

# The Effect of a Connexin43-Based Peptide on the Healing of Chronic Venous Leg Ulcers: A Multicenter, Randomized Trial

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The gap junction protein, connexin43 (Cx43), has critical roles in the inflammatory, edematous, and fibrotic processes following dermal injury and during wound healing, and is abnormally upregulated at the epidermal wound margins of venous leg ulcers (VLUs). Targeting Cx43 with ACT1, a peptide mimetic of the carboxyl-terminus of Cx43, accelerates fibroblast migration and proliferation, and wound reepithelialization. In a prospective, multicenter clinical trial conducted in India, adults with chronic VLUs were randomized to treatment with an ACT1 gel formulation plus conventional standard-of-care (SOC) protocols, involving maintaining wound moisture and four-layer compression bandage therapy, or SOC protocols alone. The primary end point was mean percent ulcer reepithelialization from baseline to 12 weeks. A significantly greater reduction in mean percent ulcer area from baseline to 12 weeks was associated with the incorporation of ACT1 therapy (79% (SD 50.4)) as compared with compression bandage therapy alone (36% (SD 179.8);  $P=0.02$ ). Evaluation of secondary efficacy end points indicated a reduced median time to 50 and 100% ulcer reepithelialization for ACT1-treated ulcers. Incorporation of ACT1 in SOC protocols may represent a well-tolerated, highly effective therapeutic strategy that expedites chronic venous ulcer healing by treating the underlying ulcer pathophysiology through Cx43-mediated pathways.

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## INTRODUCTION

Venous leg ulcers (VLUs) develop as a result of chronic venous insufficiency, leading to venous hypertension and small vessel damage. Persistent inflammation within the extracellular matrix of the wound bed and the detrimental upregulation of connexins contribute to dysfunction of wound fibroblasts and keratinocytes and the underlying pathophysiology associated with an impaired wound-healing response in chronic VLUs (Brandner *et al.*, 2004; Charles *et al.*, 2008; Mendoza-Naranjo *et al.*, 2012; Kim *et al.*, 2014). In industrialized countries, VLUs affect ~3% of people over 65 years of age (Fletcher *et al.*, 1997; Bergan *et al.*, 2006). Given that therapeutic intervention extending

beyond 1 year is common, and recurrence rates are ~60–70%, VLUs present a substantial economic and societal burden on the individual, family, and health-care system (de Araujo *et al.*, 2003; Abbade and Lastoria, 2005; O'Meara *et al.*, 2012).

Conventional treatment protocols involving infection control, nonadherent wound dressing, and limb compression remain the cornerstone of conservative treatment, healing between 30 and 75% of VLUs (O'Meara *et al.*, 2009). The Wound Healing Society suggests incorporation of adjunctive therapies in the treatment of VLUs that remain unresponsive to standard-of-care (SOC) treatment beyond 4 weeks (Tang *et al.*, 2012). Randomized controlled trials demonstrate the potential of advanced wound care matrices, such as Apligraf (Organogenesis Inc., Canton, MA). However, such living skin equivalents remain cumbersome, time-consuming and expensive, offer modest improvement over SOC, and may not address the underlying pathophysiology of chronic ulcers (Falanga *et al.*, 1998; Hankin *et al.*, 2012).

Gap junction (GJ) proteins have critical roles in the pathogenesis of chronic wounds, and targeting GJ signaling offers therapeutic opportunity (Qiu *et al.*, 2003; Gourdie *et al.*, 2006; Ghatnekar *et al.*, 2009; Churko *et al.*, 2012; Marquez-Rosado *et al.*, 2012; Mendoza-Naranjo *et al.*, 2012; Wright *et al.*, 2013; Grek *et al.*, 2014). Connexins are the channel-forming component of GJs that directly couple the cytoplasm between cells, permitting the exchange of small molecules (< 1000 Da) and facilitating electrical propagation in excitable

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Abbreviations: AE, adverse event; Cx43, connexin43; GJ, gap junction; ITT, intent-to-treat; PP, per protocol; SOC, standard of care; VLU, venous leg ulcer  
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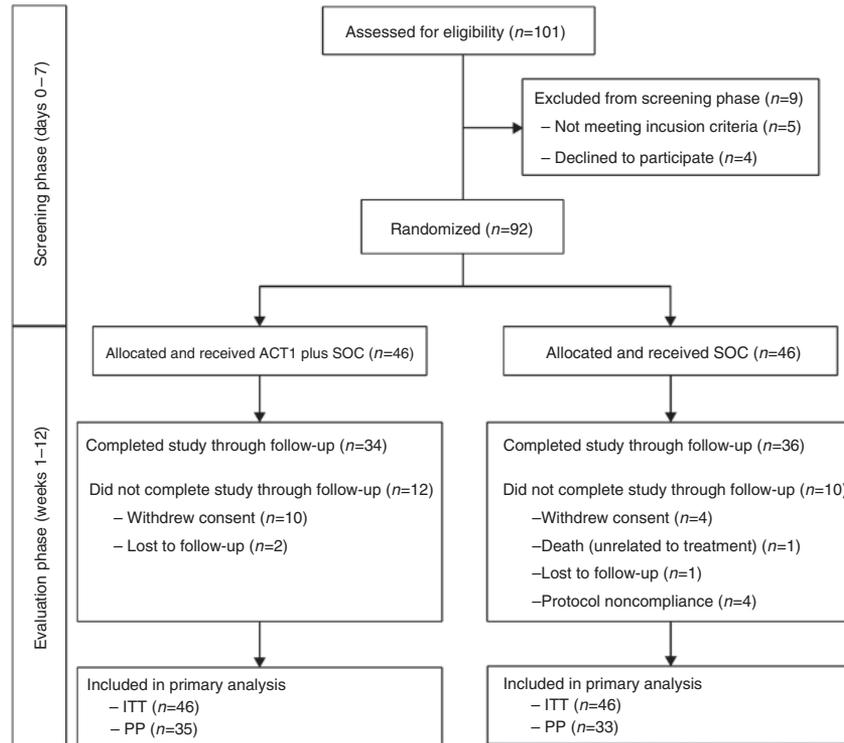


Figure 1. CONSORT flow diagram of participants. ITT, intent-to-treat; PP, per protocol; SOC, standard of care.

tissues. Connexin43 (Cx43) has critical roles in the regulation of inflammatory, edematous, and fibrotic processes following tissue injury and during healing, and is abnormally upregulated at the epidermal wound margins of chronic VLU (Brandner *et al.*, 2004; Mendoza-Naranjo *et al.*, 2012).

ACT1 is a 25 amino acid synthetic peptide containing the carboxy-terminal PDZ binding domain of Cx43 that directly interacts with Cx43 binding partners, thus offering specific and reversible modulation of GJ communication. When applied topically on rodent and porcine skin wounds, ACT1 accelerates the wound closure rate, reduces inflammatory neutrophil infiltration, and reduces scar tissue formation (Gourdie *et al.*, 2006; Rhett *et al.*, 2008; Ghatnekar *et al.*, 2009). ACT1’s mechanism of action with regard to tissue regeneration and the dampening of inflammatory responses is independent of Cx43 expression and is linked to mediated increases in the size of GJ channel aggregates and the sequestration of hemichannels from the perinexal region surrounding GJs (Hunter *et al.*, 2005; Rhett *et al.*, 2011). This study evaluated the efficacy and safety of ACT1 in accelerating the healing of chronic VLUs when incorporated into conventional treatment protocols.

**RESULTS**

Between 5 October 2011 and 10 May 2012, 101 patients were screened and a total of 92 patients were randomly assigned 1:1 to a treatment protocol involving ACT1 and conventional SOC protocols involving the maintenance of a moist wound environment and the application of compression bandage therapy or a control treatment of conventional therapy alone (Figure 1). Randomized groups showed similar

baseline patient demographics in terms of mean age, race, mean weight, mean body mass index, ankle blood pressure, baseline mean ulcer area, and ulcer location (Table 1). At study outset, the participant population had an average ulcer size of 3.5 cm<sup>2</sup> lasting about 17 months.

Of the 92 participants who were assigned to randomization, 14 withdrew consent during the course of the study, 3 were lost to follow-up, 4 were determined to be in noncompliance post-facto, and 1 participant died as a result of myocardial infarction unrelated to intervention, resulting in a total of 70 participants who completed the study. The final analysis sample sizes consisted of an intent-to-treat (ITT) participant population (n=92) and a per protocol (PP) participant population (n=68) made up of participants lacking any major protocol deviation and present for all ulcer evaluations (Figure 1). ITT analyses avoid bias associated with the nonrandom loss of participants and included all participants with a baseline visit. The PP population excluded participants who had died, had withdrawn consent, were noncompliant to the protocol, or had major protocol violations such as missed visits/treatments and lack of wound evaluation data. Of note, the number of missing observations was relatively high but was similar in the control and treatment groups. Selection of the PP population was made according to the ICH-GCP guidelines, with complete information on post-randomization exclusion provided to avoid bias.

**Primary and secondary ulcer healing outcomes**

Preliminary assessment for normality confirmed a non-normal distribution of data (P<0.001), supporting the application of

nonparametric statistical analyses. In both ITT and PP populations, mean percent reduction in ulcer area and incidence of 100% ulcer reepithelialization at week 12 were significantly greater in the treatment group incorporating ACT1 as compared with the control group receiving conventional therapy alone (Table 2). ACT1 application was associated with a significantly greater reduction in mean percent ulcer area from baseline to 12 weeks (ITT: 79% (SD 50.4); PP: 79% (SD 51.1)) compared with compression bandage therapy alone (ITT: 36% (SD 179.8),  $P=0.024$ ; PP: 29% (SD 189.2),  $P=0.007$ ). Representative images demonstrating VLU healing from baseline to week 12 in participants treated with ACT1 and participants receiving SOC-alone are presented in Supplementary Figure S1 online.

The categorical analysis of incidence of 100 and 50% ulcer closure at week 12 was conducted, where participants with 100 and 50% ulcer closure were considered responders. The combined incidence of 100% ulcer closure at week 12 for all study centers was significantly greater in the ACT1 treatment group (57% (ITT) and 74% (PP)), as compared with participants in the SOC-alone group (28% (ITT) and 30% (PP); ITT:  $P=0.006$ ; PP:  $P<0.001$ ,  $\chi^2$  analyses), indicating that standard treatment protocols that incorporated ACT1 had a higher incidence of 100% reepithelialization. Although the percentage of responders in the ACT1-treated group (63%) reaching 50% closure just failed to reach significance ( $P=0.060$ ) over those receiving SOC-alone (43%), for those participants adherent to protocol without missing data points (PP population) the incidence of ulcers exhibiting 50% closure in ACT1-treated participants significantly exceeded those in SOC-alone (80 vs. 51%;  $P=0.013$ ; Table 2).

Participants in both the ITT and PP populations showed that ACT1 incorporation in SOC protocols was associated with a shorter time to 100% ulcer closure during the 12 weeks of efficacy assessments (ITT:  $P=0.041$ ; PP:  $P=0.008$ , Cox hazard ratio), and participants who received ACT1 were approximately three times more likely to obtain 100% ulcer closure than were participants not receiving ACT1 (ITT, 2.3; PP, 3.2; Table 2 and Figure 2). ACT1 treatment was also associated with a significantly reduced time-to-completion of 50% ulcer closure (ITT:  $P=0.014$ ; PP:  $P=0.006$ ), and participants in the treatment group had ~2.5 times higher likelihood of reaching 50% closure than did participants in the SOC control group (ITT: 2.3; PP: 2.6). Ulcer duration, baseline-ulcer depth, and body mass index were not significant factors affecting ulcer closure.

There was no statistically significant difference in the number of responders center-wise between ACT1-treated populations and SOC-alone populations (ITT:  $P=0.238$ ; PP:  $P=0.516$ , Cochran–Mantel–Haenszel analysis with Breslow–Day).

#### Recurrence, pain evaluation, and safety analyses

Recurrence, pain, and safety analyses were performed on all participants with a baseline visit. Over the duration of the entire study (through follow-up phase), ulcer recurrence was reported in 10 participants (5 in each treatment group; 11% total). Of these participants, recurrence occurred during the

**Table 1. Baseline characteristics of participants**

	ACT1 + SOC (n = 46)	SOC (n = 46)	Overall (n = 92)
<i>Clinical characteristics</i>			
<i>Age (years)</i>			
Mean (SD)	48.2 (12.5)	51.5 (12.9)	49.8 (12.7)
95% Confidence interval	(44.4 – 51.9)	(47.7 – 55.3)	(47.2 – 52.4)
<i>Sex</i>			
Male	44 (96%)	39 (85%)	83 (90%)
Female	2 (4%)	7 (15%)	9 (10%)
<i>Race</i>			
Indian	46 (100%)	46 (100%)	92 (100%)
<i>Weight (kg)</i>			
Mean (SD)	72.2 (15.0)	68.8 (14.7)	70.5 (14.9)
95% Confidence interval	(67.7 – 76.6)	(64.4 – 73.2)	(67.4 – 73.6)
<i>BMI</i>			
Mean (SD)	25.1 (4.8)	24.5 (4.4)	24.8 (4.6)
95% Confidence interval	(23.7 – 26.5)	(23.5 – 25.8)	(23.9 – 25.7)
<i>Clinical history</i>			
<i>Baseline-ulcer area (cm<sup>2</sup>)</i>			
Mean (SD)	3.5 (3.7)	3.6 (3.7)	3.5 (3.7)
95% Confidence interval	(2.3 – 4.6)	(2.5 – 4.7)	(2.7 – 4.3)
<i>Ulcer location, n</i>			
Anteromedial	0 (0%)	1 (2%)	1 (1%)
Anterior side of leg	11 (24%)	9 (20%)	20 (22%)
Lateral malleolus	2 (4%)	6 (13%)	8 (9%)
Lateral side of leg	12 (26%)	8 (17%)	20 (22%)
Medial malleolus	3 (7%)	5 (11%)	8 (9%)
Medial side of leg	17 (37%)	13 (28%)	30 (33%)
Posterior side of leg	1 (2%)	4 (9%)	5 (5%)
<i>Ulcer duration (weeks)</i>			
Mean (SD)	62.7 (119.5)	74.0 (175.9)	68.4 (149.7)
95% Confidence interval	(27.2 – 98.2)	(21.8 – 126.3)	(37.4 – 99.3)
<i>Ankle Brachial Pressure Index</i>			
Mean (SD)	1.1 (0.1)	1.1 (0.1)	1.1 (0.1)
Range (min:max)	(0.9:1.4)	(0.7:1.4)	(0.7:1.4)

Abbreviations: BMI, body mass index; SOC, standard of care.

follow-up phase after completion of the treatment phase in two participants in each group (4% total). There was no statistically significant difference in ulcer recurrence during or after the study period between treatment and control groups ( $P\geq 1.00$ ).

Self-assessment of pain was recorded on a visual analog scale of 1–10 (1 = “no pain” and 10 = “extreme pain”) from baseline to the end of study, including during follow-up visits. There was no statistical difference in mean intensity of pain

**Table 2. Effect of ACT1 on ulcer area and ulcer closure from baseline**

	ACT1 + SOC (n = 46), intent-to-treat	SOC (n = 46), intent-to-treat	ACT1 + SOC (n = 35), per protocol	SOC (n = 33), per protocol
<i>Mean % area reduction + difference at week 12</i>				
Mean percent wound closure <sup>1</sup> m (SD)	79.3 (50.4)	36.3 (179.8)	79.0 (51.1)	28.6 (189.2)
% Difference between treatment groups	43%	—	50%	—
P-value	0.02	—	0.01	—
<i>Incidence of 100% ulcer closure at week 12</i>				
Number	26 (57%)	13 (28%)	26 (74%)	10 (30%)
P-value	0.01	—	<0.001	—
<i>Incidence of 50% ulcer closure at week 12</i>				
Number	29 (63%)	20 (43%)	28 (80%)	17 (52%)
P-value	0.06	—	0.01	—
<i>Kaplan–Meier weeks to 100% ulcer closure</i>				
Median weeks (90% confidence interval)	6.0 (4.0 – 8.0)	12.1 (10.1 – NA)	6.0 (4.0 – 7.0)	NA (12.0–NA)
P-value	<0.001	—	<0.001	—
<i>Kaplan–Meier weeks to 50% ulcer closure</i>				
Median weeks (90% confidence interval)	2.9 (2.1 – 3.0)	6.9 (5.0 – 9.1)	2.9 (2.0 – 3.0)	8.0 (5.1 – 9.9)
P-value	<0.001	—	<0.001	—
<i>Cox HR for 100% ulcer closure</i>				
HR (95% confidence interval)	2.3 (1.0 – 5.0)	—	3.2 (1.4 – 7.3)	—
P-value	0.04	—	0.01	—

Abbreviations: HR, hazard ratio; NA, not achieved; SOC, standard of care.

<sup>1</sup>Number of subjects having non-missing observations: intent-to-treat (n = 62); per protocol (n = 58).

from baseline to week 12 between the two treatment groups for the ITT (ACT1: 0.46 (SD 1.07); SOC-alone: 0.34 (SD 0.71);  $P=0.839$ ) and PP (ACT1: 0.18 (SD 0.39); SOC-alone: 0.48 (SD 0.80);  $P=0.119$ ) populations.

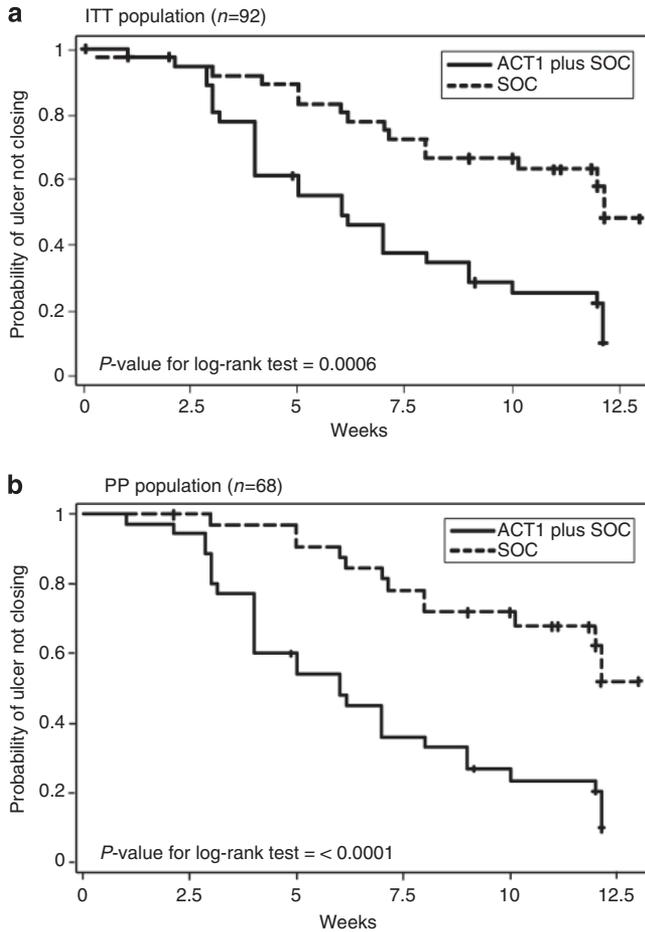
Overall, 28 (30.4%) of the 92 participants reported 40 adverse events (AEs; ACT1: 24 events; SOC-alone: 16 events) that were classified from mild to moderate, including instances of infection, pain, and wound complications (described by inflammation, ulcer irritation, and minor changes in ulcer size). AEs did not segregate in terms of type or number by the study group, and there were no statistically significant differences between the two groups in terms of complications, conditions, or disorders ( $P\geq 1.00$ ; Table 3). The number of participants reporting at least one AE was equal (14 participants per group) for both treatment and SOC-alone groups. Of the 40 events, 35 (87%) recovered and 1 (2%) event recovered with sequelae. Wound complications for the ACT1 treatment group were mild to moderate and were treatment unrelated. Two (2%) serious AEs, death by myocardial infarction and deep vein thrombosis, were reported in the SOC control group. With the exception of one participant who died prior to study completion, none of the participants withdrew from the study because of an AE. No AEs were judged related to the study medication. Immunologic testing for peptide is a crucial aspect in determining the safety profile

of ACT1. Anti-ACT1 peptide antibodies were not detected in the serum from any participant at screening or at the end of the study at week 12.

## DISCUSSION

The results of this clinical efficacy study show that application of the Cx43 peptide mimetic, ACT1, is safe and effective in promoting the healing of chronic VLU and validate the clinical potential of wound-healing therapeutics that target connexin-mediated pathways. ACT1 incorporation into conventional VLU treatment protocols involving the maintenance of a moist wound environment and the application of compression therapy was associated with a significantly greater mean percent ulcer closure, median time-to-healing, and proportion of healing than were VLUs treated with standard treatment protocols alone.

The reported increased incidence of 100% ulcer closure at week 12 in ACT1-treated participants (improvements of 28% (ITT) and 44% (PP) over SOC-alone control) is among the highest rates of clinical response reported in VLU trials (Kirsner *et al.*, 2012; Greer *et al.*, 2013; Harding *et al.*, 2013). An average ulcer size of 3.5 cm<sup>2</sup> lasting about 17 months, as described in our study population at the outset of study, describes wounds that are significantly larger and persistent than those incorporated in studies that describe



**Figure 2.** Kaplan–Meier plot of time to 100% ulcer closure with ACT1 and standard of care (SOC) compared with standard of care alone. (a) Intent-to-treat (ITT) population. (b) Per protocol (PP) population.

appropriate surrogate markers for VLU healing (Gelfand *et al.*, 2002). On the basis of a model derived from a cohort study of 20,000 patients, ulcers fitting into the category of our study population would have ~52% likelihood of healing by 24 weeks with compression bandage SOC (Margolis *et al.*, 2004). ACT1 incorporation into SOC protocols significantly enhanced this estimated prognosis, where VLUs treated with ACT1 healed completely, with a median time of 6 weeks (ITT, PP) as compared with 12 weeks (ITT) or not achieved (PP) in the control SOC group.

Given the critical role that connexin signaling has in cell and tissue homeostasis and angiogenesis, and, by default, in a slew of pathological processes (Vinken *et al.*, 2006; Pfenniger *et al.*, 2011), concern with regard to acute or systemic toxicity is reasonable. In accordance with preclinical animal efficacy and toxicology data (Gourdie *et al.*, 2006; Rhett *et al.*, 2008; Ghatnekar *et al.*, 2009; O’Quinn *et al.*, 2011), ACT1 application was not associated with immunogenicity, nor with a significantly increased proportion or distribution of AEs. Furthermore, the AEs reported were treatment unrelated. Clinically speaking, the absence of AEs is encouraging and may be linked to the relatively short half-life of the peptide.

Although statistical significance in the incidence of ulcer closure and limited associated risk demonstrates clinical relevance, this investigation has a few limitations with regard to the study sample size and uniformity, the lack of information with regard to pre-enrollment treatment protocols (i.e., length and type of compression therapy received prior to enrollment), and the lack of a longer study follow-up period to evaluate ulcer recurrence and patient compliance. Studies with larger sample sizes are needed (Greer *et al.*, 2013), and the reported predictions require confirmation in clinical testing involving larger cohorts of patients. The male-biased gender gap is the result of cultural bias that may prove challenging to overcome and again emphasizes the need for larger study sizes as well as the need for global trials. Increasingly stringent enrollment criteria designed to ensure a refractory ulcer population, including an extended run-in period to exclude patients for whom carefully monitored standard care would obviate the need for advanced therapy, will likely provide further information into the clinical efficacy of ACT1 in additional clinical trials.

VLU recurrence is of significant concern, and future studies require recurrence evaluation beyond 6 months. In preclinical studies, ACT1-treated wounds consistently showed marked reductions in granulation/scar tissue deposition, increased number of rete pegs in the healed wound epidermis, and increases in the tensile properties of the healed skin (measured by stress and strain measurements) as compared with vehicle control treatments (Ghatnekar *et al.*, 2009). Although recurrence data indicated no significant difference between ACT1 and control-treated VLUs, an increase in tensile stretch suggests future analyses involving larger sample sizes, and an extended study duration (to incorporate long-term recurrence data) may likely reveal treatment durability. Furthermore, despite evidence that hydroxyethyl cellulose placebos do not have a significant impact on wound healing (Ghatnekar *et al.*, 2009; Rodgers *et al.*, 2011; Balingit *et al.*, 2012), future studies may benefit from the incorporation of additional vehicle control arms. It is anticipated that study limitations will be addressed in future clinical trials involving multiple arms, larger and more gender and ethnically diverse patient populations, extended run-in periods, and accountability for the relapsing nature of VLUs.

ACT1 represents a first-in-class drug that targets the connexin signaling pathways involved in wound healing that contribute to the underlying pathophysiology of chronic VLUs. Attempts to modulate the signaling pathways involved in the healing of cutaneous wounds have been evaluated in studies that exogenously apply polypeptide growth promoters, such as interleukins, and growth factors, but evidence of clinical efficacy remains limited. Clinical success has been limited to a number of randomized, double-blind studies evaluating the therapeutic application of platelet-derived growth factor-BB homodimers, where enhanced wound healing in stage III and IV ulcers has been attributed to an increase in macrophage recruitment during the early phases of wound healing (Robson *et al.*, 1992; Mustoe *et al.*, 1994). Global matrix metalloproteinase modulators aimed at preventing global matrix metalloproteinase-mediated destruction of local growth

**Table 3. Summary of adverse events by MedDRA system organ class and preferred safety population (n= 92)**

	ACT1 + SOC (n= 46)		SOC (n= 46)		Overall (n= 92)		P-value <sup>1</sup>
<b>Subjects with at least 1 AE</b>	<b>14 (30%<sup>2</sup>)</b>		<b>14 (30%)</b>		<b>28 (30%)</b>		<b>≥1.00</b>
Subjects reporting 1 AE	8 (17%)		12 (26%)		20 (22%)		0.31
Subjects reporting > 1 AE	6 (13%)		2 (4%)		8 (9%)		0.27
<b>Adverse events</b>	<b>n</b>	<b>No. of AEs</b>	<b>n</b>	<b>No. of AEs</b>	<b>n</b>	<b>No. of AEs</b>	
<i>Serious adverse events</i>							
Lethal myocardial infarction	0 (0%)	0	1 (2%)	1	1 (1%)	1	—
Deep vein thrombosis	0 (0%)	0	1 (2%)	1	1 (1%)	1	—
<i>Other adverse events</i>							
Pyrexia	0 (0%)	0	2 (4%)	2	2 (2%)	2	—
Wound infection	2 (4%)	2	1 (2%)	2	3 (3%)	4	≥1.00
Wound complication	7 (15%)	11	5 (11%)	5	12 (13%)	16	0.54
Blister	1 (2%)	1	0 (0%)	0	1 (1%)	1	—
Cough	1 (2%)	1	0 (0%)	0	1 (1%)	1	—
Pneumonitis	1 (2%)	1	0 (0%)	0	1 (1%)	1	—
Venous ulcer pain	6 (13%)	6	4 (9%)	4	10 (11%)	10	0.74
Dermatitis allergic	1 (2%)	1	0 (0%)	0	1 (1%)	1	—
Pruritus	1 (2%)	1	0 (0%)	0	1 (1%)	1	—
Bleeding varicose vein	0 (0%)	0	1 (2%)	1	1 (1%)	1	—
<b>Total AEs in 28 subjects</b>		<b>24</b>		<b>16</b>		<b>40</b>	<b>0.21</b>

Abbreviations: AE, adverse event; SOC, standard of care.

<sup>1</sup>P-value is calculated by comparing two treatment groups using  $\chi^2$ -test.

<sup>2</sup>Percentage is calculated by taking respective column header group count as denominator.

factors report clinical success when incorporated into wound dressings (Meaume *et al.*, 2012). To our knowledge, the present study is the first peer-reviewed, published report of a clinical trial describing connexins (i.e., Cx43) as biological targets for wound healing.

Chronic VLUs remain stalled in the initial inflammatory phase of wound healing and fail to heal with standard SOC protocols (Eberhardt and Raffetto, 2005; Charles *et al.*, 2008; O'Meara *et al.*, 2009). Anti-inflammatory flavonoid medications have shown clinical efficacy in the treatment of VLUs, putatively via protection from hypoxic damage and free radicals, and affecting the expression of granulocyte adhesion molecules (Scallon *et al.*, 2013). In preclinical animal studies, ACT1 shortened and reduced the amplitude of the initial inflammatory phase of wound healing, reduced neutrophil infiltration, reduced wound gape and edema, and accelerated the rate of wound closure (Ghatnekar *et al.*, 2009). Molecular studies have determined that ACT1 selectively inhibits interaction between Cx43 and the PDZ-2 domain of ZO-1, independent of Cx43 expression, resulting in the release of Cx43 hemichannels in the cell membrane from the perinexus, where they are then sequestered into GJ aggregates (Rhett *et al.*, 2011; Rhett and Gourdie, 2012). Hemichannels provide a paracrine route for intercellular communication and regulate wound-healing processes associated with inflammation, edema, and

fibrosis. Reduction of Cx43 hemichannel activity tempers inflammatory responses, shortens the inflammatory phase, and enables the tissue injury process to shift toward a healthy regenerative healing process (Hunter *et al.*, 2005; Gourdie *et al.*, 2006; Rhett *et al.*, 2008; Ghatnekar *et al.*, 2009; Rhett *et al.*, 2011; Mendoza-Naranjo *et al.*, 2012). This mechanism of action in association with studies that identify abnormal upregulation of Cx43 in VLUs supports a molecular role for ACT1 in enhancing the healing rate of chronic VLUs. Further evidence is relayed in studies where targeting Cx43 with polynucleotide antisense DNA indicates wound-healing potential via the acceleration of fibroblast migration and proliferation (Mendoza-Naranjo *et al.*, 2012).

Recent studies summarizing 20 trials involving 9 advanced therapies for VLUs report potential in the application of Apligraf and keratinocyte products in terms of reducing time to ulcer healing, but also highlight the need for additional clinical evidence supporting enhanced healing rates and research involving cost-effective analyses (Greer *et al.*, 2013). Apligraf, in particular, has been associated with concerns of cost, shelf-life, and the possibility of disease transmission (Kim *et al.*, 2007). The significantly enhanced healing rates and incidence of ulcer closure observed in ACT1-treated chronic VLUs have global significance and could translate into significant pharmacoeconomic

benefits by substantially decreasing VLU-related health care costs and improving patient quality-of-life. Further investigation of ACT1 in additional, pivotal clinical studies that directly compare ACT1 with alternative advanced therapies is warranted.

## MATERIALS AND METHODS

### Design overview

A randomized, prospective, observer-blinded, parallel group, multi-center trial targeting patients with a stage II or III VLU, as defined by the International Association of Enterostomal Therapists, was conducted (Clinical Trials Registry India: CTRI/2011/09/001985). The study was designed to assess the clinical efficacy and safety of ACT1 in accelerating the healing of chronic VLUs in participants whose ulcers had remained unresponsive to SOC protocols for at least 4 weeks. The study was designed, conducted, recorded, and reported in compliance with the International Conference on Harmonisation Guidelines, the principles of the Declaration of Helsinki, and with approval from the office of the Drug Controller General of India and Independent Ethics Committees/Institutional Review Boards. Participants were informed by site investigators of the risks and benefits and the option to withdraw from the study at any time, and they signed informed consent forms before enrollment. Participants were randomly assigned (1:1) to treatment protocols with ACT1 or without ACT1 and were treated until ulcer closure or up to 14 topical ACT1 applications. Total study duration was 12 months and involved individual patient enrollment for 6 months, incorporating a total of 18 evaluation visits.

### Study procedure

Study procedures were divided into three phases: screening phase (day: -7 through day 0), treatment phase (day 0 through week 12), and follow-up phase (month 4 through month 6). Safety and efficacy were evaluated weekly through the treatment phase and then monthly through the follow-up phase. During visit 1 (day -7), ulcers were debrided, without undermining, to remove fibrin slough or necrotic tissue. Screening procedures incorporated documentation of medical history, a physical examination including neurovascular examination, vitals, and electrocardiogram, laboratory blood tests for hematology, serum chemistry and immunologic testing, evaluation of adherence to inclusion and exclusion criteria, and ulcer evaluation. Participants remained in the treatment phase until 100% ulcer reepithelialization or for 12 weeks, whichever occurred first. At each visit during the treatment phase, ulcers in both groups were cleaned, irrigated, photographed, traced, assessed for closure, and dressed, and the self-assessment of pain and AEs were recorded. For each participant designated in the treatment group, during SOC protocols ACT1 (100  $\mu\text{M}$ ) was applied topically to the ulcer on day 0 (baseline visit) and day 3 and then weekly from week 1 to week 12. The ACT1 treatment regimen was designed on the basis of alignment with currently used SOC protocols and preclinical studies revealing ACT1 mechanism of action (Hunter *et al.*, 2005; Gourdie *et al.*, 2006; Ghatnekar *et al.*, 2009; Rhett *et al.*, 2011). Participants whose target ulcer healed completely entered the follow-up phase, during which AEs and ulcer status/recurrence were assessed. Safety variables included incidence of treatment-related AEs and the determination of ACT1 immunogenicity.

### Setting and participants

Male or female patients, 18 years or older, with existing VLUs were recruited and screened by the site investigators at 10 academic centers located in South Asia. Inclusion criteria (Supplementary Table S1 online) included having a Stage II or III surface ulcer (as defined by the International Association of Enterostomal Therapists) measuring between 0.5 and 40  $\text{cm}^2$  post debridement that was present at least 4 weeks prior to initial screening (i.e., chronic). Stage II ulcers are defined by partial-thickness skin loss involving the epidermis, dermis, or both. Stage III ulcers are characterized by full-thickness skin loss involving damage or necrosis of subcutaneous tissue that may extend down to, but not through, underlying fascia. Ulcers were clinically determined to be of venous origin by the positive venous reflux test (venous refilling <20 seconds) using Doppler ultrasound. Participants were excluded if there was a change in ulcer size by >30% during a 7-day screening period, the patient was unable to tolerate or comply with compression therapy, showed signs of ulcer infection, had an Ankle Brachial Pressure Index <0.65, or the ulcer was heavily exudative (requiring daily dressing changes). In the presence of multiple ulcers, the largest ulcer was considered as the target ulcer and assessed in treatment protocols.

### Randomization and interventions

Baseline assessments (Table 1) were completed on patients who met the initial inclusion/exclusion criteria (Supplementary Table S1 online) and were registered by the site investigator to receive treatment assignment. A central block randomization (size 2) list was prepared by an independent statistician using a validated computer program (Statistical Analysis Software (SAS v9.1.3 (SAS Institute, Cary, NC)). Using the Interactive Web Response System, participants were randomized 1:1 to receive either 100  $\mu\text{M}$  ACT1 topical formulation plus SOC or SOC therapy alone. Baseline ulcer sizes and ulcer duration are well-established markers for predicting the likelihood of healing (Margolis *et al.*, 2004). To avoid bias due to these effects, ulcers were further stratified by size (<10  $\text{cm}^2$  and >10  $\text{cm}^2$ ). An unblinded coordinator designated by the site investigator received treatment assignments through the Interactive Web Response System. The trial sponsor, trial monitors, statisticians, investigators, and the observer who performed area closure measurements of ulcer photographs were blinded to treatment assignments.

The intervention (ACT1 gel formulation) is manufactured as a clear topical gel formulation (1.25% hydroxyethyl cellulose, manufactured by Dow Pharmaceutical Sciences, Petaluma, CA) containing ACT1 (100  $\mu\text{M}$ ) and was spread evenly over the ulcer surface. Preclinical studies have validated the efficacy and safety of a 100  $\mu\text{M}$  concentration of ACT1 (Ghatnekar *et al.*, 2009). ACT1 dosing strategies were further optimized in a Phase 1, double-blind, single center, controlled clinical study designed to evaluate the safety profile and tolerability of 20, 50, 100, and 200  $\mu\text{M}$  concentrations of ACT1 versus a hydroxyethyl cellulose gel vehicle control in 48 healthy participants following punch biopsy. This study recapitulated previous preclinical studies that indicated optimal safety (no AEs related to study medication) and therapeutic efficacy (in terms of accelerating wound closure) at 100  $\mu\text{M}$  ACT1 concentration and laid precedence for the 100  $\mu\text{M}$  ACT1 formulation applied in the current Phase 2 trial. The hydroxyethyl cellulose formulation was designed to optimize

ACT1 stability and ulcer application. Hydroxyethyl cellulose placebos did not have a significant impact on wound healing, an observation that is supported by recent reports (Ghatnekar *et al.*, 2009; Rodgers *et al.*, 2011; Balingit *et al.*, 2012).

ACT1 treatment was administered by trial-site-specific research-trained nurses not acting as investigators. For each participant designated in the treatment group, during SOC protocols ACT1 (100  $\mu$ M formulation) was applied topically to the ulcer on day 0 (baseline visit) and day 3 and then weekly from week 1 to week 12. No modifications to ACT1 formulation or application protocol were made during the course of the study. Ulcers were cared for and dressed in an identical manner in both groups. SOC procedures were maintained throughout all trial phases and included thorough irrigation with sterile saline, bleeding control, and application of a saline-soaked moist nonadherent dressing, which extended 1.27 cm beyond the ulcer perimeter and inflamed skin margins, followed by a non-occlusive dressing (fine mesh gauze), either folded or rolled as a bolster, and the application of a Elastocrep 3M four-layer compression bandage from the metatarsals up to the tibial plateau to ensure efficient therapeutic compression to the ulcer site. All bandages were applied by experienced research nurses using a spiral technique. Maintaining wound moisture is a critical component of VLU healing and was a priority in both treatment protocols.

### Outcomes and follow-up

Enrolled participants were considered completed for statistical analysis of efficacy end points at 12 weeks. End point analyses and study duration were chosen for practical clinical relevance and prognostic utility and were based on implemented protocols/results from pertinent published clinical studies (Veves *et al.*, 2001; Kirsner *et al.*, 2012; O'Meara *et al.*, 2012; Harding *et al.*, 2013). The primary efficacy end point was mean percent ulcer closure (reepithelialization) from baseline to week 12. Evaluation of ulcer closure was performed by the clinically qualified site investigators and independently evaluated by a central evaluator blinded to the treatment through computerized planimetry of digital photographs of the ulcers using public domain software ImageJ (U.S. National Institutes of Health, Bethesda, MD).

Secondary efficacy end points included time to 50 and 100% reepithelialization. Self-assessment of pain as measured on a visual analog scale was also completed at all visits. Incidence of 50% and complete closure at week 12 were incorporated as exploratory end points. The inclusion of incidence of 100% ulcer closure by the end of study in secondary end point analyses is considered as a primary end point for marketing approval by regulatory authorities (Guidance for Industry: Chronic Cutaneous Ulcer and Burn Wounds-Developing Products for Treatment, 2006).

Safety was determined by vitals, laboratory testing, and AE reporting. Laboratory and immunogenicity tests obtained at baseline served as a reference and were compared with tests taken during the study, at study completion, and during follow-up procedures. Laboratory evaluations included those required as per the standard for VLU patient monitoring, including hematology (hemoglobin, hematocrit, RBC, WBC, and platelets) and blood chemistry (HbA1c, creatinine, uric acid, blood urea nitrogen, potassium, sodium, chloride, bicarbonate, albumin, aspartate transaminase, alanine transaminase, total cholesterol). In accordance with good clinical practice, the investigator was permitted to obtain laboratory tests as needed to assess any

AE. Immunologic testing for ACT1 peptide was performed at baseline and at week 12 using a validated ELISA for the detection of anti-ACT1 antibodies (WuXi Aptec, Philadelphia, PA).

### Statistical analysis

The sample size enrolled ( $n=92$ ), with 40 patients needed per treatment group, was calculated with reference to the primary end point (comparing the reduction in wound size as measured by the mean percentage decrease from baseline to week 12), assuming a 25% difference in favor of participants treated with ACT1. Calculations were based on using a conservative healing rate of 20% for control and 45% for ACT1 at a power of 80%, a significance of 95% (two-sided), a SD of 40%, and adjusted for a 15% dropout rate. At least 72 total evaluable participants at week 12 were anticipated for a primary efficacy analysis.

Statistical tests were carried out as two-sided on a 5% level of significance. Primary and secondary ulcer closure end points were analyzed using the analysis of covariance Mixed-Model with Repeated Measure at 95% confidence interval. As a response variable, mean percent ulcer area reduction from baseline to week 12 was adjusted for strata, ulcer duration, viable tissue (granulation), exudate level, ankle circumference, and body mass index as covariates, with treatment group, treatment visit, and visit as factors. Clinical assessment of variables not associated with a defined measurable outcome was carried out on a scale of 1–5 (1 = "much worse"; 2 = "worse"; 3 = "same"; 4 = "improved"; 5 = "much improved"). The Wilcoxon Mann-Whitney *U*-test was used for data in which the normality assumption was tested by Shapiro-Wilk and Q-Q plots. For time-to-event end points (100 and 50% ulcer closure), the distribution was estimated by the Kaplan-Meier method, compared by the log-rank test for statistical significance, and median time (i.e., ulcer closure for 50% of enrolled participants) was calculated using a 90% confidence interval to avoid missing the upper limit, in the event that 50% of the participants did not achieve complete ulcer closure by week 12. Individual and joint effects of covariates on time to 100% ulcer closure were evaluated using Cox Proportional Hazard. The exploratory end points (i.e., incidence of 50 and 100% ulcer closure at week 12) were analyzed overall and by center, assessed by Fisher's Exact test (two-tailed), and followed by the Cochran-Mantel-Haenszel test after adjusting for pooled-center. The Breslow-Day test was used in conjunction with the Cochran-Mantel-Haenszel test to determine statistical significance of treatment by pooled-center interaction. Participant self-assessment of intensity of pain at all visit time points starting at visit 0 was analyzed by the Wilcoxon Mann-Whitney *U*-test.

Sensitivity analysis was performed to establish whether the conclusions drawn from the primary analysis were robust. Primary analysis was performed using Proc Mixed in SAS in which missing values were accounted for using the analysis of covariance method with repeated measures and the Last Observation-Carried-Forward approach was used for performing sensitivity analysis. If the ulcer healed and all further visits were missed, 100% reduction was carried forward to missing visits until any recurrence.

The site investigators were responsible for ensuring that all AEs, defined in accordance with ICH E6:1.2, observed or reported, were properly recorded using precise medical terminology. AEs were tabulated as per Medical Dictionary for Regulatory Activities v15.1, indicating number and percentage of participants and number of AEs.

AEs were evaluated in relation to study drug, seriousness, severity, action taken, and outcome. Analyses were performed by an independent statistician using SAS v9.1.3.

### CONFLICT OF INTEREST

FirstString Research Inc, Mount Pleasant, SC was responsible for the study design, interpretation of data, preparation, review, and approval of the manuscript. Gautam Ghatnekar and Robert Gourdie are co-inventors of ACT1 and co-founded FirstString Research. FirstString Research has an exclusive, worldwide license for all fields of use for ACT1. Ghatnekar is President and CEO of FirstString. Grek is an employee of FirstString Research. Gourdie and Armstrong are members of the Scientific Advisory Board of FirstString Research. Ghatnekar, Grek, Gourdie, and Armstrong have stock options issued by the company.

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### SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/jid>

### REFERENCES

- Abbate LP, Lastoria S (2005) Venous ulcer: epidemiology, physiopathology, diagnosis and treatment. *Int J Dermatol* 44:449–56
- Balingit PP, Armstrong DG, Reyzelman AM *et al.* (2012) NorLeu3-A(1-7) stimulation of diabetic foot ulcer healing: results of a randomized, parallel-group, double-blind, placebo-controlled phase 2 clinical trial. *Wound Repair Regen* 20:482–90
- Bergan JJ, Schmid-Schonbein GW, Smith PD *et al.* (2006) Chronic venous disease. *N Engl J Med* 355:488–98
- Brandner JM, Houdek P, Husing B *et al.* (2004) Connexins 26, 30, and 43: differences among spontaneous, chronic, and accelerated human wound healing. *J Invest Dermatol* 122:1310–20
- Charles CA, Tomic-Canic M, Vincek V *et al.* (2008) A gene signature of nonhealing venous ulcers: potential diagnostic markers. *J Am Acad Dermatol* 59:758–71
- Churko JM, Kelly JJ, Macdonald A *et al.* (2012) The G60S Cx43 mutant enhances keratinocyte proliferation and differentiation. *Exp Dermatol* 21: 612–8
- de Araujo T, Valencia I, Federman DG *et al.* (2003) Managing the patient with venous ulcers. *Ann Intern Med* 138:326–34
- Eberhardt RT, Raffetto JD (2005) Chronic venous insufficiency. *Circulation* 111:2398–409
- Falanga V, Margolis D, Alvarez O *et al.* (1998) Rapid healing of venous ulcers and lack of clinical rejection with an allogeneic cultured human skin equivalent. Human Skin Equivalent Investigators Group. *Arch Dermatol* 134:293–300
- Fletcher A, Cullum N, Sheldon TA (1997) A systematic review of compression treatment for venous leg ulcers. *Br Med J* 315:576–80
- Gelfand JM, Hoffstad O, Margolis DJ (2002) Surrogate endpoints for the treatment of venous leg ulcers. *J Invest Dermatol* 119:1420–5
- Ghatnekar GS, O'Quinn MP, Jourdan LJ *et al.* (2009) Connexin43 carboxyl-terminal peptides reduce scar progenitor and promote regenerative healing following skin wounding. *Regen Med* 4:205–23
- Gourdie RG, Ghatnekar GS, O'Quinn M *et al.* (2006) The unstoppable connexin43 carboxyl-terminus: new roles in gap junction organization and wound healing. *Ann N Y Acad Sci* 1080:49–62
- Greer N, Foman NA, Macdonald R *et al.* (2013) Advanced wound care therapies for nonhealing diabetic, venous, and arterial ulcers: a systematic review. *Ann Intern Med* 159:532–42
- Grek CL, Rhett JM, Ghatnekar GS (2014) Cardiac to cancer: connecting connexins to clinical opportunity. *FEBS Lett* 588:1349–64
- Guidance for Industry: Chronic Cutaneous Ulcer and Burn Wounds-Developing Products for Treatment (2006). [www.fda.gov](http://www.fda.gov)
- Hankin CS, Knispel J, Lopes M *et al.* (2012) Clinical and cost efficacy of advanced wound care matrices for venous ulcers. *J Manag Care Pharm* 18:375–84
- Harding K, Sumner M, Cardinal M (2013) A prospective, multicentre, randomised controlled study of human fibroblast-derived dermal substitute (Dermagraft) in patients with venous leg ulcers. *Int Wound J* 10:132–7
- Hunter AW, Barker RJ, Zhu C *et al.* (2005) Zonula occludens-1 alters connexin43 gap junction size and organization by influencing channel accretion. *Mol Biol Cell* 16:5686–98
- Kim MH, Gorouhi F, Ramirez S *et al.* (2014) Catecholamine stress alters neutrophil trafficking and impairs wound healing by beta2-adrenergic receptor-mediated upregulation of IL-6. *J Invest Dermatol* 134: 809–17
- Kim PJ, Heilala M, Steinberg JS *et al.* (2007) Bioengineered alternative tissues and hyperbaric oxygen in lower extremity wound healing. *Clin Podiatr MedSurg* 24:529–46
- Kirsner RS, Marston WA, Snyder RJ *et al.* (2012) Spray-applied cell therapy with human allogeneic fibroblasts and keratinocytes for the treatment of chronic venous leg ulcers: a phase 2, multicentre, double-blind, randomised, placebo-controlled trial. *Lancet* 380:977–85
- Margolis DJ, Allen-Taylor L, Hoffstad O *et al.* (2004) The accuracy of venous leg ulcer prognostic models in a wound care system. *Wound Repair Regen* 12:163–8
- Marquez-Rosado L, Singh D, Rincon-Arango H *et al.* (2012) CASK (LIN2) interacts with Cx43 in wounded skin and their coexpression affects cell migration. *J Cell Sci* 125:695–702
- Meaume S, Truchetet F, Cambazard F *et al.* (2012) A randomized, controlled, double-blind prospective trial with a Lipido-Colloid Technology-Nano-OligoSaccharide Factor wound dressing in the local management of venous leg ulcers. *Wound Repair Regen* 20:500–11
- Mendoza-Naranjo A, Cormie P, Serrano AE *et al.* (2012) Targeting Cx43 and N-cadherin, which are abnormally upregulated in venous leg ulcers, influences migration, adhesion and activation of Rho GTPases. *PLoS One* 7:e37374
- Mustoe TA, Cutler NR, Allman RM *et al.* (1994) A phase II study to evaluate recombinant platelet-derived growth factor-BB in the treatment of stage 3 and 4 pressure ulcers. *Arch Surg* 129:213–9
- O'Meara S, Cullum N, Nelson EA *et al.* (2012) Compression for venous leg ulcers. *Cochrane Database Syst Rev* 11:CD000265
- O'Meara S, Cullum NA, Nelson EA (2009) Compression for venous leg ulcers. *Cochrane Database Syst Rev* CD000265
- O'Quinn MP, Palatinus JA, Harris BS *et al.* (2011) A peptide mimetic of the connexin43 carboxyl terminus reduces gap junction remodeling and induced arrhythmia following ventricular injury. *Circ Res* 108: 704–15
- Pfenniger A, Wohlwend A, Kwak BR (2011) Mutations in connexin genes and disease. *Eur J Clin Invest* 41:103–16
- Qiu C, Coutinho P, Frank S *et al.* (2003) Targeting connexin43 expression accelerates the rate of wound repair. *Curr Biol* 13:1697–703
- Rhett JM, Ghatnekar GS, Palatinus JA *et al.* (2008) Novel therapies for scar reduction and regenerative healing of skin wounds. *Trends Biotechnology* 26:173–80
- Rhett JM, Gourdie RG (2012) The perinexus: a new feature of Cx43 gap junction organization. *Heart Rhythm* 9:619–23
- Rhett JM, Jourdan J, Gourdie RG (2011) Connexin 43 connexon to gap junction transition is regulated by zonula occludens-1. *Mol Biol Cell* 22:1516–28
- Robson MC, Phillips LG, Thomason A *et al.* (1992) Platelet-derived growth factor BB for the treatment of chronic pressure ulcers. *Lancet* 339:23–5

- Rodgers K, Verco S, Bolton L *et al.* (2011) Accelerated healing of diabetic wounds by NorLeu(3)-angiotensin (1-7). *Expert Opin Investig Drugs* 20:1575–81
- Scallan C, Bell-Syer SE, Aziz Z (2013) Flavonoids for treating venous leg ulcers. *Cochrane Database Syst Rev* 5:CD006477
- Tang JC, Marston WA, Kirsner RS (2012) Wound Healing Society (WHS) venous ulcer treatment guidelines: what's new in five years? *Wound Repair Regen* 20:619–37
- Veves A, Falanga V, Armstrong DG *et al.* (2001) Graftskin, a human skin equivalent, is effective in the management of noninfected neuropathic diabetic foot ulcers: a prospective randomized multicenter clinical trial. *Diabetes Care* 24:290–5
- Vinken M, Vanhaecke T, Papeleu P *et al.* (2006) Connexins and their channels in cell growth and cell death. *Cellular Signal* 18:592–600
- Wright CS, Berends RF, Flint DJ *et al.* (2013) Cell motility in models of wounded human skin is improved by Gap27 despite raised glucose, insulin and IGFBP-5. *Exp Cell Res* 319:390–401



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