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CLINICAL TRIALS

Angiogenic Precursor Cell Treatment of Critical Limb Ischemia Decreases Ulcer Size, Amputation and Death Rate: Re-Examination of phase II ACP NO-CLI Trial Data

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Abstract

Introduction: Critical limb ischemia has a prevalence in the US of 1.33%, with mortality 15-20% and major amputation 10-40% per year. Stem cell treatment has emerged as a treatment option for the 45% of patients for whom revascularization procedures are not possible.

Objective: This study re-examines the data of the Phase II clinical treatment of no option Critical limb ischemia with Hemostemix' angiogenic cell precursors, focusing upon ulcer wound healing, amputation and death rate of this cohort.

Methods: Primary endpoints were changes in ulcer size and major amputation or death within one year of treatment. The secondary endpoint was change in pain level.

Results: From 2015 to 2021, 67 patients with no option Critical limb ischemia were allocated to treatment with ACP-01 (46/67) or placebo (21/67). From this data, only patients who presented with wound ulcers before administration of ACP-01 were reviewed (21 treatment, 8 placebo). Ulcer size in the treated group decreased from a mean of 1.46 cm^2 to 0.48 mm^2 ($p = 0.01$) by 3 months. There was no significant decrease in the size of the ulcers of the placebo group ($p < 0.54$). At one year there were no complications related to treatment. The treatment group had one amputation (4.8%) and one death (4.8%); the placebo group had 2 amputations (25%) and 1 death (12.5%). Change in pain was not significant in either group at 3 months, but at 1 year was improved in the placebo group ($p = 0.01$).

Conclusion: The administration of ACP-01 within a program of careful patient follow up is safe and associated with reduced ulcer size and decreased rate of amputation and death. Consideration should be given to re-administration of stem cell treatments every 3-6 months to optimize improvement of Critical limb ischemia. Further studies, more appropriately powered, are warranted.

Abbreviations

Ac-LDL: Acetyl-Low Density Lipoprotein; ACP-01: Autologous Angiogenic Cell Precursors; BMMSC: Bone Marrow Derived Mesenchymal Stem Cells; CLI: Critical Limb Ischemia; no surgical option CLI: NO-CLI; CXCL 8: Interleukin 8; HSCs: Hematopoietic Stem Cells; PAD: Peripheral Artery Disease; PBMCs: Peripheral Blood Mononuclear Cells; SCP:

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Synergetic Cell Precursor; VAS: Visual Analogue Scale; VEGF: Vascular Endothelial Growth Factor

Introduction

Peripheral Arterial Disease (PAD) in humans is defined by impaired macro- and micro-circulation of the extremities. The condition, manifesting as trophic skin changes, ulcers of the hands and feet, sensory changes and hair loss, is disabling and imposes a significant economic burden upon the family and society. The incidence of PAD in the general population is 3% to 10% but rises to 20% in people over 70 years of age [1]. PAD is more common in obesity and diabetes; half of the patients with diabetes-related foot ulcers have PAD, and PAD significantly worsens the prognosis in those patients with diabetes-related foot ulcer with decreased healing rates, increased recurrence of ulceration, increased major limb amputation and decreased long-term survival [2]. The early stages of PAD are frequently missed, and diagnosis is often delayed for up to a decade. At least 8% of patients with PAD develop Critical Limb Ischemia (CLI) for a prevalence in the US of 1.33%. For these, the mortality rate is 15-20% within 6 months, and may exceed 50% at 5 years. The amputation rate is 10-40 %, and if the patient is diabetic, the rate of amputation is 50% [3].

The general understanding on the underlying pathophysiology of CLI is that of vasoconstriction resulting from atherosclerotic and inflammatory changes in the vessel walls, the risk factors for which include advanced age, nicotine use, diabetes mellitus, hypercholesterolemia and hypertension. The process of atherosclerosis is usually chronic, and often asymptomatic. Injury of the endothelium, from hypertension, trauma, infection or subclinical inflammation- initiates a cycle of increased permeability of the inner lining of blood vessels, and subsequent lipid build up in the wall of the blood vessel. Subsequent oxidative stress and inflammation then result in the release of cytokines and matrix metalloproteinases and eventual destruction of matrix components, plaque destabilization and plaque rupture [3,4].

Half of CLI patients undergo revascularization interventions, such as bypass or endovascular re-vascularization by angioplasty. However, these options are limited to 45% of patients, due to severe arterial narrowing or serious comorbidities that proscribe a surgical procedure [4]. The increasing interest in reversing limb ischemia by stem cell therapy has been supported by recent meta-analyses which have shown that autologous cell therapy may improve angiogenesis, ulcer healing, amputation rates, and pain-free walking [5,6].

Autologous Angiogenic Cell Precursors (ACP-01) are autologous hematopoietic stem cell derivatives, transdifferentiated from the Synergetic Cell Precursor (SCP). ACP-01 secrete an array of angiogenic factors and cytokines that foster angiogenesis, recruitment of systemic stem cells, engraftment and support of tissue survival and regeneration [7,8]. A previous phase II, open-label, randomized clinical trial of 20 PAD/CLI patients treated with ACP-01 found a significant improvement in hemodynamic parameters, pain, walking ability, wound healing rate and lowering of major amputations [9]. In addition, the safety and efficacy of ACP-01 was demonstrated in patients with severe ischemic cardiomyopathy and non-ischemic dilated cardiomyopathy [8]. More recently, a the randomized, blinded, multi-center, Phase II trial of patients with severe CLI for whom there were no surgical options (NO-CLI), compared intramuscular injections of autologous (ACP-01) with placebo, and showed no significant differences between the treated and control groups at 12 months [Bhuiyan, et al, submitted]. In order to assess the effect of the ACP-01 on wound healing, the present study analyses only that subpopulation of patients who presented with ulcerous wounds prior to treatment with ACP-01. Prior to treatment, ulcers were present in only 21/46 treated patients, and 8/21 of placebo patients. Moreover, given that the predominant paracrine effects of stem cells occur within the first 3 months after transplantation [10], this study focuses upon ulcer healing in the early treatment period, and follows the same subpopulation over the year for amputation and death rate.

Materials and Methods

Study design

A Phase 2 prospective, randomized, double-blind study (HS 12-01 Clinical Trial. Clinical Trials.Gov is NCT02551679) to determine safety and efficacy of ACP-01 for NO- CLI patients was completed (Bhuiyan I, Misskey CS, Sarel I, Henderson FC, Sr., Wang Y, Tuchman K, Argent-Katwala M, Smeenk T, Hsiang Y. Report from a phase 2 prospective randomized placebo controlled trial of autologous stem cells to treat patients with no option critical limb ischemia.", submitted for publication to *J*

Vasc Surg). These patients were not candidates for standard revascularization because of failed previous revascularization attempts, lack of run-off vessels to the foot or because they were at significant mortality risk. Patients met the TASC II definition of CLI, with rest pain, ischemic ulcer, or gangrene, a systolic ankle pressure below 70 mmHg and a systolic toe pressure below or equal to 50 mmHg and had received standard-of-care medical therapy for PAD. Exclusion criteria included major amputation within 4 weeks, life expectancy less than 6 months, uncorrected aorto-iliac occlusive disease, active infection of the lower extremity or advanced CLI [ischemic ulcers >10 cm^2 at or below the malleoli, severe aortic stenosis (grade 3)], diagnosis of malignancy within 3 years of the study's onset, uncontrolled myocardial ischemia, persistent NYHA class IV heart failure. The patients were randomized 2:1 to either treatment or placebo, and underwent a full medical history, physical examination, ankle-brachial index, toe brachial index, CT or MRI angiography, pain assessment using the Visual Analogue Scale (VAS), ulcer measurement and photography, followed monthly for 1 year. During each visit, patients were assessed for pain using the VAS, size of ulcers, and measurement of ankle-brachial and toe-brachial indices. The ulcer lesions were measured at each visit by ruler, measuring the greatest width and length. Area was calculated by multiplying the width x length. The following analysis is restricted to that subpopulation who presented with measurable ulcers at the commencement of the study. Those patients (ACP-01 and placebo groups) who developed ulcers after treatment or placebo were not part of this analysis.

Medical management of patients

The patients underwent standard wound care practice, each surgeon treating wounds at every visit with regular dressing changes, topical iodine, and debridement as necessary. Topical and oral antibiotics were allowed. Skin grafts and external compression were not performed.

ACP-01 processing

A simple blood draw of 250 ml from each patient yielded Peripheral Blood Mononuclear Cells (PBMCs), isolated by Ficoll gradient (Figure 1), subjected to enrichment media and cultured for five days with specific growth factors [7]. ACP-01 were analyzed for expression of $CD34^+$, $CD133^+$, and $CD117^+$ markers typical of multipotent hematopoietic stem cells (HSCs), as well as the KDR, Tie2, $CD144^+$, vWF and $CD31^+$ endothelial cell markers. The $CD31^+$ and $CD34^+$ were the percentage of cells with bright intensity $CD31^+$ Bright and $CD34^+$ Bright respectively. For Acetyl-Low Density Lipoprotein (Ac-LDL) uptake, cells were incubated in the presence of Ac-LDL (Alexa Fluor488 Ac-LDL or Ac-LDL-DiI) and stained with FITC- or PE- conjugated $CD31^+$.

Patients underwent injection of ACP-01, or placebo, in 30 locations: 24 on the posterior aspect of the calf in the gastrocnemius muscle, and 6 on the dorsum of the foot - for a total volume of 30ml. Each cell treatment injectate was 1.0cc. The entire ACP-01 treatment contained at least $5x10^6$ CD31+ bright/AcLDL cells (ACPs) and at least $1x10^6$ CD34+ cells, with the total number of cells not exceeding 2×10^8. Both treatment and placebo were delivered to a depth of 1.5 cm. The placebo consisted of the same medium without cells.

Statistical analysis

Primary end-points were ulcer size, major amputation in the treated limb and death. Secondary end-points were changes in VAS pain scores. The selection of patients who presented with ulcer wounds on the lower extremities resulted in a sample

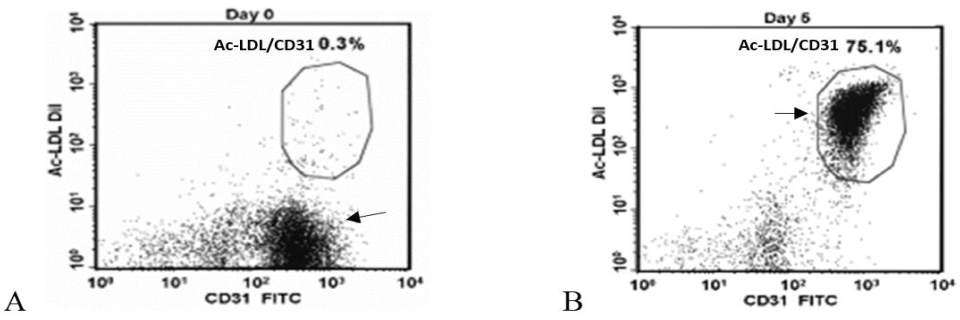

Figure 1 Flow cytometry analysis of ACP-01 demonstrating initial expression of anti-CD31+FITC before culture (Panel A), and concomitant increased uptake of Ac-LDL after incubation (Panel B).

size of 30 wounds in 21 patients who received ACP-01 and 11 wounds in 8 patients receiving the placebo. Pre-procedure and post-procedure ulcer sizes were treated as continuous variables, expressed as probability density functions, and presented as mean ± standard deviation. The ulcer sizes data did not fulfill the assumption for parametric testing (normal distribution) and therefore the Wilcoxon signed-rank test was used to compare mean pre-operative and postoperative ulcer sizes. An initial *p*-value of < 0.05 adjusted for multiple (three) comparisons was considered significant. Major amputation and death were treated as ordinal data and underwent a Chi square analysis. Non-parametric statistical hypothesis testing with the Chi square-test was used. Pain level (VAS) was treated as a continuous variable (scale of 1 to 100), expressed as a mean ± standard deviation. Paired t-test was used to compare mean preoperative and postoperative pain at 3 months. A *p-value of < 0.05* was considered significant.

Results

Patient characteristics

Of 67 patients originally randomized (2:1) to receive treatment (47/67) or placebo (21/67), two patients met exclusion criteria (one was diagnosed with cancer shortly after initial evaluation, and one had an ulcer measuring >20 square centimeters). These two patients were not included in the results. Of the patients in the treatment group, 21/45 (45.7%) patients had ulcers (*n = 30*) prior to cell treatment. Of the patients in the placebo group 8/21 (38.1%) had demonstrable ulcers (*n = 11*). The baseline characteristics of the patients were predominantly Caucasian and male (Tables 1,2). Of the treated group, 13/21 (62%) patients had diabetes mellitus, type one or type two. Of the placebo group, 8/8 patients (100%) had DM.

Ulcer response to treatment

Only the ulcers present at the time of initial treatment were included in the analysis (Tables 3,4). Ulcer size in the treated group decreased from initial average of 1.46 cm^2 to 0.60 mm^2 at 1 month (*n = 29, Z = -2.98, p = 0.003),* 0.40 mm^2 (*n = 25, z = -3.18, p = 0.002)* at 3 months and 0.42 mm^2 at final follow up (*n = 30, z = -2.87, p = 0.003*). In contrast, there was no significant decreases in the size of the ulcers of the placebo group. Mean wound area of the placebo group (Table 4) decreased from initial average of 1.58 mm^2 to 1.25 mm^2 at 1 month (*n = 11, z = -1.60, p > 0.05*) to 1.13 mm^2 at 3 months (*n = 9, z = -0.42, p > 0.05)* and 0.89 mm^2 at final follow up *(n = 11, z = -1.58, p > 0.05*). The *p* values reflect the analyses of available patient data rather than overall group means ((Tables 3,4), measurements were not taken at every time-point in a few instances).

Survival

At 8 months there was one death (1/ 21, 4.8%) in the treatment group (patient #15-510), at 247 days (Table 1), due to osteomyelitis, unrelated to treatment. One death (patient # 31-502) occurred in the placebo group (1/8, 12.5%) at 126 days (Table 2) due to multi-organ failure, unrelated to treatment. The difference in death rates were not significant *(p = 0.46)* (Tables 1,2).

Amputations

In the treatment group, 1/21 patients (4.8%) underwent a major amputation (patient #30-501), at 73 days that was unlikely related to treatment (Table 1). In the placebo group, one patient (1/8, 12.5%) (patient # 15-504) underwent a major amputation at 59 days; a second patient (patient # 28-501) underwent amputation at 261 days (Table 2) , unrelated to treatment, for an overall amputation rate of 25%. The differences in amputation rates were not significantly different *(p = 0.11*).

Visual analog pain scores (VAS)

For the treatment group, the mean initial VAS was 38.9 (*n = 12*), and the mean pain VAS by 3 months was 32.4 (*n = 12, p = 0.76*) and final pain score at average 263 days had decreased to 26.0 (*n = 20, p = 0.15*). For the placebo group, mean initial VAS was 64.5 (*n = 4*), the mean VAS by visit 7 was 40 (*p = 0.08*), and at final visits (average 301 days) the VAS score had decreased to 34.0 (*n = 8, p = 0.019*). There was no significant difference between the treated and the placebo groups (*p = 0.52*).

Discussion

Ulcer healing

Up until now, there has been little to offer "no-option" patients with CLI, in whom conservative therapeutic management has failed, or for whom surgical interventions are contraindicated. The ACP-01 treatments in this study conferred salutary benefits in terms of ulcer size. At the 3month visits, the treatment group demonstrated a significant

Table 1: Demographics ACP-01 Treatment Group.

Subj ID	Age/ Sex	BMI	Race/ Ethni-city	Amputation/ Death?	Medical History	Related Surgical History
12 - 504 12 - 504b	86 M	34.3	W	N/A	ASCVD, hx BCCA, CAD, cardiac arrythmia, hx colon Ca, DLD, HTN, hypothyroidism, hx SCCA, hx stroke, T1DM, T2DM, Vit D def	CABG, L carotid endarterectomy
13 - 505 13 - 505b	35 F	25.0	W	N/A	ASCVD, hx stroke	Femoral Thrombectomy/ endarterectomy, femoral-peroneal bypass
13 - 509b	45 F	26.1	H/L	N/A	ASCVD, DLD, T1DM	Peripheral percutaneous transluminal angioplasty, atherectomy
15 - 507 15 - 507b	78 M	33.6	W	N/A	ASCVD, hx bladder Ca, borderline CKD, HLD, HTN, hx lymphoma, PAD, hx prostate Ca	LE arteriogram and vein graft angiograms (Lx1, R x2), percutaneous coronary intervention, bypass x4
15 - 510 15 - 510b	70 F	33.1	W	Died osteomyelitis247 days post-tx	ASCVD, cerebral artery occlusion w/ infarction, CKD, CHF, DLD, HTN, hx MI, neuropathy, PAD, hx PE, T1DM	atherectomy x2, bypass, peripheral percutaneous, transluminal angioplasty x2, percutaneous coronary interventions (stents), R LE angiogram
15 - 512	76 F	16.7	W	N/A	ASCVD, hx suspected breast Ca, hx suspected cervical Ca, CKD stage III, HTN	left common femoral endarterectomy, left common femoral to below knee popliteal bypasses using PTFE and cryovein; L femoral-anterior tibial bypass with cryovein; superficial artery and R common femoral endarterectomy and patch angioplasty; L iliac angioplasty and stent placement, L iliac and proximal bypass stent placement, L leg open thrombectomy of cryovein bypass, balloon angioplasty of distal anastomosis, L LE angiograms, thrombectomy of cryovein bypass and tibial; Peripheral percutaneous transluminal angioplasty
15 - 513	84 M	26.4	W	N/A	ASCVD, Cardiac arrhythmia, carotid stenosis, CAD, CKD, DLD, HTN, pulmonary emphysema, T2DM	Thrombin injection right groin for pseudoaneurysm, hx amputation L 2nd toe and R transmetatarsal, CABG, Coronary PCI, TAVR, carotid endarterectomy, Peripheral percutaneous transluminal angioplasty
17 - 511 17 - 511b	72 M	36.4	W	N/A	ASCVD, CAD, CHF, CKD stage II, DLD, edema, emphysema, HTN, hypothyroidism, hx MI, PAD, T1DM	Right Femoral Graft, Coronary Artery Bypass Graft, bypass
21 - 503 21 - 503b	68 M	35.6	H/L	N/A	ASCVD, DLD, HCL, HTN, hypothyroidism, hx MI, peripheral neuropathy, T1DM	hx transmetatarsal amputation, stenting x3
22 - 501	42 M	28.4	W	N/A	ASCVD, DLD, HTN, T2DM	Peripheral percutaneous transluminal angioplasty x4, atherectomy x3, stenting x4
24 - 503 24 - 503b	58 F	31.5	W	N/A	ASCVD, DLD, HTN, T2DM	atherectomy, stenting, bypass
25 - 504	78 M	33.5	W	N/A	ASCVD, aortic valve stenosis, cardiac arrythmia, CAD, CKD, HLD, HTN, MI, skin Ca, T1DM	hx amputation L 4th toe, 5th metatarsal bone, foot transmetatarsal, amputation R 4th toe, other toe, Forefoot, Foot, CABG

30 - 501	86 M	25.9	W	L below knee amp. 73 days post-op	ASCVD, Cardiac Arrhythmia, DLD, HTN, Idiopathic peripheral neuropathy	Peripheral percutaneous transluminal angioplasty, Left femoral angiogram - failed angioplasty
30 - 502	85 M	22.0	W	N/A	ASCVD, hx L below knee amputation, DLD, HTN, hx stroke	Left-to-right femoral crossover bypass, Thrombectomy of femoral crossover, R axillofemoral bypass, Iliofemoral stent graft, stenting
30 - 511	72 F	23.9	W	N/A	ASCVD, DLD, HTN, T2DM	Peripheral percutaneous transluminal angioplasty
30 - 512 30 - 512b 30 - 512c	70 M	38.1	W	N/A	ASCVD, Cardiac Arrythmia, DLD, HTN, T2DM	Peripheral percutaneous transluminal angioplasty, neuropathy
30 - 514	52 M	29.6	W	N/A	ASCVD, CAD, Cardiac Arrhythmia, DLD, HTN, systolic murmur	Peripheral percutaneous transluminal angioplasty
30- 521	73 F	29.7	W	N/A	ASCVD, other x3	Peripheral percutaneous transluminal angioplasty, other x2
31 - 503b	66 M	20.9	W	N/A	ASCVD, CAD, HTN, hx Stroke, T1DM	coronary bypass, amputation L 4th toe, first toe, 3rd toe and 3rd, 4th and 5th metatarsals, 2nd toe, amputation R 2nd toe, attempted R peroneal artery and TP trunk angioplasty (failed), Peripheral percutaneous transluminal angioplasty, Bypass
31-504	66 M	33.1	W	N/A	ASCVD, CKD, DLD, HTN, DLD, hx stroke	bypass x2
32 - 506	77 F	33.7	B	N/A	ASCVD, DLD, HTN, hx stroke, T1DM	Peripheral percutaneous transluminal angioplasty
Avg.	68.5 38% F, 62% M	29.4	4.8% B 9.5% H/L 85.7% W	4.8% Died 4.8% Amputated		

Abbreviations: ASCVD = Atherosclerotic Cardiovascular Disease; BCCA = Basal Cell Carcinoma; B=Black/African American, not Hispanic/Latino; Ca= Cancer; CABG= Coronary Artery Bypass Grafting; CAD = Coronary Artery Disease; CHF= Congestive Heart Failure; CKD = Chronic Kidney Disease; DLD= Dyslipidemia; H/L= White, Hispanic/Latino; HLD = Hyperlipidemia; HTN = Hypertension; LE = Lower Extremity; MI = Myocardial Infarction; PAD = Peripheral Artery Disease; PE = Pulmonary Embolism; PTFE = Polytetrafluoroethylene; SCCA = Squamous Cell Carcinoma; T1DM = Type 1 Diabetes Mellitus; T2DM = Type 2 Diabetes Mellitus; W = White not Hispanic/Latino.

Table 2: Demographics and co-morbidities of the placebo group.

Subj. ID	Age/ Sex	BMI	Race/ Ethnicity	Amputation/ Death?	Medical History	Related Surgical History
13 - 507	50 F	37.9	B	N/A	ASCVD, DLD, HTN, hx stroke x3, T2DM	Peripheral percutaneous transluminal angioplasty x6, stenting, bypass x3
15 - 504 15 - 504b	69 M	43.9	W	R below knee amp. 59 days post-tx	ASCVD, CAD, CKD, claudication, DLD, HTN, hypothyroidism, lymphedema LE, MI, T1DM	percutaneous coronary intervention
28 - 501	60 F	26.1	W	L below knee amp. 261 days post-tx	ASCVD, CAD, DLD, HTN, T1DM	CABG, Peripheral percutaneous transluminal angioplasty, stenting x2, bypass, thrombectomy
30 - 510 30 - 510b	70 M	31.3	W	N/A	ASCVD, DLD, HTN, peripheral neuropathy, T2DM	Peripheral percutaneous transluminal angioplasty, hx L below knee amputation Other
30 - 513	72 M	25.4	W	N/A	ASCVD, DLD, HTN, peripheral neuropathy, hx non-metastatic prostate Ca, hx stroke, T1DM	Other x3

30 - 519 30 - 519b	70 M	28.4	W	N/A	ASCVD, DLD, HTN, T1DM	Peripheral percutaneous transluminal angioplasty, Bypass, R tibial angioplasty
31 - 501	68 M	28.2	W	N/A	ASCVD, CAD, DLD, HTN, hx stroke, T1DM	Peripheral percutaneous transluminal angioplasty, Amputation L 2nd toe, R 5th toe and R great toe
31 - 502	86 F	23.6	B	Died Multi-organ failure 126 days post-tx	ASCVD, CAD, CHF, DLD, HTN, hx MI, T2DM	Amputation R 3rd toe and great toe, dual chamber permanent pacemaker implant, pacemaker pocket revision, attempted L leg angioplasty (failed), Peripheral percutaneous transluminal angioplasty, bypass
Avg.	68.1 37.5%F, 62.5% M	30.61	25% B 75% W	12.5% Died 25% Amputated		

Abbreviations: ASCVD = Atherosclerotic Cardiovascular Disease; BCCA = Basal Cell Carcinoma; B = Black/African American, Not Hispanic/Latino; Ca = Cancer; CABG = Coronary Artery Bypass Grafting; CAD = Coronary Artery Disease; CHF = Congestive Heart Failure; CKD = Chronic Kidney Disease; DLD = Dyslipidemia; HCL = Hypercholesterolemia; H/L = White, Hispanic/Latino; HLD = Hyperlipidemia; HTN = Hypertension; LE= Lower Extremity; MI = Myocardial Infarction; PAD = Peripheral Artery Disease; PE = Pulmonary Embolism; PTFE = Polytetrafluoroethylene; SCCA = Squamous Cell Carcinoma; T1DM= Type 1 Diabetes Mellitus; T2DM = Type 2 Diabetes Mellitus; W = White Not Hispanic/Latino.

Table 3: Ulcer wound area of the group treated by ACP-01.

Subj. ID	Area Initial	Area Visit 6 (1month)	Area Visit 7 (3 months)	Area at last visit	Time to last visit (days)
12 - 504	1.2	0	0	0	87
12 - 504b	0.48	0.25	0	0	
13 - 505	1.8	0	0	0	260
13 - 505b	0.09	0	0	0	
13 - 509b	0.04	0.18	_	0.18	45
15 - 507	0.04	0	0	0	86
15 - 507b	0.01	0	0	0	
15 - 510	2.5	_	0	0	188
15 - 510b	0.06	0	0	0	
15 - 512	1	0.56	0.56	1	359
15 - 513	1	1	3.08	3.08	97
17 - 511	4.25	2	1.7	1.4	437
17 - 511b	0.15	0	0	0	
21 - 503	2	0	0	0	364
21 - 503b	1.5	0	0	0	
22 - 501	0.5	4	_	4	100
24 - 503	0.25	1	0.3	0	183
24 - 503b	2	0.04	0.09	0.8	
25 - 504	6	6	2.5	0	197
30 - 501	1.43	0	0	0	85
30 - 502	1	0	0	0	371
30 - 511	4	0.09	0.09	0	204
30 - 512	0.25	1.04	1.1	1.1	76
30 - 512b	0.2	0.15	0.04	0.04	
30 - 512c	0.35	0.15	0.02	0.02	
30 - 514	0.09	0	_	0	303
30 - 521	0.04	0.04	0.48	0.48	114

31 - 503b	0.09	0	_	0	97
31 - 504	7.5	0.64	0	0	363
32 - 506	4	0.36	_	0.36	29
N	**30**	**29**	**25**	**30**	**30**
Avg.	**1.46**	**0.60**	**0.40**	**0.42**	**193.4**
STD	**1.91**	**1.33**	**0.83**	**0.94**	**127.0**
SEM	**0.35**	**0.25**	**0.17**	**0.17**	**23.6**

Table 4: Change in wound area of placebo group.

Subj. ID	Area Initial	Area Visit 6 (1 month)	Area Visit 7 (3 months)	Area at last visit	Time to last visit (days)
13 - 507	1.2	1.1	0	0	84
15 - 504	0.06	0	0	0	378
15 - 504b	0.5	2.8	0	0	
28 - 501	0.5	0.05	1.2	4.84	260
30 - 510	4	4	7	3	170
30 - 510b	1	1	1	1	
30 - 513	1	0.56	0	0	366
30 - 519	6.25	4.16	_	0	294
30 - 519b	1.8	0	_	0	
31 - 501	0.8	0.04	0	0	377
31 - 502	0.25	0	1	1	90
N	11	11	9	11	11
Avg.	1.58	1.25	1.13	0.89	260.1
STD	1.89	1.63	2.26	1.60	114.5
SEM	0.60	0.52	0.80	0.51	36.2

improvement of ulcer size ($p = 0.002$), as compared to the placebo group in which there was no significant decrease in ulcer size ($p = 0.54$). The results of autologous ACP-01 are congruent with the literature, in which preliminary trials of intravascular or muscle injections of stem cells provide optimism for the treatment of CLI. A meta-analysis of 23 studies (962 patients) demonstrated a 73% increased probability of ulcer healing and 41% decreased risk of amputation; subgroup analysis showed greater efficacy among the autologous stem cell transplantation patients [6]. In a second meta-analyses of randomized, controlled trials, in which autologous bone marrow stem cell transplantation was used to treat CLI, the authors demonstrated improvement in pain, functional capacity, ulcer healing rate, arterial blood flow, and pain-free walking when compared to conventional treatment [11]. A third meta-analysis including 1186 patients demonstrated increased efficacy of autologous cells in ulcer healing rate, Ankle brachial index, TcO_2 and pain-free walking distance [12]. In the present study, significant reduction in wound size was apparent at 3 months ($p = 0.002$) and remained unchanged to the 6-month period time point ($p = 0.003$). Others report efficacy of cells at 6 months [14]. We suggest that future studies evaluate the efficacy of repeated implantations every 3 to 6 months in those who continue to have ulcers to determine the optimal benefit of ACP-01 administration.

Survival and amputation

The 1-year death rate in the treated (4.8%) and placebo (12.5%) groups was substantially less than U.S. Medicare statistics, which report a death rate of 15%-20% within 6 months of CLI diagnosis and 50% within 5 years of diagnosis. Moreover, the amputation rate of 4.8% in the treatment group compares favorably with the literature, the latter variably reporting amputation rates of 10%–40% per year [3,13-18]. Furthermore, the literature would predict that a high rate of diabetes in the treatment group (62%) would normally be associated with a 50% amputation rate at one year [3]. The patients

of the placebo group - all of whom had a diagnosis of diabetes- saw an overall 25% amputation rate. The outcome of the present study aligns with another prospective, randomized, double-blinded, placebo controlled, multicenter study (RESTORE-CLI) in which 86 patients with NO-CLI showed a significant increase in time to treatment failure and amputation-free survival in treated patients. In that study, major amputation occurred in 19% of treated patients compared to 43% of controls, and there was improved wound healing in the treated patients [19]. Others have shown that cell therapy reduced the risk of amputation by 37%, amputation-free survival by 18%, and improved wound healing by 59%, but not affecting mortality [5]. In contradistinction to the low amputation and death rate seen in the present study using autologous hematopoietic derived cells, other reviews of transplantation of bone marrow derived cells for patients with NO-CLI has shown little benefit in terms of amputation free survival [20,21]. In addressing the cause of the low death rate and amputation rate of the present study, the authors attribute some beneficial effect to the greater medical attention administered to the patients within the treatment protocol. That is to say, the efficacy of stem cell treatment is enhanced within a program of regular attentive care.

Pain relief with ACP-01

Improvement of ulcer size in the treatment group was not paralleled by significant improvement of pain at 3 months ($p < 0.68$) - and there was no significant difference compared to the placebo group. This contrasts with other studies, in which pain-free walking distance significantly increased in cell therapy [6]. The CD34+ signature of ACP-01 may confer earlier ischemia relief in CLI subjects, as demonstrated in one randomized, single-blinded, non-inferiority trial, in which trial patients were divided 1:1 receiving either purified CD34+ cells or PB-MNCs. In that trial, the CD34+ group achieved faster rest-pain relief and overall earlier ischemia relief than the PB-MNCs group [22]. The efficacy of pain relief is complicated, however, and may be variably influenced, either positively or negatively, by altered regulation of sensory nerve function and interneuronal activity, and associated changes of n-methyl-d-aspartate receptors.

Mode of administration

While some authors have recommended both intra-arterial and intramuscular injections to maximally reach target ischemic areas [23], the investigators of this study believed that femoral artery occlusion would preclude the cells from reaching ischemic areas. Moreover, the Rejuvenating Endothelial Progenitor Cells via Transcutaneous Intra-arterial Supplementation (JUVENTAS) trial - a randomized, double blinded, placebo-controlled trial of CLI patients ($n = 160$) [24] - convincingly demonstrated that *intra-arterial* infusion of BM-MNCs was non-effective. On the other hand, a study of NO- CLI patients ($n = 96$) who underwent local intra-muscular transplantation of bone marrow concentrate for treatment of ischemic foot ulcers, demonstrated improvement in limb salvage [25], and meta-analyses have also shown that intramuscular transplantation had greater efficacy than intra-arterial injection [6].

The molecular biology of Angiogenic Cell Precursor (ACP) promotes angiogenesis, migration and decreased scarring ACP-01 enhances angiogenesis

ACP-01 improves microcirculation in 4 major ways (Figure 2). First, ACP-01 are programmed to form endothelial cells, the major constituent of blood vessels. Second, ACP-01 exert a potent paracrine effect, Secreting Vascular Endothelial Growth Factor (VEGF) and angiogenin, directly promoting angiogenesis and the generation of new capillaries through proliferation and luminogenesis [26]. Potent levels of VEGF and angiogenin are secreted by ACP-01 [8]. Third, ACP-01 increase expression of Interleukin 8 (CXCL 8) which enhances angiogenesis through the Ras-MAPK/PI3K activation and the AP-1/NF-kB axis ([8]. Fourth, elevated CXCL8 mobilize an additional reservoir of endothelial cell progenitors, such as peripheral $CD34^+$ precursor cells, which amplify the angiogenic response [27,28]. The importance of $CD34^+$ was demonstrated in a study of NO-CLI patients treated with bone marrow mononuclear cells; those responding with limb salvage and wound healing (33/55 patients) had a significantly higher $CD34^+$ cell dose ($p = 0.001$) compared with non-responders (22 of 55) that required limb amputation [29,30].

Fifth, ACP-01 promote migration [7]. CXCR4 receptors present on ACP-01 home toward specific alpha-chemokine CXCL12 (ligand), expressed in injured or ischemic tissue, as exemplified in preclinical models of cardiac ischemia where ACP-01 promoted cell migration and repopulation [31]. Sixth, ACP-01, via upregulation of CXCL8 and its

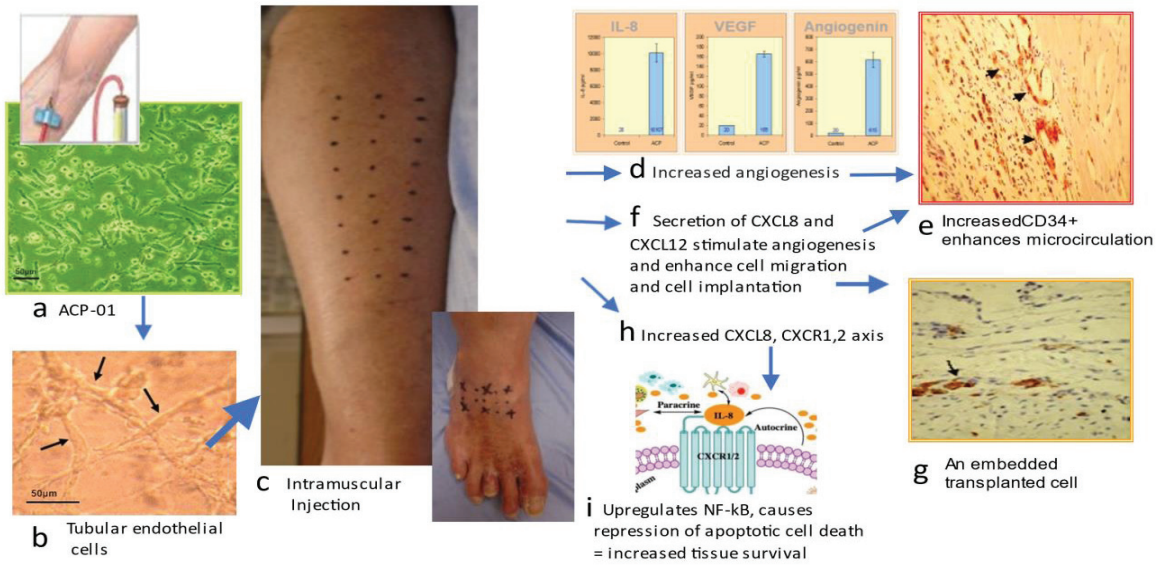

Figure 2 Intramuscular administration of ACP-01 for treatment of ulcers in CLI

Autologous multipotent hematopoietic cells obtained by a blood draw undergo a process of trans-differentiation to ACP-01.

(a) (50 μm), which are programmed to form tubular, endothelial cells.

(b) (50 μm). ACP-01cells undergo intramuscular implantation.

(c) Cytokine expression of CXCL8, VEGF, and angiogenin.

(d) Promote angiogenesis.

(e) Small black arrows indicate neo-angiogenesis (photomicrograph X400). CXCL8 also enhances cell migration and cell implantation. Injured tissue expresses CXCL12 ligand.

(f) Which attracts the CXCR 4 receptors on ACP-01, enhancing migration.

(g) To ischemic tissue (photomicrograph of myocardium X400). Elevated CXCL8 / CXCR1,2 axis.

(h) Expressed by ACP-01 repress apoptosis through upregulation of the NF-kB axis.

(i) These function collectively to improve tissue healing and survival.

downstream activation of Nuclear Factor Kappa B, exert a pro-angiogenesis influence and pro-survival influence by transcription of anti-apoptotic factors [32-35]. Seventh, the expression of CXCL8 [8] modulates the inflammatory process via downstream recruitment of monocytes and secretion of matrix metalloproteinases to effect degradation of extracellular matrix and facilitation of phagocytosis, and preferential selection of the M2 phenotype, with the consequence of decreased tissue scarring. The M2 "alternatively activated" macrophage releases anti-inflammatory mediators, protecting and promoting repair and homeostatic functions [36-39].

Bone marrow derived Mesenchymal stem cells (BMMSC)

ACP-01 are hematopoietic derived cells obtained from peripheral blood by a simple blood draw, as oppose to Bone Marrow Derived Mesenchymal Stem Cells (BMMSC), which are abundant, possess low immunogenicity, produce numerous paracrine cytokines, promote capillary growth, and are associated with few complications. However, there may be some drawbacks with BMMSC: the implantation of MSC is poor in conditions of impaired microcirculation, hypoxic micro-environment, and cell to cell interactions [40]. BMMSC injected into the blood stream suffer a short existence, with fewer than 1% of cells surviving 4 days [41]. Notwithstanding absence of the major histocompatibility complex class II antigens, BMMSC may still suffer immune rejection from alloreactive antibodies [42] and stored allogeneic MSCs appear to be less effective than fresh autologous cells [43].

The safety profile

The trial demonstrated no complications referable to the injections, notwithstanding the many co-morbidities of the CLI patients (Tables 1,2) [9,15]. Clinical studies have demonstrated the safety of autologous and allogeneic stem cells. Minor symptoms such as transient rash and sometimes fever

may accompany cell transplantation [6]. However, infusion toxicity, organ system complications, infections, death or malignancy, and complications relating to stem cells are almost unknown. ACP-01 transplantation for cardiomyopathy in four clinical studies was associated with no complications relating to the cells [8,44].

Study limitations

The present study focuses on sub-group data of The Phase II Clinical trial of ACP-01 in the treatment of NO-CLI patients. Significantly, the study was underpowered. The study examined one known subset of CLI- comprised of those patients presenting with ulcerative wounds. Although there is evidence of stem cells activity at 6 months [12,31,38], the predominant effects of implanted stem cells are generally considered to occur over the first few months. The authors believe that the primary and secondary endpoints of the Phase II trial should have been more attentive to outcomes at earlier times. Notwithstanding cost concerns, the durability of a single treatment injection is questioned [14]. Quality of life and performance level of the patients were not measured, missing the opportunity to capture other aspects of improvement. Patient satisfaction scores were not recorded. In the analysis of wound healing, extreme variability in wounds and wound healing further contributed to under powering of the study [14].

Conclusion

This re-examination of the data of a Phase 2 study of NO-CLI patients demonstrates that the use of ACP-01 in the subset of CLI patients who presented with ulcerous wounds was technically safe, and associated with a significant improvement in wound healing, and lessening of both amputation and death rates. The primary modes of action are thought to reside in amplification of angiogenesis, cell migration, and recruitment of non-inflammatory macrophages and CD34+ cells to the injury sites.

The very low amputation and death rate of the treated patients is attributed to a combined effect of the autologous cells administration within a program of careful surveillance and attentiveness to medical treatment of the patients. Significantly, the study was underpowered, but nevertheless, supports the optimistic findings of other meta-analyses, and warrants further clinical investigation. Future studies should include the evaluation of the efficacy of re-administering cell treatments within the 3-6 month time-interval, particularly in those patients who do not heal within that time period.

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Author contributions

Professor Fraser Henderson Sr wrote and edited the manuscript. Dr. Ina Sarel was involved in the conception of the clinical study, data verification and analysis, wrote parts of the manuscript, and edited the whole document. Professor York Hsiang was senior author, involved in the conception and execution of the clinical study, treatment of many of the patient , and edited the manuscript. Professor Stephen Lewis reviewed, advised and approved the scientific and statistical methods, and assisted in statistical analysis, and editing the manuscript. Kelly Tuchman assisted in the clinical data analysis and performed statistical analyses and formation of tables and figures, and also participated in writing and editing the manuscript.

Disclosure statement

Professor Henderson is a practicing, academic neurosurgeon who serves as Chief Medical Officer, and has stock in *Hemostemix, Inc.* Dr. Ina Sarel is the Chief Scientific Officer for *Hemostemix Inc.*, and has stock in the corporation. Professors York Hsiang and Stephen Lewis and Kelly Tuchman have no financial interest or conflict of interest. Kelly Tuchman was paid for her work by *Hemostemix Inc.* Dr. Hsiang is on the Scientific Advisory Board for *Hemostemix, Inc.*

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Data Availability Statement

The clinical study data for the HS 12-01 Clinical Trial. Clinical Trials.Gov is NCT02551679) is freely available upon application to Dr. Ina Sarel, Chief Scientific Officer, *Hemostemix Inc.*

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