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Enzymatic debridement: is HA-collagenase the right synergy? Randomized double-blind controlled clinical trial in venous leg ulcers

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Abstract. – OBJECTIVE: The aim of this study is to evaluate the efficacy and safety of a new ointment containing Hyaluronic Acid and collagenase from non-pathogenic *Vibrio alginolyticus*.

PATIENTS AND METHODS: Double blind, multicenter, controlled clinical trial (no. ISRCTN71239043) conducted to demonstrate the superiority of Hyaluronic Acid-Collagenase applied once a day over placebo in mean reduction of devitalized/fibrinous/slough tissue after 15 days of treatment. 113 patients with venous ulcers were enrolled and randomized to receive active treatment therapy or vehicle preparation. Both arms also received compression therapy. Subjects were assessed at baseline and at 4 different clinical study visits up to a maximum of 30 days. Outcome measures included mean percentage debridement evaluated by digital planimetry, pain during change of dressing measured on a visual analogue scale and adverse event assessment for tolerance.

RESULTS: After 15 days the debridement rate in the active group was 67.5% compared to 59% in the placebo group ($p = 0.0436$). A significantly higher number of patients in the treatment group achieved 100% debridement by day 15 ($p = 0.0025$) than in the control group, and a higher percentage also demonstrated complete debridement at every other time point. Pain perception was similar in both groups with low levels during medication. No differences in tolerance were observed between groups.

CONCLUSIONS: Chronic venous ulcers treated with this novel compound of Hyaluronic Acid and collagenase resulted in a significantly higher debridement rate at Day 15 *vs.* the control group. Hyaluronic Acid-Collagenase was well tolerated and a low degree of pain was perceived during dressing change. The preparation of 0.2% of Hyaluronic acid-collagenase shows significant benefits in the management of chronic ulcers.

Key Words:
Collagenase, Enzymatic debridement, Hayluronic acid, Leg ulcer.

Abbreviations

VAS = visual analogue scale; VLU = venous leg ulcers; DVT = deep vein thrombosis; LMW = low molecular weight; HA = Hyaluronic Acid; ITT = Intention-to-Treat; PP: Per-Protocol; SP: Safety Population; SDS-PAGE = sodium dodecyl-sulfate electrophoresis; WBP = wound bed preparation.

Introduction

Venous leg ulcers (VLU) are a medical condition of important clinical relevance and high social impact. They affect 0.3-2% of the general population and 2-4% of patients with chronic venous insufficiency[1,2]. Values may reach 5%

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when considering patients over 65 years of age[3]. Up to one-third of ulcerations become chronic and need continuous treatment. Poor healing is patient-related (advanced age, comorbidities, increased BMI, diabetes mellitus, history of deep venous thrombosis, non-compliance with compression therapy) and local factor-related (ulcer dimensions, oxygenation status, duration of the ulcer)[4]. These considerations lead to the need for a full understanding of the pathophysiological mechanisms that are at the basis of the lesion to reach a fast and effective diagnosis and identify appropriate therapies. The two main causes of venous ulceration are primary degenerative disease and/or post-thrombotic disease. One-half to two-thirds of venous ulcers are due to slowly progressive primary reflux disease that begins as varicose veins[5] and can lead to ulceration. The risk of this kind of progression and development of VLU increases as patients reach 60-70 years. The other one-third to one-half of venous ulcers develop after deep vein thrombosis (DVT) and are prone to advance more rapidly to the ulcer stage in periods from 6 months to several years after the DVT event[5]. The clinical appearance of ulcers, due to primary venous reflux disease and post-thrombotic deep vein changes, are so similar that diagnosis of the ulcer's cause requires an imaging examination by duplex scanning in every case for confirmation. Due to their chronicity and relatively high prevalence, the impact of chronic venous ulcers on healthcare costs and patient quality of life is quite significant. Venous hypertension influences the microcirculation triggering an inflammatory process with a reduction of the shear stress (tangential force imposed by the flow of blood on the endothelial surface) leading to an interaction between endothelial cells and leukocytes. The latter releases proteolytic enzymes, inflammatory cytokines, interleukins and tumor necrosis factor-alpha in the interstitial space[6]. Tumor necrosis factor-alpha contributes to fibrin deposition because it inhibits fibrinolysis in patients with venous leg ulcers[7]. Although the gold standard in the treatment of venous leg ulcers is compression therapy, debridement is recognized as a fundamental aspect for removing devitalized/fibrinous tissue. Debridement may be surgical, mechanical, biological, autolytic, enzymatic or chemical. Surgical debridement using scalpels is the fastest method to remove necrotic tissue. Mechanical debridement involves the use of special dressings or ultrasounds to remove the eschar of a stable chronic wound. Larval biologic debridement therapy is based on the use of larvae for medical use that feed on the necrotic tissue and clean the wound by removing bacteria. Autolytic debridement promotes autolysis through enzymes produced by the body and the white blood cells. It is much more effective in patients with compromised immune systems. Enzymatic debridement is a highly selective method consisting in the use of particular enzymes, which promote the removal of necrotic tissue. Topical administration of collagenase has been shown to increase the effects of macrophage collagenase. This allows wound debridement by breaking down proteins in the wound eschar. In the case of enzymatic debridement, the standard treatment uses a collagenase derived from *Clostridium histolyticum*[8]. A new enzymatic debrider has recently been marketed. It is a semi-solid preparation consisting of a hydrophobic ointment for topical application containing 0.2% of low molecular weight (LMW) Hyaluronic Acid (HA) as the principal component and a novel collagenase as the enzymatic component (bacterial collagenase from non-pathogenic *Vibrio V. alginolyticus* >2.0 nkat/g ointment). Collagenases are proteolytic enzymes with a high specificity for native and denatured collagen. In particular *V. alginolyticus* collagenase performs its action by cleaving the Y-Gly bond of the sequence -Pro-Y-Gly-Pro-, while the more commonly used *C. histolyticum* collagenase cleaves the Y-Gly bond in the sequence Y-Gly-X (where Y and X are, respectively, a neutral or any aminoacid). *V. alginolyticus* collagenase is characterized by high purity (> 99.0%) and does not contain non-specific proteases, migrating as a single 82 kDa band on SDS-PAGE. This collagenase preparation shows the highest activity at pH between 7 and 9, decreasing significantly at pH<7 and completely loses activity at pH<5.5, where a transition in the protein folding pattern occurs. This property enables *V. alginolyticus* collagenase to act in a selective manner purely on non-healing wounds, since chronic wounds are characterized by alkaline pH values between 7.15 and 8.9, while healing wounds present pH in mildly acidic range[9]. HA is an endogenous glycosaminoglycan consisting of repeating disaccharide units of N-acetyl-glucosamine and glucuronic acid distributed in the extracellular matrix of most tissues, and particularly concentrated whenever rapid tissue proliferation, regeneration and repair occur[10]. HA has important mechanical

and structural functions and plays a key role in wound healing processes[11]. Its addition maintains an optimal moist environment reducing crusting, discomfort and swelling. Moreover, the LMW-HA fraction specifically enhances the healing process as it acts at the cellular level by increasing the migration and proliferation of fibroblasts and the formation of new blood vessels (neo angiogenesis) and favors re-epithelialization[12,13]. Collagenase was added to the HA-based device as a debriding agent to make the treatment more adequate for the management of ulcers in the first phase of wound bed preparation. The presence of collagenase offers a series of advantages as it combines wound cleaning action with the beneficial effect of hyaluronic acid in the preparation of the wound bed and makes this formulation suitable for the management of chronic ulcers, particularly those with areas of necrosis and slough. Very few placebo-controlled studies comparing enzymatic debridement *vs.* placebo are available[14] and the quality of many studies in the field remains poor[15]. Considering that chronic wounds are a marked healthcare expense, there is an urgent need for efficient and evidence-based treatment guidelines[16]. For this reason, this double-blind placebo-controlled study was designed to evaluate the safety and efficacy of the recently marketed LMW-HA-Collagenase product.

Patients and Methods

Patients

This was a perspective, randomized, double-blind, controlled study *vs.* placebo.

Patients affected by chronic venous ulcers, who fulfilled the study inclusion/exclusion criteria as described in Table I, were recruited at ten Italian renowned specialized referral Wound Healing Centers for the management of VLU.

The purpose of this comparative investigation was to demonstrate the superiority of the LMW-HA-Collagenase device (Bionect Start®, Fidia Farmaceutici S.p.A., Abano Terme, Italy) applied once a day to the comparator in terms of mean reduction in devitalized/fibrinous/slough tissue after 15 days of treatment in patients affected by chronic venous ulcers. The primary study outcome was the mean debridement rate (regarding percentage change in the devitalized/fibrinous/slough tissue area) measured by digital planimetry at Day 15. Secondary objectives included the assessment of the device's efficacy in terms of reduction of pain during change of medication, reduction in wound size and improvement in periwound skin status.

This project was developed in cooperation with the "Associazione Italiana Ulcere Cutanee" (AIUC) and the study protocol was approved by

Table I. Inclusion and exclusion criteria.

Main Inclusion Criteria
1. Both sexes, all ethnic backgrounds, both ambulatory and hospitalized subjects, between 18 and 85 years of age. **2.** Subjects with a diagnosis of chronic venous ulcers (CEAP classification: C6) with devitalized/fibrinous/slough tissue comprising more than 40% of the lesion. **3.** Subjects who have had a venous leg ulcer for at least 6 months. **4.** Subjects who have a target wound which is between 5 cm squared to 30 cm squared in area at the baseline assessment. **5.** Subjects who have given their written informed consent in accordance with the relevant provisions of the Declaration of Helsinki (revised October 2008) and GCP for medical devices.
Main Exclusion Criteria
1. Subjects who have exposed bone, tendon or fascia visible around the target wound. **2.** Subjects who have an Ankle Brachial Pressure Index lower than 0.8 (ABPI < 0.8) measured by Doppler sonography, absent pulses and peripheral arterial disease. **3.** Concomitant use of local antibiotics, hydrogels, hydrocolloids (the administration of oral antibiotics is allowed in the presence of infection). **4.** Concomitant use of detergents, hexachlorophene, acid solutions, antiseptics containing heavy metal ions or soaks containing metal ions or acidic solutions. Concomitant use of disinfectants containing quaternary ammonium. **5.** Subjects with a known hypersensitivity to collagenase or Hyaluronic acid. **6.** Immunocompromised Subjects; known seropositivity to HIV virus. **7.** Subjects affected by severe renal, dismetabolic or hepatic failure which represents a risk to the subjects; presence of underlying medical conditions that might interfere with study completion. **8.** Females who are pregnant, lactating or who have not reached menopause and are not abstinent or practising an acceptable means of birth control as determined by the Investigator for the duration of the study. **9.** Subjects unlikely to be compliant/cooperative during the study, in the judgment of the Investigator.

the Italian Competent Authority and by the Ethics Committees of all recruitment centers. The clinical trial is registered in the ISRCTN registry with the identification number ISRCTN71239043. One hundred and thirteen patients were recruited from November 2011 to October 2013. Written informed consent was obtained from each enrolled patient.

Methods

Patients who were eligible to participate were randomized to the intervention group or the control group following a 4 balanced-block randomization list kept in sequentially numbered sealed opaque envelopes. Randomization was generated with a prevalidated computer program. The double-blind design was made possible thanks to a placebo ointment that was identical to the test product in terms of appearance, color, consistency, packaging and mode of administration. Furthermore, both products had the same composition with the difference that the test ointment also contained the active ingredients: LMW-HA (0.2% w/w) and collagenase (≥ 2.0 nkat/g of ointment). Both ointments were provided free of charge by the manufacturer. Treatment group patients received LMW-HA-Collagenase, the control group received the comparator, both products were topically self-administered on the wound once a day (except the day of the visits when the product was applied by the investigator). Before application, the wound was cleansed of debris by gently rubbing with a gauze pad saturated with normal saline solution. Dry wounds had to be moistened with physiological saline (0.9% NaCl) or glucose solution before treatment. The wounds were covered with a layer of about 2 mm of product, as per clinical practice and as indicated in the manufacturer user's instructions. The same dose of treatment has previously been used in other clinical trials[17,18]. Test preparations were covered using a non-occlusive dressing to ensure contact with the wound surface. The investigator instructed subjects, or their care-givers, on the proper treatment administration procedure that had to be followed once a day at home. Treatment continued for up to 1 month or until complete wound debridement.

Whatever arm of the patient was assigned to compression therapy, the standard of care for venous ulcers, was guaranteed to all subjects by a double layer compression stocking (UlcerKit®, PRO, 40 mmHg, Gloria Med S.p.A., Menaggio, Como, Italy). Both groups were assessed at baseline and at 4 different clinical study visits up to a maximum of 30 days. Clinical data including demographics, medical/surgical history, medication, wound characteristics (i.e., total lesion area, area of devitalized tissue, presence of infection, ulcer duration) and status of the wound bed were collected by trained study staff at days 1 (baseline), 7 (visit 1), 15 (visit 2), 21 (visit 3), and 30 (final visit) using case report forms. Wounds were measured and the devitalized area was assessed using a digital planimetry system: the margins of the reference ulcer were traced on an acetate paper grid that was then transferred to a portable digital tablet to calculate both total lesion area and devitalized area. Pain during target wound medication was assessed by means of a visual analogue scale (0-100 mm) at each visit; no medication influencing the evaluation of pain was allowed within 24 hours before each visit (48 hours if systemic corticosteroids were used in addition to analgesics to treat pain). Local and systemic tolerability was also assessed: participants were monitored throughout the duration of the trial for serious and non-serious adverse events, which were recorded as they occurred by the assigned investigator.

Statistical Analysis

A power estimate of 80% suggested that a minimum sample size of 110 participants was required (significance level of 0.05). Enrollment was closed with 113 patients.

All data summaries and listings were performed using the SAS System version 9.2 under Windows 7 Ultimate. Continuous variables were summarized by descriptive statistics [number of cases, mean, standard deviation, median, minimum, maximum, first (Q1) and third quartile (Q3)]. Continuous variables were summarized using counts of patients and percentages. For continuous endpoints, differences between treatment arms were tested using the X^2-test or Fisher's Exact Test, where applicable, at the 5% significance level. Two-sided 95% confidence intervals for proportions were also calculated. The analysis of the primary efficacy endpoint was performed using the analysis of covariance on ranks due to the non-normality of the primary endpoint. Normality assumption was verified with the Shapiro-Wilk test. The model included the treatment arm as an independent factor and the area of devitalized/fibrinous/slough tissue at baseline as a covariate. Treatment difference was estimated using least squares means and a corresponding 95% confidence interval was derived from the same mod-

el. The percentage of patients that reached full debridement at each time point was compared between groups using the X^2-test.

The analysis populations defined for this study were:

- Intention-to-Treat (ITT): all randomized patients who received at least one application of HA-Collagenase/comparator and had at least one post-baseline measurement for debridement.
- Per-Protocol (PP): extracted from the ITT population after the exclusion of subjects with major protocol violations and subjects who withdrew from the study for reasons not related to treatment performance and tolerability.
- Safety Population (SP): all randomized patients who received at least one application of HA-Collagenase/comparator.

The analysis of primary and secondary efficacy endpoints was performed using the Intention-To-Treat population. Analysis of the primary efficacy endpoint was also repeated on the Per-Protocol population. The results obtained on the PP population were seen as supportive.

Analysis of safety was performed on the SP set: data were summarized with descriptive statistics. No statistics tests were applied.

Results

One hundred and thirteen patients were recruited between November 2011 and October 2013; then they were randomly allocated, 58 to the LMW-HA-Collagenase group and 55 to the comparator group. Ninety-nine patients overall completed the study, while 14 patients in total, 8 in the treatment group and 6 in the control group, prematurely discontinued the study. Figure 1 shows the reasons for study discontinuation in the randomized population. The treatment groups were comparable in all baseline characteristics (Table II).

Observations on the Primary Outcome Measure

The main endpoint examined across the two groups was the mean percentage debridement after 15 days of daily ointment application. At Day 15 LMW-HA Collagenase treated ulcers (Figure 2A and 2B) had a significantly higher debridement rate compared with the control group (Figure 3A and 3B).

The wound characteristics after 15 days of treatment with placebo (Figure 3B) presented more fibrinous tissue than lesions in patients af-

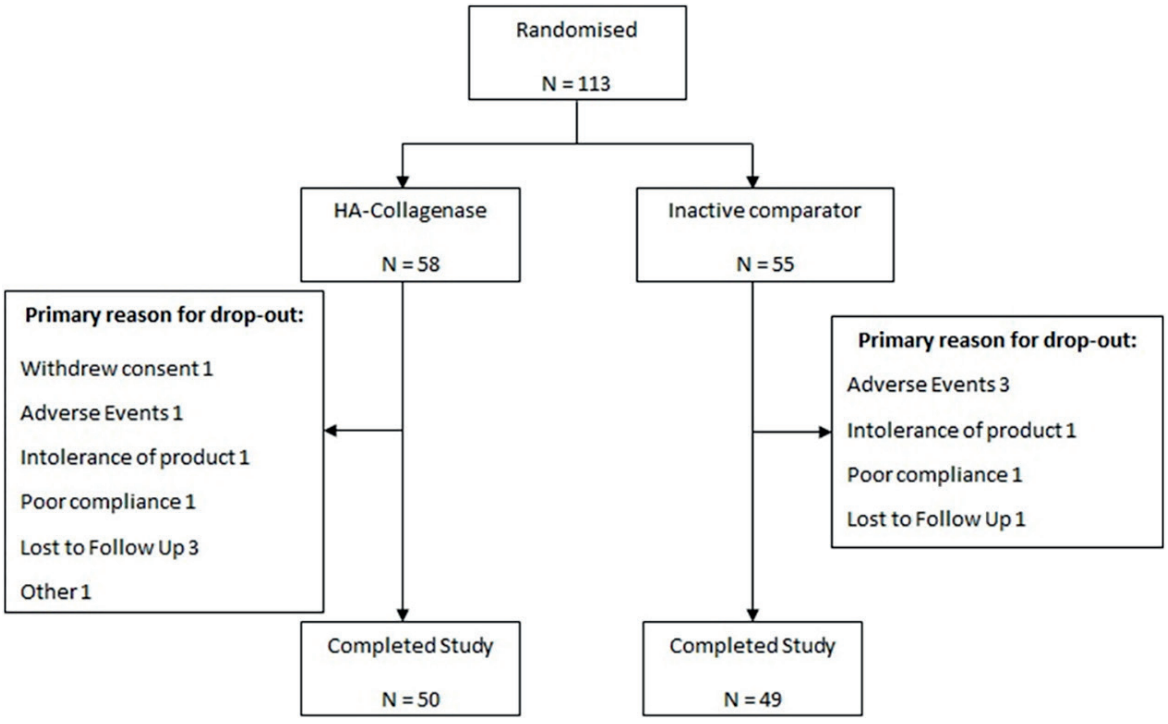

Figure 1. Study flow diagram.

Table II. Baseline characteristics of participants.

		HA-Collagenase (N = 58)	Inactive comparator (N = 55)	*p*-value
Age (years)	Mean (SD)	66.48 (13.64)	65.13 (15.81)	0.6260
	Median (range)	70 (28-86)	70 (28-93)	
Gender				0.6622
Male	N (%)	24 (41.4)	25 (45.6)	
Female	N (%)	34 (58.6)	30 (54.5)	
BMI (kg/m^2)	Mean (SD)	29.42 (4.331)	31.07 (7.01)	0.1320
	Median (range)	29.9 (21-38.6)	30 (20-48.5)	
Time suffering from the target ulcer (months)	Mean (SD)	15.47 (23.75)	29.73 (55.58)	0.0762
	Median (range)	8 (2-166)	11 (6-310)	
ABPI/ABI	Mean (SD)	0.982 (0.077)	0.972 (0.067)	0.4593
	Median (range)	1 (0.8-1.2)	1 (0.8-1.2)	
Total lesion area (cm^2)	Mean (SD)	11.83 (9.93)	12.48 (8.429)	0.7095
	Median (range)	7.55 (2.1-48.2)	8.2 (2.0-42.6)	
Devitalized/fibrinous/slough tissue (cm^2)	Mean (SD)	8.188 (7.696)	7.929 (5.535)	0.8385
	Median (range)	5.35 (0.8-34.4)	6.1 (1.9-25)	
Devitalized area (%)	Mean (SD)	68.99 (22.9)	66.24 (21.33)	0.5108
	Median (range)	64.35 (26.7-100)	60.4 (24.8-100)	
Presence of ulcer infection				0.4169
No infection	N (%)	45 (77.6%)	46 (83.6%)	
Infection	N (%)	13 (22.4%)	9 (16.4%)	
Administration of oral antibiotic therapy				0.4169
No	N (%)	45 (77.6%)	46 (83.6%)	
Yes	N (%)	13 (22.4%)	9 (16.4%)	

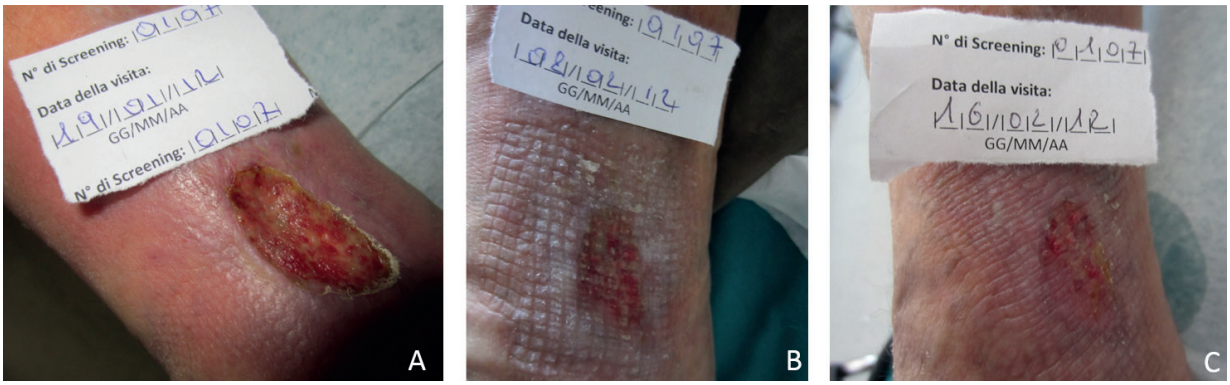

Figure 2. Treatment group. ***A,*** Pre-treatment aspect of the wound; ***B,*** Aspect after 15 days of HA-Collagenase treatment; ***C,*** Aspect after 30 days of treatment.

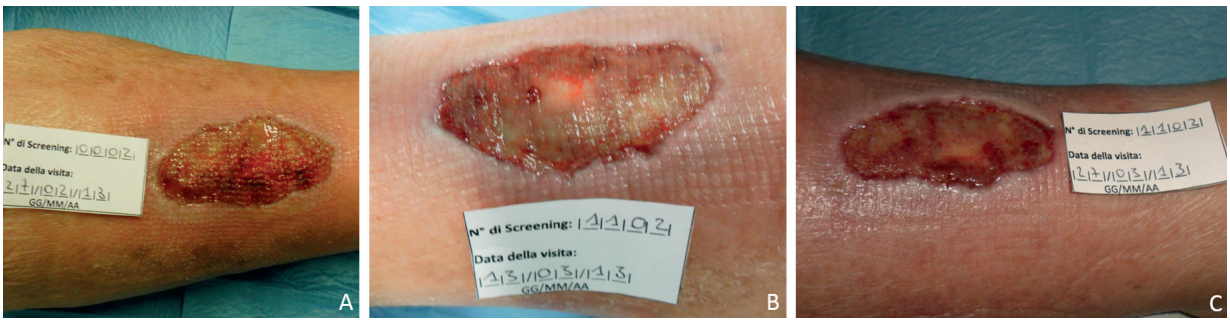

Figure 3. Placebo Group: ***A,*** Pre-treatment lesion; ***B,*** Lesion after 15 days of treatment with placebo ointment; ***C,*** Lesion after 30 days of treatment.

ter 15 days of LMW-HA-Collagenase treatment (Figures 2B and 2C). Consequently, after 30 days wounds treated with placebo ointment (Figure 3C) showed delayed healing. The debridement rate was 67.5 ± 6.7% (median 86.9%, range -148 to 100%) in the device group and 59.0±7.6% (median 74.4%, range -253 to 100%) in the placebo group (Figure 4). The comparison between groups showed that the difference between the adjusted means of the treatment and the control group was 11.816% (95% CI: 0.348 to 23.283%), thus showing the difference was statistically significant (p = 0.0436) in favour of the LMW-HA-Collagenase group (Figure 4). The same analysis was also repeated in the PP population and it was found to be confirmatory with a debridement rate of 79.5% in the treatment group and 66.9% in the control group (p = 0.0031).

Observations on Secondary Outcome Measure

The mean debridement rate was higher in the LMW-HA-Collagenase group than in the control group at all other time points (Day 7, 21 and final visit). The LMW-HA-Collagenase group had more than three times the number of ulcers that achieved complete wound debridement by 15 days post-baseline visit compared to those in the control arm, with 20 (39.2%) and 6 (12.5%) ulcers completely debrided respectively (p = 0.0025). Even at Day 7 and at the final visit the number of completely debrided wounds was higher in the LMW-HA-Collagenase group than in the comparator group. This same analysis repeated in the PP population confirmed the LMW-HA-Collagenase superiority at 15 days (p = 0.0002) and at the other time points, with 18.9% of ulcers completely debrided at Day 7 in the treatment arm *vs.* 4.8% in the control arm (p = 0.0481) and 63.2% at the final visit in the treatment arm *vs.* 39.5% in the control arm (p = 0.0338) (Figure 5). The mean total lesion area decreased from baseline at all post-baseline time points in both groups. The number and percentage of patients with at least a 50% reduction in wound size at the different time points were analyzed. Although the reduction in total lesion area ≥ 50%, ≥ 75% and equal to 100% was generally observed in higher percentages of patients in the treatment group than in the control group at any time point in the overall sample, the differences between the groups were not statistically significant in either the ITT or the PP population. Pain intensity during dressing change decreased from baseline at all post-baseline time points in both groups. The mean decrease from baseline was comparable in the two groups. The

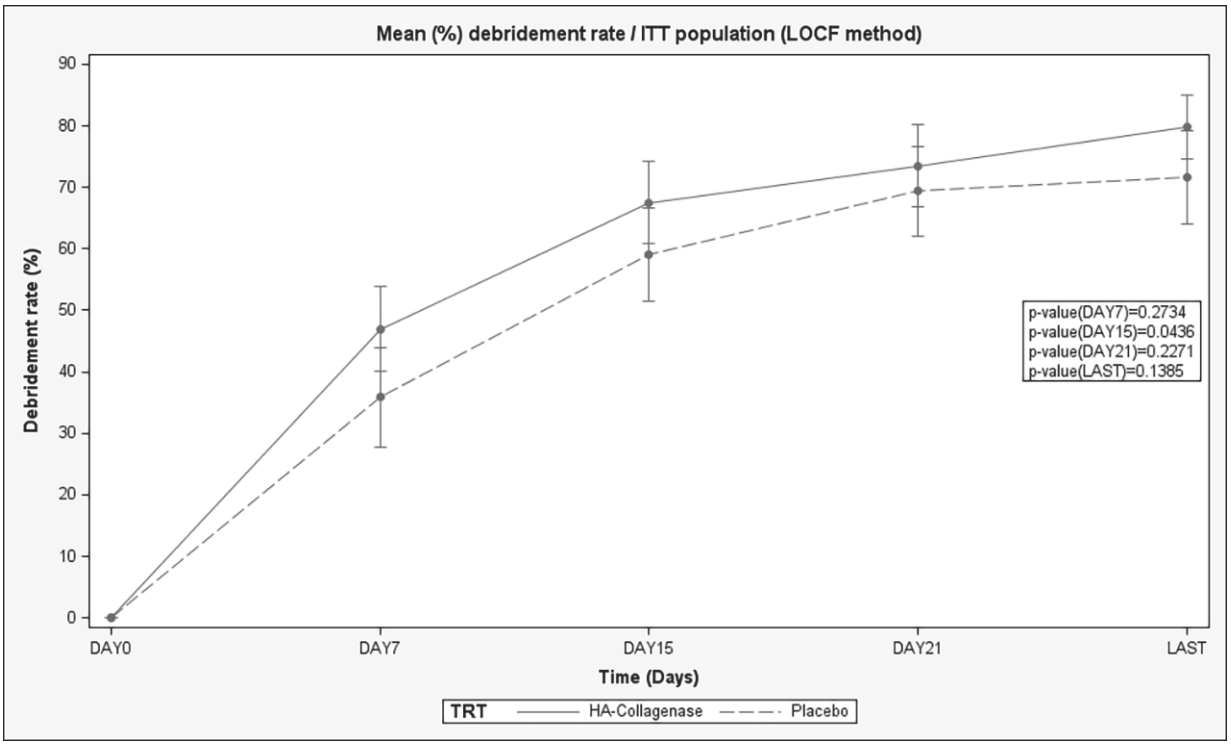

Figure 4. Primary efficacy endpoint: Mean percentage debridement at day 15, ITT population.

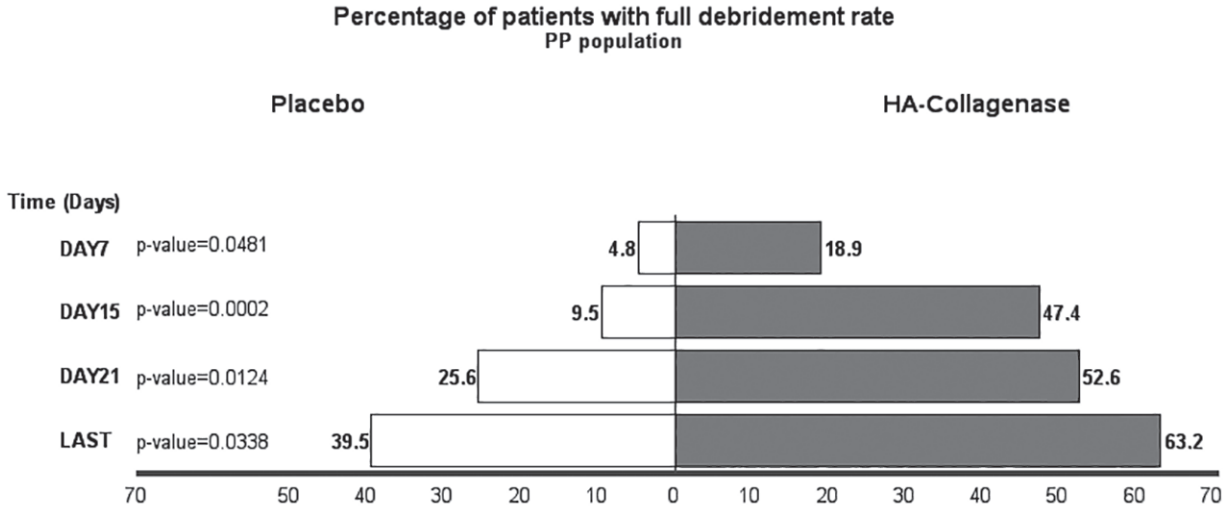

Figure 5. Proportion of patients with complete debridement at different time points (PP).

mean decrease from baseline to the last visit was -4.4 in the LMW-HA-Collagenase group and -2.5 in the placebo group. The frequency of adverse events and serious adverse events was very similar in the treatment and control groups and we recorded no significant differences between the two. As an additional measure of tolerability, the status of periwound skin was observed at each visit: the number and rate of patients with the presence of erythema/redness in the periwound skin decreased from baseline to the last visit in both groups, with no statistically significant difference between the two arms. Furthermore, there were no cases of treatment-related serious adverse events in either group (Table III).

Discussion

Managing skin ulcers is based primarily on a holistic approach to the patient, with the identification of the disease responsible for the injury and the implementation of fast and effective diagnostic-therapeutic treatments. A successful out-

Table III. Summary of treatment related adverse events, by SOC and PT (Safety Population).

Primary System Organ Class (SOC)	Preferred Term (PT)	Bionect (N = 58)	Placebo (N = 55)
Number of patients with at least one related AE	n (%)	7 (12.1%)	8 (14.5%)
Skin and subcutaneous tissue disorders	n (%)	5 (8.6%)	3 (5.5%)
Erythema	n (%)	3 (5.2%)	1 (1.8%)
Venous ulcer pain	n (%)	2 (3.4%)	2 (3.6%)
Skin disorder	n (%)	1 (1.7%)	
Injury, poisoning and procedural complications	n (%)	3 (5.2%)	2 (3.6%)
Thermal burn	n (%)	2 (3.4%)	1 (1.8%)
Wound secretion	n (%)	1 (1.7%)	
Wound complication	n (%)	1 (1.8%)	
General disorders and administration site conditions	n (%)	2 (3.4%)	1 (1.8%)
Pain	n (%)	2 (3.4%)	1 (1.8%)
Infections and infestations	n (%)	1 (1.7%)	2 (3.6%)
Infected skin ulcer	n (%)	1 (1.7%)	1 (1.8%)
Erysipelas	n (%)	1 (1.8%)	
Musculoskeletal and connective tissue disorders	n (%)	1 (1.8%)	
Pain in extremity	n (%)	1 (1.8%)	

come is dependent on the presence of a multidisciplinary team working closely together. Wound healing is a complex series of events that are interlinked and dependent on each other[19]. The first step in wound bed preparation (WBP) entails the global and coordinated management of the wound performed under an appropriate diagnostic protocol to identify the cause of the wound, treat it and meet the patient's needs. Subsequently, the problems related to the wound are addressed and effective actions undertaken to facilitate endogenous wound healing processes. In this context, WBP is essential for accelerating endogenous healing and facilitating the effectiveness of other therapeutic measures when the wound does not heal spontaneously. The recommendations contained in the European Wound Management Association position document have become a touchstone in WBP literature on continuous wound debridement, moisture balance and resolution of bacterial overload. In brief, debridement is a fundamental act performing all the actions described above: it allows the removal of necrotic tissue and devitalized and senescent cells, reduces the bacterial overload and excess exudate and exerts a stimulating action on keratinocytes of the lesion edges. Enzymatic, autolytic, mechanical and surgical methods can be used and the choice of the debridement technique should be related to clinical and management criteria. Enzymatic debridement can be considered as a first choice due to its selectivity of action, the lack of pain following application, the low cost, and the relative speed of action. The clinical examination of the ulcer is the guide: the presence of fibrin and devitalized tissue, without excessive exuding and signs of infection, is the indication for the use of enzymes. Proteolytic enzymes have been used for wound debridement for many years; the main advantage of their use in the debridement of patients with chronic wounds is their easy, safe handling. Therapies are bloodless and generally considered quite painless. Because of the highly selective mode of action, this type of debridement can be appropriate to use in long-term care facilities and in homecare settings[20]. This study evaluated the activity of a novel collagenase product *vs.* placebo and demonstrated superiority of this new debriding agent. Based on the results of this trial, the LMW-HA-Collagenase device appears to have a rapid onset of action and achieves complete debridement in a shorter time compared to its comparator. Patients treated with LMW-HA-Collagenase achieved a faster wound cleansing: after 15 days of treatment not only was the percentage debridement significantly higher in the treatment group ($p = 0.0436$) but complete wound debridement was also obtained by a significantly higher number of subjects ($p = 0.0025$). At baseline, the two arms of the study were statistically comparable overall, although a small, albeit non-significant, difference between the ulcer duration of the two groups was observed (15.5 months in the treatment group 29.7 months in the placebo group [p=0.07]). This difference may have influenced the clinical evidence. The physician's choice of a debriding agent is influenced by multiple factors including patient pain and the tolerability profile of the device; HA-Collagenase was well tolerated by patients: the prevalence and severity of adverse events were comparable to the comparator. Furthermore, it is well known that products containing proteolytic enzymes can lead to irritation of the periwound skin, with clinical signs of inflammation or discomfort. The present study demonstrated that LMW-HA-Collagenase is also safe from this point of view, which can probably be attributed in part to the HA protective action on skin surrounding the ulcer. The purity and substrate selectivity of *V. alginolyticus* collagenase could partly be the reason why *V. alginolyticus* collagenase /LMW-HA ointment seems to be milder on healthy tissue and able to ensure the protection of the periwound skin. This is consistent with the findings obtained in a recent study[21] that compared two different collagenase-based ointments with mechanical debridement and that furthermore suggested a more rapid action of *V. alginolyticus* collagenase compared to *C. histolyticum* collagenase. Also, it must be mentioned that LMW-HA, apart from maintaining an optimal moist environment, promotes the healing process with proangiogenic effects, facilitating the migration and proliferation of endothelial cells[10] and fibroblasts[11]. Concerning keratinocytes, the main cell type involved in wound re-epithelialization, the role of LMW HA is even more interesting: an averaged 200kDa HA fraction has been demonstrated to induce a significant migration and proliferation of primary human keratinocyte cultures in an *in vitro* wound closure model, while higher or lower MW HA fractions were not effective[22]. Similarly, a topical application of a 0.2% 200kDa HA cream caused a significant skin thickening in patients with age- or corticosteroid-related skin atrophy[23]. Both responses were completely eliminated when hyaluronan receptor binding was blocked using

specific antibodies and/or inhibitors. In our study, a tendency to enhanced re-epithelialization was found in the LMW-HA–Collagenase group, but the difference compared to the placebo group was not statistically significant. In any case, it must be underlined that the present study did not have a follow-up period to investigate long term ulcer healing and once complete debridement was achieved, patients left the study and no additional observation was scheduled. In all likelihood, LMW-HA achieves a better tolerability profile compared to products containing *C. histolyticum* collagenase alone, where the biological behavior of the collagenase is more likely to be associated with periwound skin irritation and increased aggressiveness on the wound edges. As to pain experienced by the patient, the study results show that levels of perceived pain during change of medication at the target wound, evaluated by means of a visual analogue scale, were quite low (mean value: 8.5) at each visit and similar in both arms. This is in contrast with general clinical experience where change of medication proves to be the most painful moment for patients (Pain at Wound Dressing Changes, EWMA, 2002). Nevertheless, Gravante et al[17] had already reported that 87% of patients treated in his study with 0.2% HA-Collagenase referred no pain during dressing removal. These findings suggest a relationship with the ointment composition of both the active product and its vehicle, which has a softer texture than other collagenases resulting in less pain for the patient and more frequent and easier applicability[19]. A weakness of this study may be the absence of follow up. Moreover, it is worth remembering that, for ethical reasons, compression therapy, which is the mainstay of treatment of venous leg ulcers[24], was guaranteed to all subjects. Compression is known to be a treatment that plays an essential role in itself in promoting healing and prolonging the recurrence-free period after complete healing[25] and this should be taken into consideration when evaluating the study healing outcomes. For all these reasons it is likely that a follow-up period would add validity to the healing frequency and this aspect needs to be considered for future trials. For this study, the quality of the wound fluid was assessed and the evaluation was made by a clinician. Wound assessment was performed by the use of a digital planimetry system. Arguably a more effective and accurate monitoring of skin lesions should be performed measuring the complete status and evolution of the wound in an objective, precise, and reproducible manner. This level of monitoring could be achieved by using 3-dimensional scanners and systems based on active optical approaches[26-28]. However, these kinds of devices are generally not yet present in most outpatient clinics.

Conclusions

In this study, the percentage reduction in devitalized tissue was statistically significantly greater in patients randomized to LMW-HA-Collagenase therapy compared to vehicle after 15 days of treatment. The improvement was observed regardless of the age of the wound, the size of the wound, or compounding comorbidities reported in this cohort. Participants treated with LMW-HA-Collagenase also reported a statistically significant higher complete debridement rate within 15 days of treatment and an improved healing rate, though not significant, over those treated with the comparator. Performing fast and effective debridement, as well as avoiding pain and periwound skin irritation, can be considered the first step towards successful healing. Thus, with better effectiveness, good tolerability and less pain for patients, the preparation of 0.2% of LMW-Hyaluronic Acid and *V. alginolyticus* collagenolytic enzyme used in the study holds great promise for the management of chronic ulcers.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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