into a 3 ml purple cap (EDTA tube) Vacutainer tube and then into a 7ml and 10 ml purple top tube on the day of the harvest (following anesthetic but prior to aspiration). Similar draws will also take place 30 minutes after study product injection (day 0) and at the 1 week, 1 month, and 6 month visits. This blood will be sent to CCTRN biorepository for analysis of circulating progenitor cells using colony-forming assay and FACS (Fluorescence-Activated Cell Sorting). Plasma isolated from these samples will be profiled for circulating microRNAs. All surplus cells from marrow and blood will be cryopreserved.

6.5 Follow-up Evaluations

6.5.1 Follow-up windows

The timeline for follow-up will begin on the day of injection (Day 0). The time windows for each of the subsequent follow-up visits will be, as follows:

1. The 1 week visit will be 7 days (between 5 days and 12 days of SPI)
2. The 1 month visit will be 30 days ± 7 days
3. The 3 month visit will be 90 days ± 14 days
4. The 6 month visit will be 180 days ± 30 days
5. The 12 month telephone contact will be 365 ± 30 days

6.5.2 Scheduling and Lost to Follow-up

Randomized subjects will be followed for efficacy for 6 months and for safety up to one year. Subjects will be considered lost to follow-up after 3 consecutive failed telephone contacts AND one certified letter returned to the site. Contact attempts will be documented in the patient's study chart.

7.0 Event Monitoring and Reporting

The safety monitoring program is a comprehensive, data driven program that provides ongoing capture and analyses of safety data and issues timely notifications, event specific reports, and scheduled cumulative trial reports of safety issues to appropriate study personnel, the National Heart Lung and Blood Institute (NHLBI) Project Officer, the Data Safety and Monitoring Board (DSMB), and the Food and Drug Administration (FDA). The program complies with applicable U.S. law, regulations, and guidance.

7.1 Definitions Related to Adverse Events

The following definitions arise from recently modified FDA reporting regulations and International Conference on Harmonization (ICH) guidelines for use in this study:

7.1.1 Adverse Events (AEs)

An *adverse event* (AE) is defined as any untoward medical occurrence associated with the use of a drug in humans, whether or not considered drug related. An AE can be any unfavorable and unintended sign (including an abnormal laboratory finding), symptom, or disease temporally associated with the study. The event does not need to have a causal relationship with treatment.

7.1.2 Suspected Adverse Reaction (SARs)

A *suspected adverse reaction* (SAR) is defined as any adverse event for which there is a reasonable possibility that the drug caused the adverse event. For the purposes of IND safety reporting, "reasonable possibility" means there is evidence to suggest a causal relationship between the study product and the adverse event.

7.1.3 Serious Adverse Events (SAEs) or Serious Suspected Adverse Reaction (SSAR)

A *serious adverse event* (SAE) or *serious suspected adverse reaction* (SSAR) is defined as an AE/SAR which, in the view of the Investigator or Sponsor, results in: 1) Death; 2) a life-threatening event (i.e. an event in which the subject was at risk of death at the time of the event; it does not refer to an event which hypothetically might have caused death if it were more severe); 3) inpatient hospitalization of > 24 hours or prolongation of existing hospitalization; 4) a significant disability/incapacity; or 5) a congenital anomaly/birth defect. Other important medical events may be considered SAEs/SSARs if, in the opinion of the Investigator or DCC, they jeopardize the subject or require intervention to prevent one of the other outcomes listed above.

7.2 Role of Abnormal Test Findings and Hospitalizations in Classifying an Event

7.2.1 Abnormal Test Findings

If a test result is associated with accompanying symptoms, and/or the test result requires additional diagnostic testing or medical/surgical intervention, and/or the test result is considered to be an AE/SAR by the Investigator or Sponsor it should be reported as an adverse event. NOTE: Merely repeating an abnormal test, in the absence of any of the above conditions, does not constitute an AE/SAR. Any abnormal test result that is determined to be an error does not require reporting as an AE/SAR.

7.2.2 Hospitalizations

AE/SARs associated with hospitalization or prolongation of hospitalization is considered serious. Admission also includes transfer within the hospital to an acute/intensive care unit (e.g., from the cardiac wing to the medical floor for an infection, or from the medical division to the neurologic unit for a stroke).

Hospitalization does not include rehabilitation facilities, hospice facilities, respite care

(i.e., caregiver relief), skilled nursing facilities or homes, routine emergency room admissions, or same day surgeries (as outpatient/same day/ambulatory procedures).

Hospitalization or prolongation of hospitalization in the absence of a precipitating, clinical AE/SAR is not in itself an SAE/SSAR.

7.3 Reporting Responsibilities of the Investigator

For all events (AE/SAR and SAE/SSAR), monitoring and reporting to the DCC begins at the time that the subject provides informed consent, which is obtained prior to the subject's participation in the study, i.e., prior to undergoing any study related procedure and/or receiving investigational product, through and including 30 calendar days after the subject completes the study. Events should be recorded on the Adverse Event eCRF. **Do not delay the initial reporting of an event in order to obtain resolution or follow up information**.

For all events, the Investigator must pursue and obtain adequate information both to determine the severity and causality of the event. For events with a causal relationship to the investigational product, follow-up by the Investigator is required until the event or its sequelae resolve or stabilize at a level acceptable to the Investigator, and the DCC concurs with that assessment.

In the rare event that the Investigator does not become aware of the occurrence of a SAE/SSAR immediately (i.e., if an outpatient study subject initially seeks treatment elsewhere), the Investigator is to report the event within 24 hours after learning of it and document the time of his/her first awareness of the event.

7.3.1 Severity Assessment

The DCC uses the Common Terminology Criteria for Adverse Events (CTCAE), version 4.0, for detailed descriptions of Severity Grades. The CTCAE schema is classified by body system and event using the MedDRA hierarchy and provides descriptions of events that qualify under each severity rating.

The following table contains general descriptions of Adverse Event Severity Grades.

Please note: Grade 1 (Mild) AE/SARs are not entered in the electronic CRF in the CCTRN database.

CTCAE Severity Grading Scale

Severity Grade	Description
1	Mild. Asymptomatic or mild symptoms; clinical or diagnostic observations only; intervention is not indicated.
2	Moderate. Minimal, local, or non-invasive intervention indicated or limiting activities of daily living (i.e. preparing meals, shopping for groceries/clothes, managing money, using telephone, etc.)
3	Severe or medically significant but not immediately life-threatening. Hospitalization or prolongation of hospitalization indicated OR disabling OR limiting self-care (e.g. bathing, dressing, feeding self, using toilet, taking medications, etc.)
4	Life-threatening consequences; urgent intervention indicated.
5	Death. Death related to adverse event.

Notice that severity and seriousness are different concepts. For example, a headache may be severe (interferes significantly with subject's usual function) but would not be classified as serious unless it met one of the criteria for SAE/SSARs (see section 7.1.3 above).

7.3.2 Causality Assessment

The DCC nomenclature for assessing the causal relationship between the study product/procedure and an event is listed in the following table.

Adverse Event/Suspected Adverse Reaction Relationship to Study Product/Procedure

Unrelated	No temporal association to study product An alternate etiology has been established.
Unlikely	Clinical events that are likely to be caused by patient's clinical state, environment or administration of other therapies or exposure to toxins
Possibly related	Reasonable temporal relationship to study product. Connection to study product cannot be ruled out
Probably related	There is a reasonable temporal association with the study product. There is a high degree of certainty that the event is related to the study product.
Definitely related	There is a direct temporal relationship to the study product. The event follows a known pattern of response to the study product.

The Investigator chooses the category that overall best describes the relationship between the event and the study product and records the evaluation on the Adverse Event eCRF. Note: If the Investigator does not know whether or not the study product caused the event, then the event will be handled as "possibly related to investigational product" for reporting purposes.

7.3.3 Expectedness Assessment

The DCC nomenclature for assessing whether an event is expected or unexpected with regard to the study product is listed in the following table.

Expected	Any event for which the nature or severity is consistent with information in study Investigator Brochure
Unexpected	Any event for which the nature or severity is <u>not</u> consistent with information in study Investigator Brochure

7.4 Reporting Responsibilities of the Sponsor (DCC)

7.4.1 Safety Monitoring Program and Reporting

The Safety Monitoring Program uses a combination of, notifications, event specific reports, and scheduled cumulative trial reports to keep the Executive Committee (EC), NHLBI, and DSMB informed about real and potential safety issues.

Notifications are comprised of an email to the EC, NHLBI, and DSMB with available information on the date and nature of the event, the site Investigator's evaluation of the severity, expectedness, and relatedness to study product; and a Sponsor assessment of the event given the information known at the time of the initial reporting.

Event specific reports are formal written reports providing the details of the event (including circumstances surrounding the event, laboratory testing, concomitant medications, and any formal diagnoses made via medical intervention). These reports include a full sponsor assessment of the severity, expectedness, and relatedness to study product as well as any available status update on the patient.

Scheduled cumulative trial reports are prepared semi-annually by the DCC. These are used by the DSMB to assess recruitment, subject safety, and continued trial feasibility. These reports include total numbers of AE/SARs and SAE/SSARs experienced in the overall trial. The information provided includes both new events reported since the last DSMB meeting and cumulative events reported during the life of the trial.

7.4.2 Sponsor Reporting Requirements to the EC, NHLBI and DSMB

Once the event has been reported to the DCC by the Investigator, the DCC uses the Medical Dictionary for Regulatory Activities (MedDRA) System Organ Classification (SOC) to classify all AEs/SARs (including SAEs/SSARs assessed by Investigator or DCC). Additional supporting documentation is requested from the site Investigator and his/her team to enable the DCC Safety Officer to accurately assess the event for reporting.

Reportable Events:

All unexpected events, all events related to study product, and all events with a severity grade of 3 or higher are reported by the DCC to the EC, NHLBI, and DSMB.

Reporting Timeframe:

For events which are unexpected AND associated with study product, the DCC will notify the EC, NHLBI, and DSMB within 72 hours of learning of the event; with an event specific report filed no later than 7 days post notification.

For all other reportable events, the DCC will notify the EC, NHLBI, and DSMB no later than 15 days of learning of the event; with an event specific report filed no later than 30 days post notification.

Cumulative trial reports (which include all events) will be generated for review by the EC, NHLBI, and DSMB at semi-annual DSMB meetings.

7.4.3 Sponsor Reporting Requirements to FDA

Once the DCC has been notified of a SAE/SSAR the following are the DCC's reporting requirements to the FDA:

Event type	Report to	Timeframe
Fatal or life-threatening, unexpected, and associated with study product	FDA	MedWatch submitted within 7 calendar days of learning of event
Other SAE/SSARs that are non-fatal or life-threatening but are unexpected and associated with study product	FDA	MedWatch submitted within 15 calendar days of learning of event

7.5 Unanticipated Problems (UPs)

An UP is an incident, experience, or outcome that specifically causes increased risk to the study or to its participants which may be of medical or non-medical etiology, and meets the following criteria:

- Unexpected (in terms of nature, severity, or frequency), given (a) the research procedures that are described in the protocol-related documents, such as the IRB-approved research protocol and informed consent document; and (b) the characteristics of the subject population being studied;

- Definitely, probably, or possibly related to participation in the research (i.e., there is a reasonable possibility that the incident, experience, or outcome may have been caused by the procedures or materials involved in the research); and

- Suggests that the research places patients or others at a greater risk of harm (including physical, psychological, economic, or social harm) than was previously known or recognized.

All UP reporting will follow the same guidelines as noted above for SAE/SSAR reporting, and must include a corrective action plan/measures to prevent recurrence.

7.6 Guidelines for Canceling Study Product Administration

The events listed below will follow the same reporting criteria for SAE/SSARs as it relates to the investigational sites as well as the DCC and are to be considered sufficient to halt the study procedure. If subject develops any of the following conditions within 24 hours of planned injection, study product delivery will be halted:

1. Hemodynamically unstable
2. Fever (Temperature increase to ≥100.4^{o}F)
3. Significant bleeding from bone marrow harvest site

7.7 Holding Rules

The expected number of SAE/SSARs in this small trial is anticipated to be too small to support formal statistical stopping rules. However, the following criteria are provided as guidelines under which this clinical trial may be put on hold pending a detailed investigation by the DSMB. The assessment of the event shall include the relationship to the trial procedures as unrelated or possibly related. If possibly related, an indication of causality to a specific aspect of the study product/procedures will be determined: (e.g. bone marrow harvest related, vascular access related, cell delivery related, or other procedure related).

7.8 Holding Criteria

The study will be placed on hold if any of the following events occur during the course of the study.

- 1 case of malignant tumor growth at the site of study product injection
- 3 cases of cellulitis/vasculitis in treated (index) limb
- 3 cases of deep vein thrombosis (DVT)
- 1 case of pulmonary embolism
- 1 case of progression of disease to critical limb ischemia (CLI)
- 1 case of compartment syndrome

As soon as any of the above is identified, the centers will inform the DCC, and the DCC will inform the NHLBI Project Office and the DSMB. In addition, the DSMB will monitor the distribution of all SAE/SSARs, and the Network will be responsive to all DSMB concerns regarding SAE/SSARs that are not part of holding criteria listed above.

In addition, the DSMB may also recommend stopping the trial for the performance-related issues that would prevent the study from meeting its scientific objectives, such as:

- Failure to recruit and enroll patients
- Inability to meet protocol requirements for delivery of cells at specified times
- Inability to prepare study product which meets quality specifications

8.0 STATISTICAL PROCEDURES

8.1 Randomization

Randomization to treatment assignment will be conducted using a web access database created and maintained by the Data Coordinating Center (DCC). After the research coordinator verifies that baseline testing is complete and the inclusion and exclusion criteria for the study have been satisfied, an unblinded member of the local stem cell laboratory will have secured access to the patient's treatment assignment. Patients will be randomized to the active or placebo group, using variable block sizes of 2 and 4, randomly selected. Patients will be stratified by center. When a patient is consented, the clinic will be given an identification (ID) number and acrostic and a list of procedures to be completed at the baseline. A patient-specific schedule of visits and procedures will be displayed for printing locally. The DCC will monitor patient recruitment by providing reports to the Core Laboratories and the NHLBI Project Office as appropriate during the recruitment phase. Updated reports will be maintained on an Internet site accessible to all units of the study. The recruitment reports will provide data on recruitment of women and minorities (African-Americans, Hispanics, and Asians). Goals for recruitment will be set and will be reviewed by the DCC and the NHLBI Project Office.

8.2 Statistical Analyses

Biostatisticians at the DCC, with the assistance of scientific programmers, have adapted or developed a number of statistical programs for analyzing study data. Data are analyzed for both data monitoring purposes, as described above, and for the purpose of detecting beneficial or adverse treatment effects. The DCC uses standard statistical packages such as SAS, S-PLUS, R and Stata to perform statistical analyses.

8.3 Baseline Analyses

Although the stratified (by clinical center) random assignment of patients to the various treatments should ensure comparability with respect to known and unknown variables, imbalance may occur by chance. Descriptive statistics for baseline characteristics known or suspected to be associated with outcomes will be prepared for the various treatment groups. The variables considered in such a description can be categorized as: 1) demographic characteristics; 2) medical history; 3) physical examination; and 4) laboratory data. Exact testing for categorical variables and Student *t* testing for continuous variables will be used to evaluate the differences in baseline variables between treatment groups.

8.4 Outcome Analyses

8.4.1 Primary and Secondary Endpoints

There are four primary endpoints: PWT, anatomy, vascular flow, and perfusion MR. Each will be tested at the 0.05 level. Each of these is a continuous endpoint. There are seven secondary endpoints: Change in pre-exercise ABI, change in post-exercise ABI, change in claudication onset time (COT), change in WIQ score, change in PAQ score, PWT at 3 months, and the assessment of the relationship between PWT and the three imaging based primary endpoints.

The four primary endpoints and seven secondary endpoints listed above are each continuous and will be assessed individually using a simple unweighted general linear model to evaluate the effect of therapy on the change from baseline to six months in the primary and each of the secondary endpoints.

$$E[\Delta y_i] = \beta_0 + \beta_1 w_i + \beta_2 x_i \qquad (0.0)$$

where the dependent variable, Δy_i is the change in PWT (follow-up – baseline). The predictor variables are the baseline measure of PWT, w_i (continuous) and the effect of therapy, x_i (dichotomous). The effect of therapy is based on a hypothesis test for the coefficient β_2 H_0:$\beta_2 = 0$ versus H_a:$\beta_2 \neq 0$. In addition, the general linear model will be used to adjust for the influence of important covariates (e.g., age, duration of claudication). A dichotomous variable will be created that assigns the value of zero to every patient in the placebo group and one to every patient in the cell delivery group. Both unadjusted and adjusted treatment effects will be computed; adjustments will be for baseline covariates whose association with the dependent variable is generally accepted. If we let y_{ijk} be the measurement or relative measurement of the outcome for treatment *i* of patient *j* at time *k*, then y_{ijk} can be represented as a general linear mixed model:

$$y_{ijk} = \mu + \alpha_i + \tau_k + (\alpha\tau)_{ik} + d_{ij} + e_{ijk}$$

where $\mu, \alpha_i, \tau_k, (\alpha\tau)_{ik}$ are fixed effects due to overall treatment, follow-up time, and interaction between treatment and follow-up time, respectively, and d_{ij} is the random effect due to patient *j* in treatment *i* group and e_{ijk} is the error term. Although we do not anticipate there will be interaction between treatment and follow-up time, we will perform the analysis. More generally, the above model can be written in the following matrix form

$$Y = X\beta + Zu + e$$

where *X* is the design matrix and $\mu, \alpha_i, \tau_k, (\alpha\tau)_{ik}$ are elements of β, Z is the design matrix for the random part, d_{ij} are elements of *u*. We can assume *u* to be a multivariate normally-distributed random variable with a mean vector of zero and variance-covariance matrix G, where *e* is the error vector with elements of e_{ijk}. Various forms of correlation structure for *e* will be investigated in statistical modeling. Should it be necessary, transformations of the measurements will be considered (e.g., natural log, square root, and reciprocal transforms).

In addition, an analysis will be conducted in those patients who had pre-exercise and post-exercise ABI measurements. Within this subcohort the effect of therapy will be assessed in patients who had a 20% fall in ABI between rest and exercise pressures and those who did not.

A second subanalysis to be executed is the effect of therapy in patients with suprapopliteal disease and infrapopliteal disease compared to the effect of therapy in patients with only infrapopliteal disease

Anatomy (contrast enhanced MR)
A Mann Whitney U statistic will be computed to compare the difference between the change in the number of patent vessels in the treated group to that in the placebo group.

8.4.2 Subgroup Evaluations

The effect of subgroup stratum on the relationship between cell delivery and the endpoints (both primary and secondary) will be assessed. If a treatment effect is demonstrated, it is not likely to behave identically among all important subgroups. The subgroups of interest are age, gender, race, baseline ABI, diabetes, hypertension, cilostazol, statins, LDL, smoking status, BMI, and characteristics of the bone marrow and study product, including function of progenitor cells (hematopoietic and mesenchymal), expression of surface markers, RNA and protein expression, growth kinetics and metabolic patterns, and the number of cells delivered. These additional analyses can sometimes be helpful in identifying extreme differences in the effects of treatment among subgroups, although the literature wisely warrants that caution be used in interpreting subgroup analyses.

8.5 Additional Analyses

We will use the three month PWT to assess the change of PWT from baseline from three months to six months, assessing if this trajectory is related to study product using mixed model analysis of variance.

We will evaluate the relationship among the four primary endpoints. Specifically, we will assess the relationship between the change in PWT and each of the three remaining primary endpoints, adjusting for important baseline covariates.

We will adjust the primary analyses for clinical center effects and important baseline covariates.

We will examine the number of subjects who walked for greater than 11 minutes on their baseline PWT by therapy group and adjust the treatment effect for the influence of duration of walking.

8.6 Multiple Comparisons

In this study, no adjustments are made for multiple comparisons. Measures of effect will be effect size, the standard error of the effect size, the 95% confidence interval for the effect size and the *p*-value. *P*-values will be interpreted at nominal 0.05 levels.

9.0 TRIAL MANAGEMENT

9.1 Institutional Review Board (IRB) Review and Informed Consent

This protocol and the informed consent document and any subsequent modifications will be reviewed and approved by the IRB or ethics committee responsible for oversight of the study. A signed consent form will be obtained from the subject. The consent form will describe the purpose of the study, the procedures to be followed, and the risks and benefits of participation. A copy of the consent form will be given to the subject and this fact will be documented in the subject's record.

9.1.1 Informed Consent Process

Potential participants will be approached by one of the study investigators or research coordinators. Information regarding study participation will be provided to the potential participant. The informed consent includes descriptions of all study related procedures, all possible risks to participant, and the time commitment involved with participating. All consent forms will have IRB approval. Individuals who agree to participate will receive a copy of the signed informed sent. The research staff member obtaining consent will document the informed process in the patient's chart for monitoring purposes.

9.1.2 Risks Associated with the Procurement, Processing, and Injection of the Study Product

Bone Marrow Aspiration

Possible risks of bone marrow aspiration include: bruising, bleeding, infection, hematoma (a swelling filled with blood) at site of biopsy, brief discomfort in your hip area and faintness from the procedure. There is a possibility of a fat embolism (fat tissue passing into the bloodstream and blocking a blood vessel). Patients who are taking anticoagulation medications at the time of the bone marrow aspiration, may experience a temporary interruption; during which time the patient may be at an increased risk of a stroke. The patient should be advised to inform the research team immediately of any symptoms of dizziness, light-headedness, blurred vision, slurred speech, facial drooping, decrease sensations anywhere on your body, or weakness or a decrease in strength of the arms or legs. The patient will be closely monitored during any interruption in anticoagulation therapy, such as the bone marrow aspiration for the events listed above.

Cell Processing Procedure

Processing the cells is done under strict sterile conditions; however, there is a rare chance that the cells could become contaminated while being processed. Testing will be done on the cells, and if the tests reveal contamination, the patient will be notified and instructed on whether or not he/she should be treated with antibiotics. The subject will keep a daily temperature log for 7 days to help determine the development of an infection before the test results are known. If the patient notes a fever, he/she will be requested to notify the investigator/study team.

Study Product Injection

Cell injection procedure will be performed after prepping the skin and treatment with local anesthetic creams. Risks associated with the study product and its injection include bleeding associated with needle penetration, hematoma (a swelling filled with blood), fever, infection, rash, skin ulcer (skin breaks), swelling, redness, inflammation of muscle or skin (myositis, fasciitis, cellulitis), pain or pressure in the leg or at the site of injection, and lack of blood flow and oxygen to the leg. The most likely possible risk is an allergic reaction around the injection sites in the leg. Less likely risks include nerve damage, decreased blood volume, dizziness or fainting, chest pain, muscle spasms, seizures, reduced body temperature in your leg, chest pain, blood clots in the veins of the leg, and decreased sensitivity to touch. In very rare cases, injections can cause excessive bleeding in your calf or lower thigh muscles leading to swelling in your lower leg which could require surgery to correct. Participants will be asked to take their temperature daily and to notify the study team immediately if they experience any of the above symptoms. Participants will be checked 1 week after the procedure for any developing complications.

Study product will be administered according to the injection guidelines in the study product injection manual (Appendix E)

9.1.3 Adequacy of Protection Against Risks

The precautionary measures mentioned in the last two sections will minimize the risk associated with bone marrow aspiration and cell injection for patients. Overall the study procedures are low risk and in our previous similar trials there was no complications related to bone marrow aspiration and cell delivery.

9.1.4 Potential Benefits of the Proposed Research to the Patients and Others

The potential benefits of this research for patients include increase in leg blood flow which will have therapeutic effects such as increase in pain-free walking.

This project will also provide mechanistic insight into cell therapy which will be useful for finding new treatments for other diseases.

9.1.5 Risk Benefit Analysis

The administration of autologous ALDH^{br} cells offers a new option to patients with intermittent claudication. The goal of this therapy is to improve ameliorate the progressive nature of this disease by stimulating the development of new blood vessels. Having highly trained experts deliver and oversee the therapy with close study monitoring substantially reduces the likelihood of AEs. The potential risks to the patients remain reasonably low in relation to the possible benefit of improving their claudication symptoms above which can be obtained with standard of care treatment regimens.

9.1.6 Importance of the Knowledge to be Gained

The knowledge to be gained from this clinical trial is significant in that 1) patients with intermittent claudication may benefit from the delivery of a promising cell type that heretofore has demonstrated few risks to the subject. The trial has been designed to address critical limitations in the previous published trials which are all in the critical limb ischemia patient population. The risks to the subjects are reasonable in relation to the knowledge gained from this study since this therapy may potentially reduce the progressive and debilitating effects of intermittent claudication.

9.1.7 Data Safety Monitoring Board (DSMB)

The Data and Safety Monitoring Plan has been outlined in Section 7 above

9.2 Clinical Monitoring

9.2.1 Pre-Investigation Visits

The DCC team assures the Investigator clearly understands and accepts the obligations incurred in undertaking a clinical investigation:

Prior to the initiation of a clinical investigation, the DCC team will train the site of the clinical investigation to assure that the Investigator:

1. Understands the investigational status of the test article and the requirements for this accountability.
2. Understands the nature of the protocol or investigational plan.
3. Understands the requirements for an adequate and well-controlled study.
4. Understands and accepts his or her obligations to obtain informed consent in accordance with 21 CFR Part 50. The monitor should review a specimen of each consent document to be used by the Investigator to assure that reasonably foreseeable risks are adequately explained.
5. Understands and accepts his or her obligation to obtain IRB review and approval of a clinical investigation before the investigation may be initiated and to ensure continuing review of the study by the IRB in accordance with 21 CFR Part 56, and to keep the sponsor informed of such IRB approval and subsequent IRB actions concerning the study.
6. Has access to an adequate number of suitable patients to conduct the investigation.
7. Have adequate facilities for product preparation and conducting the clinical investigation.
8. Has sufficient time from other obligations to carry out the responsibilities to which the Investigator is committed by applicable regulations.
9. Understands periodic monitoring visits will occur.

9.2.2 Interim site visits

The monitor should visit the Investigator at the site of the investigation frequently enough to assure that:

1. The facilities used by the Investigator continue to be acceptable for purposes of the study.
2. The study protocol or investigational plan is being followed.
3. Changes to the protocol have been approved by the IRB and/or reported to the sponsor and the IRB.
4. Accurate, complete, and current and current records are being maintained.

5. Accurate, complete, and timely reports are being made to the sponsor and IRB.
6. The Investigator is carrying out the agreed-upon activities and has not delegated them to other previously unspecified staff.
7. Review of subject records will take place.

9.2.3 Monitor Role

The monitor should compare a representative number of subject records and other supporting documents with the Investigator's reports to determine that:

1. The information recorded in the Investigator's report is complete, accurate, and legible.
2. There are no omissions in the reports of specific data elements such as the administration to any subject of concomitant test articles or the development of an intercurrent illness.
3. Missing visits or examinations are noted in the reports.
4. Patients failing to complete the study and the reason for each failure are noted in the reports.
5. Informed consent has been documented in accordance with 21 CFR Parts 50 and 56.

9.2.4 Monitor Recording

The monitor should maintain a record of the findings, conclusions, and action taken to correct deficiencies for each on-site visit to an Investigator. Such a record may enable the FDA to determine that a sponsor's obligations in monitoring the progress of a clinical investigation are being fulfilled. The record may include such elements as:

1. The date of the visit;
2. The name of the individual who conducted the visit;
3. The name and address of the Investigator visited;
4. A statement of the findings, conclusions and any actions taken to correct any deficiencies noted during the visit.

9.3 Investigator Responsibilities

9.3.1 Investigator Performance

Prior to enrolling the first subject, each Investigator must read and understand the protocol. Additional requirement that must be met are:

1. Signed Protocol Signature Page
2. Current medical license
3. Financial disclosure
4. CV, signed and dated, for all primary Investigators and sub-Investigators
5. Local stem cell processing lab certified
6. Completed site training
7. Follow all Good Clinical Practice requirements for clinical research

9.3.2 Site Requirements:

Prior to enrollment of the first subject, the Investigator and institution will be asked to provide the following documents:

1. Executed study contract between NHLBI and the clinical center
2. IRB approved informed consent form
3. IRB approved final protocol
4. Current laboratory certification for all associated laboratories
5. Current laboratory normal ranges

9.3.3 Institutional Review Board Approval

Prior to enrolling the first subject, the Investigator must obtain written approval from the IRB. The approval must contain the date the study was approved, the version of the informed consent that

was approved and the signature of the IRB chairperson. The primary investigator and their staff will follow all Good Clinical Practice (GCP) requirements.

9.3.4 Informed Consent

The DCC must review and approve all informed consent forms prior to submitting to the IRB. All study patients must provide written informed consent using an IRB- approved informed consent document.

9.3.5 Reporting Requirement of the Sites

See Investigator reporting responsibilities in section 7 above.

9.4 Sponsor Responsibilities

9.4.1 Introduction

The DCC will act as the study Sponsor, and thus have overall responsibility for the conduct of the study, including assurance that the study follows all standards and regulatory requirement of the U.S. Food and Drug Administration. The DCC will adhere to Sponsor general duties as outlined by 21 CFR Subpart D; Part 312.50-312.70.

9.4.2 Routine Duties

The DCC is responsible for obtaining and reviewing copies of IRB approvals. They are responsible for setting up all training for each site and reviewing all certification of their local laboratories for handling of study products. The DCC will ensure that the study is conducted according to Good Clinical Practice (GCP), the Declaration of Helsinki, the Study Protocol, and any other applicable regulatory agency requirement. The DCC will also ensure proper clinical site monitoring.

9.4.3 Site Training

The DCC will be responsible for the setting up all training required in the protocol and will establish a schedule for site initiation visits (SIVs).

9.4.4 Site Monitoring

The DCC will be responsible for monitoring each site throughout the course of the study by following the FDA Guidelines for monitoring of a clinical trial (revised 1998). Source document review will be performed against entries on the CRF and a quality assurance check will be performed to ensure that the Investigator is complying with the protocol and regulations. At the time of the completion of the study, a close out monitoring visit will take place to ensure all trial materials and subject data are properly documented.

9.4.5 Reporting to the FDA

The DCC will hold the IND for this study and submit proper filings to obtain and maintain the IND. The DCC will submit all appropriate reports and fillings to the FDA as required by regulations. This includes unanticipated adverse events, withdrawal of IRB approval, and withdrawal of FDA approval, annual progress reports to the FDA and all final reports. The DCC will maintain all records according to Good Clinical Practice Guidelines (GCP).

All clinical centers and core labs will comply with 21 CFR, part 312.62 with regard to record retention.

9.5 Database

The DCC will maintain the CCTRN study database in a web-accessible electronic format. Detailed documentation of study variables will be prepared and available to study Investigators, and

where necessary, to external scientists. Appropriate confidentiality and security of these files will be maintained at all times.

9.5.1 Framework

The DCC will develop and maintain a web-based online application for data entry using the state-of-the-art, Microsoft .NET framework. A secure environment, requiring user login and authentication, will be maintained for the entry of and/or access to patient data. The data collected from Clinical Centers will be stored on a secure database in the DCC computer facility. Training will be provided and DCC staff will be available to answer questions and resolve issues. Extensive data verification and validation will be implemented on the web application to check for data accuracy, completeness, and consistency within patients.

9.5.2 Security

Several levels of security will be implemented to protect the confidentiality of the data. All authorized users will be provided a unique name/password and will be given access as identified by the Principal Investigator. The server on which the data is stored will be behind a firewall and will be in the most secure zone (100) with no direct access to the internet. In addition, data will be protected through the use of Secure Socket Layers, (SSL), the current standard for encrypting data between a client and a server as it is passed across the Internet. In addition to these layers of security, every connection to a secured site will be recorded with data indicating which person connected, the time of the connection, and the area accessed. The user's password will be stored in binary, hashed format within the database for additional security. Access to secure areas of the website will be logged with the users ID and the date and time of access. This audit table will be maintained throughout the life of the studies. The servers that host the Network database are enrolled in the automated virus and operating system patch management system to protect against any virus attacks. The database will be backed up nightly, and backup will be stored at an off-site University on-line storage facility that is secure and has restricted access.

9.5.3 Follow-up

The DCC will provide online web-based forms for follow-up data collection. All the standards and security guidelines that were set for baseline forms will be implemented for these forms as well. Data will be stored on a secure database and access will be limited and secure. Training and documentation will be provided by DCC staff to all the CCs on the data entry process. DCC staff will also be available to answer questions and help resolve issues as necessary. Reports for follow-up data will also be made available.

9.5.4 Laboratory Data Processing Support

The DCC will develop and maintain online web forms for the laboratories for data collection, both for baseline and annual follow-up. The data will be validated with extensive edit rules and the CCs/Lab will be able to correct errors real time. Access will be limited and will require secure log-in authentication. The DCC will provide training and documentation to laboratory personnel on the data entry process and will be available to answer question and resolve issues as necessary. The data collected will be stored on a secure database in the DCC and will be backed up every night. Reports will be generated as necessary with real-time data.

9.5.4.1 File transfers

Provisions will be made for those sites that prefer to transfer files in a batch mode. Files with data from the laboratory will be transferred to a secure server residing in the computer facility of the DCC. Users transferring this data will be provided with user identification numbers and passwords for restricted and secure access. Data transmitted will then be processed and checked for validity and completeness. Only data that passes these edits will be stored in the

database. The rejected records will be sent back to the centers/lab for correction and re-transmittal.

9.5.5 Data Quality

The case report forms used for data entry are created by the DCC project and programming staff in conjunction with the research personnel at each clinical site. Once developed, individual forms are unit tested by the programming team and released to a test server. The forms are then tested by both DCC and clinical site personnel for accuracy and utility. Continuity and acceptance testing will be done by the clinical site research and laboratory personnel. An iterative process of suggestions/corrections/retesting will occur until the application is accepted. Personnel accessing the application for data submission will receive training on the web based system prior to the randomization of patients. There will be defined a minimum data set that constitutes completeness. All data will have to pass through range and logical checks in addition to intra- and inter-form checks for consistency. The sequence of events will be enforced by allowing subordinate forms to become accessible only after its primary form has been submitted. If a response to a question on a form requires ancillary forms to be completed, the user will receive reminder messages within the application to complete the proper form. Weekly reports on the Clinical Center's data entry and completeness will be generated. If a Clinical Center has problems, action will be taken from retraining through phone calls to a site visit, if necessary

9.5.6 Computing Infrastructure

The University of Texas School of Public Health network consists of a fiber optic backbone using gigabit technology to provide the fastest and most state-of-the-art network communications possible. A backbone of Cisco switches provides for client access to backend resources and servers at 100 megabits per second. Aside from providing simple network access, Information Technology staff has real-time monitoring capabilities to diagnose and correct potential network problems. The campus has also implemented a four-tier network firewall to protect all workstations and servers with varying degrees of security, based on the device's security level within the organization.

9.5.7 Backup Procedure

The study data will be backed up on a nightly basis and the backup will be stored offsite at a University on-line storage facility that is secure and has restricted access.

9.6 Dissemination

The overall usefulness of scientific research depends not only on the importance of the findings, but also on its eventual reach and effect on population health. Therefore, research projects must integrate ways to promote the eventual diffusion of the results into their research plans. CCTRN will work with professional associations to access health care providers like the NHLBI has done for a number of initiatives including asthma and hypertension. CCTRN will use three general dissemination methods that will be tailored for the target audiences.

9.6.1 Web Site

The web site will be created from the beginning of the project with objectives targeted to the study audience. The CCTRN web site will serve as one method of distribution of information about stem cell research in cardiovascular disease in general and about the specific study protocols. For the general lay public, the goal is to promote a hospitable context for the research by informing the public about the kinds of research being done, including the source of the stem cells; what this research is and what it isn't; plans for studies; study findings; and the potential for new treatments. Physicians need information about the research that is closely tied to clinical trial opportunities and potential treatments for patients. This information should be tied to the normal places practi-

tioners seek such resources. For the researcher audience, the web site will provide more in-depth technical information and published works.

9.6.2 E-network

To develop a dissemination network or linkage system for the beginning of the research, the DCC will recruit participation in two networks. These interactive networks will build support for distribution of information as it becomes available. The first is the public-service network. These participants would be liaisons from voluntary health associations such as the American Heart Association. This type of organization has a mission of public information and can serve as an effective link to public media sources. The second network will comprise liaisons from professional health care provider associations. These organizations will be identified by the NHLBI and project committees based on the model of successful programs at the NHLBI such as Asthma Education and Prevention Program. The organizations will recruit liaisons who will receive periodic updates about ongoing studies and results and who will be available to provide feedback about the implications of study findings for practitioners and the barriers to patient participation in protocols. As studies are initiated and as results become available, the CCTRN will work with the clinical sites and the NHLBI press office to coordinate the release of this information.

9.6.3 Manuscripts and Presentations

A primary task of the DCC will be to provide data analyses for all manuscript proposals and presentations approved by the SC. The CCTRN Investigators will take the lead in presenting study data at major scientific meetings and in the writing, preparation, and submission of manuscripts to appropriate peer-reviewed journals. In addition, the Network Investigators will actively enlist the participation of junior Investigators in manuscript writing and presentations at scientific meetings. The DCC will also make data sets available to the Clinical Centers (CCs), Cell Processing and other Cores, will provide consultation and assistance to the CCs regarding the appropriate data analysis methods, and will perform independent data analysis in order to verify the Investigators' findings.

The DCC will play an active role in preparing study publications in collaboration with other study Investigators and the NHLBI Project Office. The DCC will prepare all manuscripts for submission to the journals and will serve as the liaison between the lead author, and the journal. A Publications and Ancillary Studies Committee will organize and monitor writing committees and provide oversight on what presentations and publication have priority within the study. The DCC will maintain and distribute a progress report on the status of all active papers, as well as a study bibliography including abstracts, presentations, letters, editorials, etc.

9.6.4 Methodologic Developments

In addition to providing statistical support to PIs at CCs and NHLBI, Investigators at DCC will take leading role in developing possible new statistical methods that may have the potential to improve statistical analysis for projects in CCTRN and beyond. These new discoveries will be presented to scientific meetings and in statistical journals as peer-reviewed articles.

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Appendix A- MRI Assessments

MRI assessments for the protocol include the following elements. Standard operating procedures can be found in the MRI core lab manual provided to each participating clinical center.

- **Contrast-Enhanced MR Angiography (CE-MRA) for Evaluating Vascular Morpholgy**
  The morphology of the peripheral vascular tree at the level of the thighs and calves will be determined using time-resolved MRA procedures using a dual-bolus strategy. Gadolinium contrast (Magnevist ONLY) doses of 0.05 mmol/kg and 0.10 mmol/kg will be injected to produce contrast enhancement. These images will be used to identify arterial stenosis and collateral artery development.

- **Phase Contrast MRA for assessment of bulk flow**
  The bulk macrovascular blood flow in the popliteal artery will be measured using a MR phase contrast method. Using a cardiac gated MR phase contrast technique, the bulk blood flow across the popliteal artery will be measured. The velocity encoding value will be set at 100 cm/s and if aliasing was observed, then the value will be increased to 150 cm/s. The acquired spatial resolution will be less than 1.25 x 1.25 x 5 mm, and the temporal resolution will be less than 50 ms. This will be performed before and after induced hyperemia. Hyperemia will be induced using a thigh-cuff inflated to supra-systolic pressures that will be inflated for a maximum of 5 minutes or for as long as the patient can tolerate. The cuffs will be rapidly deflated (< 5 s). Imaging will be performed pre-cuff inflation to measure resting flow and for up to 10 minutes post-cuff deflation. The analysis of the data (at the core lab) includes measuring peak flow at rest and hyperemia from the symptomatic leg.

- **Dynamic Contrast Enhanced MRI (DCE MRI):**
  The microvascular blood flow to the calf muscle will be assessed using DCE-MRI method. The provocation of post-ischemic reactive hyperemia on both legs will be accomplished using a cuff-inflation paradigm similar to that described above. The cuffs will be placed at the mid-thigh level and inflated to supra-systolic values. The two cuff inflations will be separated by at least 15 minutes after deflation of the cuff for the previous experiment to ensure full recovery.
  The longitudinal relaxation time (T1) of the calf muscle will be measured using a variable-flip angle T1 mapping method before the contrast administration. The cuff will be inflated, and an extravascular Gd-chelate based MR contrast administration will be administered (0.05 mmol/kg). The data acquisition will commence at 30 seconds prior to cuff deflation (to bring the MR signal to steady state), and will continue for 300 seconds after cuff deflation. MRI data will be acquired using a 3-D T1 weighted spoiled gradient echo technique.
  Data analysis, performed at JHU includes analysis of the time-intensity curve of the signal evolution to determine the influx constant and the area under the curve after cuff release in the symptomatic leg.

- **Data Transfer**
  MRI technologists burn the study images onto a CD and give it to the Clinical Site Coordinator who will upload to a secure website at Johns Hopkins. The Clinical Site Coordinator retains the CDs with other study forms/documents. The MRI images will also be stored on the local PACS system.

Appendix B- GARDNER SKINNER TREADMILL TEST PROCEDURES

Each study participant will complete a treadmill familiarization session and four treadmills [two at baseline TMET-1 and TMET-2 at least 72 hours apart, 3 months, and 6 months] at 2 mph using a graded treadmill protocol (Gardner-Skinner protocol described below) with ECG monitoring. Both baseline TMET PWTs must be under the 12 minute exclusionary cut-off and the higher of the two PWTs will be used as the Baseline PWT. Participants that have a PWT ≥12 minutes, in either TMET-1 or TMET-2 will be excluded from the study. The primary endpoint treadmill testing session will occur at the 6 month follow-up visit.

For each of these tests, the following information will be reported:

- Description of the participant's typical leg pain/claudication (to determine COT)
- COT (min/sec)

Onset of any exercise-limiting symptoms, including:

- PWT (min/sec)
- Most symptomatic leg (which will be the index leg)
- Treadmill grade when exercise terminated
- Stability of the ECG during exercise (absence of ST-segment changes and arrhythmias to document safety)
- Reason for stopping test (e.g., general fatigue, chest pain, shortness of breath, claudication pain)

Terms

Peak Walking Time (PWT) is defined as the maximum time in minutes and seconds walked by a participant on a treadmill under standardized conditions. The participant should continue the test until walking can no longer be tolerated because of claudication symptoms or safety concerns such as chest pain or shortness of breath. It is critical that the participant **not** stop walking when they normally would do so. The participant should be asked to continue to walk until they feel they must stop due to maximally-tolerated claudication symptoms.

Claudication Onset Time (COT) is defined as the time in minutes and seconds walked by a participant on a treadmill under standardized conditions before the onset of claudication symptoms, regardless of whether this is manifested or characterized as muscle pain, ache, cramp, numbness or fatigue. This does not include joint pain or other pain not associated with claudication.

The Index Leg is that leg which is most symptomatic for the participant, and will be the treated (injected) limb. The index leg will be determined through participant self-report at baseline physical/vascular exam, as well as by the leg that stopped them walking on the treadmill during baseline treadmill testing.

Treadmill Set up

The treadmill must be programmed with the attached Gardner-Skinner protocol.

It is critical that the treadmill be functioning accurately throughout the trial. If the treadmill is self-calibrating or only requires calibration after being moved or having maintenance performed, document this appropriately and follow the specifics for your treadmill. Otherwise the treadmill function must be assessed by an appropriate technician using appropriate methodology to check the accuracy of the speed and gradient. The treadmill speed and gradient must be within manufacturer specifications as verified by a qualified assessment prior to commencement of the study and reassessed annually during the study.

The treadmill room should be free of distractions that might interfere with the treadmill test. These distractions include, but are not limited to, televisions, other staff present that are not involved in the treadmill testing, other procedures being performed on other participants and general background noise. There must be a gurney or exam table next to the treadmill to accommodate pre-exercise and post-exercise ABI testing. The treadmill should be situated such that the staff is able to assist the participant if they have difficulty while walking on the treadmill.

Treadmill Familiarization

A short familiarization session on the treadmill will take place at the first screening visit, then the first baseline treadmill exercise test (TMET-1) should take place later that day, at least 30 minutes after the familiarization session. The second baseline (TMET-2) should take place at least 72 hours apart from TMET-1. More details regarding the treadmill familiarization session will be available in the Treadmill Core Lab Manual.

Pre-Exercise Ankle Brachial Index (ABI)/Toe Brachial Index (TBI)

It is important that ABI be obtained immediately prior to, and for the index limb, following TMET-1 This measurement will be reviewed by the Treadmill Core Lab for inclusion in the study. If the ABI is < 0.90 and approved by the Treadmill Core Lab, the ABI does not need to be repeated for TMET-2. If the ABI is ≥ 0.90 or not acceptable to the Treadmill Core Lab, both the pre and post measurements will need to take place at TMET-2. If ABI is >1.3, a TBI can be obtained by photoplethysmography (PPG). TBI must be <.0.70 for patient to quality for study.

The arm and ankle/toe blood pressures will be obtained with the participant supine. The participant should be supine for at least 10 minutes prior to the pre-exercise ABI/TBI.

Treadmill Exercise Test (TMET)

All treadmill tests should be completed at the same time of day within a four hour window. Participants should be instructed to fast for at least 2 hours prior to the treadmill test except for clear decaffeinated liquids and should refrain from smoking for at least 2 hours prior to the treadmill test. There should also be at least 2 hours between MRI testing and treadmill testing on any visit where both occur. Any sedatives given to the participant on the day of a treadmill test (for example, during the MRI) will need to clear the system before completing the treadmill test.

The treadmill controller timer should be used to measure PWT and COT. Time should be recorded in _min _sec format as this is the format used by most treadmill controllers.

Continuous ECG testing is required during treadmill testing. If the PI or designee observes a clinically significant abnormality in the ECG, assess whether the participant is appropriate to continue the treadmill test. A 12-lead printout should be done to document the COT and the PWT.

Remember that “severe” claudication (“5” on the Claudication Rating Scale) does not mean that the participant should stop walking. Many participants can continue walking even though their symptoms are severe.

If the participant experiences shortness of breath, chest pain, dizziness, significant ECG changes or any other significant sign or symptom that makes the site staff concerned for the participant’s safety, **STOP** the treadmill test **IMMEDIATELY** and take appropriate medical intervention.

The detailed instructions for performing the Treadmill Exercise Test will be included in the Treadmill Core Lab Manual.

Post-Exercise ABI

Once the participant stops walking, transfer them as quickly as reasonably possible to a stretcher and obtain systolic blood pressure (use either the dorsalis pedis or posterior tibial artery, whichever gave the HIGHER reading at rest if pre-exercise pressure was obtained by ABI) for the Index Leg, **WITHIN 2 MINUTES** after exercise is stopped. Since the pressures should be obtained within 2 minutes after completion of the treadmill test, it is important to have the stretcher or bed nearby and set up for the participant ahead of time. For some participants, the "immediate" ankle pressure in the index leg will be non-detectable due to absence of Doppler signals—this result should be entered as "zero" ("0"). If the ABI is zero in the Index Leg, continue to check pressure every 2 minutes for up to 10 minutes until pressure goes up to confirm it was zero post exercise.

It may be helpful to mark the location of the Doppler signal pre-exercise to increase confidence post-exercise that the location is correct if Doppler signals are not detected. Usually, if this occurs, Doppler signals will return in a couple of minutes. The highest resting brachial pressure should also be measured and recorded at the same intervals post-exercise.

If pre-exercise TBI was used to qualify the subject, no post-exercise ABI or TBI will need to be collected.

Gardner Skinner Treadmill Protocol

Stage	Speed (mph)	Elevation (% grade)	Duration (min)
Rest/Recovery*	2.0	0	--
1	2.0	0	2 minutes
2	2.0	2	2 minutes
3	2.0	4	2 minutes
4	2.0	6	2 minutes
5	2.0	8	2 minutes
6	2.0	10	2 minutes
7	2.0	12	2 minutes
8	2.0	14	2 minutes
9	2.0	16	2 minutes
10	2.0	18	2 minutes
11	2.0	18	At least 20 minutes

** On some treadmills this stage may be called Sitting, Supine and/or Standing. Other treadmills may not have this stage. The purpose of this stage is to get the belt up to 2.0 mph prior to the participant stepping on the belt. The participant should not straddle the belt for longer than necessary.*

Appendix C- Bone Marrow Aspiration Standard Operating Procedure

The following Standard Operating Procedure (SOP) is for carrying out bone marrow aspirations for patients recruited in the Cardiovascular Cell Therapy Research Network (CCTRN) protocols.

CCTRN patients will undergo bone marrow aspiration to harvest cells for the protocol.

Purpose:
Bone marrow aspiration is a scheduled procedure performed by a trained Physician (e.g., hematologist, pathologist, or hematopathologist). Only physicians with substantial experience in carrying out bone marrow harvesting procedures (more than forty previous successful procedures) will perform the procedure. Other medical personnel trained in bone marrow aspiration procedures (e.g. physician assistants, registered nurses, nurse practitioners, and medical technologists) will assist in the collection to ensure proper sample collection, preparation and processing of the specimen. The bone marrow aspiration is indicated for research regarding cell therapy for cardiovascular conditions.

Scope:
This SOP refers to bone marrow collections at the seven cell therapy centers and their associated satellite facilities involved in the CCTRN. The seven centers are as follows:

1. Texas Heart Institute Stem Cell Center
2. Minneapolis Heart Institute Foundation
3. University of Florida Department of Medicine
4. Stanford University School of Medicine
5. University of Miami Miller School of Medicine
6. Indiana University School of Medicine
7. University of Louisville School of Medicine

PROCEDURE

Supplies and transportation:

1. Bone marrow aspiration supplies will comply with the site-specific institutional procedures and practices.
2. All equipment, supplies, and reagents used in the process of bone marrow collection must be sterile with a lot number and date of expiration noted and able to be recorded on site-specific institutional data forms.
3. Designated unblinded study personnel will notify the site-specific cell processing lab and the designated processing personnel at manufacturer at the following time points: 1) when a patient is enrolled and randomized to the active arm, 2) when a patient's bone marrow aspiration has been scheduled, 3) when the bone marrow aspiration has begun.
4. Bone marrow aspiration specimen transportation to the cell processing laboratory will be treated as a STAT procedure.

Patient preparation and specimen collection performed by Physician:

1. Verify patient identification with the patient.
2. Explain the risks and benefits of bone marrow aspiration and anesthesia. Give patients an opportunity to ask questions and verbalize understanding. Document the informed consent process by having the patient sign informed consent forms for bone marrow aspiration and anesthesia.
3. Sedative and analgesic medication for the bone marrow aspiration procedure, including conscious sedation will be left to the discretion of the performing or supervising physician per institutional guidelines for procedures of this volume with the exception of general anesthesia which will not be paid for by the study.
4. Patients on aspirin and/or Plavix (clopidogrel) at the time of consent should remain on aspirin

and/or Plavix (clopidogrel) for the bone marrow aspiration procedure. Continuance or discontinuance of other anticoagulation medications at the time of bone marrow aspiration, (e.g. warfarin and heparins) are left to the discretion of the study physician.

5. All collection procedures must be performed with universal precautions and sterile aseptic technique.

Bone marrow aspiration procedures:

1. The media container and/or heparin vials must be opened with sterile technique and media prepared with the appropriate amount of anticoagulant. The final concentration of heparin will be 10-25 units of heparin/mL of bone marrow.
2. Position the patient in a prone or partial prone position. Evaluate pressure points with special attention to avoid pressure on arms, brachial plexus, breasts, genitalia, knees, vascular structures or other body parts.
3. Verify location of posterior iliac crest.
4. Prep and drape the location in sterile manner using institutionally approved preoperative skin antiseptic (e.g., chlorhexidine gluconate (ChloraPrep ®), betadine, isopropyl alcohol, alcohol 60) and sterile draping. Allow for the antiseptic to dry before applying local anesthesia.
5. Begin induction sedation and analgesic medications (e.g. Propofol, Versed, Ativan, or morphine).
6. After evidence of induction effect, apply local anesthesia (e.g., lidocaine 1% or bupivacaine) to the skin above the posterior iliac crest.
7. With a longer needle (e.g., spinal needle) apply local anesthesia (e.g., lidocaine 1% or bupivacaine) to the periosteum of the posterior iliac crest region
8. Holding the bone marrow aspiration needle and stylet in place, puncture skin and advance through subcutaneous tissue, periosterum and into the marrow cavity using a steady, controlled pressure with a twisting motion. When the needle is firmly in the bone and slight give in pressure is felt, the cavity has been entered.
9. Remove the stylet and quickly attach the prepared syringe to the needle hub.
10. Apply a strong, quick suction and obtain 5-10 mL of bone marrow.
11. Rotate the aspiration needle by 60 to 90 degrees, and aspirate 5-10 mL of bone marrow**.**
12. Repeat the rotation a total of two to six times per puncture, totaling 20 – 30 mL of bone marrow aspirate per puncture.
13. Re-insert the stylet and remove the needle from the bone with a light twisting motion.
14. While keeping the aspiration needle in the subcutaneous tissue, reposition the aspiration needle in an adjacent site of the posterior iliac crest.
15. The target aspiration volume is 180 mL (± 10 mL). Therefore a total 18-36 aspirations (5-10 mL) will be made. Since 2-6 aspirations are made in each puncture, then a total of 3-8 punctures will be required. Typically this is performed with only 1 to 2 skin punctures.
16. Physicians will perform the aspiration on both the left and right side to reduce the likelihood of venous blood contaminating the sample and trauma to the patient.
17. In the event that no marrow is aspirable, then pressure will be applied to the injection site until hemostasis is achieved.
18. A sterile dressing will be applied to all puncture sites. A sterile pressure dressing (e.g., Elastoplast) will be applied if persistent venous oozing is present.
19. Rotate the patient into a supine position and maintain that position for a minimum of 30 minutes.
20. The dressing over the puncture site should be checked after the 30 minutes of supine positioning to ensure no hemorrhage. The dressing may be removed 24 hours after the procedure and the patient should observe for signs of infection, bleeding or any other drainage. The patient should notify the study coordinator if evidence for these signs. It is usual for the patient to observe bruising and feel aching for several days after the procedure. This may be relieved with a warm pack. The patient should notify the study coordinator if the pain persists beyond several days or wors-

ening pain.

21. Documentation of the procedure should be made by the Physician.
22. All bone marrow collections will be sent to the site's cell processing laboratory using site-specific institutional transportation procedures. Bone marrow aspiration transportation to the cell processing laboratory will be treated as a STAT procedure and arrive at the cell processing lab as soon as possible following the bone marrow aspiration procedure.

Reporting requirements:

1. Label the CCTRN Study Product Infusion form and all specimens with the patient acrostic, study ID, date and time of collection (including start and completion of procedure), and label the form with the amount aspirated.
2. Site-specific chain of custody forms must be used to document the chain of custody of the bone marrow aspirate from the site of the procedure to the cell processing laboratory (if applicable) and back.

Appendix D- PAD Classifications

Rutherford Classification

Grade	Category	Clinical
0	0	Asymptomatic
I	1	Mild claudication
I	2	Moderate claudication
I	3	Severe claudication
II	4	Ischemic rest pain
III	5	Minor tissue loss
IV	6	Ulceration or gangrene

Reprinted from ACC/AHA 2005 Guidelines for the Management of Patients with Peripheral Arterial Disease, Hirsch AT, Haskal ZJ, Hertzer NR, et al. *J. Am. Coll. Cardiol.* 2006;47;1-192

Fontaine Classification

Stage 1	No symptoms
Stage 2	Intermittent claudication subdivided into:
2a	without pain on resting, but with claudication at a distance of greater than 200 meters
2b	without pain on resting, but with claudication at a distance of less than 200 meters
Stage 3	Nocturnal and / or resting pain
Stage 4	Necrosis (death of tissue) and / or gangrene in the limb

Reprinted from Management of peripheral arterial disease (PAD). TASC Working Group. TransAtlantic Inter-Society Consensus (TASC). Dormandy JA, Rutherford RB. *J Vasc Surg*. Jan 2000;31(1 Pt 2):S1–S296.

Appendix E – Study Product injection Procedure

Patient Injection Procedure Instructions

1. Make the patient comfortable in the prone position.
2. Confirm which leg is receiving injections.
3. Apply local anesthetic cream to the areas to be injected. (detailed instructions in MOP Section 6)
4. Prep the whole calf area and lower half of posterior thigh with alcohol swab, then wait until dry.
5. Mark the 10 injection sites with permanent marker according to the injection pattern illustration; injection points should be **at least 1 inch apart**. (see picture below)
6. Take a picture of the injection sites, if possible.
7. Insert the needle at a 90° angle to the skin at each injection point.
8. Depth of injection may vary according to the trophic state of the leg to be treated. The objective of delivery is to place the cell product at a depth corresponding to the muscular mass of the calf (gastrocnemius) and lower thigh (semimembranosus and biceps femoris).
9. After insertion, aspirate the syringe to avoid intravascular injection and then proceed to perform each 1mL injection slowly (over a 45-60 second time period for each injection).
10. After each injection is completed apply gentle pressure.
11. Place bandages over each injection sites.

Injection Pattern Illustration

Prepare lower extremity and mark injection points according to patient injection procedure instructions.

Place:

a) 8 – 1mL injections in the calf to target the gastrocnemius according to the illustration.

b) 2 – 1mL injections in the posterior aspect of the lower thigh to target semimembranosus and biceps femoris according to the illustration.