The influence of patient and wound variables on healing of venous leg ulcers in a randomized controlled trial of growth-arrested allogeneic keratinocytes and fibroblasts

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Objective: To examine patient and wound variables presumed to influence healing outcomes in the context of therapeutic trials for chronic venous leg ulcers.

Methods: This double-blind, vehicle-controlled study was conducted with randomized assignment to one of four cell therapy dose groups (n = 46, 43, 44, 45) or vehicle control (n = 50). A 2-week run-in period was used to exclude rapid healers and those with infection or uncontrolled edema. This was a multicenter (ambulatory, private, hospital-based and university-based practices, and wound care centers in North America) study. Adults ≥18 years old with chronic venous insufficiency associated with an uninfected venous leg ulcer (2-12 cm^2 area, 6-104 weeks' duration) were included in the study. Excluded were pregnant or lactating women, wounds with exposed muscle, tendon or bone, patients unable to tolerate compression bandages, or patients who had exclusionary medical conditions or exposure to certain products. Exclusion during run-in included patients with infection, uncontrolled severe edema or with healing rates ≥0.349 cm/2 wk. Screen fail rate was 37% (134/362), and the withdrawal rate was ~10% (23 of 228). Growth-arrested neonatal dermal fibroblasts and keratinocytes were delivered via pump spray in a fibrin sealant-based matrix, plus a foam dressing and four-layer compression bandaging. Treatment continued for 12 weeks or until healed, whichever occurred first. Patient demographic and wound-related variables were evaluated for influence on complete wound healing in all patients, as well as the subsets of treated and control patients.

Results: Wound duration (*P* = .004) and the presence of specific quantities of certain bacterial species (*P* < .001) affected healing in the vehicle group, while healing in the cell-treated groups was influenced by wound duration (*P* = .012), wound area (*P* = .026), wound location (*P* = .011), and specific quantities of certain bacterial species (*P* = .002). Age, sex, race, diabetes, HbA1C, peripheral neuropathy, and serum prealbumin did not significantly affect healing. Body mass index was positively associated with healing in cell-treated patients.

Conclusions: Wound duration is a quantifiable surrogate for one or more undefined variables that can have a profound negative effect on venous leg ulcer healing. Although cell therapy overcame barriers to healing, the only specific barrier identified was the presence of certain bacterial species. Interventional trials of potentially effective new therapies can be most informative when patients with suspected barriers to healing are included. The specific measurement of candidate barriers such as microbial pathogens, wound inflammatory state, and fibroblast function should be considered in future randomized trials to improve our understanding of the basis for chronicity. (J Vasc Surg 2013;58:433-9.)

Venous leg ulcers (VLUs) do not universally heal following standard therapies.[1] Reducing reflux or correcting obstruction may address pathologies that lead to ulceration, but such therapy failed to improve healing in the ESCHAR (Effect of Surgery and Compression on Healing and Recurrence) trial compared with compression therapy alone.[2] The mainstay of treatment for VLU is therefore compression bandaging, often used together with debridement, infection control, and management of wound moisture. A variety of more advanced therapies

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using living or nonliving biologic materials may be employed, however, a considerable proportion of wounds are relentlessly unresponsive.[3]

Identifying variables, which predict a poor response, may prompt earlier consideration of appropriate advanced therapies for certain patients.[4] Variables previously identified include initial wound size, wound duration, coexistent arterial insufficiency, increased body mass index (BMI), history of deep vein thrombosis (DVT), limited range of motion at the ankle (calf pump dysfunction), and poor compliance with compression. Recurrent DVT and poor arterial supply are potentially modifiable,[5-7] as are general mobility, ankle movement, obesity, and presence of fibrin in the wound bed[5,6] while patient age, sex, and history of hip/knee replacement surgery are nonmodifiable variables found by some to influence response to standard care. The two most commonly predictive factors, ulcer size and duration, may in fact be surrogates for potentially unidentified underlying pathologies.[5,6,8,9]

A prospective randomized VLU trial evaluating a novel allogeneic cell-based bioformulation found the therapy to be significantly more effective than standard care.[10] A unique feature of this nonpivotal, dose-response trial design was that trial exclusion criteria intentionally allowed patients to enroll who would be expected to fail standard care, on the assumption that the influence of certain variables on the probability of not healing would be successfully modified by the investigational new therapy.[11]

We report here an analysis of the impact of measured variables on wound closure following up to 12 weeks of cell treatment or vehicle, with both groups receiving four-layer compression therapy. As part of the protocol design, a 2-week run-in with standard care screened out rapid healers and, as might be expected, factors known to influence response to standard care in an unselected population appeared less important when standard care was again applied to those who passed the run-in. In contrast, cell therapy achieved a greater proportion of wound closures, and we found healing was influenced primarily by wound duration. At least one variable, wound bioburden, was specifically found to correlate with duration and to differentially affect responses to standard care and cell therapy. Our findings have implications for enrollment criteria in randomized controlled trials of potential new VLU therapies.

METHODS

Study design. HP802-247 is an investigational allogeneic living cell bioformulation consisting of neonatal keratinocytes and fibroblasts in a fixed ratio of 1:9, maintained through growth arrest using gamma irradiation.[12] The living cells are stored frozen and are rapidly thawed for administration via a pump spray. A full description of the study is published elsewhere.[10] Briefly, this multicenter double-blind, vehicle-controlled study was conducted using Institutional Review Board-approved protocol and informed consent documents, with consent obtained from all patients before screening. The primary goal was to examine dose and frequency of application of the test material for safety and efficacy in reducing the size of VLU, relative to standard care plus vehicle. Complete wound closure, defined as re-epithelialization, absence of drainage, and lack of need for a wound dressing persisting for 2 weeks, occurred in a significantly greater proportion of subjects treated with cells and serves as the endpoint of interest for this report.

Two hundred five (205) of 228 randomized patients completed the 12-week study at 28 sites, with 23 dropouts being attributed to adverse events (five), withdrawal of consent (seven), disease progression (four), protocol deviation (three), physician decision (one), loss to follow-up (two), and other (one). Inclusion required duplex-confirmed venous disease and a VLU of 6-104 weeks' duration and 2-12 cm^2 area (the maximal area covered with one spray of HP802-247) prior to debridement. Patients were excluded for pregnancy or lactation, >3 ulcers on the target limb, serum HbA1C > 108 mmol/mol, serum prealbumin ≥150 mg/L, history of Hashimoto's thyroiditis, nontoxic nodular Goiter, or Grave's disease (due to use of Cadexomer iodine dressing during the run in phase); prior diagnosis of systemic lupus erythematosus with elevated anti-DNA antibody titers; clinical impression of ulcer bed infection; documented history of osteomyelitis at the target wound location; or refusal of or inability to tolerate compression therapy.

Patients who had successful surgical correction or intervention aimed at improving venous return in the target limb within 1 month preceding the screening visit were also excluded. No limits were placed on wound bioburden, BMI, or peripheral neuropathy but they were prospectively collected. The limits on counts of red and white blood cells, platelets, glycosylated hemoglobin, hemoglobin, and prealbumin were set generously (eg, hemoglobin cutoff value 8.0 g/dL). The need for weekly debridement was at the discretion of the investigator. A run-in phase of treatment for 2 weeks with four-layer compression and a Cadexomer iodine dressing allowed exclusion of rapid healers. At each weekly visit, wounds were treated by study personnel using four-layer compression bandaging plus either one of four doses of HP-802-247 or vehicle for up to 12 weeks. No anticoagulation therapy was required by the protocol.

Variables evaluated. Patient interviews, review of medical records, and physical examinations were used to determine age, sex, race, body weight and height, wound duration and location, and history of diabetes mellitus type I or II. BMI was calculated as weight (kg)/height^2 (m). Wound area was determined using the ARANZ Silhouette (ARANZ Medical, Ltd, Christchurch, New Zealand), which combines a fixed focal-length digital camera with dual lasers. Computer software integral to the hand held device calculated wound area by combining laser line interpretation with on-screen wound margin tracings. Serum was separated from venous blood via centrifugation using serum separator tubes, which were sent via overnight mail to North Coast Clinical Laboratory, Inc (Sandusky, Ohio) for measurement of prealbumin and HbA1C using standard clinical laboratory methods. Peripheral neuropathy was

determined using a single 10-g monofilament probe of the dorsum of the foot while the patient's gaze was averted. Ankle goniometry was performed at baseline.

Wound bioburden was measured by quantitative bacteriologic culture of a 4-mm punch tissue sample taken from the cleansed wound bed during the screening visit, prior to run-in. Standard microbiology testing was performed by Pathology Laboratories Inc (Toledo, Ohio), with an assay lower limit of quantitation of 1.0×10^3 colony-forming units (cfu)/g. Weekly sharp debridement was allowed but not required. To ascertain organisms specifically associated with impaired healing, wounds that yielded any organism at $\geq 1.0 \times 10^4$ cfu/g, or $>1.0 \times 10^3$ cfu/g for *Streptococcus agalactiae* or *S dysgalactiae* (β-hemolytic Strep) were identified. For each genus of bacteria, the proportion of culture-positive wounds that healed was compared against the average healed for all cell-treated patients (60%).

Statistical analyses. Distribution of baseline characteristics was tested using analysis of variance. Prospectively defined statistical analysis utilized a Cochran-Mantel-Haenszel test, adjusted for pooled site, for the proportion of subjects achieving complete wound closure by treatment group during the 12 weeks of treatment. Missing outcomes were imputed using the last observation carried forward. Wound survival analysis was similarly evaluated based on a prospective plan using Cox proportional hazard, adjusted for baseline wound area. The Cochran-Mantel-Haenszel test with adjustment for pooled site was used to examine treatment effects on the number of patients who had debridement performed at each weekly visit, performing the test separately for each pair of an active group vs vehicle. For the purpose of this report, post hoc analyses of patient and wound variables were conducted using all patients combined, patients treated with any dose of cells, and patients treated with vehicle. Quantitative continuous variables (wound area, wound duration, BMI, HbA1C) were tested using parametric analysis (*t*-test) or, if not normally distributed (based on the Shapiro-Wilk test), by nonparametric analysis (Mann-Whitney rank-sum test). Categorical variables were dichotomized as "0" or "1" where appropriate (not healed vs healed, white vs non-white, ankle location vs leg location, monofilament sensation no vs yes). Dichotomous categorical variables were tested using χ^2 with Yates correction. Both continuous and coded dichotomous variables were tested using multiple logistic regression, using maximum likelihood estimation. No adjustments were made for multiplicity. The relationship between wound bioburden and median wound duration was tested using Kruskal-Wallis one-way analysis of variance on ranks. Prospective analyses were performed using SAS 9.1.3 for Windows (SAS Institute Inc, Cary, NC), with SigmaPlot 12.0 (Systat Software, Inc, San Jose, Calif) for post hoc analyses.

RESULTS

Baseline characteristics. Demographic and wound variables were similar among treatment groups at baseline, with no statistical imbalances (Table I).

Multivariate analysis: Age, sex, race, wound area, wound duration, and wound location. Multivariate analysis using a multiple logistic regression model to test the influence of age, sex, race, area, and duration on complete wound closure found only duration to be significant ($P < .001$). Baseline wound area was a significant single covariate in the Cox proportional hazard analysis on number of days to wound closure (hazard ratio, 0.914; 95% confidence interval, 0.858-0.974; $P = .0058$). A shallow linear relationship was seen between wound duration (independent variable) and wound area (dependent variable), but it did not reach statistical significance (slope, 0.0124; $R^2 = 0.0126$; $P = .091$). Increases in either wound duration or wound area showed a negative effect on wound closure, but wounds of any duration or area responded better to treatment with standard care plus cells than with standard care plus vehicle.

Wounds were nearly evenly distributed between the leg and ankle, with slightly more located on the leg. Combining the 228 patients across all treatment assignments, the proportion of healed leg wounds was significantly greater than the proportion of healed ankle wounds (68% vs 51%; $P = .018$). The model was adjusted to include treatment (cells or vehicle) and wound location, while removing age, sex, and race. In this adjusted model, wound location ($P = .012$), duration ($P \leq .001$), and treatment ($P = .004$) significantly affected complete healing, whereas baseline wound area did not ($P = .106$).

Univariate analyses. Within the cell-treated group, baseline wound area was significantly smaller in those who healed compared with those whose wound remained open (*t*-test, $P = .026$). A total of 104 patients were tested for HbA1C, including 63 patients with a history of diabetes mellitus (62 measured at screening or run-in, one at end of treatment) and 41 patients with no history of diabetes. Three patients with a positive history were not tested. The HbA1C levels were similar among patients with a history of diabetes, whether treated with vehicle (50.27 mmol/mol ± 8.57) or with cells (54.10 mmol/mol ± 15.87; $P = .224$). There was no evidence of a significant effect of the HbA1C level on ulcer healing, either in the control or the cell-treated groups.

Most patients reported perceiving a monofilament probe (87.7%, 200/228). Those who lacked perception were regarded as having peripheral neuropathy. Among 104 patients for whom HbA1C values had been determined, those with peripheral neuropathy had significantly higher baseline HbA1C values than those who reported feeling the monofilament (57.49 mmol/mol ± 19.87 vs 44.63 mmol/mol ± 10.79; $P = .0105$). There was no significant difference in the incidence of wound closure in the presence of neuropathy, as 74.1% of those reporting lack of sensation healed compared with 58.5% of those with normal sensory perception ($P = .179$).

Among patients treated with cells, BMI was significantly greater for those who healed (median, 34.0; range, 18.6-63.0) than for those who did not heal (median, 29.4; range, 18.4-65.5; $P = .007$). Among patients treated with

Table I. Patient demographics

Category	Total (N = 228)	Vehicle (n = 50)	Low dose, cells Biweekly (n = 46)	Low dose, cells Weekly (n = 43)	High dose, cells Biweekly (n = 44)	High dose, cells Weekly (n = 45)
Age, years	62.0 (61.6 ± 15.2) [22-94]	59.5 (62.1 ± 14.1) [33-91]	63.0 (61.7 ± 15.7) [24-88]	64.0 (62.6 ± 15.4) [28-93]	61.0 (59.8 ± 15.0) [22-89]	62.0 (61.8 ± 16.1) [24-94]
Male	135 (59.2%)	33 (66.0%)	26 (56.5%)	29(67.4%)	22 (50.0%)	25 (55.6%)
Female	93 (40.8%)	17 (34.0%)	20 (43.5%)	14 (32.6%)	22 (50.0%)	20 (44.4%)
White	164 (71.9%)	37 (74.0%)	34 (74.0%)	30 (69.8%)	30 (68.2%)	33 (73.3%)
Black	48 (21.1%)	9 (18.0%)	10 (21.7%)	9 (20.9%)	12 (27.3%)	8 (17.8%)
Other	16 (7.0%)	4 (8.0%)	2 (4.3%)	4 (9.3%)	2 (4.5%)	4 (8.9%)
Area, cm^2	4.9 (5.5 ± 3.1) [1.1-19.2]^a	4.7 (5.4 ± 3.1) [1.1-14.7]	5.0 (5.1 ± 2.5) [1.1-12.1]	4.6 (5.4 ± 2.7) [1.5-11.9]	5.2 (6.0 ± 3.4) [2.0-17.4]	4.7 (5.9 ± 3.6) [1.9-19.2]
Duration, weeks	24.0 (33.5 ± 1.9) [6-104]	17.7 (29.5 ± 24.6) [6-104]	22.0 (34.8 ± 29.1) [6-104]	23.9 (36.1 ± 31.3) [6-104]	25.0 (34.1 ± 29.2) [6-104]	24.0 (33.8 ± 27.0) [6-104]
Weight, kg	100 (103 ± 34)	102 (108 ± 39)	93 (96 ± 26)	100 (102 ± 34)	95 (101 ± 29)	104 (107 ± 38)
Ankle	103 (45.2%)	23 (46.0%)	20 (43.5%)	24 (55.8%)	18 (40.9%)	18 (40.0%)
Lower calf	110 (48.2%)	26 (52.0%)	22 (47.8%)	17 (39.5%)	21 (47.7%)	24 (53.3%)
Upper calf	15 (6.6%)	1 (2.0%)	4 (8.7%)	2 (4.7%)	5 (11.4%)	3 (6.7%)
Diabetes	66 (28.9%)	19 (38.0%)	14 (30.4%)	10 (23.2%)	15 (34.1%)	8 (17.8%)
Neuropathy	27 (11.8%)	7 (14.0%)	6 (13.0%)	4 (9.3%)	6 (13.6%)	4 (8.9%)

Values given as median, (mean ± SD), and [range]. No statistically significant differences (imbalances) were found between groups for any variable listed.
^{a}Baseline values at the end of a 2-week run-in period, during which time wounds may have increased (debridement or deterioration) or decreased (healing) in size since screening.

vehicle, BMI was greater but not statistically different, for those who healed (median, 33.0; range, 23.1-82.5) than for those who did not heal (median, 30.3; range, 16.7-62.0).

Slightly more than one-half of all patients (55%) underwent debridement at the baseline visit. There were no statistically significant differences between groups in the proportion of patients having wound debridement at any visit. Patients for whom no debridement was recorded during the study had outcomes indistinguishable from those who had debridement performed routinely. For example, 43% (9/21) of vehicle-treated patients and 72% (48/67) of cell-treated patients with no debridement at either of the first two run-in visits achieved wound closure, compared with 48% (14/29) and 60% (66/110), respectively, having undergone debridement at either or both of the first two visits. Based on analysis of debridements performed after screening, during run-in, and during the treatment phase, comparing those who healed to those who did not heal, no relationship between debridement and healing could be found for either vehicle-treated or cell-treated patients. Overall, ankle goniometry showed no strong correlations with outcome.

Wound bacteriology. All 228 patients were free of clinically evident wound infection at the time of randomization. Only 227 patients are analyzed here, as one patient died prior to first treatment. Quantitative bacteriology assessment of baseline wound bed tissue yielded positive cultures in 192/221 patients (87%), with biopsies not performed in six patients. Most (110) positive cultures yielded a single species, with 60 each yielding two, 18 yielding three, four yielding four different species, whereas 29 cultures were negative. Among patients with negative cultures, 40% healed with vehicle treatment (2/5) compared with 67% with cell treatment (16/24) (P = .339), for 62% overall

healing. The positive cultures identified 37 species and 20 genera. No culture yielded 1×10^6 or greater cfu/g. Total quantities of bacteria were similar among patients whose wounds healed (median, 3.5×10^4 cfu/g; mean, $9.5 \pm 11.5 \times 10^4$ cfu/g) and those whose wounds remained open (median, 3.0×10^4 cfu/g; mean, $8.3 \pm 10.3 \times 10^4$ cfu/g). Bacteria associated with a lower than average proportion healed (<60%) were tabulated according to treatment group, giving 71 patients with 92 positive cultures (Table II). We termed these bacteria "inhibitory bacteria," which were all facultative anaerobes, with the exception of *Pseudomonas* species. Thirty-three percent of patients (59/177) in the cell-treated groups harbored one or more of these bacteria compared with 24% in the vehicle-treated group (12/50). The concentrations of bacterial species, inhibitory or noninhibitory, were not different between the cell-treated and vehicle control groups.

To assess the effect of bacteriology on wound healing, the number of those bacteria with $\geq 1 \times 10^4$ cfu/g was calculated, respectively, for both the inhibitory and noninhibitory bacterial families as identified in Table II and was used as the "bioburden index" of the corresponding bacteria family. A regression model, including treatment (cells vs vehicle) and both the inhibitory and noninhibitory bioburden indices as the predictors, was performed on wound healing, using logistic analysis. The same regression analysis was further performed, respectively, for cell-treated subjects and vehicle-treated subjects. A significant, positive effect on wound healing was seen for treatment (slope, 0.8721; P = .0096), whereas a significant, overall negative effect on wound healing was seen for inhibitory bioburden index (slope, −0.6803; P = .0021). The effect of noninhibitory bioburden index on wound healing was not statistically significant (slope, 0.3499; P = .1625).

Table II. Proportion of wounds closed in the presence of inhibitory bacteria

Having (+) culture for	All patients (n = 228)	Vehicle (n = 50)	Cells (n = 177)
Noninhibitory			
Acinetobacter spp. $\geq 1 \times 10^4$	75% (3/4)	0% (0/1)	100% (3/3)
Serratia spp. $\geq 1 \times 10^4$	86% (6/7)	67% (2/3)	100% (4/4)
Providencia spp. $\geq 1 \times 10^4$	50% (2/4)	0% (0/1)	67% (2/3)
Staphylococcus spp. $\geq 1 \times 10^4$	62% (60/97)	50% (10/20)	65% (50/77)
Staphylococcus aureus $\geq 1 \times 10^4$	60% (53/88)	50% (10/20)	63% (43/68)
Proteus spp. $\geq 1 \times 10^4$	67% (4/6)	100% (1/1)	60% (3/5)
Inhibitory			
Strept. beta-hemolytic $\geq 1 \times 10^3$	52% (11/21)	0% (0/2)	58% (11/19)
Morganella morganii $\geq 1 \times 10^4$	50% (3/6)	—	50% (3/6)
Pseudomonas spp. $\geq 1 \times 10^4$	47% (7/15)	33% (1/3)	50% (6/12)
Enterococcus spp. $\geq 1 \times 10^4$	39% (7/18)	0% (0/1)	41% (7/17)
Klebsiella spp. $\geq 1 \times 10^4$	38% (6/16)	25% (1/4)	42% (5/12)
Enterobacter spp. $\geq 1 \times 10^4$	36% (4/11)	0% (0/2)	44% (4/9)
Escherichia coli $\geq 1 \times 10^4$	20% (1/5)	0% (0/3)	50% (1/2)
Any below the dashed line[a]		8% (1/12)	49% (29/59)

[a]The denominators reflect several cases of individual patients with more than one bacterial species.

For the cell-treated subjects, a significant, negative effect on wound healing was seen for the inhibitory bioburden index (slope, -0.6199; $P = .0086$). For the vehicle-treated subjects, a more negative but, likely because of sample size, not statistically significant effect on wound healing was seen for the inhibitory bioburden index (slope, -1.1478; $P = .0811$). Although the association between measured bacterial concentration and healing did not differ significantly between the groups, an association existed between failing to heal and inhibitory bioburden, as vehicle-treated wounds harboring inhibitory bacteria were more likely to remain open at the end of treatment (92%) compared with cell-treated wounds (51%) (odds ratio, 10.63; 95% confidence interval, 1.29-87.69; $P = .0096$). Wound bioburden was associated with increasing wound duration (Fig), with wounds harboring inhibitory species having significantly greater duration compared with other wounds ($P < .001$, Mann-Whitney). A summary of the influence of variables tested is presented in Table III.

DISCUSSION

In the setting of controlled trials, the use of infection control, debridement as necessary, wound moisture control, and application of a high-compression bandage system results in VLU closure in 30%-75% of patients over a 12- to 24-week time period.[1,13-16] In line with our findings, a number of published studies noted that larger wounds, wounds of greater duration, and wounds below the ankle have a reduced probability of healing. Phillips reported that wound size and duration were important variables, whereas sex, race, age, skin condition, and clinically evident infection had no prognostic value.[17] Using multivariate analysis of more than 20,000 patients, Margolis found age, sex, number of wounds, and wound depth to be minor contributors, whereas wound size and duration were major contributors to a risk of not healing.[11]

Patients with persistent wounds may be recruited into clinical trials of potential new therapies on the assumption
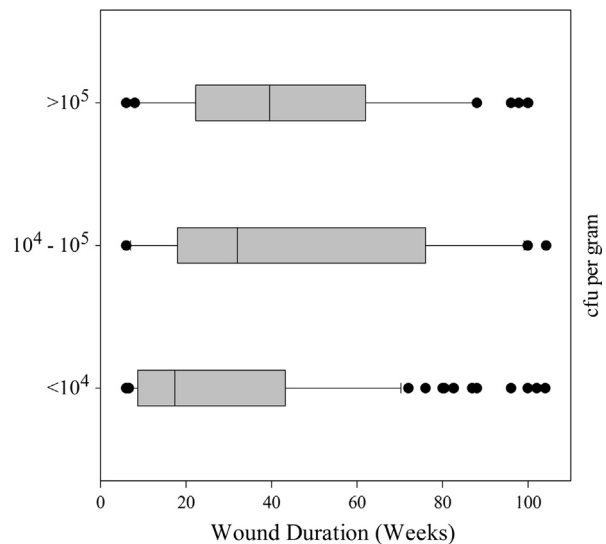

Fig. Wound bioburden and duration. Box and whisker plot showing the median, 25th percentile, 75th percentile (*box*), and 95% confidence intervals (*whiskers*) of wound durations by quantities of certain bacterial species recovered from a punch biopsy specimen taken at the screening visit. Bioburden levels were significantly greater in wounds of longer duration ($P < .001$). *cfu,* Colony forming units.

that variables, which contributed to initial failure, will continue to limit responses in the standard care control group, while yielding to the intervention.[11] Thus, for example, wounds of relatively small size may be enrolled on the presumption that something other than size resulted in failure to heal with standard care alone. Despite this, VLU interventional trials typically exclude patients who have conditions assumed to be barriers to healing, such as poorly controlled diabetes. The potential benefit of the intervention may, thus, be underestimated or missed entirely, and generalizeability of positive results becomes limited by each additional exclusion criterion.[18]

Table III. Influence of tested variables on complete healing

Category	More likely to heal when	P value Cell group	P value Vehicle group
Age	No effect	.748	.922
Sex	No effect	.393	.891
Race	No effect	.142	.203
HbA$_{1C}$	No effect	.300	.360
MF sensation	No effect	.202	.689
BMI	Greater	.007	.206
Location	Leg	.011	.570
Area	Smaller	.026	.444
Duration	Younger	.012	.004
Quant. bact.	$\leq 1 \times 10^4$ select species	.002	<.001

BMI, Body mass index.

In the context of our trial, healing outcomes were unaffected by age or sex. Area and location influenced healing in the control group, but the effect did not reach significance. We found that certain variables, often presumed to impair healing (history of diabetes mellitus, elevated HbA1C level, peripheral neuropathy, decreased serum prealbumin), did not influence outcomes. Patients with high BMI did well in this study, suggesting that the conditions of the study and perhaps cell therapy in particular, overcame this presumed impediment to healing. No evidence was found to suggest that wound bed sharp debridement performed routinely influenced healing regardless of clinical indication.[19]

An unexpected finding was that modest quantities of certain bacteria correlated with poor response to treatment, and to a greater degree, in patients receiving vehicle compared with patients treated with cells. Although the reason for these findings is not entirely clear, the identified bacterial species are likely to elaborate virulence factors and/or influence host inflammatory responses in a manner detrimental to healing. To the extent that bacterial counts tended to increase with increasing wound duration, it is possible that colonization is secondary to chronicity rather than causally related. Our study did not assess whether bacterial counts were reduced, or species eliminated, prior to the initiation of healing, and thus, we cannot conclude with certainty that these species reinforce unresponsiveness. In fact, healing rates were lower with standard care than with cells even among patients with no bacteria recovered from the wound bed. We did not evaluate biofilms.

As noted, it is possible that wound duration, and perhaps wound area, are surrogates for impediments to healing that may (eg, extent of venous disease)[20] or may not (eg, bacterial colonization with certain species)[21] be routinely measured. Additional potential impediments not routinely measured include local innate immune responses, which are inappropriately active[22] or inactive,[23] fibroblast senescence induced by oxidative stress,[24] or wound bed fibrosis.[25] Measurement of such variables is far from practical in a clinical setting, as local tissue immune responses, biofilm assays, and fibroblast senescence are not readily assayable. Still, greater duration of a wound at presentation may suggest a need to rule out arterial or vasculitic components,[4] or to initiate earlier aggressive therapy rather than to wait for failure of standard care therapies.

Our findings are in agreement with a recent comprehensive cohort design study in a population of 250,000.[4] Among 113 patients with 138 wounded limbs, large ulcer size, age > 65, diabetes, varicose eczema, and atrophie blanche were not associated with delayed closure, whereas duration greater than 18 months, wheelchair or bedbound status, poor ankle mobility, equinus deformity, evidence of DVT, and superficial thrombophlebitis as well as the presence of lipodermatosclerosis were associated with delayed healing. Bacterial burden showed interesting trends; presence of clinical cellulitis, of *Staphylococcus aureus* or β-*Streptococcus* were associated with delayed healing. This association, however, was not statistically significant.

Several limitations affect interpretation of our results. The trial was not designed to prospectively test any of the observed correlations, thus, causal conclusions should not be drawn. Wounds were limited in size to 2-12 cm^2, which does not account for the small proportion of VLU that are larger than 12 cm^2.[9] Combining all cell-treated patients together creates an imbalance in statistical power between the treated and control groups, raising caution in the interpretation of *P* values. Some variables were measured with greater precision than others. For example, wound area was determined using digital imaging, whereas in some cases, wound duration was based on patient recall.

In summary, cell therapy was able to overcome one or more impediments to healing in the population studied. Our findings imply that future interventional trials may benefit from stratified randomization on wound duration and should avoid excluding subjects for the presence of variables not known to influence outcome.

AUTHOR CONTRIBUTIONS

Conception and design: WM, RK, YZ, TL, DC, HS
Analysis and interpretation: WM, RK, YZ, DC, HS
Data collection: JL, AF, WM, RK
Writing the article: JL, WM, AF, RK, YZ, TL, DC, HS
Critical revision of the article: JL, WM, AF, RK, YZ, TL, DC, HS
Final approval of the article: JL, WM, AF, RK, YZ, TL, DC, HS
Statistical analysis: YZ, HS
Obtained funding: HS
Overall responsibility: HS

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