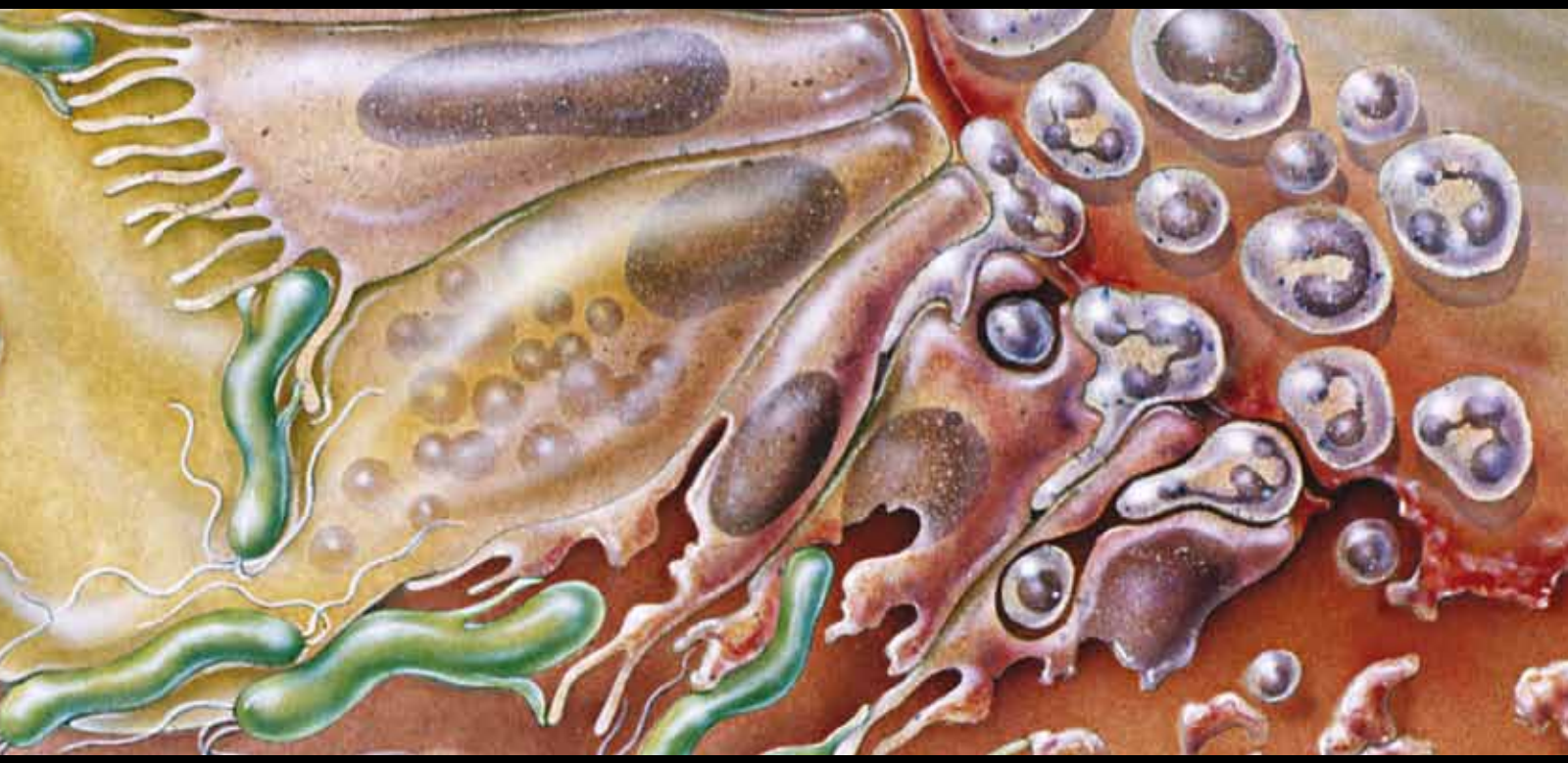
Chronic Ulcers: Updating Epidemiology, Physiopathology, and Therapies

Guest Editors: Marco A. C. Frade, Pranab K. Das



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Editorial

Chronic Ulcers: Updating Epidemiology, Physiopathology, and Therapies

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I, as the Lead Guest Editor and co-editors would like to convey our sincere thanks to the Editorial Board of Ulcers, for the privilege rendered to ourselves in addressing the journal readers.

Primary goal for compiling this special issue was to encourage researchers (both clinical and non-clinical) engaged directly with care for patients suffering from with chronic leg ulcers to publish their experiences in the form of articles. Based on this goal the present edition has been compiled. We sincerely hope that it will promote an update knowledge covering the multiple angles on the topic of ulcers, which is nowadays a worldwide public health problem with high morbidity but still neglected.

Among the submitted manuscripts papers were particularly selected: (i) studies focusing on the epidemiology of risk factors for chronic ulcers, (ii) new concepts about etiology and physiopathology of leg ulcers, (iii) impaired wound healing associated with the hereditary factors, (iv) biofilm formation and bacteria resistance, (v) new methods to access osteomyelitis in diabetic foot, and so forth. Lastly, but not the least, several review articles about many other aspects of chronic ulcers have also been included in order to bring together the important knowledge about their treatment.

In the future, considering the importance of the theme "leg ulcers and healing" and the huge number of aspects related to it, the publishing of the new special issues about it should be stimulated, mainly involving experimental models, new treatments and technologies, and cell therapy.

Acknowledgments

This brief editorial has also to acknowledge our sad feeling for the death of my friend, Joaquim Coutinho-Netto, in August 2012. Dr. Coutinho was a distinguished medical doctor and associate professor at the University of São Paulo, Brazil. He published several papers on wound healing, and he was also invited to be one of our guest editors for this special issue. We hope that the readers will join me in expressing our condolence to the friends and family of Dr. Coutinho. Finally, we would like to thank the reviewers who invested their valuable time to support us and make this issue possible. Most importantly we sincerely hope that this issue will serve a useful reference for the teachers and researchers in the field of ulcers.

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Pranab K. Das.

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Review Article

What Is New in the Understanding of Non Healing Wounds Epidemiology, Pathophysiology, and Therapies

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Chronic wounds are a growing socioeconomic problem in the western world. Knowledge on recalcitrant wounds relies on *in vitro* studies or clinical observations, and there is emerging evidence on the clinical impact of bacterial biofilm on skin healing. Chronic wounds are locked in the inflammatory state of wound healing, and there are multiple explanations for this arrest with the theory of exaggerated proteolysis as the most commonly accepted. Previously, there has not been enough focus on the different etiologies of chronic wounds compared to acute, healing wounds. There is an urgent need to group chronic wounds by its cause when searching for possible diagnostic or therapeutic targets. Good wound management should therefore consist of recognition of basic wound etiology, irrigation, and debridement in order to reduce microbial and necrotic load, frequently changed dressings, and appropriate antimicrobial and antibiofilm strategies based on precise diagnosis. Representative sampling is required for diagnosis and antimicrobial treatment of wounds. The present review aims at describing the impact of biofilm infections on wounds in relation to diagnosing, treatment strategies, including experimentally adjuvant approaches and animal models.

1. Introduction

A practical classification of a nonhealing wound is one that fails to heal spontaneously within 3 months [1]. Emergence of chronic wounds is a substantial health problem as 1% of western population will suffer from it. Common chronic types of wounds are venous leg ulcers, ischemic wounds, diabetic foot ulcers, and pressure wounds [2].

Socioeconomically, management of chronic wounds reaches a total cost of 2–4% of the health budget in western countries [3]. This estimate is expected to rise as a natural consequence of an increasing population of the elderly and the diabetic and obesity epidemic. Complications to nonhealing wounds are vast, and patients are at risk of severe pain, septicaemia, hospitalization, and in some cases amputations.

Microbiological findings in chronic wounds vary depending on the mode of sampling (swab versus biopsies) and the diagnostic method used (culturing, PCR methods, and microscopy preceded by PNA-FISH). The most common bacteriological findings in human chronic wounds are also present on the skin, in faeces and water: *Staphylococcus aureus* (SA), coagulase-negative staphylococci, *Enterococcus faecalis*, *Proteus species*, anaerobic bacteria, and *Pseudomonas aeruginosa* (PA) [4].

All the studies of chronic wounds so far agree on the almost universal presence of SA [5–8]. Also, most studies agree on the PA being present in around half of the investigated wounds and that the deep dermal tissues of all chronic wounds harbor multiple bacterial species [4, 5, 9]. The organization and distribution of these two species in the chronic wound bed has been elucidated by two studies [9, 10]. Two specific PNA probes for FISH analysis, one for SA and one for PA in combination with a universal bacterial probe, were used in both. The observations revealed that the different bacterial species might be present in the same wound but they seemed not to mix. Very few aggregates of different bacteria were observed in close proximity of each other. Aggregates of mixed species were observed by James et al. [6],

Table 1: Predisposing factors for developing a chronic wound.

Age
Venous insufficiency
Arterial insufficiency
Diabetes
Neuropathy
Renal impairment
Systemic morbidity (fibrosis, atherosclerosis, edema, sickle cell disease)
Malignancy
Lymphoedema
Trauma
Rheumatological morbidity
Malnutrition
Pressure over prominent bone
Use of corticosteroids
Vasculitis
Immune suppression
Pyoderma gangrenosum

with both rod- and cocci-shaped bacteria in close proximity to one wound.

Growing evidence supports that chronic wounds can be attributed to an adversely combination of structural damage and establishment of a chronic biofilm infection, inducing host responses, further structural damage, and thereby generation of a vicious circle [6, 9, 11, 12].

A critical review of current literature on wound management is needed considering the increasing evidence of bacteria being present as biofilms resistant to antibiotics and the defense mechanisms of the host.

2. Pathophysiologies of Wounds

Wound healing is comprised of a series of complex events with different time spans, which are not fully understood. Different pathogenetic mechanisms cause the establishment and maintenance of nonhealing wounds and may explain the divergence in existing literature on chronic wounds. Compromised venous flow, atherosclerosis, age, diabetes, renal impairment, lymphoedema, rheumatological disease, poor nutritional status, local pressure over prominent bone, and ischemia-refusion injury as a result of trauma are all possible causes of chronic wounds (Table 1). Most of such wounds have more than one microbial etiology, and this has to be taken in consideration in the clinical care.

In order to optimize treatment the pathogenesis has to be illuminated for each different category of wound. Unfortunately, we have no knowledge on the differences or similarities in different categories of chronic wounds as most of the previous literature compares chronic wounds of different microbial etiologies to healing wounds.

In normal skin wound healing is divided into four spatial and temporal integrated phases which occur in a tight regulated modus: hemostasis following a structural damage to the skin, inflammation, proliferation, and tissue remodeling [13].

Angiogenesis and proliferation of endothelial cells and granulation tissue are stimulated by local cytokines like IL-1β, IL-8, and TNF-α and will in normal wound healing follow the proteolysis of a temporary wound matrix.

Chronic wounds are thought to persist in the inflammatory state of wound healing [14]. The theory of exaggerated proteolysis in wound fluids from patients with chronic venous ulcers is also dominating [15, 16]. The current understanding is that locally elevated levels of proteolytic enzymes in the hypoxic microenvironment of the wound bed degrade beneficial growth factors and thereby prevent the wound from progressing into the proliferative phase with laying of granulation tissue and a provisional matrix as a precursor for tissue remodelling and healing.

Histologically, chronic wounds are infiltrated by T cells and macrophages in the dermis, and this causes a cascade of tissue toxicity or local oxidative stress caused by cytokines, proteases, and free oxygen radicals of leucocyte [17]. Bacteria may also play a role in immunoregulation locally [18].

The prolongated inflammation is possibly also induced by local biofilm infection, which causes upregulated cytokines and reduced growth factors. In humans the loss of skin barrier as a consequence of a structural damage to the skin will cause microorganisms to colonize the damaged area and successive formation of biofilm. This transformation from planktonic to biofilm mode of growth *in vivo* is not fully understood. From *in vitro* studies using type strains various physiological changes and mutations have been shown to be involved, all depending on the species and the experimental set up. It has also been shown that this is a dynamic process, where biofilm growing bacteria can reverse in to the planktonic mode of growth to leave the biofilm probably due to lack of nutrients, a so-called dispersion. Whether this is possible in the wound bed is not know. Biofilms can be formed by virtually all kinds of bacteria and fungi including commonly found PA and SA in nonhealing wounds [4]. *Ex vivo* studies show that bacterial aggregates are surrounded by debris, pus, and inflammatory cells (Figure 1). The biofilms in chronic wounds do not possess the highly structured organization that has been described for *in vitro* biofilms, but they resist antibiotics and the host defense nevertheless [12, 19, 20]. The background for this transformation to biofilm mode of growth is believed to be survival mechanisms of the microorganisms, and *in vivo* this is due to evasion of the host responses [21]. The result is adaptation to the chronic phenotype, which is the biofilm lifestyle, in contrast to the acute phenotype, which is the planktonic lifestyle. The former is also found in other chronic infections [22].

In the last decade there has been a focus on bacteria and their role in promoting a continuous inflammatory response probably adding to the tissue damage and preventing wound healing [12]. This is especially a problem since when the biofilm has established, it enables the bacteria to resist antibiotics and other antimicrobial agents such as silver and the host

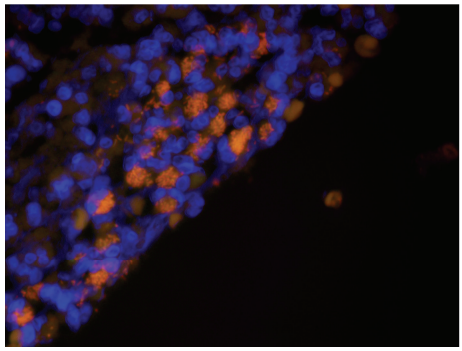

Figure 1: Biopsy showing numerous biofilm aggregates of PA (identified by a specific PNA FISH probe (red stain)) surrounded by host cells (DAPI (bluestain)), in a chronic nonhealing wound (magnification ×1000).

defense. The biofilm resists antibiotic concentrations 1000 times higher than the planktonic counterpart [23–26]. This implicates that if the bacteria succeed in forming a biofilm in the wound bed, they will be extremely difficult to eradicate. In a study by Kirketerp-Møller et al. [9] chronic wounds samples obtained from 22 different patients, all allegedly infected by PA, were investigated. These wound samples were investigated by both standard culturing methods and peptide nucleic acid-based fluorescence in situ hybridization (PNA-FISH) for direct identification of bacteria. By means of the classic culturing methods, SA was detected in the majority of the wounds, whereas PA was in only 2. In contrast, by visualizing the bacteria using PNA-FISH, it was observed that a large fraction of the wounds in fact harbored PA. The visual observations revealed the structural organization of bacteria in the samples. It appeared that PA was aggregated and imbedded in the matrix component alginate. The matrix is one of the hallmarks of the biofilm mode of growth. The biofilms of PA were detected in the wound bed, whereas SA biofilms, when present, were detected on the surface of the wounds. This is supported by other observations demonstrating that SA appears in biofilms on the surface of the wound bed [9]. In the study by James et al. [6], an elevated presence of microbial aggregates in chronic wounds compared to acute wounds by usage of scanning electron microscopy (SEM) was observed.

There is now evidence that bacteria, and especially PA biofilm, contribute to the lack of healing in recalcitrant wounds [4, 9, 27], and research in animal models of chronic PA biofilm infections in wounds supports these findings [28, 29]. In a chronic wound model PA biofilms kept the wounds in a polymorphonuclear (PMN) dominated inflammatory state (Trøstrup, Thomsen et al., WRR, resubmitted, 2012).

In the biofilms the aggregates of bacteria are embedded in an extracellular matrix consisting of proteins, polysaccharides, and extracellular DNA (eDNA). Especially the eDNA can be the source of exchange of antibiotic resistance caused by mutations in target genes. It is known that the biofilm phenotype promotes higher mutation rates than when the bacteria are in the planktonic phenotype [30]. Another characteristic of biofilms is slow growth of the bacteria and the so-called persisters which are highly resistant to antibiotics [31]. Both the matrix and the slow growth are believed to be major contributors to the increased tolerance to antibiotics, disinfectants and host responses [32].

In order to understand the pathogenesis of a given type of wounds, patient populations have to be comparable regarding comorbidity and age and their wounds and the microbial species in the wounds also have to be comparable.

Collection of wound fluids followed by careful analysis in all categories of patients is an easy and noninvasive means to obtain knowledge of the cellular microenvironment of wounds. The composition of wound fluid reflects the temporal processes taking place in the tissue of a wound. A standardized method to collect and examine the protein content in chronic wound fluids is needed [33].

In 2011 we compared wound fluids from chronic venous ulcers collected and standardized over a time period of 4 weeks, to fluids from acute, open granulating wounds in order to map the differences in proteins of interest. We expected to find elevated levels of proteinases in the chronic wound fluids (CWF) compared to the acute (AWF) in accordance with the current paradigm claiming an excessive proteolytic local environment in chronic ulcers [34, 35] resulting in degradation of extracellular molecules like fibronectin and growth factors locally [36, 37]. Surprisingly, we found no significant different levels of neither proteolytic nor proinflammatory, proangiogenic, growth factor or antimicrobial peptides in the two compared groups. As expected, we found histologically a surplus of mononuclear cells in the chronic wound edges. The only protein quantitatively differing between chronic and healing wounds was increased S100A8/A9 in the latter [38]. S100A8/A9 is a proinflammatory neutrophilic derived heterodimer involved in cell proliferation, redox reaction, and wound healing [38, 39].

Previously, we also found that in chronic venous ulcers, the duration of wound fluid collection influences levels of IL-1β, IL-1α, and IL-8. Cytokine levels increase with collection time, but surprisingly, the longer the collection time, the lesser the ability to stimulate human dermal fibroblasts [40]. Clinically, the nonproliferative property of 24 h wound fluids may have important consequences for practical wound fluid management [41]. For example a beneficial effect of irrigation could be explained by the continuous removal of deleterious wound fluid factors [42]. Besides the intrinsic factors, local wound environment is loaded with PMNs and their toxic oxygen radicals and degrading enzymes as a result of the persisting inflammation, and these may also participate in the maintenance of the wound in a chronic state [43, 44].

There is an urgent need for identifying possible target molecules for diagnostic or prognostic markers of healing. As for pressure ulcers, proteomic technology has recently been used on wound fluids for detection of the content of multiple proteins centrally and in the periphery of such ulcers compared to healing ulcers. Twenty-one proteins were found to distinguish between healed and chronic wounds, and 19 proteins were differentially expressed between the interior and periphery of wounds [45].

3. Therapies

Individual Design of Therapy Based on the Individual Pathophysiology. Randomized clinical trials of optimal treatment of wound healing are scarce [46, 47] presumably because of the heterogeneity and multimorbidity of these patients [48]. Every category of wound has its own standard multimodal and multidisciplinary treatment regime with local and systemic treatment. Clinically, the emergence of granulation tissue is the criteria of success of managing chronic wounds. Other criteria for wound healing include decrease in size or complete reepithelialization. There are no systemic or other local markers of healing that currently are used in clinical practice for diagnosis or evaluation of treatment response to treatment.

Compression therapy, surgical debridement, antibiotic treatment when there are clinical signs of infection and maintenance of a moist wound environment are all cornerstones in venous leg ulcer therapy. Skin grafting of chronic venous ulcers improves healing rate [49] unless there is chronic PA infection locally at the time of surgery [27]. A bioengineered skin equivalent also seems effective [50]. Surgical correction of superficial venous reflux in addition to compression bandaging did not improve ulcer healing in a controlled, randomized clinical trial, though it may reduce the recurrence of venous leg wounds [51].

Standard of care for diabetic foot ulcers includes offloading, attentive debridement, maintenance of a moist wound environment, and, if infection is present, systemic antibiotics. As for the risk of amputation in diabetic patients with wounds, this was reduced both by the use of hyperbaric oxygen therapy (HBOT) [52] and adjuvant topical treatment with granulocyte-stimulating factor (G-CSF). Unfortunately, however, no beneficial effect on healing was found by the use of G-CSF in these patients [53]. Maggot therapy may be an alternative to surgical debridement, especially in diabetic foot ulcers [54, 55].

As for pressure ulcers, standard treatment consists of pressure relief, enzymatic and surgical debridement, maintenance of a clean, moist wound environment, and in some cases osteotomy. Monitoring and optimizing the nutritional status of unconscious or paralysed patients are also of critical importance.

Ischemic wounds are caused by arterial insufficiency and are often very painful. Standard treatment regime is vascular surgery to restore circulation (if possible), good pain control and moist dressings on open wounds, and no debridement unless there is an active infection. Dressings containing antimicrobial or pain relieving substances used beneath compression bandages are currently being developed [56] and are typically used for chronic wounds of vascular origin. No significant difference in healing rates was found when comparing different types of dressings beneath appropriate compression bandages in a Cochrane study, where the authors compared hydrocolloids, foam dressings, alginates, low-adherent dressings, and hydrogels [57].

Topical silver or silver dressings are used in infected wounds of all origins, but evidence for their efficacy is lacking [58], and this is probably due to different microbial etiologies.

Application of topical growth factors is an adjuvant to standard care of treatment to nonhealing wounds. Depletion of growth factors shows delayed wound healing rate *in vitro*, but unfortunately substitution of single growth factors shows disappointing results in daily clinical practice. For example, topical application of recombinant basic fibroblast growth factor (FGF) has no advantage over placebo in healing potential of chronic neuropathic diabetic ulcers of the foot [59]. Topical application of epidermal growth factor (EGF) to nonhealing venous ulcers did not promote reepiteliazation [60]. The latter may be due to the degradation of EGF and PDGF in the extracellular matrix [61], since this degradation was reversed when applying matrix metalloproteinase inhibitors in chronic ulcers; however, clinical studies must take the current state of the particular wound treated in to consideration. Furthermore, physicians must be sure that the amount of growth factor and treatment duration is sufficient to produce a biologic response [62]. Assessment tools for these matters unfortunately do not exist.

An experimental tool used in combination with standard wound care, topically applied working platelet concentrate or plasma (PRP), may be used to boost chronic inflammatory wounds into the state of proliferation and healing as they release multiple growth factors and cytokines into the wound mimicking natural healing conditions [63, 64]. In addition, PRP shows antimicrobial activity towards *Escherichia coli* and SA, but not PA [65]. Recombinant platelet-derived growth factor (Regranex) is currently the only approved exogenously applicable drug for chronic wounds, showing promising results in wound healing of diabetic foot ulcers [66]. With respect to the microbial etiology, it is important to reduce local biofilm load locally in the wound for optimal healing [67].

Regarding an autologous platelet-rich fibrin patch, promising results exist as it increases formation of granulation tissue in a heterogenic group of problem wounds; however, further randomized and controlled studies are needed [68]. In a prospective trial, complete closure was observed in 66.7% of patients with venous leg ulcers in 7.1 weeks with an average of two applications of autologous platelet-rich fibrin matrix membrane per patient [69]. However, one randomized prospective double-blind placebo-controlled study (1991) investigated the use of autologous platelet-derived wound healing formula and did not find significantly improved healing in the patients with lower extremity wounds of predominantly diabetic origin [70].

There are multiple emerging trends in the management of the different categories of chronic wounds. Currently, there is a focus on stem cell therapy in treatment of problem wounds [71]. A recent publication showed improved wound healing, neovascularization, and endothelial progenitor cell recruitment in a murine diabetic wound model [72]. No gain in reepithelialization was, however, found in treating cutaneous lesions of diabetic mice with combination of PRP and autologous mesenchymal stem cell transplant versus PRP alone [73].

Negative pressure therapy with devices absorbing detrimental exudates and transudates and promoting vascularization may reduce surface area in some kind of wounds [74–76].

Low-frequency ultrasound (US) has been used clinically for many years in order to promote healing; however, its efficacy remains to be proven. Cullum et al. report no evidence of a benefit associated with low-frequency US in chronic venous leg ulcers [77], but others find a possible positive effect of US on wound area in the same category of patients [78].

There is no current clear evidence of laser therapy and improvement of wound healing [79].

As evident from the previously mentioned no single treatment or handling of chronic wounds has been convincing. An important reason for the unsuccessful management of chronic wounds is missing consideration of biofilm physiology in the antibiotic and previously mentioned treatments of chronic wounds. Apart from increased demands to sampling and analysis as stated previously, treatment of biofilms has to include knowledge of the background for the tolerance of biofilms.

Anwar and Costerton were among the first to report an up to 1000-fold increased minimal inhibitory concentrations (MICs) of biofilm growing PA as compared to planktonic growing PA [80]. Instead of using traditional diffusion testing of antibiotic susceptibility, biofilm resistance testing has revealed significantly increased MICs of several antibiotics [81–83]. Recent *in vivo* studies on PK/PD dynamics when treating biofilm infections have revealed that biofilm growing bacteria in general follows the same PK/PD parameters (time-, concentration-, or area-under-the-curve-dependent killing) as planktonic growing bacteria when analyzing outcome of antibiotic treatment [84]. However, an interesting observation was an element of concentration-dependent killing of biofilm growing PA of beta-lactam treatment probably due to high MICs and a concentration gradient in the biofilms [84]. Moreover, a time dependent killing element was observed in the treatments with colistin [84]. Thus the parameter best correlating to elimination of biofilm growing PA in the lungs was the area under the curve (AUC) versus the minimal biofilm inhibitory concentration (MBIC) [84].

However, the consequence of these observations is that nonobtainable concentrations (due to toxicity) of antibiotics are necessary to eradicate the biofilms. This augments the difficulties for many antibiotics to penetrate into poorly vascularized tissue of many patients with chronic wounds.

Experience of treating biofilm related infections is particularly obtained from handling chronic lung infections in cystic fibrosis patients, periprosthetic joint infections, and colonization of tunnelated central venous catheters. A possible solution is to combine several different treatment strategies, both antibiotic and non-antibiotics strategies. Some of the latter, for example, the surgical debridement to remove dead and infected tissue has been mentioned above. Concerning antibiotic use of high doses, long-term combination therapy with two (or more—especially in cases of multiple-species biofilms) antibiotics with different mode of action is now a well-established strategy [85–87]. Hereby different physiological niches of the biofilm are reached and development of antibiotic resistance is prevented [87–89]. Antibiotics penetrating well into the tissue have to be selected.

Another used strategy is adding local antibiotic treatment achieving higher antibiotic concentrations at the site of infection [87]. This mode of administration is especially used in patients with cystic fibrosis, where inhalation of antibiotics like colistine tobramycin, and aztreonam is now available in special formulations. Likewise, local application of antibiotics during treatment of periprosthetic joint infections is routine practice at some institutions [85]. Local treatments can also be agents not suitable for systemic use as mentioned previously. Finally, adding of quorum sensing inhibiting agents like macrolides in cystic fibrosis can be considered.

All experiences, both *in vitro* and *in vivo*, report significantly improved effect of antibiofilm treatments if these are initiated early and on young biofilms. In contrast, older more established biofilms, which may also have resulted in substantial tissue degradation, are more difficult to treat. Even though implementation of these antibiofilm strategies hopefully can improve outcomes of chronic wounds for many patients, some may remain infected. In these patients where the biofilms cannot be eradicated completely the strategy by means of suppression therapy with antibiotics may be an option and is used in several cystic fibrosis centers, as opposed to only treating patients when they are experiencing exacerbations. Based on accurate microbial sampling and diagnosing in cystic fibrosis, this approach is used by inhalation of antibiotics (especially colistin) alternating with routine intravenous antibiotic courses every three months to reduce bacterial load and thereby the biofilm induced inflammation [87]. Utilizing information and experiences from handling biofilm infections in one host niche probably can be translated to other host niches such as chronic wounds. The exact composition of the treatments modified to chronic wounds has to be established based on trials and records of the outcomes and animal experiments.

4. Conclusion

Chronic nonhealing wounds remain to be a clinical challenge with room for improvements. The increasing recognition that different categories of wounds have to be regarded and handled diversely is a big step forward in the treatment of chronic wounds. Hopefully our growing understanding of the complex bacteriology of chronic wounds will result in optimized treatment regimes. Involvement of representative animal models is a promising approach where the numerous bewildering factors can be compensated for.

Including the impact of biofilm infections with its chronic induction of the host responses in handling of nonhealing wounds is an auspicious area. Especially this kind of infection demands particular antibiotic treatment strategies, like higher doses and combination of antibiotics. In addition, antibiotic penetration in the skin is somewhat unpredictable, especially if blood circulation is comprised. Finally, more alternative antibiofilm strategies may be implemented in chronic wound care as has been suggested for other chronic biofilm infections [85, 90, 91].

With the enhanced knowledge larger, clinical and randomized trials may be more attractive to perform in order to evaluate the different treatments for the benefit of this increasingly large group of patients.

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Review Article

Chronic Leg Ulcers: Epidemiology, Aetiopathogenesis, and Management

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Chronic leg ulcer is defined as a defect in the skin below the level of knee persisting for more than six weeks and shows no tendency to heal after three or more months. Chronic ulceration of the lower legs is a relatively common condition amongst adults, one that causes pain and social distress. The condition affects 1% of the adult population and 3.6% of people older than 65 years. Leg ulcers are debilitating and greatly reduce patients' quality of life. The common causes are venous disease, arterial disease, and neuropathy. Less common causes are metabolic disorders, hematological disorders, and infective diseases. As many factors lead to chronic lower leg ulceration, an interdisciplinary approach to the systematic assessment of the patient is required, in order to ascertain the pathogenesis, definitive diagnosis, and optimal treatment. A correct diagnosis is essential to avoid inappropriate treatment that may cause deterioration of the wound, delay wound healing, or harm the patient. The researchers are inventing newer modalities of treatments for patients with chronic leg ulceration, so that they can have better quality life and reduction in personal financial burden.

1. Introduction

Chronic leg ulcer (CLU) also known as chronic lower limb ulcer is a chronic wound of the leg that shows no tendency to heal after 3 months of appropriate treatment or is still not fully healed at 12 months [1]. The incidence of ulceration is rising as a result of the ageing population and increased risk factors for atherosclerotic occlusion such as smoking, obesity, and diabetes. Ulcers can be defined as wounds with a "full thickness depth" and a "slow healing tendency". Ulcers of skin can result in complete loss of the epidermis and often portions of the dermis and even subcutaneous fat [2]. Chronic ulceration of the lower legs is a relatively common condition amongst adults, and ulcer symptoms usually include increasing pain, friable granulation tissue, foul odor, and wound breakdown instead of healing. This results in social distress and considerable healthcare and personal costs [3, 4]. Since numerous factors lead to lower leg ulceration, it is essential that health professionals adopt an interdisciplinary approach to the systematic assessment of the individual in order to ascertain the pathogenesis, a definitive diagnosis, and optimal treatment required. A correct diagnosis is essential to avoid inappropriate treatment that may delay wound healing, cause deterioration of the wound, or harm the patient.

CLU is reported to have impact on virtually every aspect of daily life: pain is common, sleep is often impaired, mobility and work capacity tend to be restricted, and personal finances are often adversely affected. It is also known that social activities are restricted due to fear of injury and negative body image. CLU is usually associated with significant morbidity, high cost of healthcare, loss of productivity, and reduced quality of life [1–12].

2. Epidemiology

Chronic leg ulcers affect 0.6–3% of those aged over 60 years, increasing to over 5% of those aged over 80 years. CLU is a common cause of morbidity, and its prevalence in the community ranges from 1.9% to 13.1% [6]. It is thought that the incidence of ulceration is rising as a result of aging population

and increased risk factors for atherosclerotic occlusion such as smoking, obesity, and diabetes. In the course of a lifetime, almost 10% of the population will develop a chronic wound, with a wound-related mortality rate of 2.5% [4].

According to the Wound Healing Society, about 15% of older adults in the US suffer from chronic wounds, including predominantly venous stasis ulcers, pressure ulcers (bedsores), and diabetic (neuropathic) foot ulcers. Every year 2 to 3 million more Americans are diagnosed with various types of chronic wounds [7]. Estimate of annual incidence of leg ulcer in the UK and Switzerland are 3.5 and 0.2 per 1000 individuals, respectively. The prevalence of vascular ulcer in the US is estimated at 500,000 to 600,000 and increases with age [8, 9].

According to the study in Ireland the prevalence was 0.12% but it was 1.03% in the patients aged 70 years and over. Women were twice as likely to be affected. Venous disease accounted for 81% of ulcers and arterial disease for 16.3%, while ulceration due to diabetic neuropathy and rheumatoid vasculitis was unusual. Leg ulcers are an important source of morbidity in our ageing population [10].

In Brazil, a study conducted in Botucatu, São Paulo, reported a 35.5% prevalence of varicose veins and 1.5% prevalence of severe chronic venous insufficiency with an ulcer or ulcer scar [11]. The peripheral artery disease, the circulatory disease commonly associated with nonhealing wounds, affects about 8 million Americans and 12–20% of Americans of age group 65–72 years. It is estimated that there are over 7.4 million pressure ulcers in the world where estimation was possible, that is, excluding the vast number of developing countries [12].

In Western Australia (WA) in 1994, leg ulcers were found to affect 1.1 per 1000 population (0.11% point prevalence). This study demonstrated that 24% of the ulcers were present for 1 year, 35% had a problem of ulceration for 5 years, 20% had experienced 10 or more episodes of ulceration, and 45% of sufferers were housebound [13].

According to a study carried out in Germany, venous insufficiency was the dominating causative factor in 47.6% and arterial insufficiency in 14.5%, and 17.6% of ulcers were due to combined arterial and venous insufficiency. Rarer causes included vasculitis (5.1%), exogenous factors (3.8%), and pyoderma gangrenosum (3.0%) [14].

While there are few Indian studies on the epidemiology of chronic wounds, one study estimated the prevalence at 4.5 per 1000 population. The incidence of acute wounds was more than double at 10.5 per 1000 population [15].

According to data from epidemiological studies, the incidence of chronic ulcers in surgically hospitalized patients in China is 1.5% to 20.3%. In one study, of the 580 wound areas in 489 patients, 366 or 63% were ulcers on the lower extremities [16, 17].

The period prevalence of leg ulcers in New Zealand has been estimated at 79 per 100,000 per year, although capture-recapture analysis suggests a more accurate estimation, which is between 393 and 839 per 100,000 per year [18]. Prevalence of leg ulceration increases dramatically with age, although ulcers can occur in quite young people and there are records of people suffering with venous ulcers for up to 60 years.

Table 1: Causes of leg ulcers [21].

Vascular	Venus
	Arterial
	Mixed
Neuropathic	Diabetes
	Tabes
	Syringomyelia
Metabolic	Diabetes
	Gout
	Prolidase deficiency
Haematological	Sickle cell disease
	Cryoglobulinemia
Trauma	Pressure
	Injury
	Burns
Tumors	Basal cell carcinoma
	Squamous cell carcinoma
Infection	Bacterial
	Fungal
	Protozoal
Panniculitis	Necrobiosis lipoidica
	Fat necrosis
Pyoderma	Gangrenosum
Special	Hypertensive ulcer

3. Aetiopathogenesis

It has been reported that ulcers related to venous insufficiency constitute 70%, arterial disease 10%, and ulcers of mixed etiology 15% of leg ulcer presentations [19]. The remaining 5% of leg ulcers result from less common pathophysiological causes, and this latter group comprise considerable challenges in diagnosis, assessment, and management [20].

In the Western world, leg ulcers are mainly caused by venous insufficiency, arterial insufficiency, neuropathy, diabetes, or a combination of these factors (Table 1) [21]. Venous ulcers are the most common type of leg ulcers, accounting for approximately 70% of cases. Arterial disease accounts for another 5% to 10% of leg ulcers; most of the others are due to either neuropathy (usually diabetic) or a combination of those diseases [21, 22]. The study from India shows that etiology of chronic wounds included systemic conditions such as diabetes, atherosclerosis, tuberculosis, and leprosy. Other major causes included venous ulcers, pressure ulcers, vasculitis, and trauma. The study report stated that inappropriate treatment of acute traumatic wounds was the most common cause of the chronic wound [15]. Chinese study shows that the principle etiology (67%) of ulceration is trauma or traumatic wounds compounded by infection. Diabetic ulcers, venous ulcers, and pressure ulcers accounted for 4.9%, 6.5%, and 9.2%, respectively. The majority of these wounds were seen in farmers and other agricultural workers [16, 17].

It is useful to divide leg ulcers into those occurring in the gaiter area and those occurring in the forefoot because

the aetiologies in these two sites are different. At least two aetiological factors can be identified in one third of all lower limb ulcers. Venous ulcers most commonly occur above the medial or lateral malleoli. Arterial ulcers often affect the toes or shin or occur over pressure points. Neuropathic ulcers tend to occur on the sole of the foot or over pressure points [23, 24].

Patients with reduced mobility or obesity may develop ulceration in the gaiter area because of venous hypertension resulting from inadequate functioning of the calf muscle pump. The commonest causes of vasculitis ulcers are rheumatoid arthritis, systemic lupus, and polyarteritis nodosa. The blood dyscrasias that most commonly lead to leg ulceration are sickle-cell disease, thalassaemia, thrombocythaemia, and polycythaemia rubra vera [23]. Other hematological disorders associated with the development of leg ulcers include leukaemia, hereditary spherocytosis, thrombotic thrombocytopenic purpura, granulocytopenia, and polyclonal dysproteinaemia [6]. Leg ulcers related to hematological disorders generally result from microcirculatory occlusion [25].

Microcirculatory and vascular disorders that can result in atypical leg ulceration include Raynaud's phenomenon, Martorell's ulcers, and cutaneous vasculitis. There are numerous disorders that can result in neuropathy of the lower legs and associated ulceration due to insensate injury, burns, or pressure ulcers, for example, leprosy, alcoholic neuropathy, and tabes dorsalis [6].

According to a recent report, chronic kidney disease (CKD), hypertension, and myocardial ischemia may also be associated with increased risk of developing foot ulcers including severe ulcers that necessitate amputation. Additionally, there are reports of higher rates of malnutrition and deficiencies of vitamins and minerals such as zinc in patients with chronic venous leg ulcers compared to the general population [5].

4. Pathogenesis of Chronic Leg Ulcers

4.1. Venous Ulcers. The association between ulceration at the ankle and venous disorders of the lower limbs has been known for more than 2000 years. Venous circulation of the lower extremities progresses from the superficial to perforating to deep veins, with valves in each system to ensure unidirectional blood flow. As the calf muscles contract, the pumping action causes the blood to flow from the deep veins into the inferior vena cava. Disease of these pathways results in venous insufficiency. Venous insufficiency is the most common cause of lower-leg ulcers, accounting for nearly 80% of all cases. Of the approximately 7 million people in the United States with venous insufficiency, approximately 1 million develop venous leg ulcers [25]. Approximately 1% of the population will suffer from leg ulceration at some point in their lives. Chronic venous leg ulceration has an estimated prevalence of between 0.1% and 0.3% in the United Kingdom. Prevalence increases with age. The overall prevalence of venous ulcers in the United States is approximately one percent. Venous ulcers are more common in women and older persons. The primary risk factors are older age, obesity, previous leg injuries, deep venous thrombosis, and phlebitis. Venous ulcers are often recurrent, and open ulcers can persist from weeks to many years. Severe complications include cellulitis, osteomyelitis, and malignant change [26]. Patients who develop chronic venous ulcer before their 50th birthday appear to represent a distinct group in terms of aetiology, natural history, and prognosis.

In venous disease, ulcers are usually located in the gaiter area between the ankle and the calf, often on the medial aspect of the leg. Venous ulcers arise from venous valve incompetence. Valvular incompetence in the deep veins causes the vessels to become distended and stretch to accommodate the additional blood flow. The valves are not able to effectively close, which results in retrograde blood flow and venous hypertension [27]. The venous hypertension, leads to leakage of fluid out of the stretched veins into the tissues, causing deposition of a brownish/red pigment in the gaiter area of the leg. Venous ulceration occurs in the gaiter area in 95% of cases especially around the malleolar (the rounded protuberances on the ankle) region [28]. Veins can be damaged by surgery, trauma, or DVT, which causes a backflow of blood in the venous system at the point of damage. Other causative factors include multiple pregnancies, obesity, congenital vein abnormalities, and varicose veins.

Another factor that influences the development of venous leg ulcers is calf muscle pump failure. Calf pump failure arises from paralysis, immobility, sleeping in a chair with legs dependant for long periods of time, and fixed ankle joints. The calf muscle, through contraction and relaxation, aids in the flow of blood back to the heart through the veins. Failure of this mechanism causes stasis of blood and increased venous pressure [29].

There are three major theories of how ulceration develops. (1) Fibrin cuff theory: fibrinogen leaks from dilated capillaries of the epidermis forming a pericapillary fibrin cuff. This is then responsible for a reduced diffusion of oxygenated blood to the tissues resulting in ulceration. (2) Leukocyte entrapment theory: venous hypertension reduces the pressure gradient between the arteriolar and venular end of the capillaries. This results in sluggish movement of the blood within these capillaries and increases the adherence of blood cells to the endothelium. Inflammatory mediators (ICAM-1, VCAM-1) and reactive oxygen species are then released resulting in the obliteration of functioning capillary loops aggravating ischemia and result in ulceration. (3) Microangiopathy theory: it has been demonstrated that some of the capillaries in patients with venous leg ulcers are occluded by microthrombi or exhibit long intracapillary stasis. This in turn can reduce nutrition and oxygenation of the skin, predisposing to ulceration [30].

Venous ulceration is a chronic disease, which is characterized by periods of exacerbation and remission. Venous ulcers often take a long time to heal, which results in physical and psychological discomfort and negatively affects a patient's functional status [11].

4.2. Arterial Ulcers. Arterial leg ulcers occur as a result of reduced arterial blood flow and subsequent tissue perfusion [31]. Arterial or arteriolar occlusion due to any cause can

result in ischemia of the skin and subcutaneous tissues which might lead to ulceration. Peripheral vascular disease due to atherosclerosis, diabetes with microvascular or macrovascular disease, and/or vasculitis could lead to ischemic leg resulting in ulceration [30, 31]. A reduction in blood supply causes death of tissue in the area being fed by the affected artery. Ulcer development is often rapid with deep destruction of tissue. The limb looks pale, and there is a noticeable lack of hair.

There are three mechanisms involved in the pathophysiology of ischemic leg ulcer: (1) extramural strangulation (2) mural thickening or accretion, and (3) intramural restriction of blood flow. There is often considerable overlap, and the exact pathogenesis cannot be always well defined. Most acute forms of vasculitis and some subacute and chronic forms are likely to cause leg ulceration due to tissue hypoxia and exudation of fibrin-like substances [17].

Arterial ulceration typically occurs over the toes, heels, and bony prominences of the foot. The ulcer appears "punched out" with well-demarcated edges and a pale, nongranulating, and necrotic base [31].

4.3. Diabetic Foot Ulcer. Diabetic foot ulcers are common and estimated to affect 15% of all diabetic individuals during their lifetime. For instance, an estimated 18% of diabetic patients over the age of 65 in the US have nonhealing foot ulcers [7]. It is now appreciated that 15–20% of patients with such foot ulcers go on to need an amputation. Almost 85% of the amputations are preceded by diabetic foot ulcers [32]. Worldwide, it is estimated that a lower limb is lost every 30 seconds as a result of diabetic wound infection [7].

Diabetic patients are at higher risk for arterial diseases and neuropathy, therefore, can develop ulcers due to both entities. In addition, hyperglycemia poses the risk of ulcers secondary to neuropathic impairment of sensory, motor, and autonomic function, typically in the hand and foot, or "stocking and glove" distributions [24]. The etiology of diabetic foot ulcers usually has many components [33]. The major underlying causes are noted to be peripheral neuropathy and ischemia from peripheral vascular disease. Other factors in ulceration are trauma, deformity, callus formation, and edema [32, 33].

4.4. Pressure Ulcer. Pressure ulcers are, as their name implies, caused primarily by unrelieved pressure. They usually occur over bony prominences such as the sacrum or the heel but can occur on any part of the body subjected to pressure. Approximately 70% of all pressure ulcers occur in the geriatric population. Pressure ulcers can be a major source of infection and lead to complications such as septicemia, osteomyelitis, and even death. Prevention of pressure damage to the skin and the underlying tissue is an essential part of treatment in at-risk patients [1].

5. Management of Chronic Leg Ulcers

An ideal management plan for patients with chronic leg ulcers should involve an early strategic and coordinated approach to delivering the correct treatment option for each individual patient, based on accurate assessment of the underlying pathophysiology [34].

Table 2: Assessment of lower limb ulcers [24, 34].

Patient	History of ulcer development
	Past and current medical problems
	General health status
	Nutrition
	Social, occupation
	Mobility problem
	Limitations to self care
	Obesity
Skin changes	Arterial
	Malignant
	Autoimmune
Vascular assessment	Pedal pulses
	Ankle Brachial Pressure Index
Limb factors	Oedema
	Circumferences
	Lymphoedema
	Orthopaedic problems
	Sensation and pain
Ulcer	Site-venous, arterial, pressure
	Appearance
	Size-measure
	Wound base
	Exudate level
	Surrounding skin

The management of leg ulcers should include a detailed history of the onset of the problem, examination of the legs and skin, investigations, and modalities of treatments. Successful management of leg ulcers requires a clear diagnosis, establishment of a treatment plan, accurate monitoring, and adherence to the plan as the ulcer decreases in size. Education and training is vital for all those involved in caring for patients with chronic ulceration.

5.1. Clinical Assessment

5.1.1. History. The first step toward diagnosis of any leg ulcer is to compile a comprehensive history and assessment of the patient (Table 2) [35]. This should include general health status, social and occupational situation, past and current medical history of relevant diseases (such as deep vein thrombosis, diabetes, autoimmune disorders, inflammatory bowel disease, and connective tissue disease), condition of the skin, current vascular status, limb size and shape, and history and status of the ulcer [35]. The patient should be asked about lower extremity pain, paresthesia, anesthesia, and claudication [24]. It is important to determine the duration of ulceration and whether it is a first episode or recurrent. Pain is a major problem for patients with leg ulcers unless there is

Table 3: Assessment of leg ulcers: The difference between venous and arterial disease [29].

Assessment criteria	Venous disease	Arterial disease
Presenting history, physical and social risk factors	Previous history of DVT Varicose veins Reduced mobility Traumatic injury to the lower leg Obesity Pregnancy Nonhealing ulceration Recurrent phlebitis Previous vein surgery	Diabetes Hypertension Smoking Previous history of vascular disease Obesity Inability to elevate limb
Position of ulceration	Gaiter area of the leg Common site is medial aspect	Lateral malleolus and tibial area are common sites as well as toes and feet Over pressure points
Pain	Throbbing, aching, and heavy feeling in legs Improves with elevation and rest	Intermittent claudication Can be worse at night and at rest Improves with dependency
Ulcer characteristics	Shallow with flat margins Often presents with slough at the base with granulation tissue Moderate to heavy exudate	Punched out, occasionally deep Irregular in shape Unhealthy appearance of wound bed Presence of necrotic tissue or fixed slough Low exudate unless ulcers infected
Condition of the lower leg	Haemosiderin staining Thickening and fibrosis Dilated veins at the ankle Crusty, dry, and hyperkeratotic skin Eczematous, itchy skin Pedal pulses present Normal capillary refill (less than three seconds) Limb edema is common	Thin, shiny, and dry skin Reduced or no hair on lower leg Skin feels cooler to touch Pallor on leg elevation Absence or weak pedal pulses Delayed capillary refill (greater than three seconds) Development of gangrene

a neuropathic component. Lack of pain, therefore, suggests a neuropathic aetiology. Patients should also be asked about their mobility [23].

Clinical course of the ulcer can suggest its etiology. Possible considerations to rule out include diabetes; hypertension; hyperlipidemia; coronary artery disease; alcohol and tobacco use; thyroid, pulmonary, renal, neurologic, and rheumatic diseases; peripheral vascular disease; deep vein thrombosis; specifically cutaneous factors including cellulitis, trauma, and recent surgery [24].

5.1.2. Examination. The examination of the leg should include palpation of pulses and a search for the signs of venous hypertension, including varicose veins, haemosiderin pigmentation, varicose eczema, atrophie blanche, and lipodermatosclerosis. The range of hip, knee, and ankle movement should be determined, and sensation should be tested to exclude a peripheral neuropathy [23].

The ulcer examination should include site, size, appearance, wound base, exudates level, and surrounding skin (Table 2) [35]. The surrounding region should be examined for pain, edema, erythema, warmth, induration, discoloration, maceration, dryness, scarring from previous wounds, hair pattern, gangrenous digits, clubbing, cyanosis, capillary refill, and varicose veins. It is important to bear in mind that venous and arterial disease may coexist in the same patient [24].

The venous ulcers considerably differ from arterial ulcers (Table 3) [29] and other ulcers of lower extremity (Table 4) [26]. An irregular ulcer border, black necrosis, erythema, or bluish or purple discolorations of adjacent skin are suggestive for ulcer due to vasculitis [2]. A painful leg ulcer with violaceous borders suggests pyoderma gangrenosum.

Investigations. (1) The Ankle Brachial Pressure Index (ABPI) using a handheld Doppler ultrasound and sphygmomanometer can be carried out for more accurate assessment of arterial perfusion. The results are used to determine the likelihood of arterial insufficiency and can be used to guide the management plan (Table 5) [28].

When Doppler tests indicate arterial insufficiency, arterial duplex ultrasonography will (noninvasively) provide accurate anatomic and haemodynamic information on the site and extent of the arterial disease [34]. When indicated, further detailed anatomic information for treatment planning can be obtained from magnetic resonance angiography, computer tomographic angiography, or digital subtraction angiography [34].

(2) Accurate and regular measurement of the wound is important to give an objective assessment of the effectiveness of the current management plan. The Leg Ulcer Measurement Tool (LUMT) is a validated tool that has been developed to quantify leg ulcer assessment and can be used to track change in wound status over time [36].

Table 4: Common lower extremity ulcers [25, 26].

Ulcer type	General characteristics	Pathophysiology	Clinical features
Venous	Most common type; women affected more than men; often occurs in older persons	Venous hypertension	Shallow, painful ulcer located over bony prominences, particularly the gaiter area (over medial malleolus); granulation tissue and fibrin present Associated findings include edema, venous dermatitis, varicosities, and lipodermatosclerosis
Arterial	Associated with cardiac or cerebrovascular disease; patients may present with claudication, impotence, and pain in distal foot; concomitant with venous disease in up to 25 percent of cases	Tissue ischemia	Ulcers are commonly deep, located over bony prominences, and round or punched out with sharply demarcated borders; yellow base or necrosis; exposure of tendons Associated findings include abnormal pedal pulses, cool limbs, femoral bruit, and prolonged venous filling time
Neuropathic	Most common cause of foot ulcers, usually from diabetes mellitus	Trauma, prolonged pressure	Usually occurs on plantar aspect of feet in patients with diabetes, neurologic disorders, or Hansen disease
Pressure	Usually occurs in patients with limited mobility	Tissue ischemia and necrosis secondary to prolonged pressure	Located over bony prominences; risk factors include excessive moisture and altered mental status

Table 5: ABPI symptoms: management correlation guide [28].

Index	Symptoms	Severity of disease	Management
>0.8–0.95	None/mild intermittent claudication	Mild arterial disease	Modify risk factors, stop smoking, regular exercise, and consider antiplatelet therapy
>0.5–0.8	Intermittent claudication	Moderate arterial disease	As for patients with ABPI between 0.8 and 0.95, together with routine referral to a vascular surgeon. Possible arterial duplex scan/angiogram
>0.3–0.5	Severe intermittent claudication and rest pain	Severe arterial disease	As for patients with ABPI between 0.8 and 0.95, together with urgent referral to a vascular surgeon. Possible arterial duplex scan/angiogram
0.3 or below or ankle systolic pressure of less than 50 mmHg	Critical ischaemia (rest pain for greater than 2 weeks duration) with or without tissue loss (ulcer, gangrene)	Severe arterial disease; risk of losing limb	Urgent referral to the vascular emergency on-call team and possible surgicalor radiological intervention
Abnormally high ABPI (greater than 1.3)	Variable	Vessel calcification	As for patients with ABPI between 0.8 and 0.95, together with referral to avascular surgeon

(3) Blood investigations such as complete blood count, erythrocyte sedimentation rate, blood sugar, lipid profile, renal function tests, and liver function tests are essential in patients with chronic leg ulcers. The plain radiography of the foot along with CT and MRI should be done to rule out osteomyelitis and malignancy.

(4) Laboratory screening tests for vasculitis: urine analysis for proteinuria, hematuria, cylindruria, routine and immunohistopathology of skin biopsies, antinuclear antibodies, rheumatoid factor, complement C4, circulating immune complexes, paraproteins, immunoglobulin fractions, antineutrophil cytoplasmic antibodies, serological tests, and cultures for underlying infections [9].

(5) Laboratory screening tests for clotting disorders: activated partial thromboplastin time, prothrombin time, thrombin time, factor V (Leiden) mutation (506R fi 506Q), factor II (prothrombin) mutation (20210G fi 20210A), antithrombin III, protein C and protein S, and lupus anticoagulant anticardiolipin [9].

(6) Venography may be performed as an investigational procedure prior to valvular surgery. Lower extremities arteriography is indicated in patients with ischemic rest pain, intolerable claudication, impending gangrene, or the presence of nonhealing ulcers of suspected arterial origin [23].

(7) Color duplex ultrasound scanning which is becoming the *de facto* standard for evaluation of venous obstruction is also used to assess the location and extent of reflux in venous ulcers [37].

(8) Plethysmography and venous pressure data are important in determining the need for surgical bypass or valve replacement. Quantitative data on venous obstruction, calf muscle pump ejection fraction, and reflux are provided by air plethysmography, whereas venous pressure studies assess the physiological importance of anatomic obstruction because the collaterals may or may not provide adequate compensation for an obstructed pathway [4].

(9) A quantitative bacterial culture is more specific and should be performed once wound infection is suspected [37]. This is performed by curetting or biopsying the bed of the ulcer. The quantitative biopsy is the current gold standard for assessing the quality and quantity of microbial pathogens within wound [37, 38]. Quantitative biopsies containing greater than 10^5 organisms per gram of tissue are considered significant, and systemic antibiotic therapy should be considered. If osteomyelitis is suspected, representative cultures need to be obtained from the bone or deepest tissue layers [6].

(10) Ulcer biopsy is important in making a correct diagnosis and to rule out malignancy as these ulcers are prone to malignant transformation [39]. This requires taking a deep wedge of tissue from the ulcer edge and can usually be performed under local anesthesia [34]. Chronic ulcers are sometimes biopsied for experimental protocols: (A) to obtain information regarding the wound bed or the wound edge. (B) to grow cells in vitro from nonhealing wound [40].

(11) The clinical application of gene variant analysis and evaluation in patients with venous leg ulcers implies that the high risk minority of patients could be identified in advance by means of a simple blood test that would act as a genetic screening device [41].

Table 6: Treatment options for common leg ulcers [25, 26].

Ulcer type	Treatment options
Venous	Leg elevation, compression therapy, aspirin, pentoxifylline (Trental), surgical management
Arterial	Revascularization, antiplatelet medications, management of risk factors
Neuropathic	Off-loading of pressure, topical growth factors; tissue-engineered skin
Pressure	Off-loading of pressure; reduction of excessive moisture, sheer, and friction; adequate nutrition

5.2. Treatments. The treatment of chronic ulcers of the lower extremities presents a therapeutic challenge. There is clear evidence suggesting that causal treatment should have priority. A comprehensive diagnostic evaluation including vascular, metabolic, and physical aspects as mentioned above is essential at the start of treatment.

The basic principles of treatment are to remove or treat precipitating cause, for example, surgical intervention, to promote circulation and improve venous return, for example, compression therapy, to promote healing, for example, wound care, lifestyle changes, symptom management, and to promote preventative care, for example, health education, current treatments for CLU include surgery, sclerotherapy, compressive therapy (conventional therapy), and adjuvant pharmacotherapy [26]. Vowden [42] has outlined four basic therapeutic strategies that can be employed singularly or in combination to enhance healing and improve outcomes when surgical intervention is not an option. He has also discussed neurovascular interventions such as lumbar sympathectomy or spinal cord stimulation; systemic therapy with hyperbaric oxygen or intervenous therapy with agents such as prostaglandins; local mechanical therapy such as negative pressure wound therapy (NPWT), electromagnetic stimulation or enhanced local oxygen therapy; finally, topical therapy with vaso-active growth factors or tissue-engineered skin products. The various treatment options for different types of ulcers are as shown in Table 6 [25, 26].

5.2.1. Recent Advances in Management. Several researchers are still discovering other modalities of treatment.

(1) The discovery of miRNAs has opened up vast therapeutic opportunities. The knowledge of miRNA function in the regulation of wound healing and developing improved miRNA modulation techniques in the skin will help in translating this knowledge into more effective therapies [43, 44].

(2) The clinical practices could be strongly influenced by the results of the HFE genetic test. The presence of C282Y mutation would strengthen the indications and priorities for surgical correction of superficial venous insufficiency [41].

(3) Chronic wounds are characterized by changes in cell receptors (integrins). The activation or inhibition of integrin receptors by various agents may provide an excellent means of influencing wound healing [45].

(4) Venous leg ulcers can be healed with a spray formulation of allogeneic neonatal keratinocytes and fibroblasts without the need for tissue engineering, at an optimum dose of 0.5×10^6 cells per mL every 14 days [46].

(5) The regenerative medicine is utilizing therapeutic potential of the stem cells to promote skin regeneration. The promise of regenerative medicine lies in the ability to understand and regulate these stem cell populations to promote skin regeneration, and biomaterials will continue to play a central role in regenerative medicine by providing the framework upon which to reconstruct functional niches [47]. Stem cell-based therapies offer tremendous potential for skin regeneration following injury and disease. Functional stem cell units have been described throughout all layers of human skin, and the collective physical and chemical microenvironmental cues that enable this regenerative potential are known as the stem cell niche. Stem cells in the hair follicle bulge, interfollicular epidermis, dermal papillae, and perivascular space have been closely investigated as model systems for niche-driven regeneration. These studies suggest that stem cell strategies for skin engineering must consider the intricate molecular and biologic features of these niches. Innovative biomaterial systems that successfully recapitulate these microenvironments will facilitate progenitor-cell-mediated skin repair and regeneration [47].

(6) According to Frade et al., the natural biomembrane of latex extracted from Hevea brasiliensis proved to be safe as a dressing, for it did not induce hypersensitivity reactions among the volunteers who underwent the patch test or among users of the natural biomembrane, as it was clinically and immunologically demonstrated by IgE levels [48].

The vegetal biomembrane was important for the induction of the healing, especially on the inflammatory stage, confirmed by the abundant exudation and debridement of the ulcers in relation of the control treatment of chronic venous ulcers, which seems to be directly related to the intense vascular formation followed by reepithelialization [49].

(7) Authors report that a 115-aa fragment of secreted Hsp90α (F-5) acts as an unconventional wound healing agent in mice. Topical application of F-5 peptide promoted acute and diabetic wound closure in mice far more effectively than did PDGF-BB [7].

6. Conclusions

An ulcer which is present for more than three months is considered as chronic ulcer. The majority of chronic leg ulcers are caused by venous insufficiency followed by arterial ulcers. A comprehensive assessment of the patient, limb, and ulcer is required to determine etiology and to formulate an appropriate management plan. Management of patients with chronic ulcers has to be multidisciplinary and should include detail history, physical examination, investigations, basic and newer treatment modalities, and educating patients on issues of correct foot care and the importance of seeking early medical advice.

Conflict of Interests

The author declares that there is no conflict of interests.

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Research Article

Screening for Osteomyelitis Using Thermography in Patients with Diabetic Foot

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One of the most serious complications of diabetic foot (DF) is osteomyelitis, and early detection is important. To assess the validity of thermography to screen for osteomyelitis, we investigated thermographic findings in patients with both DF and osteomyelitis. The subjects were 18 diabetic patients with 20 occurrences of DF who visited a dermatology department at a hospital in Tokyo and underwent evaluation by magnetic resonance imaging (MRI) and thermography between June 2010 and July 2012. Osteomyelitis was identified by MRI. Thermographs were taken of the wounds and legs after bed rest of more than 15 minutes. Two wound management researchers evaluated the range of increased skin temperature. There were three types of distribution of increased skin temperature: the periwound, ankle, and knee patterns. Fisher's exact test revealed that the ankle pattern was significantly more common in the group with osteomyelitis than in the group without osteomyelitis ($P = 0.011$). The positive predictive value was 100%, and the negative predictive value was 71.4%. Our results suggest that an area of increased skin temperature extending to the ankle can be a sign of osteomyelitis. Thermography might therefore be useful for screening for osteomyelitis in patients with DF.

1. Introduction

Diabetic foot (DF) is defined as infection, ulceration, and/or destruction of deep tissue associated with neurological abnormalities and various degrees of peripheral vascular disease in the lower limb [1]. The prevalence of foot ulcers is 4% to 10% in patients with diabetes mellitus (DM) [2]. DF severely affects a patient's physical condition, long-term prognosis [3, 4], and quality of life [5, 6]. Therefore, early healing of DF is important.

One of the most serious complications of DF is osteomyelitis, and its diagnosis and treatment (surgery and/or long-term antibiotics) have been long-standing controversies [7–9]. Therefore, early detection of osteomyelitis is important. Although swelling and warmth are associated with osteomyelitis, it is difficult to diagnose the presence of osteomyelitis in DF by physical examination because the plantar skin has an especially thick layer of stratum corneum. Furthermore, inflammatory pain may occasionally be overlooked in diabetic patients due to sensory disturbance. Although biopsy or magnetic resonance imaging (MRI) is the gold standard for diagnosing osteomyelitis [7–9], it is accompanied by disadvantages such as invasiveness, a high cost, and non-real-time diagnosis.

One of the possible tools for detecting inflammation or infection in DF is thermography. Several previous studies have indicated the usefulness of thermometry both for early detection of inflammation and for prevention and home monitoring of ulceration risk. Armstrong et al. [11] showed that monitoring skin temperature reduced the risk of diabetic foot ulceration. This study was further supported by multiple independent randomized controlled trials reporting similar findings and approximately 4- to 10-fold reductions in reulceration for patients using home-based thermometry devices [12, 13]. Compared with a conventional device, such as the contact infrared skin thermometer (TempTouch, Xilas

Table 1: Standard values used for nerve conduction velocity exam in the study.

	Median nerve	Tibial nerve	Peroneal nerve	Sural nerve
M-wave amplitude (mV)	4–25	7–40	—	
Latency (ms)	<4.5	<7.5	<7.0	
MCV (m/s)	45–65	40–60	40–60	
SNAP (μV)	10–60			5–30
SCV (m/s)	45–58			40–60

MCV: motor nerve conduction velocity; SNAP: sensory nerve action potential; SCV: sensory nerve conduction velocity.

Medical Inc., Texas, USA) [11–13] for measuring the skin temperature of local points, we consider that thermography has an advantage in that it can visualize morphological patterns of temperature distribution [14–16]. Thermography is also noninvasive and quite easy to use by clinicians of various backgrounds. We previously reported a case study of a patient with both DF and osteomyelitis, detailing the thermographic findings [10]. A high temperature area was observed, not only in the wounds but also in the ankles. This case suggested that thermography might be useful for screening for DF with osteomyelitis.

Therefore, the purpose of this study was to investigate thermographic findings in patients with both DF and osteomyelitis in a larger number of patients and to assess the validity of screening for osteomyelitis using thermography.

2. Methods

This was a cross-sectional study. The subjects were patients with DF who visited a dermatology department at a hospital in Tokyo and underwent evaluation by MRI and thermography between June 2010 and July 2012.

Osteomyelitis was identified by MRI. Interpretations of diagnostic images, including MRI scans, by radiologists were collected from medical records. The criteria for diagnosing osteomyelitis were hypointense signal within the bone on T1 weighted images and hyperintense signal within the bone on T2 weighted images, in direct continuity with abnormal high signal in the surrounding soft tissues of the ulcer [17]. Thermographs were taken of the wounds and legs after bed rest of more than 15 minutes using a Thermotracer TH7800N (NEC Avio Infrared Technologies Co., Ltd., Tokyo, Japan) or a Thermo Shot F30S (NEC Avio Co., Ltd.). We adjusted temperature intervals in the thermographs at 1.5°C using NS9200 software (NEC Avio Co., Ltd.). Two wound management researchers evaluated the distribution of increased skin temperature. For evaluation, the investigators were blinded to data other than the thermographic images and location of the diabetic foot. Inflammation in the soft tissue was identified by MRI based on interpretations by a radiologist, and angiopathy was identified by the ankle-brachial index (ABI) and the toe-brachial index (TBI) as influential factors of skin temperature. The criteria for diagnosing angiopathy were as follows: (1) ABI no more than 0.9, or (2) ABI no less than 1.4, and TBI no more than 0.7 [18]. Evaluation of peripheral neuropathy was performed by means of nerve conduction velocity. Median, tibial, and peroneal nerves were tested for motor nerve conduction velocity, and median and sural nerves were tested for sensory nerve conduction velocity. Table 1 gives the standard values used in hospital for the exam. The final diagnosis was made with the neurology specialists by assessing patient's nerve conduction velocity results against these standard values. Retinopathy, renal dialysis, and history of myocardial infarction were determined using the medical records.

Table 2: Characteristics of subjects.

	$N = 18$
Age, years	66.8 ± 15.0
Sex	
Male	15 (83.3)
Female	3. (16.7)
Duration of diabetes, years	22.4 ± 13.0
HbA1c (%)*	8.4 ± 2.6
Angiopathy**	10 (55.6)
Neuropathy***	13 (72.2)
Renal dialysis	4. (22.2)
Retinopathy*	16 (94.1)
History of myocardial infarction	3. (16.7)

Mean ± SD, n (%). *$n = 17$. **The criteria for diagnosing angiopathy were as follows: (1) ABI no more than 0.9, or (2) ABI no less than 1.4, and TBI no more than 0.7. ***Diagnosis of neuropathy was made according to the findings from the nerve conduction velocity exams.

2.1. Statistical Analysis. Quantitative data are expressed as means ± standard deviation. The relationships between the thermographic findings and osteomyelitis were analyzed using Fisher's exact test. To assess the validity of the thermographic findings for screening for osteomyelitis, the sensitivity, specificity, and positive and negative predictive values were calculated. Statistical analysis was performed using IBM SPSS Statistics 20 (IBM, Armonk, NY, USA). The level of statistical significance was $P = 0.05$.

2.2. Ethical Considerations. This research was approved by the Ethics Committee at National Center for Global Health and Medicine Hospital. All patients gave their written informed consent.

3. Results

Eighteen patients with 20 occurrences of DF were included in this study (Tables 2 and 3). Ten occurrences of DF were

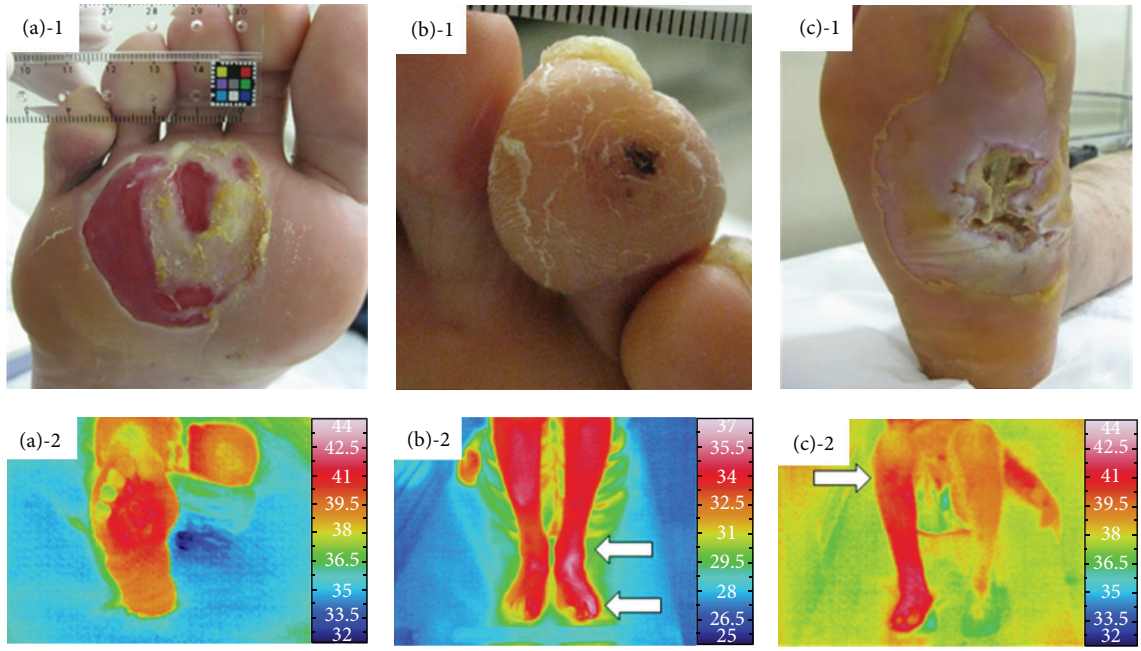


Figure 1: Thermographic patterns in patients with diabetic foot. (a): Periwound pattern, (a)-1: example of the periwound pattern: ulcer in the sole. (a)-2: Thermographic image of (a)-1. Distribution of increased skin temperature is found in the periwound area. (b): Ankle pattern, (b)-1: example of the ankle pattern: ulcer in the fourth toe. (b)-2: Thermographic image of (b)-1. Distribution of increased skin temperature extends from the fourth toe to the ankle (arrows). (c): Knee pattern, (c)-1: example of the knee pattern: ulcer in the sole. (c)-2: Thermographic image of (c)-1. Distribution of increased skin temperature extends to the knee (arrow). (b)-1 and (b)-2 are reproduced from [10], with permission of the Journal of Wound Care.

Table 3: Characteristics of diabetic foot.

	$N = 20$
Diabetic foot	
Ulcer	11 (55.0)
Gangrene	6. (30.0)
Cellulitis	3. (15.0)
Location of diabetic foot	
Toe	5. (25.0)
Sole	12 (60.0)
Entire foot	3. (15.0)
Evaluation of MRI	
Osteomyelitis (+) inflammation of soft tissues (−)	4. (20.0)
Osteomyelitis (−) inflammation of soft tissues (+)	6. (30.0)
Osteomyelitis (+) inflammation of soft tissues (+)	6. (30.0)
Osteomyelitis (−) inflammation of soft tissues (−)	4. (20.0)

n (%).

Table 4: Thermographic findings and osteomyelitis.

	Osteomyelitis		*P*
	Yes ($n = 10$)	No ($n = 10$)	
Periwound pattern			1.000
Yes	0. (0.0.)	1. (10.0.)	
No	10 (100.0)	9. (90.0.)	
Ankle pattern			0.011
Yes	6. (60.0.)	0. (0.0.)	
No	4. (40.0.)	10 (100.0)	
Knee pattern			0.170
Yes	2. (20.0.)	6. (60.0.)	
No	8. (80.0.)	4. (40.0.)	

n (%), Fisher's exact test. Periwound pattern: increased skin temperature is observed in the periwound area. Ankle pattern: the area of increased skin temperature extends to the ankle. Knee pattern: the area of increased skin temperature extends to the knee.

complicated by osteomyelitis. There were three types of distribution of increased skin temperature: the periwound, ankle, and knee patterns (Figure 1). The periwound pattern indicated that increased skin temperature was observed in the periwound area. The ankle pattern indicated that the area of increased skin temperature extended to the ankle. The knee pattern indicated that the area of increased skin temperature extended to the knee. The room temperature when thermography was performed was controlled at 27.3 ± 2.8°C.

We compared the types of distribution of increased skin temperature between the group with osteomyelitis and the group without osteomyelitis. Fisher's exact test revealed that the ankle pattern was significantly more common in the group with osteomyelitis than in the group without osteomyelitis (Table 4). The sensitivity of the positive ankle pattern to recognize osteomyelitis was 60.0%, the specificity was 100%, the positive predictive value was 100%, and

Table 5: Thermographic findings and the site of osteomyelitis.

ID	Site of osteomyelitis	Thermographic findings
1	1st distal phalange	Ankle pattern
2	4th proximal and middle phalange	Ankle pattern
3	5th metatarsal bone	Knee pattern
4	1st proximal phalange	Ankle pattern
5	1st proximal phalange and metatarsal bone	—*
6	5th proximal, middle and distal phalange, and metatarsal bone 4th metatarsal bone	Ankle pattern
7	1st proximal, middle, and distal phalange	—*
8	2nd proximal phalanges and metatarsal bone	Ankle pattern
9	5th proximal phalange	Knee pattern
10	1st metatarsal bone	Ankle pattern

* No observation of increased skin temperature.

the negative predictive value was 71.4%. Table 5 shows the site of osteomyelitis and the types of distribution of increased skin temperature in each case. The site of osteomyelitis and the types of distribution of increased skin temperature seem to be unrelated, as far as we can observe from this result.

4. Discussion

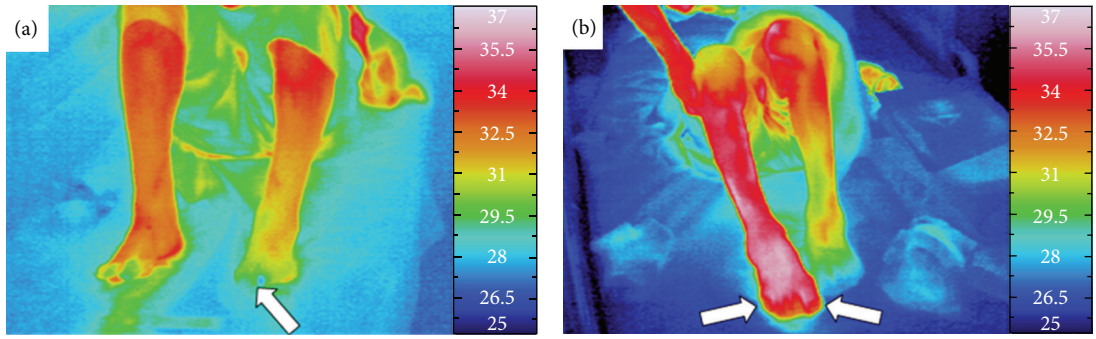
The present study is the first to show a correlation between the ankle pattern in thermography (area of increased skin temperature extending to the ankle) and the presence of osteomyelitis in multiple DF patients. Furthermore, the positive predictive value of the ankle pattern was high, indicating the high validity of this finding. This result suggests that thermography is useful for screening for DF with osteomyelitis.

In this study, we considered that skin temperature increase detected by thermography was due to inflammation of the tissues, including the bone. Fever is one of the signs of inflammation [19]. A number of studies have previously highlighted the usefulness of thermometry in monitoring skin temperature to identify inflammation or infection of the lower extremities in patients with DM. Armstrong et al. [20] reported that there were differences in skin temperature between the affected feet and the contralateral feet in patients with Charcot's arthropathy (8.3°F) and in patients with neuropathic ulcer (5.6°F), with no difference identified in patients with asymptomatic sensory neuropathy. Our previous study showed that signs of inflammation were detected by thermography and ultrasonography in 10% of the calluses in the diabetic group without ulcers [21].

We designated the thermographic finding as "the ankle pattern" when the area of increased skin temperature extended to the ankle. We believe that "the ankle pattern" is a thermographic indicator for screening for osteomyelitis in DF patients, as we reported previously in a single case [10]. It is noteworthy that the specificity and the positive predictive value of the ankle pattern were 100% for the presence of osteomyelitis, indicating the surprisingly high validity of this thermographic finding. As stated above, thermography has an advantage over conventional thermometry in that the morphological patterns of temperature distribution can be obtained [14–16]. In this sense, thermography may be the best way to detect such a specific temperature distribution. In the majority of cases, osteomyelitis in the DF results from the contiguous spread of infection from the adjacent soft tissue to the bone [8]. We also speculate that skin temperature in the patients with osteomyelitis was increased due to inflammation in the adjacent deep tissues, such as the tendons. However, it is unclear in this study why the ankle pattern was specifically observed in the patients with osteomyelitis even in regions other than the ankle. Further investigation of this point might be necessary.

In the present study, there were four patients who had osteomyelitis without the ankle pattern. It was considered that angiopathy or inflammation in soft tissue influenced their skin temperature. The two patients with osteomyelitis had no area of increased skin temperature (Figure 2(a)); rather, they had severe angiopathy. It is known that the skin temperature of patients with ischemia is low [22]. Furthermore, skin temperature might be insensitive to inflammation due to insufficient blood supply [17]. The other two patients showed the knee pattern (Figure 2(b)). In these patients, inflammation of soft tissues was identified by MRI. The area of increased skin temperature due to extensive inflammation of soft tissues might mask the ankle pattern of osteomyelitis.

This study had several limitations. Morphological evaluations of thermographic patterns are quite subjective and can be easily affected by environmental conditions, expertise of the investigators, and possible bias of the patients' information. A more controlled method should be established for the collection and interpretation of thermographic data. In the present study, the diagnosis of osteomyelitis was based on MRI findings; biopsy of the bone or surrounding tissues was not able to be performed. Therefore, we cannot clearly understand the pathophysiological status underlying "the ankle pattern." We could not investigate grade of severity of angiopathy for application of this method because the sample size was small. There were no patients with Charcot arthropathy or reflex sympathetic dystrophy in the present series. A further study is needed to show if the ankle pattern would be less significant in these other conditions. This study



(a) (b)

Figure 2: Examples of irregular thermographic patterns of diabetic foot patients with osteomyelitis. (a) Thermographic image of a patient with angiopathy. An increased skin temperature is not seen in any area. Arrow indicates the location of diabetic foot with osteomyelitis. (b) Thermographic image of a patient with inflammation in the soft tissue. The area of increased skin temperature extends to the knee due to cellulitis. Arrows indicate the locating of diabetic foot with osteomyelitis.

was cross-sectional, and the causal relationship between the ankle pattern and osteomyelitis could not be substantiated. A longitudinal study will be needed for investigation of the causal relationship, as well as the effectiveness of this method for prognostic evaluation.

5. Conclusions

We investigated thermographic findings in DF patients with and without osteomyelitis and assessed the validity of screening for osteomyelitis using thermography. An area of increased skin temperature extending to the ankle was seen in DF patients with osteomyelitis. Furthermore, this thermographic finding (the ankle pattern) was shown to be quite valid, with high positive predictive value. Thermography might therefore be useful for screening for osteomyelitis in patients with DF.

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Research Article

Chronic Nonhealing Wounds: Could Leg Ulcers Be Hereditary?

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Background. A number of well-known acquired and putative inherited etiological factors contribute to the development of venous leg ulcer (VLU). *Aim*. In this study we set out to perform a meta-analysis of putative genetic and acquired factors predisposing to VLU development. *Methods*. VLU patients ($n = 157$) were divided into three subgroups in accordance with their acquired etiological factors. The frequencies of four genetic factors were determined: the R506Q (Leiden) mutation of the F5 gene, the G20210A mutation of the F2 (prothrombin) gene, the 2451 A/G SNP of the fibroblast growth factor receptor 2 (FGFR2) 3′ UTR, and the −308 G/A SNP of the tumor necrosis factor α (TNFA) promoter. *Results*. The −308 TNFA SNP exhibited a higher frequency among VLU patients without known acquired predisposing factor in their history, than among patients with thrombosis or soft tissue infection in their history (Fisher $P = 0.0173$). *Conclusions*. This study has demonstrated that the group of VLU patients is heterogeneous in their genetic predisposing factors. Further large-scale studies are needed to delineate the associations among genetic and acquired etiological factors with regard to VLU development and to integrate the consequences of the already known genetic factors to the management of VLU.

1. Introduction

Venous leg ulcer (VLU) is multifactorial disease with well-known acquired and putative inherited predisposing factors [1–15]. Besides the characteristic acquired etiological factors, such as venous insufficiency, obesity, and deep vein thrombosis, case-control studies suggest putative inherited etiological factors, which may also contribute to the mechanism of delayed or pathological wound healing and hence to the development of leg ulcer. A delineation of the genetic susceptibility factors relating to pathological wound healing would therefore promote a better understanding of the molecular background of VLU and that could provide opportunities for developing causative treatment of therapy-resistant forms [1, 2].

The difficulties involved in such investigations are increased by the fact that these inherited factors form a complex multifactorial genetic background which does not follow the rules of Mendelien inheritance. Moreover, each genetic component contributes differently to the pathogenesis of VLU, and assessment of its individual relevance in the development of the disease is difficult. To investigate the putative genetic factors and to minimize statistical bias, we set out to form subgroups of VLU patients which were homogeneous in their clinical characteristics and to perform a meta-analysis of four genetic factors within the subgroups.

2. Methods

One hundred and fifty-seven VLU patients with therapy-resistant nonhealing VLU have been enrolled into the study. Diabetes and arterial leg ulcer were exclusion criteria. The female (48.41%) : male (51.59%) ratio was close to 1 : 1. The average duration of the VLU was 5.84 ± 5.12 years. The clinically relevant parameters and the clinically homogeneous subgroups of VLU patients are shown in Table 1.

Table 1: Clinical characteristics and subgroups of VLU patients.

Clinical characteristics of VLU patients (n = 157)		
Cardiac disease (49.04%, n = 77)		
Soft tissue infection (47.13%, n = 74)		
Deep vein thrombosis (29.94%, n = 47)		
Leg fracture (22.93%, n = 36)		
Atherosclerosis (20.38%, n = 32)		
Autoimmune disease (5.10%, n = 8)		
Subgroups of VLU patients		
	Leg fracture	Deep vein thrombosis or soft tissue infection
Group A (n = 72)	−	−
Group B (n = 33)	+	−
Group C (n = 52)	−	+

The frequency and putative interactions of several previously determined genetic factors (the R506Q [Leiden] mutation of the F5 gene, the G20210A mutation of the F2 [prothrombin] gene, the 2451 A/G SNP of the FGFR2 3′ UTR, and the −308 G/A SNP of the TNFA promoter) were earlier assessed in VLU patients [3–6]. The analysis was based on previous results of genotyping performed by either PCR-RFLP or PCR TaqMan methods [3–6]. Chi^2 tests and multinomial regression analyses performed by SPSS were used to determine frequency and genetic interactions.

The investigation was approved by the Internal Review Board of the University of Szeged. Written informed consent was obtained from all donors, and the study was conducted according to the Principles of the Declaration of Helsinki.

3. Results

The R506Q mutation of the F5 gene was detected in heterozygous form in 11 patients with an overall frequency of 7.85%, demonstrating a nonsignificant, higher presentation in group A and group C than in group B (data not shown). The G20210A mutation of the F2 gene occurred in only 3 patients in heterozygous form; all the others carried the wild-type allele (data not shown).

The distributions of the rare genotypes (AG and GG) of the FGFR2 gene polymorphism (2451A/G SNP at the 3′UTR) were highest in group A (ratio of homozygous mutants 18.84%, rare allele frequency [MAF] = 0.4638) and lowest in group B (ratio of homozygous rare alleles 8.82%, MAF = 0.3676, Fisher exact probability test P = 0.1227, Odds ratio 1.4876, CI 0.8804–1.8075; Figure 1). We have previously reported that the FGFR2 3′UTR 2451A/G polymorphism is associated with VLU [5], and the present analysis revealed a similar distribution in the various subgroups of VLU patients, suggesting an overall susceptibility role for this polymorphism in the development of the disease.

The −308 G/A SNP of the TNFA promoter likewise exhibited the highest frequency in group A (ratio of homozygous rare alleles 5.8%, MAF = 0.2246), while in groups B and C homozygous rare genotype was not detected; only the heterozygous rare genotype was present (group B MAF = 0.1765, group C, MAF = 0.1087; group A versus group B, P = 0.2711, odds ratio 1.352, CI 0.6988–2.3189; group A versus group C, P = 0.0173, odds ratio 2.3757, CI 1.0658–4.0073). It was previously demonstrated that the −308 A/G SNP of the promoter region of the TNFA gene is a factor predisposing to VLU development [6, 7]. Our present data indicate that the homozygous rare genotype of the −308 TNFA SNP occurred significantly more frequently among VLU patients without additional acquired predisposing factors in their history (group A: no thrombosis, fracture, or soft tissue infection) than among patients with other known etiological events in their history (group C: patients with previous thrombosis or soft tissue infection; group A versus group C Fisher exact probability test P = 0.0173; Figure 1). Previously we have reported that the −308 G/A SNP of the TNFA promoter is associated with VLU development in obese patients [6]. In the present study, the ratio of obese patients did not show significant difference within the subgroups of the VLU patients. In accordance with our previous results, the highest ratio (38%) was observed in group A, in which the −308 G/A SNP of the TNFA promoter also exhibited the highest frequency.

Our meta-analysis included an assessment of putative genetic interactions using the multinomial regression method. The R506Q mutation of the F5 gene and the G20210A mutation of the F2 gene were excluded from this analysis because of their low allele frequency. No interaction was found between the 2451 A/G SNP of the FGFR2 gene and the −308 G/A SNP of the TNFA gene. The 2451 A/G SNP of the FGFR2 gene proved to be a significantly (5-fold) stronger

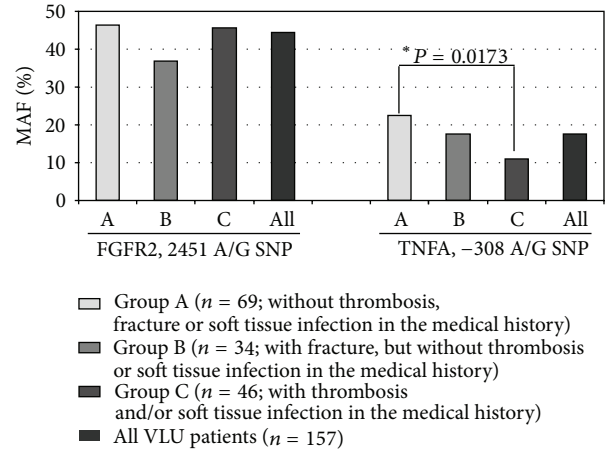

Figure 1: Rare allele frequencies of the 2451 A/G SNP of FGFR2 and the −308 A/G SNP of TNFA in the subgroups of VLU patients. The FGFR2 3′UTR 2451A/G polymorphism exhibited similar distributions among the subgroups of VLU patients, suggesting an overall role of susceptibility in the disease development. Our data also demonstrated that the homozygous rare allele of the −308 TNFA SNP occurred significantly higher among VLU patients without additional acquired predisposing factors in their history (group A) than among patients with other known etiological events in their history (group C; group A versus group C Fisher exact probability test, P = 0.0173).

Table 2: Putative genetic factors predisposing to VLU development.

	Detected genetic abnormality	Population	Author	Journal	Year
(1)	F5 gene R506Q (Leiden)*	German	Peus et al.	J Am Acad Dermatol	1996
(2)	F2 gene G20210A*	Romanian	Jebeleanu et al.	J Cell Mol Med	2001
(3)	F13A gene V34L	Italian	Gemmati et al.	Wound Repair Regen	2004
(4)	FGFR2 gene 3′ UTR A2451G*	Hungarian	Nagy et al.	J Invest Dermatol	2005
(5)	ESRB gene CA repeat D14S1026	UK	Ashworth et al.	J Steroid Biochem Mol Biol	2005
(6)	HFE gene C282Y	Italian	Zamboni et al.	J Vasc Surg	2005
(7)	TNFA gene promoter −308*	Australian	Wallace et al.	J Invest Dermatol	2006
		Hungarian	Nagy et al.	J Invest Dermatol	2007
(8)	FPN1 gene promoter −8GG	Italian	Gemmati et al.	J Vasc Surg	2009
(9)	MMP12 gene promoter −82AA	Italian	Gemmati et al.	J Vasc Surg	2009
(10)	Sex chromosome aberrations (47,XXY/48,XXXY karyotype)	Austrian	Gattringer et al.	Acta Derm Venereol	2010

* The distributions of the genotypes and the allele frequencies of these genetic factors were compared in the present study.

susceptibility factor than the −308 G/A SNP of the TNFA gene.

4. Discussion

Up to now little is known about the genetic background of VLU; however there have been several papers published in this topic. The first report on the genetic backgrounds of VLU was on the Leiden and the prothrombin gene mutation; the first findings demonstrated their association with venous thrombosis and later with postthrombotic leg ulcer development [3, 8]. The FGFR2 gene encodes keratinocyte growth factor receptor involved in the proliferation of keratinocytes and wound healing, while the TNFA gene encodes a well-known proinflammatory cytokine. The investigated SNPs of the FGFR2 and TNFA genes were previously proved to be associated with VLU [5, 6].

Other genetic factors—not investigated in this study—have been also reported to be associated with VLU (Table 2). The V34L SNP of the F13A gene was proved to be associated with the progression of VLU due to its direct effect on the activity of F13 [9]. Estrogen is a well-known accelerator of wound healing by dampening the inflammatory response; a common variant of its receptor (ESRB) increases the risk of VLU development [10]. The C282Y SNP of the HFE gene increases the risk of VLU by affecting iron protective mechanisms [11]. A DNA-array reported by Gemmati et al. (2009) revealed that the −82 A/G SNP of the MMP12 and the −8 G/C SNP of the FPN1 genes are also associated with VLU [12]. Moreover, chromosomal abnormalities have also been found in VLU patients with unusual early onset [16].

The aim of this study was to assess the relevance of already known genetic factors and their interactions in VLU development in clinically homogeneous subgroups of patients. Deep vein thrombosis, soft tissue infection, and leg fracture frequently found clinical characteristics among VLU patients, were suitable for the creation of clinically homogeneous subgroups within our study population. Cardiac disease was also frequent, but displayed a very similar distribution in the VLU patient subgroups. Of the four investigated genetic factors, the 2451 A/G SNP of the FGFR2 gene proved most relevant.

Our data further emphasize the importance of clinically homogeneous subgroups of patients for the analysis of putative genetic factors in order to assess mutual relevance, to create hierarchy, and to measure potential interactions. Further larger-scale studies are needed to assess the contributions of different putative genetic factors to the variable appearance of VLU phenotypes. Such analyses could hold the key to the understanding of VLU development. They might also serve a crucial role in the development of future causative treatment strategies through the creation of cost-effective investigation techniques for routine diagnostic assessment of putative genetic factors and causative treatment options.

Conflict of Interests

The authors have declared no conflicting interests.

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Review Article

Chronic Ulcers in Thromboangiitis Obliterans (Buerger's Disease): Updating Epidemiology, Physiopathology, and Bosentan—A Novel Strategy of Therapy

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Thromboangiitis obliterans (TAO) or Buerger's disease is associated with both distal ulcers in the extremities and the possibility of amputation. The only treatment that has been shown to be effective in TAO is complete abstention from smoking. In spite of this, the disease progresses in up to 30 percent of cases and finally results in limb amputation. Only a few pharmacological and surgical options are available to date to improve healing ulcers in TAO. The efficacy of prostaglandin analogues is controversial. This paper summarizes the current evidence for medical treatment with bosentan in chronic ulcers in TAO patients. These available data up to date allow us to conclude that the beneficial effects of bosentan on improving endothelial function, inflammatory processes, and selective vasodilatation of damaged vessels result in a clinical enhancement regarding healing and preventive digital ulcers in such patients. In any case, these promising findings have to be confirmed with larger randomised trials.

1. Introduction

Thromboangiitis obliterans (TAO) or Buerger's disease is a thrombotic, occlusive, and nonatherosclerotic segmental vasculitis that affects small- and medium-sized arteries and veins which may involve distal vessel of upper and lower extremities. As a vasculitis, it is characterized by inflammation and fibrinoid necrosis of blood vessel walls.

Classically, various mechanisms have been implicated in its etiopathogenesis including cell-mediated inflammation, immune complex-mediated inflammation, and autoantibody-mediated inflammation [1]. Recently, novel pathways have been described in physiopathology of the disease, though not completely well known. The endothelin-1 (ET-1) has been associated in these etiological processes, which can induce to endothelial cell activation causing complications such as vessel occlusion and tissue destruction [2].

ET-1 is a potent vasoconstrictor peptide, which exerts its action by targeting two transmembrane receptors (ETA and ETB). ET-1 facilitates the proliferation of vascular smooth muscle cells, promotes monocytes via activation of the ETA, and contributes to matrix remodelling leading to the abnormal thickening of vessel walls [1–3]. Raised levels of ET-1 have been described in different kind of systemic vasculitis as mixed cryoglobulinemia, secondary Raynaud's phenomenon [2], acute phase of Henoch-Schölein purpura, early stages of giant cell arteritis [1], Takayasu's arteritis, and Buerger's disease [3]. This finding supports that ET-1 may act as marker of vascular damage [2, 3]. Other nonvasculitic entities as rheumatoid arthritis, systemic lupus erythematous, systemic sclerosis, pulmonary hypertension, or artherosclerosis have been also associated with high levels of ET-1 and have been related to vascular injury. Although data are limited, there is evidence suggesting that ET-1 plays a role in the clinical manifestations of vasculitis. Accordingly, blockade of ET-1 could therefore be of therapeutic benefit in these diseases.

TAO usually occurs in people around the age of 45 and is more frequent in male smokers. In the general population of USA, its incidence has declined as tobacco use has also declined. However, as a consequence of

the increase in smoking between women, an increase in the incidence of TAO has been observed in the last 20 years [4–6]. Intermittent claudication and, in more advanced cases, pain at rest are the predominant clinical symptoms. Distal ischaemic lesions (trophic and ulcerations) are frequently observed by means of physical examination. Clinical course is characterised by alternating periods of exacerbation with periods of remission. Angiographic studies reveal a distal and segmental involvement of the vasculature of the extremities. Recanalisation is frequently demonstrated, showing a typical image (corkscrew collateral vessels) [7]. Skin disorders such as migrating phlebitis or Raynaud-like colour changes may be associated with TAO.

Tobacco use is strongly connected to the onset, progression, and prognosis of this disease. In fact, just less than 5% of patients are nonsmokers. In those small percentages of nonsmoker patients, the mechanisms that trigger the disease are not completely known. Published hypothesis state that it might be induced by cold, frostbite, traumatism of extremities, or even abuse of sympathomimetic drugs [8].

Interestingly, an impaired endothelium-dependent vasodilatation in the peripheral vasculature, even in the nondiseased limbs, has been shown in patients with TAO [9]. Although, mild perturbations in clotting have been described, there is no evidence suggesting that hypercoagulability or fibrinolytic abnormalities play a major role in the ethiopatogenia of this disease [8]. Moreover, various investigations have been also carried out with the aim of indentifying an autoimmune mechanism responsible for TAO. However, the abnormalities found have proved to be nonspecific and have not been completely confirmed [8].

Therefore, giving up smoking is the most important therapeutic measure in TAO patients [10]. In fact, it leads to dramatic improvement of the symptoms and lesions. Otherwise, drugs used to manage TAO, prostacyclin (PGI2) or its analogues (iloprost, beraprost, trepostinil sodium), aspirin, or streptokinase (as a thrombolytic) have shown an uncertain efficacy. On the other hand, revascularization by means of a bypass surgery or endovascular procedure is usually not possible as a consequence of the predominantly diffuse and distal location of the lesions in the veins and arteries involved. The fact that TAO is highly associated with both distal ulcers in the extremities and the possibility of amputation leads frequently to involve in social problems and a worsening in the quality of life of the affected patients [11]. Added to the fact that only a few pharmacological and surgical options (of controversial efficacy) are available to date, new therapeutic options with a higher efficacy than the current ones are clearly needed in order to properly manage patients affected by TAO.

ET-1 receptor blockade may be used as a therapeutic target for improvement in TAO patients. Pharmacologic ET-1 receptor blockade may be single (ETA or ETB) or dual (both, ETA and ETB) [12]. Bosentan is a dual ET-1 receptor antagonist, administered orally, which is approved by the European Union to treat pulmonary arterial hypertension in systemic sclerosis patients and to prevent the occurrence of new digital ulcers in systemic sclerosis patients with ongoing digital ulcers. Recent investigations have suggested that bosentan could have a role in healing ongoing digital ulcers in TAO [13–18].

This paper summarizes the current evidence for medical treatment with bosentan in chronic ulcers in vasculitis, especially in TAO patients.

2. Effect of Bosentan on Microcirculation Physiopathology

ET-1 is an endothelium-derived peptide, which is involved in the regulation of vascular function under normal physiologic conditions [19]. It plays a key role in vascular pathologies by exerting various deleterious effects. These include hypertrophy of vascular smooth muscle cells, cellular proliferation, fibrosis, increase of vascular permeability, activation of leukocytes, and induction of cytokine and adhesion molecule expression [19, 20]. Moreover, ET-1 is the most potent natural vasoconstrictive mediator. It has been demonstrated that its exogenous administration in healthy volunteers produces a marked dose-dependent reduction of the blood flow [21].

The effects of ET-1 are transmitted upon binding 2 cognate receptors, ETA and ETB, which are mainly expressed on endothelial cells (ET-B), smooth muscle cells, and fibroblasts [19, 20]. Elevated circulating levels of ET-1 have been repeatedly observed in scleroderma, as well as in various other pathologies in which the vascular endothelium is involved [22]. It has been also detected an increase in plasma levels of ET-1 in situations of acute or chronic limb ischemia, chronic and acute coronary syndromes, acute renal failure, and stroke [23, 24]. Nevertheless, the role of ET-1 activity as a causal factor of endothelial dysfunction and/or damage or an epiphenomenon remains not completely clear [22, 25]. Experimental studies in animal models of hypertension [26, 27] and atherosclerosis [28] have shown an improvement in the endothelial function of large arteries following short-term administration of endothelin receptor antagonists. Any case, these data point that some of the endothelin-mediated deleterious effects on the vasculature may be reversible.

Bosentan, an oral dual ET-1 receptor antagonist, can exert a selective vasodilator effect on the vascular bed. Its efficacy has been demonstrated, with a favourable safety profile, in two randomised controlled clinical trials, RAPIDS-1 and RAPIDS-2, for the treatment and prevention of digital ulcers in patients with systemic sclerosis [13, 29]. The results of such trials suggest that it may be beneficial for the treatment of Raynaud phenomenon. There is evidence that bosentan exert a selective vasodilator and anti-inflammatory effects in patients affected by TAO, comparable to the effects observed in connective tissue diseases.

Several studies have shown that can improve endothelial function after 4 weeks of treatment, indirectly demonstrated by the increasing of the flow-mediated dilation (FMD) measurements in the brachial artery in patients with systemic sclerosis, diabetes mellitus, microalbuminuria, and peripheral artery disease [17, 24, 30].

Meanwhile, Nitric oxide (NO) is considered to be another reliable marker and is involved in the homeostasis of endothelial function [31]. Endothelial dysfunction appears

as an early change in the onset stages of vasculitis [32]. An increase in ET-1 activity has also been associated to an inhibition of NO synthesis [30]. Recent investigations have suggested that an improvement in endothelial function would be achieved by enhanced NO production. Thus, treatment with bosentan could improve NO synthesis in patients with vasculitis by inhibition of the ET-1 [30]. These data allow us to hypothesize that the improvement of endothelial dysfunction, after bosentan treatment, may not only be associated with hemodynamic changes, proinflammatory processes, or activated endothelium effects, but rather may be due to the enhancement of NO production following inhibition of ET-1, as has previously been seen in pulmonary hypertension [33, 34]. These findings prove that the endothelin receptor system is an important molecular pathway that is directly involved in certain reversible aspects of vascular injury.

3. Efficacy of Bosentan on Chronic Ulcers Treatment in Buerger's Disease

Up to date, the only treatment that has been shown to be effective in TAO is complete abstention from smoking. Both clinical improvement and complete healing of the ulcers have been achieved in the majority of patients after giving up smoking. In spite of this, the disease progresses in up to 30 percent of cases and finally results in multiple limb amputation [35]. Furthermore, giving up smoking is achieved in a very low number of these patients, inferior to 30% in some studies [17]. This unsatisfactory rate, in accordance with previous reports, highlights the fact that it is extremely difficult for patients, who are heavy smokers, to give up smoking despite having strongly been advised to do so, as well as received full information about the benefits of giving up smoking, especially in terms of avoiding amputations [36].

Only a few pharmacological and surgical options (of controversial efficacy) are available to date to improve healing ulcers in TAO [8]. Vasodilators, antiplatelet agents, anticoagulants, and corticosteroids appear to be of no use [37]. Prostaglandin analogues are beneficial when administered intravenously [38], although their efficacy is controversial on oral administration [39]. A randomised clinical trial of intravenous iloprost versus aspirin [38] has shown that healing of ulcers is higher in patients who have received treatment with intravenous prostaglandins. Nonetheless, in other randomised trials, an oral formulation of iloprost has not been better than placebo with regard to this outcome [39]. Therefore, the efficacy results shown by prostacyclin analogues when used for the management of TAO are far from satisfactory.

Meanwhile, sympathectomy may alleviate the pain and improves superficial ulcers, but it does not prevent or reduce the number of amputations [37]. Surgical revascularization is not usually feasible because of the diffuse and segmental character of the disease [37]. Thus, new therapeutic options with a higher efficacy than the current ones are clearly needed in order to properly manage patients affected by TAO. In any case, the characteristics of this disease, the low incidence, and the lack of effective treatments that improve the course of the disease or correct the cause contributed to serious ethical difficulties in carrying out large prospective studies that confirm the benefits and further definitive assessment in comparative randomised trials of the efficacy of novel therapy in this particular disease.

There are few articles published regarding the treatment of TAO with bosentan. However, they have shown that bosentan therapy is associated with several clinical and endothelial function-related outcomes in patients with TAO, which may be promising.

The anti-inflammatory, antifibrotic, and selective vasodilator properties of bosentan have been shown to alleviate pain at rest and reduce the size of ischaemic ulcers caused by damage mainly to the microcirculation. Recently, a single centre clinical study has been published, where 12 patients (13 extremities) previously diagnosed with TAO received treatment with bosentan in a compassionate use programme [17]. Bosentan therapy consisted of a month treatment with 62.5 mg twice a day followed by a double dose after the first month. The full-dose regimen was maintained for the following three months or until total healing of the ulcers. Prior to the treatment with bosentan, 10 of 12 patients hve previously been treated with a 21 days prostaglandin regimen, 3 had been undergone revascularizing procedures, and 3 patients had a lumbar sympathectomy. Clinical improvement was observed in 12 extremities (92%) treated, while only 1 extremity required major amputation below the knee. 10 extremities (77%) achieved complete clinical therapeutic success (healing or complete pain relief). A minor amputation of one toe was performed with conservation of the extremity. Also, a statistical improve of the endothelial function that was assessed by means of the FMD was observed.

Several case reports have been also published in the literature. All of them provide information on TAO patients with a history of insidious necrotic ulcers with poor outcomes despite smoking cessation and conventional medical treatment, including intravenous prostaglandins [18, 40, 41]. Their results show that treatment with bosentan is able to obtain a favourable clinical response with healing of ulcers, as well as the disappearing of the rest pain. Furthermore, most patients remained asymptomatic for six months after treatment cessation. Therefore, beneficial effects of bosentan in TAO patients are not only during the acute phase of ulcers and rest pain, but also they extend over time.

Although these results are from a small study and case reports and are not comparable with those from randomised trials, they seem to be hopeful.

A possible explanation for the bosentan pharmacodynamic effect has been related on its capacity of improving endothelial function based on the endothelial function impairment observed in patients with peripheral arterial disease in general [42] and in TAO patients in particular after treatment [9]. Moreover, an elevated serum ET-1 level has been observed in patients with TAO, supporting a possible mechanistic explanation of the clinical benefit of bosentan in these patients [3, 43]. Additionally, bosentan can exert a selective vasodilatory and anti-inflammatory effect on the vascular bed in patients affected by TAO, comparable to

the effects observed in connective tissue diseases such as scleroderma with the added complication of digital ulcers.

Summarizing, bosentan should be further investigated with regard to TAO patient management. The hypothesis that bosentan treatment in TAO patients results in an improvement of clinical, angiographic, and endothelial function outcomes is supported by the results of a small pilot study and several case reports that have been recently published. However, larger prospective studies and comparative randomised trials are needed to confirm them.

4. Treatment of Other Types of Vasculitis Digital Ulcers with Bosentan

The use of bosentan in Europe is approved for the treatment of pulmonary arterial hypertension and for digital ulcers (DUs) due to systemic sclerosis (SSc). The key sources of evidence for the use of bosentan in the management of digital ulcers in scleroderma are RAPIDS-1 and RAPIDS-2 trials [28, 29].

RAPIDS-1 is a randomized, prospective, placebo-controlled, and double-blind study of 122 patients with confirmed diagnosis of SSc. The primary outcome measure in this trial was the number of new DUs developed during the 16 week study period. Secondary assessment included healing of existing DUs. This trial demonstrated a significant beneficial difference between patients with bosentan compared to patients with placebo in the primary endpoint regarding to the appearance of new DUs. This difference was greater in patients who had ulcers at baseline (63%) and in those with diffuse disease. Nevertheless, no differences were found between placebo and bosentan in the time of complete or partial healing of DUs [44].

RAPIDS-2 is a randomized, double-blind, placebo-controlled trial with 188 SSc patients with at least one active DU [29]. In this trial the two primary endpoints were the number of new DUs and the time of healing of the DUs. Over 24 weeks, bosentan treatment was significantly associated with a 30% reduction in the number of new DUs compared with placebo. This effect was greater in patients who entered the trial with more DUs. Once again, there was no difference between treatments in the healing rate of DUs [29].

In both trials, bosentan treatment has demonstrated its ability to reduce the occurrence of new DUs in patient with SSc. However, this treatment has no effect on DU period of healing. Besides, there are published small series of patients that also show the beneficial effects of bosentan on preventing DUs, confirming the evidence obtained from RAPIDS studies [14, 16, 45–47]. An open-label study of 15 patients with SSc and DUs using bosentan for a median period of 24 months has revealed that bosentan is safe and effective in these patients [45]. Tsifetaki et al. have reported the longest prospective study (until 4 years) evaluating the number of healed DUs and new ulcer formation in 30 patients with SSc. Their results have showed that healed DUs occurred in 65% of treated patients [16].

There are also references in the literature regarding successful treatment with bosentan of refractory ulcers secondary to other pathologies. Bosentan treatment has been effective in healing of refractory DUs in patients with systemic lupus erythematous [48, 49], as well as in DUs due to Werner syndrome [50].

In addition, bosentan has been also used in paediatric patients. Studies in children with pulmonary hypertension have demonstrated its safety [51]. In this group of patients bosentan has also been effective to treat digital necrosis secondary to polyarteritis nodosa [52] and SSc [53].

In conclusion, the known beneficial effect of bosentan in the prevention of DUs secondary to SSc must be added to the promising healing effect on ulcers due to other pathologies, like systemic lupus erythematosus, polyarteritis nodosa, or Werner syndrome. Bosentan may be taken into account as a treatment option in these vasculitis diseases. Nonetheless, it is necessary to design prospective, randomized, and controlled trials to confirm these amazing results drawn from open-label or noncontrolled studies.

5. Summary and Conclusions

Endothelin-1 has been associated to vascular damage responsible for vasculitis and plays a key role in its clinical manifestations.

Focused on TAO, a vasculitis that affects small- and medium-sized arteries and veins of both upper and lower extremities, distal ischaemic lesions, and digital necrotic ulcers are frequently observed. Major amputation rate among these patients is not negligible. Furthermore, increased levels of ET-1 have been proved in TAO patients. Thus, ET-1 receptor antagonists, as bosentan, should be considered as a useful treatment option in this disease.

Initial results from open-label, nonparallel groups controlled studies or case reports published articles show promising efficacy of bosentan for treatment and prevention of digital ulcers in TAO with a favourable safety profile.

This efficacy can be justified by a selective vasodilator effect on the vascular bed, improving vascular permeability in digital ischemic ulcers, and restitution of endothelial function by increasing NO levels.

The anti-inflammatory, antifibrotic, and selective vasodilator properties of bosentan have been demonstrated to alleviate pain at rest and reduce the size of ischemic ulcers due to TAO. Beneficial effects of bosentan in TAO patients have been also reported to extend over time.

On the other hand, Bosentan has been found to decrease the number of new digital ulcers, although no significant effect in the healing period duration has been observed in patients with SSc. Moreover, Bosentan have been also stated to be effective in the healing of refractory ulcers due to systemic lupus erythematous, Werner syndrome, or polyarteritis nodosa.

Lastly, in any case, Bosentan should be further investigated in TAO and vasculitis patient management. To confirm these promising findings, larger controlled randomised trials with a control group are needed. In the meantime, bosentan should be considered as a hopeful investigational agent for treating these patients.

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Research Article

Chronic Wounds, Biofilms and Use of Medicinal Larvae

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Chronic wounds are a significant health problem in the United States, with annual associated costs exceeding $20 billion annually. Traditional wound care consists of surgical debridement, manual irrigation, moisture retentive dressings, and topical and/or systemic antimicrobial therapy. However, despite progress in the science of wound healing, the prevalence and incidence of chronic wounds and their complications are escalating. The presence & complexity of bacterial biofilms in chronic wounds has recently been recognized as a key aspect of non-healing wounds. Bacterial biofilms are sessile colonies of polymicrobial organisms (bacteria, fungus, etc.) enclosed within a self-produced exopolymeric matrix that provides high levels of tolerance to host defenses, antibiotics and antiseptics. Thus, there is a need for alternative therapies to reduce biofilms in chronic wounds. In this report, we present initial findings from in vitro experiments which show that larval debridement therapy with disinfected blow fly larvae (*Phaenicia sericata*) reduced total CFUs (6-logs) of planktonic and mature biofilms of *Pseudomonas aeruginosa* or *Staphylococcus aureus* grown on dermal pig skin explants by 5-logs after 24 hours of exposure, and eliminated biofilms (no measurable CFUs) after 48 hours of exposure.

1. Introduction

Chronic wounds are a significant health problem in the USA. Chronic wounds are those wounds which fail to progress as expected through the typical healing processes in a timely manner. Health care costs related to the management and treatment of chronic wounds in the USA exceeds $20 billion annually [1–7]. For many health care providers, the treatment and management of nonhealing wounds are challenging. Traditionally, basic wound care has consisted of surgical debridement, manual irrigation, moisture retentive dressings, and topical and/or systemic antimicrobial therapy. Although there has been tremendous progress in the science of wound healing, the prevalence and incidence of chronic wounds and their associated complications continue to escalate [1]. The presence and complexity of bacterial biofilms in chronic wounds have recently been recognized as key aspects of nonhealing wounds [8–20]. Bacterial biofilms are sessile colonies of polymicrobial organisms (bacterial, fungal, and possibly, viral) which are often symbiotic. These biofilm colonies produce a protective coating to protect the colonies from host defenses. The character of this protective substance unique to biofilms is dynamic, and the production of its components seems to be triggered by hostile environments in the wound bed (such as the presence of topical antibiotics). Biofilms have been shown to have survival and defense mechanisms that inhibit the healing aspects of inflammatory cells, resist antibiotics (topical and systemic) and other therapies, and initiate cell-to-cell communication pathways (quorum sensing) which facilitate new biofilm growth, resulting in recalcitrant nonhealing wounds [2].

With the increase of drug-resistant organisms such as methicillin-resistant *Staphylococcus aureus* (MRSA) [21], there is a need for innovative therapies in the treatment of

wound biofilms. Wound larval debridement therapy (LDT) has been shown to have promise in healing chronic wounds by eradicating biofilms. In this paper, we discuss the pathogenesis of chronic wounds with a focus on biofilms. We also discuss biofilm characteristics and the clinical relevance of LDT as an important treatment option for eradicating wound biofilms. We will also present preliminary findings from significant in vitro experiments demonstrating the effects of disinfected blow fly larvae (*Phaenicia sericata)* exposed to mature biofilm models (*Pseudomonas aeruginosa* or *Staphylococcus aureus* biofilms attached to the dermis of pig skin explants).

2. Characteristics of Biofilms

A bacterial biofilm is characterized as an aggregated bacteria attached to a surface or formed at a surface interface and organized as a complex community embedded in a self-secreted extracellular polymeric substance (EPS) [2–20, 22–24]. These dynamic bacterial communities may consist predominately of single bacterial or fungal species or, more commonly, may be polymicrobial, containing multiple diverse species that are continuously changing [23]. Biofilms have been identified on various surfaces of the body including the teeth (plaque), endocardium, GI and GU mucosa, and nasal epithelium as well as foreign objects such as orthopedic prosthetics and invasive catheters [25–27]. Evidence suggests that biofilms are strongly associated with impaired wound healing in chronic skin wounds [6, 10, 12, 15, 16, 23, 24]. Wound biofilms trigger a chronic inflammatory response resulting in accumulation of neutrophils and macrophages surrounding biofilms. The neutrophils and macrophages secrete high levels of reactive oxygen species (ROS) that affect the biofilm and the surrounding tissues [15]. Inflammatory cells also secrete high levels of proteases (matrix metalloproteinases and elastase) that can help break down the attachments between biofilms and the affected tissue, dislodging the biofilms from the tissue [25, 28]. However, the ROS and proteases also have the capacity to damage the normal surrounding tissue, proteins, immune cells, and tissue cells, delaying healing. In vulnerable tissue, biofilms are created by planktonic bacteria attaching and forming a protective community before they are killed by the patient's immune system, antibiotics, or by debridement. Several conditions which impair the immune system or reduce the effectiveness of antibiotic drugs encourage the development and spread of biofilms in wounds. These include ischemia or necrosis of tissues, nutritional deficits or compromise, and comorbidities that impair the body's immune function, such as HIV, diabetes, major physical trauma, radiation treatment, or treatment with immune-suppressing drugs [1].

3. Biofilm Mechanisms

It has been suggested that the processes employed by biofilms include molecular mechanisms which enable bacteria to attach to host cells and inject proteins to reorganize host cellular pathways [25, 26]. For some bacterial species, the injected bacterial proteins reorganize the host cellular cytoskeleton and prevent migration and mitosis, and inhibit apoptosis [27, 29–33]. As bacteria begin to form a biofilm, their molecular mechanisms may attract other bacteria to form a sustainable polymicrobial system [25, 26]. A biofilm colony is thought to possess an expanded diverse gene pool representing numerous species of bacteria [16, 34]. Long-term biofilm survival is often directly related to the genetic diversity of the biofilms, resulting in chronic infections that become recalcitrant to treatment. Survival of a bacterial biofilm requires gene expression to ensure attachment to the host, cellular senescence of the host to prevent shedding and to cause local inflammation, and stimulation of the production of plasma in the wound bed to nourish the biofilm colony [35].

Microorganisms that have the ability to form biofilms also possess quorum-sensing molecules to direct the focus and organization of the biofilm [26, 36]. Directed secretion of molecules and organization of the colonies in biofilms maximize the availability of nutrients and other essential molecules while minimizing the opposing effects of waste products, toxins of competitors, and other environmental hazards on the biofilms. Polymicrobial biofilms likely incorporate quorum-sensing molecules that can regulate pathways and also perform bidirectional signaling [26]. Biofilm organisms have the ability to sense and communicate with many quorum-sensing pathways. Biofilms have numerous defenses and can be resistant to treatment, limiting the effectiveness of antibiotics [26]. Antibiotics and antiseptics kill single bacteria very easily, but the biofilm barrier blocks most antibiotics and antiseptics from reaching the bacteria, particularly towards the center of the wound matrix [26]. Wound biofilms are resistant to antibodies, antibiotics, disinfectants, and phagocytic inflammatory cells. There is strong clinical evidence suggesting that larvae therapy, a less costly continuous debridement therapy, may be useful in eradicating wound biofilms [37–39].

4. Larvae Background

Maggot or larval debridement therapy (MDT or LDT) has been utilized for medical purposes for hundreds, if not thousands of years [40–42]. Surgeons since the 1700s have documented that the larvae of certain common blow flies or greenbottle flies (*Phaenicia sericata* and *Lucilia sericata*) remove only dead tissue while promoting healthy tissue in the wound bed, helping wounds heal faster [42]. The lifecycle of the typical fly larvae is about 10–14 days from the point of hatching until becoming an adult fly. However, larvae need to pupate before maturing into an adult fly; medicinal maggots are both physically and reproductively sterile, and because they are maintained in a moist environment, they are never allowed to pupate. In addition, these fly larvae will not burrow into or remove healthy tissue; they will only degrade, liquefy, and ingest dead tissue [40, 41]. Interestingly, while the larvae secretes an enzymatic substance which may also have natural antimicrobial properties [41], they do not excrete any waste product back into the wound. Prete (1997) suggested that their secretions also stimulate the growth of granulation tissue in the wound bed [42].

The medicinal use of fly larvae to remove necrotic tissue has been referred to as biosurgery, maggot debridement therapy (MDT), larval debridement therapy (LDT), or just larval therapy. Typically, the larvae are only 2 mm long when first applied to the wound bed. They are applied using 5–10 larvae per square centimeter and are usually left in the wound for up to 4 days. These therapeutic larvae essentially continue to ingest necrotic tissue (and wound waste, such as bacteria) until they have grown to more than 4-5 times their original size, about 3-4 days, at which point they are removed and/or replaced with new larvae. Larvae applied in such a fashion have been known to ingest up to 15 grams of necrotic tissue per day [38, 40–43].

Dr. John F. Zacharias (1837-1901), a Confederate American Civil War surgeon, is recognized as the first healthcare provider in the USA who intentionally applied maggots for wound care/debridement purposes. He noted that "maggots could clean a wound better in one day" than any other agent they had at their disposal [36]. He credited maggots with saving many soldiers' lives. Likewise, Dr. William S. Baer was an orthopaedic surgeon in WW1 who recognized the efficacy of maggots on the battlefield to "clean up" compound fractures and large flesh wounds, recognizing that maggots prevented sepsis in two battlefield cases which otherwise would have certainly been fatal [36, 42]. Maggots as a medical treatment impressed Dr. Baer immensely, and in 1929, he started conducting research at Johns Hopkins University using maggots he found in the neighborhood or raised on a windowsill. Two of his patients contracted tetanus from contaminated maggots (one died), so he developed sterile maggot-raising procedures. He used maggot therapy in 21 patients with chronic osteomyelitis who had not responded to other treatment. He demonstrated rapid wound debridement of necrotic tissue, a return of the wound bed to an alkaline pH environment, the reduction of bacteria, reduced odor levels, wound closure, and complete healing of the osteomyelitis infections within six weeks [36, 43].

With the development of antibiotics in the 1940s and various skin and wound antiseptics, the use of LDT declined. Arguably, one of the biggest reasons LDT may have lost favor in clinicians' eyes was not ineffectiveness, for they remain a most effective form of debridement, but rather, the "yuck factor." Patients, their caregivers, and clinicians found it distasteful to apply small squirming worms that could crawl out of a wound. Even Dr. Baer said that "the sight was very disgusting and measures were taken hurriedly to wash out these abominable looking creatures [36]."

With the advent of antibiotic-resistant organisms and increasing drug sensitivities, there was a renewed interest in maggot therapy in the 1980s [40]. The U.S. Food and Drug Administration (FDA) cleared medicinal maggots (*P.* or *L. sericata*) for debriding nonhealing necrotic skin and soft tissue wounds including diabetic foot ulcers, pressure ulcers, nonhealing surgical or traumatic wounds, and venous stasis ulcers. In the USA, larval therapy with maggots is classified as a medical *device* [40]. However, in Europe, Canada, and Japan, maggots are classified as medicinal *drugs*.

Maggots used in the USA for larval debridement therapy are all processed under controlled laboratory conditions and are sterile (free of disease as well as unable to reproduce). Larval debridement of nonviable tissue within chronic wounds results partly from necrotic tissue and wound waste being liquefied by the proteolytic digestive enzymes (along with bacteria and biofilm) which the larvae ingest. As such, larval therapy is a most efficient and noninvasive method to debride a wound without the pain, bleeding, or inflammatory response associated with debridement [40].

Unfortunately, current larval debridement methods available in the USA have not addressed the "yuck factor" of free-roaming maggots in open wounds (or the patient's aversion to the sight of maggots). This may explain why, despite the clinically proven effectiveness of larval therapy to aid in the healing process, many US clinicians do not use this method of wound treatment. Many nurses, doctors, caregivers, and patients have voiced an aversion to handling maggots or having to "count the number that go into a wound or come out of a wound."

5. Materials and Methods

Mechanisms of action. Studies demonstrate that LDT works by mechanical as well as enzymatic debridement, has antibacterial properties, and stimulates wound healing [40–43]. However, the exact mechanisms of action require further exploration. Recently, the University of Florida Wound Research Laboratory conducted several in vitro experiments to demonstrate the efficacy of larval exposure to biofilm and document the results.

The In-Vitro Experiment Protocol included.

Step 1. Three 35 mm diameter pigskin explants were inoculated with *Pseudomonas aeruginosa* (PA01) bacteria, and three additional 35 mm pigskin explants were inoculated with *Staphylococcus aureus* (SA35556) bacteria. The pigskin explants were maintained on soft agar in a 90 mm Petri dish for 3 days. At the end of the 3 days, these explants represent a chronic wound biofilm model (demonstrating fully mature biofilm colonies on the explant). This model has provided consistent results and has been used successfully in several other in-vitro biofilm studies by the Wound Research Laboratory at University of Florida.

Step 2. In the control, colony forming units (CFU) of bacteria were determined before and after high antibiotic treatment (a wash of gentamycin, stimulating the free floating bacteria to develop into a fully mature biofilm colony $>10^5$ CFU).

Step 3. Punch biopsies (5 mm) were obtained from each explant for plating, CFU counts, and SEM (electron microscopy photos) at 24 hours prior to the addition of larvae to the Petri dishes.

Step 4. Eight-layer cotton gauze moistened with 3 mL 0.9% NaCl was applied on top of the biofilm models.

Step 5. At 3 days, the explants (with 3-day biofilm) were changed from atop soft agar to atop saline moistened gauze in the Petri dish.

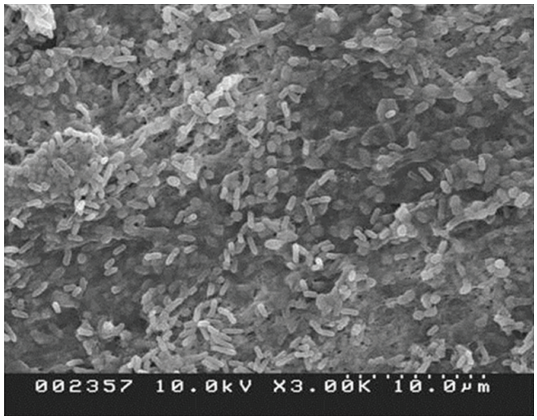

Figure 1: Three-day PA01 biofilm on pig explant (before larvae are applied).

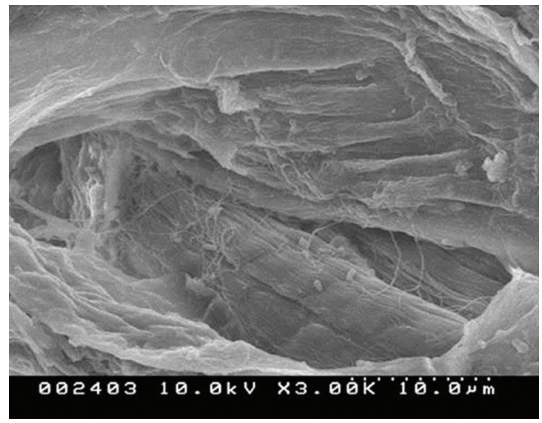

Figure 2: PA01 biofilm on explant (24 hours after larvae are applied).

Step 6. Thirty live *L. sericata* larvae were applied to the top of each pigskin explant/mature biofilm model, then the Petri dish lid was set in place and sealed with parafilm.

Step 7. Punch biopsies (5 mm) were obtained from each explant for plating, CFU counts, and SEM (electron microscopy photos) at 24 and 48 hours after the addition of larvae to the Petri dishes.

Figures 1–4 are scanning electron microscope photo documentation of the punch biopsies obtained from the 35 mm round pigskin explants (chronic wound model) inoculated with either *P. areuginosa* (PA01) bacteria or *S. aureus* (SA35556) bacteria. Figures 1 and 3 demonstrate the mature biofilm colonies present on the pigskin explants. Figures 2 and 4 demonstrate complete eradication of the biofilm (the pigskin explant was left intact) within 24–48 hours of exposure to the maggots (Table 1). The bacteria had not reappeared on the explants after 48 hours. This is the first paper, to our knowledge, that showed LDT specifically and preferentially removing biofilm attached to non-viable dermal tissue. Additionally, there was no evidence that the maggots had ingested any of the pigskin explant or each other.

6. Results

Results are illustrated in Table 1 and Figure 5.

Figures 1 through 4 show scanning electron microscope (SEM) photographs of punch biopsies obtained from 35mm diameter pigskin explants (chronic wound model) on which mature biofilms of either *P. aeruginosa* (PA01) bacteria or *S. aureus* (SA35556) bacteria were grown. Figure 1 (PAO1) and Figure 3 (SA3556) show the mature biofilm colonies present on the pigskin explants before exposure to LDT. There are clear structural features that are characteristic of biofilm community structures. For example, in Figure 1, numerous rod-shaped bacteria are present within the sheet-like structure of the exopolymeric matrix of the biofilm that collapsed during the fixation and dehydration of the SEM sample. A few *P. aeruginosa* rods are visible on the surface

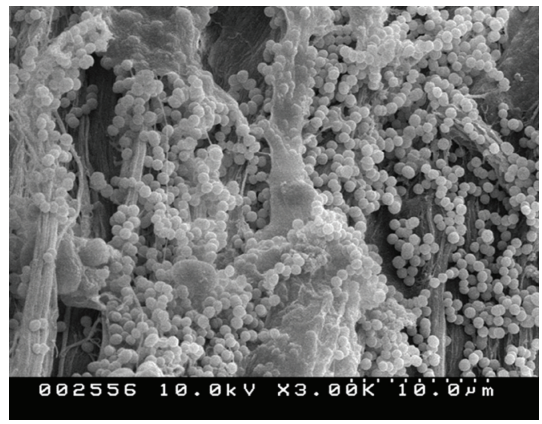

Figure 3: Three-day SA35556 biofilm on pig explant (before larvae are applied).

of the biofilm matrix. Similarly, Figure 3 shows numerous spherical shaped *S. aureus* bacteria embedded in the biofilm matrix, and some bacteria that are attached to the surface of the pig skin dermis that are not yet enclosed in a matrix.

Figures 2 and 4 demonstrate no detectable biofilm or planktonic bacteria on these areas of the surface of the pig skin explants after 24 hours of exposure to the medial maggots. The rope-like structures of collagen fibers are still visible, showing the effective debridement accomplished by the LDT.

Quantitation of levels of planktonic and biofilm bacteria in the treatment groups are presented in Table 1. Three days after inoculating the pig skin explants, there were high total bacterial counts on the explants, with approximately 3.4 million CFUs of PA01, of which 300,000 CFUs were in the biofilm and were tolerant to 24 hours of exposure to high levels of antibiotics. Similarly, there were 11.3 million CFUs of total SA35556, of which 430,000 CFUs were in the biofilm and were protected from 24 hours of exposure to antibiotics. After 24 hours of exposure to medical maggots, the levels of both bacteria were 1.7 to 3.3 CFUs per explant, which represents approximately 5-log reduction of total bacteria. After 48 hours of exposure to LDT, no bacterial growth (0 CFUs) was

Table 1: Bacterial CFU/mL at 24 hours and 48 hours after larvae exposure.

Bacterial strain	Total bacterial count (including free-floating planktonic)	Antibiotic-tolerant biofilm	1 day (24 hours after larvae treatment)	2 days (48 hours after larvae treatment)
PA01	3.4*E*06 (3.4×10^6)	3.0*E*05 (3.0×10^5)	3.3*E*00	0.0*E*00 (eradicated)
SA35556	1.13*E*07 (1.13×10^7)	4.3*E*05 (4.3×10^5)	1.7*E*00	0.0*E*00 (eradicated)

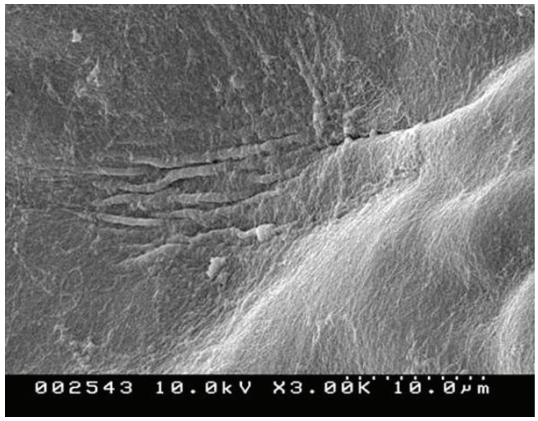

Figure 4: SA35556 biofilm on pig explants (24 hours after larvae are applied).

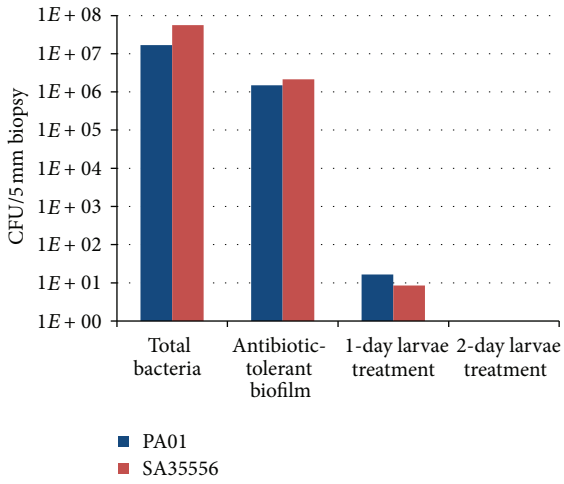

Figure 5: Graph of bacterial CFU before and after larval exposure.

recovered from the processed pig skin explants, indicating total removal of the planktonic and biofilm bacteria.

7. Discussion and Conclusions

As demonstrated, LDT shows promising effectiveness at eradicating bacterial biofilm from chronic wounds. In light of multidrug-resistant organisms [44] and drug allergies/sensitivities as well as the pain associated with traditional debridement procedures, larvae therapy may indeed be one of the most effective tools in the clinician's arsenal for treating chronic, non-healing wounds. More research is warranted to further investigate the clinical efficacy of this treatment and explore the exact mechanisms of action with regards to wound healing and the effects of larvae on the microenvironment of the wound bed. Future research is also needed to explore ways to utilize this treatment in a more aesthetically acceptable manner. Potential limitations of the experiments reported in this paper include the fact that they were conducted in a controlled in-vitro setting without a human host wound environment, limiting their generalizability. Currently, this study is being repeated in living human wound models to validate similar findings regarding the larvae's ability to eradicate biofilm within a chronic wound environment. In addition, further research should address questions regarding the length of time larvae debridement therapy should be conducted to achieve maximum therapeutic results.

8. Pearls for Clinicians

Clinical Indications for Larval Therapy. For debriding non-healing full thickness skin or soft tissue wounds with necrotic or non-viable tissue wound types that may benefit from larval therapy include; diabetic or neuropathic foot ulcers, venous stasis ulcers (where compression may be delayed a few days/weeks), pressure ulcers, and non-healing traumatic or postsurgical wounds.

Contraindications. Blind tunnels or fistulas which lead to internal organs; wounds with necrosis around major blood vessels; patient allergy to fly larvae or to products used in larvae cultivation (soy proteins and/or brewer's yeast); in or near eyes; upper GI tract or respiratory tract; wounds not exposed to the outside air; wounds that must be covered with completely occlusive dressings or compression/direct pressure such as sitting surfaces (situations where the larvae would be killed, compressed, suffocated, etc.). *Precautions.* Patients with coagulopathies (monitor closely for bleeding) or severe arterial insufficiency. See manufacturers' insert for full listing of contraindications or precautions [43]. *How to order.* At present there are limited resources where disinfected larvae or medical maggots may be purchased in the USA (more sources are available in the European market). Larvae used for this study were kindly provided by Monarch Labs [45] 17875 Sky Park Circle, Suite K, Irvine, CA 92614, USA.

Applying and Removing LDT. Follow universal precautions and good clinical practice as you would for any dressing change. Suggestions unique to larval therapy: keep the shipping container with the larvae at about 8–10 degrees Celsius. Check manufacturer guidelines about storage—they typically should be placed in the wound within one day. You may wish

to use a "cage" to keep the larvae within the wound bed or "window" the wound edges with hydrocolloid dressing cut to the exact size and shape of the wound. Place larvae in wound bed, some recommend counting each one as it goes in and counting them as they are removed. They may be applied on a moist 4"x4" cotton gauze and place larvae side down in the wound, secured with a nylon stocking or netting. The dressing should remain slightly moist but avoid excessive moisture as it will drown the larvae. Avoid putting direct tight pressure over the larvae and avoid overpacking the wound. The larvae will expand in size, and if there are too many, this may cause some discomfort to the patient. When removing them, place the old dressing/larvae in alcohol or other cleaning agent (to kill them) or sealed biohazard bag and dispose in biohazard container. Document number larvae in and out, number of days in wound, appearance of wound bed before and after LDT, and standard wound monitoring documentation.

Considerations When Using Live Biological Dressings. Consider placing a note on the patient's door ("Biological Therapy in Progress") or otherwise give advanced notice to caregivers that larval therapy is underway so they are not surprised/caught unaware and demonstrate a negative reaction.

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Clinical Study

Compression Stockings for Treating Venous Leg Ulcers

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Background. In order to treat venous leg ulcers, it is recommended to use high pressure compression (30–40 mmHg at the ankle). Compression stockings which are not operator dependant could be the best option because of their pressure control. However 30–40 mmHg compression stockings are often hard to put on. Putting two lower pressure compression stockings over each other could be a good therapeutic alternative. *Objectives*. To compare the *in vitro* pressures given by the manufacturers of 2 antiulcer kits with the *in vivo* interface pressures measured in healthy subjects and to evaluate the stiffness and friction indices from those kits based on the interface pressure in order to assess their clinical properties. *Material and Methods*. Using a Kikuhime pressure device, interface pressure was measured in 12 healthy subjects at the reference point B1. One stiffness index (Static Stiffness Index (SSI)) and a friction index have been calculated. *Results*. Mediven Ulcer kit gets the recommended pressures whereas Jobst's Ulcer Care kit does not for treating a venous leg ulcer. Jobst's Ulcer Care transmits entirely the pressure in relation to a friction index close to 1. *Conclusion*. This antiulcer kit study underlines that *in vivo* and *in vitro* pressures can be different (Jobst's Ulcer Care kit and Mediven Ulcer kit). In order not to lose pressure, it is important to take into account the friction index when superimposing two stockings.

1. Background

Compression increases ulcer healing rates compared with no compression [1, 2].

Thus to improve the healing process (recommendation grade 1B) it is recommended to treat venous or mixed venous ($0.6 > \text{ABI} < 0.9$) with high pressure. A pressure between 30 and 40 mmHg should be obtained at the ankle (professional agreement).

Multicomponent systems are more effective than single-component systems. Multicomponent systems containing an elastic bandage appear more effective than those composed mainly of inelastic constituents. Two-layer stockings appear more effective than the short-stretch bandage [3]. In fact, there are no clear differences in the effectiveness of different types of high compression.

Putting on the bandages requires a great experience and the respect of the bandage stretching rules. A pressure level from 30 to 40 mmHg may not be easy to achieve. The main criticism that can be made against the use of a multilayer bandage or short stretch is linked to bandage slippage. Slippage is a cause of adverse effects: pain, aggravation of ulcer ulceration, and necrosis [4].

The use of compression stockings seems to be the best option because of the pressure control it allows for and it is not operator dependant. However 30–40 mmHg compression stockings are often hard to put on, especially for the elderly. In this case a donning and doffing aid could be useful.

According to Amsler et al. [5] putting two lower pressure compression stockings on top of each other is the best option to get the desired pressure level. In terms of healing process, pain level, and nursing cares, compression stockings are better than bandages.

Concerning the pressure under 2 stockings on top of each other, Cornu-Thenard et al. [6] showed that the in-vitro pressure, in such conditions, is equal to the sum of the pressures that each stocking induces separately.

The pressure is different *in vivo*.

For Partsch et al. [7], the pressure under 2 stockings on top of each other is slightly inferior to the sum of the pressures that each stocking induces separately.

Benigni et al. [8] came to the same conclusions in regard to the *in vivo* pressures and the stiffness indices.

Rastel and Lun [9] agree that the loss of pressure can be explained by the added pressure resulting from two elastic yarns on top of each other. Concerning compression stockings, the yarns go on top of each other in the remaining free areas (Figure 1). Yarns do not rub uniformly on top of each other. Friction forces need to be taken into account in order to understand the loss of pressure transmitted.

The interface pressures and the *in vivo* kits stiffness must be known. By analogy with bandages they could allow to anticipate the expected clinical effects. Moreover pressure loss happening by superimposing needs to be linked with friction consequences. A better understanding of this process should result in improved kits.

2. Objectives

The aim of this paper is as follows:

(1) to compare *in vivo* interface pressures at B1 measured in healthy subjects with *in vitro* pressures of two different superimposed antiulcer 40 mmHg kits,

(2) to calculate their stiffness and friction indices based on the *in vivo* interface pressures, in order to appreciate the outcome.

3. Material and Methods

Twelve healthy subjects participated in the study (4 men and 8 women). They were aged between 52.1 ± 12 years, with an average height of 169 ± 6 cm, an average weight of 69.0 ± 8 kg with ankles of 22 ± 0.9 cm at point B and of 29 ± 3 cm at point B1. Healthy patients were randomized in 2 groups of 6.

The interface pressures were measured at point B1 (Figure 2). This point is described in the CEN document [10]. Measurements have been done both at rest and then in a standing position [11].

3.1. Compression Ulcer Kits. Mediven Ulcer Kit (Medi Bayreuth) compression stockings (kit 1) were as follows:

(i) a Mediven ulcer understocking with an ankle pressure of 20 mmHg (point B). This stocking is to be worn day and night. It is made of 71% polyamide, 28% elastin, and 1% silver (antimicrobial texture),

(ii) a Mediven ulcer plus overstocking also with an ankle pressure of 20 mmHg (point B) only to be worn during the day. It is made of 75% polyamide and 25% elastan.

In vitro pressure Mediven Ulcer kit (manufacturer) 40 mmHg at point B.

Jobst's Ulcers Care, (Jobst) compression stockings (kit 2) were as follows:

(i) an understocking for protection, made of 78% nylon/polyamide and 22% Spandex/elastane,

(ii) an overstocking with a zipper. It is made of 85% Nylon/polyamide and 15% Spandex/elastane.

(iii) *in vitro* Jobst's Ulcer Care pressure (manufacturer): 40 mmHg at point B.

The sizes of stockings were selected accordingly to the circumferences measured at ankle level (point B).

3.2. In Vivo Interface Pressure Measurements. The interface pressures were measured using the Kikuhime system (TT Medi Trade, Soleddet 15, DK 4180 Soro), which is composed of the following:

(i) a Kikuhime device (Figures 3 and 4),

(ii) this system uses two identical, oval-shaped measuring sensors, 30 × 38 mm, 3 mm thick when calibrated to 0 mmHg.

At point B1, the interface pressures were measured on the 12 healthy subjects' right leg in 2 positions (at rest and standing up). Each measurement was repeated 3 times as follows: with the understocking, then the overstocking alone, and finally the two on top of each other. 216 measurements were completed.

3.3. Stiffness Index Calculation. Static Stiffness Index (SSI) reflects the difference in interface pressures between the lying and standing positions.

We consider that a compression is stiff when the SSI is higher than 10 mmHg [11].

3.4. Friction Index Calculation. When on top of each other and moving, the knitting yarns rub each other. When stretching the two knitted pieces, the threads are not superimposed anymore and the transmitted pressures become smaller.

This index equals 2 superimposed stockings stiffness index (SI^{sup}) divided by the sum of the stiffness indices of the 2 stockings used separately (SI^{alone}):

$$IF = \frac{SI^{superimposed}}{SI^{alone} + SI^{alone}}. \quad (1)$$

4. Statistical Analysis

Measurement of the coefficient of variation, comparison of means for the interface pressure, and the Stiffness Index were performed using the Student's *t*-test.

Statview version 5 statistics software was used to perform the calculations.

5. Results

The 2 groups were comparable for sex, age, and leg circumferences.

5.1. In Vivo Pressure Measurements in mmHg and Stiffness Indices Calculation. For the kit 1 (Table 1), the *in vivo* interface pressures at B1, in the 2 situations, are within the

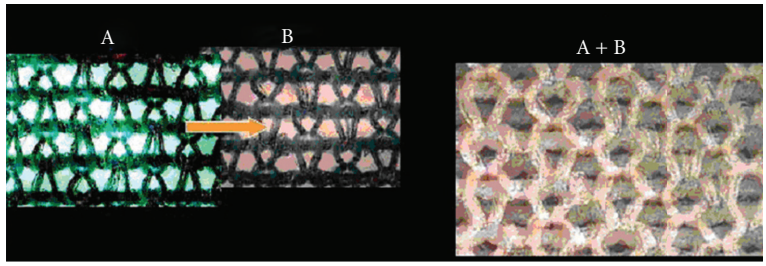

Figure 1: Compression stockings superimposition (yarn of woof and stitch, picture obtained by 2 stockings numeric superimposition).

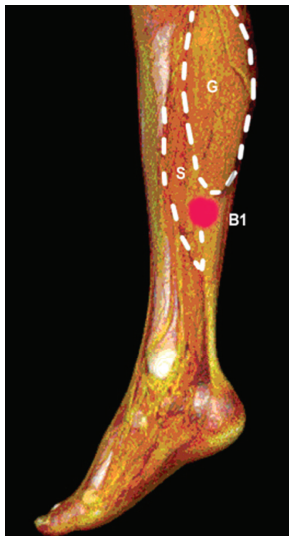

Figure 2: Point B1 (virtual dissection of the leg with a CT scan and a 3D reconstruction without contrast medium. G: medial gastrocnemius muscle; S: soleus muscle).

Figure 3: Kikuhime device.

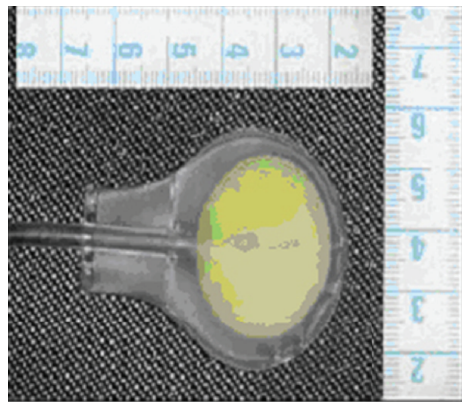

Figure 4: Pressure sensor.

limits of pressures recommended to treat a venous ulcer. On the other hand the pressures of the kit 2 stay under 30 mmHg at rest. They only exceed 30 mmHg when there is a muscular activity (Table 2).

For the kit 1, the pressures measured *in vivo,* when superimposing, are smaller than the sum of the two stockings used separately. As for kit 2, there is no significant difference.

All the pressures measured under the 2 understockings are low; hence the understockings can be kept on the leg during night, even in patients with peripheral arterial occlusive disease (with an ABI > 0.6) without ischemic risks.

The bigger the pressures get, the more the Stiffness Index (SSI) increases. Our analysis goes along previous publications [7, 8].

For the two tested kits the comparison between the *in vivo* average pressure at rest shows a noticeable difference but inferior to 10 mmHg (Table 3). None of the 2 kits are stiff.

None of the two kits are stiff between the resting and standing positions (SSI).

Concerning the kit 1, the stiffness indices are lower than the sum when the two stockings are superimposed, whereas for the second, the kit 2, there is no difference between the results of the sum of the two pressures and the superimposition.

The calculation of a friction index is necessary to explain these differences.

5.2. Friction Index. The kit 2 friction index is 1 (SSI). In other words, the kit 2 transmits all of the two stockings pressure.

Table 1: Average and standard deviation of the kit 1 of *in vivo* pressures at point B1 and stiffness indices.

In vivo	Overstocking	Understocking	Theoretical sum	Superimposition measured
At rest	19.0 (3.9)	16.8 (3.3)	35.8	33.0 (4.7)**
Standing up	25.1 (3.4)	22.2 (3.2)	47.3	41.9 (5.5)**
SSI	**6.1**	**5.4**	11.5	**8.9**

** $P < 0.05$.

Table 2: Average and standard deviation of the kit 2 of *in vivo* pressures at point B1 and stiffness indices.

In vivo	Overstocking	Understocking	Theoretical sum	Superimposition measured
At rest	15.7 (3.4)	8.3 (0.8)	24	24.2 (4.5)**
Standing up	19.8 (4.5)	12.2 (2.3)	32	32.2 (5.3)**
SSI	**4.2**	**3.9**	8.1	**8.1**

** $P < 0.05$.

Table 3: Comparison of the stiffness indices measured with 2 kits and the stiffness indices calculated based on the sum of pressures, $P < 0.05$.

	Kit 1	Kit 2	Kit 1 versus Kit 2
SSI 2 CS superimposed	**8.9 (4.1)**	**8.1 (3.9)**	**NS**
SSI sum	11.5 (4.7)**	8.1 (3.9)**	$P < 0.05$

Table 4: Friction indices.

Friction index	Kit 1	Kit 2
SSI	0.77	1

However the other kit, whose friction index is 0.77 for kit 1, underlines that they only transmit the pressure partially. The pressure loss is about 20% for this kit.

In this kit, the two superimposed stockings fibers do not come on top of each other when stretched, in contrast to kit 2 (Table 4).

6. Discussion

This underlines the importance of the friction index. In order to understand it better, one should go back to the laws of friction for materials. Pierre-Gilles de Gennes summarizes them as follow [12].

"Leonard da Vinci's work imposed itself as a cornerstone in this field. He observes that if an object—a piece of wood—is on a surface that is then raised up, it will slide along it up from a certain angle. This is a feature of static friction. In 1699, Guillaume Amontons repeats the experience and comes to the same conclusion. It is only in 1950 that the British school (T. P. Bowden and David Tabor) explained why a small surface has the same properties as a big one: the tight contact results from asperities and bumps. When using a small surface, the pressure applied increases; hence the decrease in surface is compensated by a higher density on the contact zone. The same result is obtained than on a bigger surface".

The kit 2 has the biggest friction index possible: 1 for the SSI. There is no loss of pressure, during a muscle contraction when superimposing, in relation with the number of asperities between the two stockings, although the pressures applied are smaller.

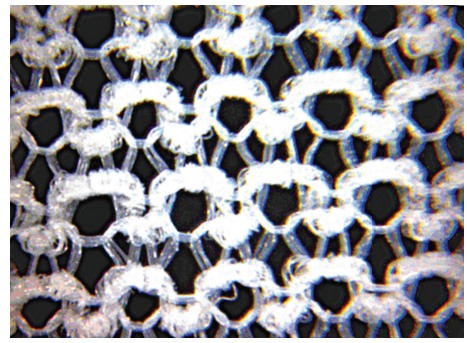

Figure 5: Stitch of the understocking from kit 2.

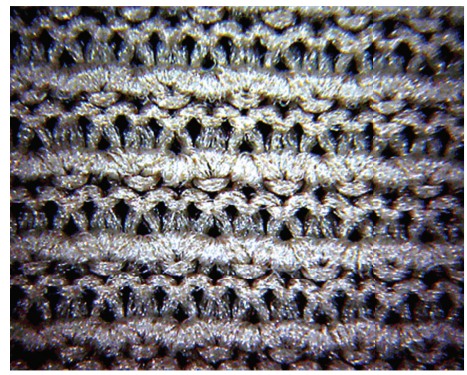

Figure 6: Stitch of the overstocking from kit 2.

In this kit, the stitch of the overstocking is very dense. Because there are a lot of asperities, the friction of the understocking on the overstocking is high. There is no free space between the yarns of woof; hence a friction index equals to 1 (Figures 5 and 6 numeric microscope).

The knitting of the other kit is completely different. There are fewer asperities; hence the friction indexes are smaller by approximately 20% (Figures 7 and 8). In the stitch, the

Table 5: Selection criteria for compression.

	Size of ulcer	Dressing stage of the wound	Dysmorphic leg	Presence of significant edema
Compression stocking kit	Small size	Granulation/epithelialisation	–	–
Bandages	Large size	Exudative/debridement	+	+

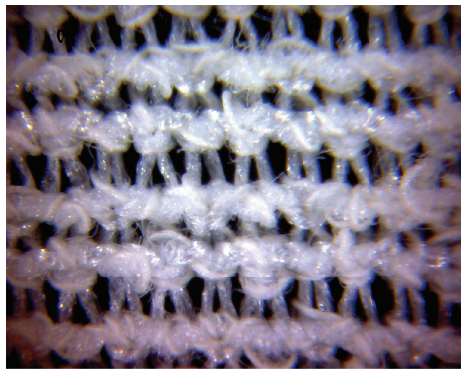

Figure 7: Stitch of the understocking from kit 1.

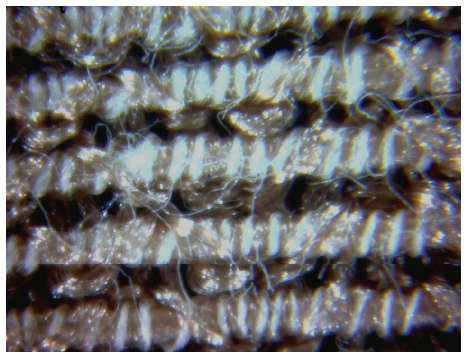

Figure 8: Stitch of the overstocking from kit 1.

yarns of woof are superimposing because of the remaining free space between them.

Therefore when superimposing stockings, using the kit 1, the real (*in vivo*) pressures obtained at rest and standing up are similar to the ones given by the manufacturers (*in vitro*).

However the kit 2 shows differences when tested at rest.

Practical Consequences. The short stretch or multilayer bandages represent an ideal in terms of pressure and stiffness. But sliding, high variation of pressures, poor interoperator and intraoperator reproducibility, and cost-effectiveness are obstacles to their daily use.

Compression stocking kits marketed must come closer to the ideal of bandages without its flaws. This trial provides a reference for an "ideal" antiulcer kit by compression stockings. Their pressure at the ankle must be higher than 30 mmHg, stiffness greater than 10 mmHg, and the pressure of the kit should be equal to the sum of the pressures of each stocking measured separately (friction index = 1).

On the other hand, an important issue to tackle is the donning of the kit. The kit 2 understocking is easy to put on. However the overstocking is not, it is hard to zip it up.

However, the choice between compression stockings and bandages cannot be reduced to a pressure problem. Some other factors play a role (Table 5):

(i) the size of ulcer,

(ii) the dressing applied depending on the stage of the wound,

(iii) the presence or absence of musculoskeletal deformities of knees, feet, or other dysmorphia,

(iv) the importance of edema.

The size of ulcer is important in the choice. A large ulcer will require a dressing that may slip or come off when threading a stocking. It is preferable to use a multitype or a short stretch bandage easier to install. If the ulcer is small, the dressing with the hand when threading can be easily maintained.

At the exudative phase, the risk of leakage and odors requires the use of a secondary absorbent dressing. The thickness of two dressings makes difficult to apply one or two stockings in superimposition. In contrast during the granulation/epithelialisation phase, wound no longer flows, the primary dressing is usually thin and changed less frequently. The use of a kit is then fully justified.

The remark is similar in case of dysmorphic leg. A bandage is more suitable.

A significant edema is a problem of a different nature.

In the initial phase of edema reduction, the use of a stiff bandage (multilayer or short-stretch) allows a rapid reduction of swelling but the bandage may slip and lose all effectiveness or sliding may cause skin disorders. The bandage should be removed every two or three days to adapt it to the volume of the leg. If the swelling is small, the compression stockings find its natural place.

7. Conclusions

This antiulcer compression stocking study underlines that *in vivo* and *in vitro* pressures can be different (Jobst's Ulcer Care kit).

In order not to lose pressure, it is important to take into account the friction index when superimposing two stockings. To that end it is more important to increase the number of asperities between the two antiulcer stockings, through their knitting, rather than considering the actual pressure applied.

In the future, bandages will only be used during the initial oedematous phase of venous leg ulcer treatment. The kits' two superimposed stockings will be used during the maintenance phase.

Disclosure Agreement

No fees paid by manufacturers to authors. The concerned laboratories provided only the necessary stockings for measurement purposes.

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Review Article

Ulcerative Lesions in Behcet's Disease

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Ulcerative lesions in Behcet's disease (BD) are regarded as important manifestation for diagnosis. Various kinds of ulcerative lesions appear in patients with BD. They present as orogenital ulcers, necrotizing vasculitis and pyoderma gengrenosum. Gastrointestinal system involvement (Gis) in Behçet's disease affects all areas from the esophagus to the anus. Most authors believe that the Gis manifestations of Behçet's disease should be confined to aphthous ulcers, which can occur throughout the Gis tract. All patients with oro-genital and Gis ulcerations should be fully investigated to establish a definitive diagnosis and eliminate the possibility of an underlying BD.

1. Introduction

BD was first defined by Behçet, a Turkish Professor of Dermatology, in 1937 as a triad of recurrent aphthous stomatitis, genital aphthae, and relapsing uveitis [1]. During the ensuing 65 years multiple systemic associations of the disease including articular, vascular, gastrointestinal, cardiopulmonary and neurologic involvement have become increasingly apparent [2–4]. Although the etiology and pathogenesis is not clearly defined, genetic predisposition, infections and immunological dysfunctions have been implicated [5]. BD has been reported worldwide, but has a distinct geographic distribution, with highest prevalences in countries along the ancient silk route. Although much has been learned during recent years on the pathogenesis and treatment of the disease, it is still an important cause of morbidity and mortality in areas where it is prevalent [3]. Young individuals are most commonly affected. Male to female ratio is usually 1 : 1. The gender predominance is different according to the prevalent countries. So M : F ratio 1 : 1 seems to be not always correct [4]. Ocular and central nervous system involvement are the basic prognostic factors in BD. Cardiovascular, pulmonary, and gastrointestinal system involvements are the major causes of mortality. In different series, high prevalence of ocular, nervous system, pulmonary system involvement, large vessel thrombosis, thrombophlebitis and patergy positivity has been found in male patients, and in view of these data a more severe course in male patients can be expected. Higher incidence of severe clinical course and systemic involvement is observed when early onset of the disease is present [2–4].

2. Ulcerative Clinical Manifestations

Ulcerative lesions in BD are regarded as important manifestation for diagnosis. Various kinds of ulcerative mucocutaneous and Gis lesions appear in patients with BD.

2.1. Mucocutaneous Ulcerative Lesions. Mucocutaneous ulcerations are the most common presenting symptoms of the disease.

Oral Ulcers. Oral ulcer (or aphthae) is localized, painful, shallow, round to oval ulcer often covered by a gray fibromembranous slough and surrounded by an erythematous halo (Figure 1). They are seen as minor or major ulcerations, sometimes with herpetiform distribution at any site in the oral cavity. International study group criteria do not permit diagnosis in the absence of oral aphthae, and oral

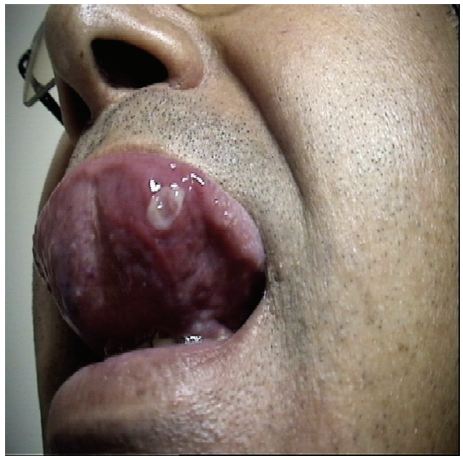

Figure 1: Oral ulcers.

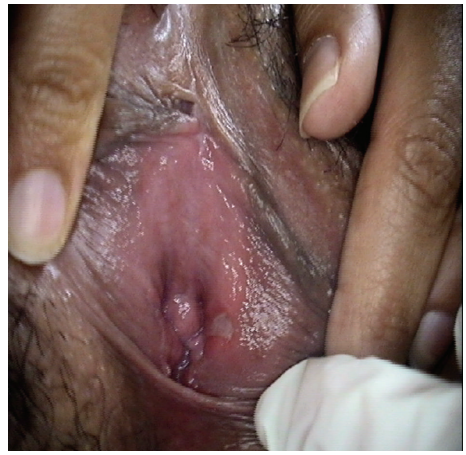

Figure 2: Genital ulcers.

aphthae was seen in all patients with BD. The vast majority of mild cases present with recurrent aphthous ulcerations of the oral mucosa which are usually the earliest and universal sign of the disease that are indistinguishable from common aphthae-canker sores in appearance and localization and has a yellowish necrotic base. This is frequently the first symptom and can precede the other manifestations of the syndrome by many years. Minor aphthous ulcers (<10 mm in diameter) are the most common type (85%); major or herpetiform ulcers are less frequent. Such mouth ulcers may be so painful that the patient is unable to eat during the attack. Aphthae may evolve quickly from a pinpoint flat ulcer to a large sore. In addition, intervals between recurrences range from weeks to months and typically may precede the onset of ocular, central nervous system, and some other systemic findings by many years. Smokers often experience a relapse of oral ulcers after quitting and nicotine replacement patches have been suggested to be useful in BD [2, 4–7].

Genital Ulcers. In previous reports the prevalence of genital ulceration (or aphthae) was found to be between 60 and 90% (Figure 2). Genital lesions were most commonly seen on the scrotum of male patients and on the vulva of female patients and tended to be larger and deeper in the female patients, sometimes even leading to perforations. The ulcers usually heal in 2–4 weeks; large ulcers frequently leave a scar whereas small ulcers and those on the minor labia heal without leaving a mark [2, 4, 5, 7]. Genital ulcers are the second most commonly observed onset manifestation and resemble their oral counterparts. However, they are larger and deeper than mouth lesions, and appear at some time during the course of the disease [2, 4].

Otherskin Ulcerations. Other skin ulcerations, such as extragenital skin ulcers in the axillary and interdigital areas, pyoderma gangrenosum, leucocytoclastic vasculitis, polyarteritis-like cutaneous lesions, true arterial lesions, subungual infarctions, are less common [8].

Extragenital Ulcers. Extragenital ulcers occur in about %3 of patients (Figure 3). They are common in children with BD and these recurrent ulcers usually heal with mild scarring [8]. Skin biopsies of extragenital ulcerations showed vasculitis [9, 10]. Extragenital ulcers look like aphthous ulcers and commonly heal leaving a round atrophic scar. They are common in children with BD [8].

Pyoderma Gangrenosum. Pyoderma gangrenosum-like lesions are extremely rare. Pyoderma gangrenosum is a neutrophilic dermatitis with the same hypersensitivity to trauma as BD. In pyoderma gangrenosum some cases are associated with bowel disease as in BD. Also, pyoderma gangrenosum can produce in some cases localization of neutrophilic lesions in other organs such as heart, lymph nodes, and central nervous system which resembles BD to some extent [11].

Necrotizing Vasculitic Ulcers. Some cases of BD with severe necrotizing vasculitis as a skin manifestation have been described. A case of BD in a 11-year-old Korean boy who had severe necrotizing vasculitis as a skin manifestation was reported [12]. Cutaneous vasculitis in BD is predominantly a venulitis or thrombophlebitis, with relative sparing of the arterial compartment. Vasculopathy [13] reported that approximately half (48%) of BD patients with cutaneous lesions had either lymphocytic (31%) or leukocytoclastic vasculitis (17%). They have suggested that vascular inflammation is the pathologic basis of the skin lesions in BD and that the histologic spectrum ranges from fully developed necrotizing vasculitis with marked fibrinoid necrosis of vessel walls to perivascular inflammation with or without a marked interstitial infiltrate [13–15]. Plotkin et al. [16] reported that a patient with chronic recurrent migratory superficial thrombophlebitis and marked cutaneous hyperreactivity (pathergy) who developed leukocytoclastic vasculitis with recalcitrant leg ulcerations 9 years after the onset of his illness. Cutaneous polyarteritis-nodosa-like lesions and necrotizing panarteritis involving small and medium-sized arteries in the dermis-subcutis junction have also been

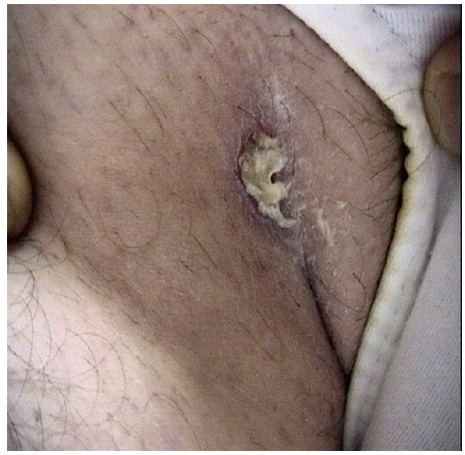

Figure 3: Extragenital ulcers.

reported rarely with BD. Vikas et al. [17] reported that their patients had both venous and arterial involvement, the former with thrombotic angiopathy and the latter with acute vasculitis.

2.2. Systemic Ulcerative Manifestations

2.2.1. Gastrointestinal System Involvement. Gastrointestinal system involvement in BD affects all areas from the esophagus to the anus. Most authors believe that the gastrointestinal system manifestations of BD should be confined to aphthae, which can occur throughout the gastrointestinal system tract. The frequency of gastrointestinal system involvement varies considerably in different studies and also between different countries. In Japan and Korea the prevalence of gastrointestinal system involvement is higher (15–45%), whereas in Turkey and Israel the prevalence is much lower (0–5%). Some patients with inflammatory bowel disease have been included in series of patients with BD. Gastrointestinal system ulcers were most commonly found in the esophagus, terminal ileum, colon and rectum, and no significant difference was noted in the frequency of gastrointestinal system involvement between the two sexes [2, 4]. The symptoms include anorexia, vomiting, dyspepsia, diarrhoea and abdominal pain. The ileocaecal ulcers have a distinct tendency to perforate. Intestinal ulcers in BD are usually multiple and tend to perforate easily, which may lead to an emergency operation [14, 18]. Transmural inflammation may give rise to fistulae. The ileocaecal region is affected frequently, but any part of the gastrointestinal tract may be involved. Distinguishing BD from inflammatory bowel disease may prove challenging. Ulcers are identical histologically to ulcerative colitis; if present, granulomata suggest Crohn's disease. Pathergy, when positive, points to a diagnosis of BD [18, 19].

2.3. Diagnostic Investigations

2.3.1. Laboratory Studies. Although there is no specific laboratory profile to diagnose BD, the key is to obtain maximal history and review of systems with detailed physical examination. A moderate anaemia of chronic disease, a slightly raised neutrophils and/or platelet count is found in around 15% of patients. The erythrocyte sedimentation rate and C-reactive protein are usually moderately elevated but do not correlate well with disease activity. Serum immunoglobulins, especially IgA and IgD, are sometimes elevated with the presence of circulating immune complexes; complement levels might also be high. Autoantibodies such as rheumatoid factor, antinuclear antibody, anticardiolipin and antineutrophilic antibodies are absent. Disease activity may be assessed by elevated status of neopterin, anti-streptolysin-O, α1-antitrypsin and α2-macroglobulin, all of which are the active components of phagocytic system of polymorphonuclear leukocytes. An elevation in the level of β2-microglobulin and myeloperoxidase, generated by activated neutrophils, have also been reported. Cryoglobulinemia, and eosinophilia may occur. HLA analysis should be performed for differential diagnosis in some cases. Abnormalities in the coagulation cascade such as increased levels of fibrinogen, plasminogen activator inhibitor-1 and circulating factor VIII have been described along with reduced fibrinolytic activity. Known thrombophilic factors such as factor V Leiden and prothrombin gene mutations and protein C and protein S deficiency also have been reported to coexist in BD patients by us [19–21].

2.3.2. Skin Tests. Pathergy describes th inappropriately excessive subacute inflammatory reaction to nonspecific injury. It is relatively specific for BD, although it can also be observed in Sweet syndrome, in patients with chronic myeloid leukemia on treatment with interferon-α, erythema elevatum diutinum, pyoderma gangrenosum, and also inflammatory bowel disease such as colitis ulcerosa and Chron disease [2, 4]. The urat crystal test has been found to be more sensitive than the formal pathergy test in the demonstration of abnormal inflammation in BD. The usual response to an intradermal injection of 2.5 mg of urate crystals is an erythematous reaction, maximal at 24 hours and mostly resolved at 48 hours. In BD, the erythematous response is exaggerted, with a greater degree of inflammation present at 24 hours and/or persistance at 48 hours. This test has been reported as having a sensitivity of %61 and a specificity of %100 for the diagnosis of BD. The greater sensitivity of the urate crystal test suggests it has clear potential as an aid to the diagnosis of BD, although a positive test may be difficult to demonstrate in patients on anti-inflammatory drugs [23–25].

3. Diagnosis

In the absence of a universally accepted diagnostic test, the diagnosis of BD remains purely clinical. In 1990, the International Study Group for BD proposed new diagnostic criteria based on the analysis of 914 patients from several countries. For patients to be classified as having BD, the patients must have recurrent oral ulcers plus at least two of the other criteria including ocular involvement, genital

Table 1: Differential diagnosis of Behçet's ulcerations.

Manifestations	Treatments
Oral ulcers	Recurrent oral stomatitis
	PFAPA (Periodic fever, aphthous ulcers, pharyngitis, adenopathy)
	Familial Hibernian fever
	Sytemic lupus erythematosus
	Ulcerative colitis
	Coeliac disease and other malabsorption states
	Iron, B12 and folate deficiency
	Human immunodeficiency virus infections
	Chickenpox
	Hand, foot and mouth disease
	Nicorandil (anal ulcers also reported)
	Bisphosphonates
	Cyclical neutropenia
	Lymphoma
	Bullous skin disease
	Syphilis
	Tuberculosis
	Lichen planus
Genital ulcers	Complex aphthosis
	Reiter's syndrome
	Mouth and genital ulcers with inflamed cartilage (MAGIC)
	Crohn's disease
	Sweet's syndrome
	Erythema multiforme
	Bullous skin disease
	Erosive lichen planus
	Fixed drug reaction
	CMV (in immunocompromised patients)
	Herpes simplex (HSV1)
	Chancroid
	Syphilis
	Scabies
	Tuberculosis
Vasculitic ulcerations	Sweet's syndrome
	Pyoderma gangrenosum
	Erythema multiforme
	Pernio
	Leukocytoclastic vasculitis
	Polyarteritis nodosa
Gastrointestinal ulcerations	Crohn's disease
	Coeliac disease
	Colitis ulcerosa [19, 22]

ulcers, skin lesions (erythema nodosum-like lesions and papulopustular eruptions), or the pathergy test in the absence of an alternative clinical diagnosis. It is important to note that a patient who fails to meet the criteria fully may still have BD [26]. Due to orogenital ulcers tendency to spontaneous healing and well-known morphology, biopsies are rarely performed, and, due to similar histopathological features of all variants of ulcers, histopathological examination has a limited value in the differential diagnosis. Lymphocytes, macrophages, and neutrophils are observed at the base of oral ulcers. The infiltrate is more pronounced around the vessels. Although classified as vasculitis, some studies report that most mucocutaneous lesions in BD do not present typical characteristics of an actual vasculitis. Fibrinoid necrosis in the vessel walls is reported to be very rare. At the periphery of the ulcer base, the infiltrate may penetrate into the epidermis. Some recently published direct immunofluorescence studies report IgM and C_3 deposits in perivascular region with or without granular C_3 deposits at the dermoepidermal junction in the perilesional skin of oral ulcers in BD patients. Also in another study, Wilhelmsen et al. evaluated perilesional skin of 23 oral ulcer patients with direct immunoflourescence and found out the immunocomplexes to be absent [9, 26] (Table 1).

4. Differential Diagnosis

It usually is not difficult to recognize the full-blown syndrome of BD, but the so-called incomplete forms sometimes cause problems. Therefore, other causes of oculomucocutaneous syndromes should carefully be excluded including autoimmune bullous skin diseases, erythema multiforme major, Reiter syndrome, seronegative arthropathies, sarcoidosis, Sweet syndrome, cicatricial pemphigoid, celiac disease, and pemphigus vulgaris. Similarly, herpes simplex virus infection, lichen planus, syphilis, systemic lupus erythematosus, ulcerative colitis, and mixed connective tissue diseases may also cause oral and cutaneous ulcers. Oral ulcers alone should be differentiated from recurrent aphtous stomatitis, erythema multiforme, toxic epidermal necrolysis, syphilis, tuberculosis orificialis, inflammatory bowel diseases, and erosive lichen planus. Genital ulcerations should be differentiated from venereal diseases such as chancroid, syphilis, scabies, and herpes simplex virus infection. Similarly, recurrent orogenital ulcerations are also seen in hypereosinophilic syndrome, myelodysplastic syndrome, Munchausen syndrome (pseudo-BD), pemphigus vulgaris, tuberculosis cutis, and acquired immunedeficiency syndrome [2, 4, 19, 22].

5. Treatment

Treatment of the various symptoms of BD remains controversial because of the heterogenity of the condition, lack of reliable laboratory markers of disease activity, and paucity of controlled clinical trials and unstandardized outcome measures for this disease (Table 2).

5.1. Therapy of Ulcerations

5.1.1. Mucocutaneous Ulcers. In mild forms of the mucocutaneous ulcerations, initial treatment consists of mild diet,

Table 2: Therapy of types/condition and Floor Chart of 1st, 2nd, 3rd line medicine.

(a) Topical treatments of ulcerations

Treatment	Dose	Used as first-line therapy	Used as alternative therapy
Topical steroids	3 times a day topically	Oral and genital ulcers	
%5 Amlexanox paste	4/day topically	Oral ulcers	
Sucralfate suspension	4/day topically for 3 months	Orogenital ulcers	
Triamcinolone acetonide 40 mg ampule	Intralesionally 5 mg/mL	Severe orogenital ulcers	
Lidocaine %2–5	4/day topically as mouthwashes, before meals	Severe and multiple oral ulcers in Behçet's patients with insufficients oral intake by pain,	
Chlorhexidine gluconate rinses %1-2	Topically as mouthwashes	Oral ulcers	
Tetracycline	250 mg in 5 mL water solution, held in mouth for 2 min once a day		Oral ulcers
rhGM-CSF 300 μg ampule	Intralesionally injection in every 2 weeks		Large genital ulcers

(b) Systemic treatments

Treatment	Dose	Used as first-line therapy	Used as alternative therapy
Systemic steroids	5–100 mg/day orally		Orogenital and gastrointestinal ulcerations
Colchicine	0.5–1.5 mg/day orally	Oral and genital ulcers	
Thalidomide	100–300 mg/day orally		Orogenital ulcers
Dapsone	100 mg/day orally		Oral and genital ulcers
Pentoxifylline	300 mg/day orally		Orogenital and leg ulcers
Levamisole	150 mg in 3 doses/day every 2 day $\times$ 1 week		Mucocutaneous ulcers
Penicilline	1.2×10^6 U/3 week		Mucocutaneous ulcers
Azithromycin	500 mg 3 times a week for 4 weeks		Mucocutaneous ulcers
Interferon-α	5 million U/day im or s.c.		Mucocutaneous ulcers
Sulfasalazine	1–3 gr/day orally	Gastrointestinal ulcers	
Surgery			Gastrointestinal ulcers
Combination therapies			
Penicilline + colchicine	Penicilline 1.2×10^6 U/3 week, colchicine 1.5 mg/day		Mucocutaneous ulcers

and avoidance of hard, spicy, or salty nutrients and chemicals. Topical treatment of oral ulcers includes caustic solutions (silver nitrate %1-2, tinctura myrrhae %5–10 w/v, hydrogen peroxides %0.5, and methyl violet %0.5) 1-2×/day, topical antiseptic and anti-inflammatory drugs (amlexanox %5 in oral paste, rebamipine, hexetidine %1, chlorhexidine %1-2 mouth-wash solutions, benzydamine, camomile extracts, and tetracycline mouth-wash) and also glycerine solution 250 mg/5 mL glycerine for 2 min, 4–6×/day, topical corticosteroids (triamcinolon mucosal ointment, dexamethasone mucosal paste, and betamethasone pastilles) 4×/day or during the night or intrafocal infiltrations with triamcinolone suspension 0.1–0.5 mL per lesion, topical anaesthetics (lidocaine %2–5, mepivacaine %1.5, tetracaine %0.5–1 gels or mucosal ointments) 2-3/day, topical sucralfate (suspension, 1 gr/5 mL) 4×/day, 3 months durations as mouthwash, topical aminosalicylic acid (%5 cream) 3×/day[5]. In daily practice, the contents of a tetracycline capsule (250 mg) can be dissolved in 5 mL of water, holding in the mouth for about 2 minutes (four times a day). BD patients with insufficient oral intake caused by pain can be treated with topical lidocaine (2–5%) applications before meals and oral anti-inflammatory rinses containing chlorhexidine gluconate (1-2%) [27–31].

In topical treatment of genital ulcers and cutaneous ulcers, corticosteroid and antiseptic creams can be applied for a short period of time like 7 days. Painful genital ulcers can be managed by topical anaesthetic in cream [5]. Topical sucralfate reduces the healing duration and pain of genital ulcers like oral ulcers. Sucralfate has been used in the treatment of orogenital ulcerations [27]. For severe ulcers, intralesional corticosteroid (triamcinolone acetonide) may be helpful. Corticosteroid injections like triamcinolone 0.1–0.5 mL/lesions can be focally applied in recalcitran ulcerations. Bacanli et al. studied the efficacy of topically applied granulocyte colony-stimulating factor in the treatment of oral and genital ulcers. It decreased the healing time and pain of both ulcers in 6 of 7 patients compared with the pretreatment period. The effectiveness of the treatment, however, did not continue during the posttreatment period [32]. In a randomized, controlled, crossover double-blind trial, zinc sulfate treatment decreased the mucocutaneous ulcerations index after the first month of therapy. After shifting to placebo treatment, the clinical index started to increase but remained significantly lower than levels before therapy [32–34].

In severe forms of the mucocutaneous ulcerations, additional systemic treatment is required. The following drugs have proven beneficial: Corticosteroids (prednisolone, initial dose 30–60 mg/day p.o. for at least 4 weeks) can be administered as monotherapy or in combination with colchicine (1-2 mg/day p.o.), dapsone (100–150 mg/day p.o.), interferon-α (3–12 million IU/3× week s.c.), or azathioprine (initial dose 100 mg/day p.o.). Nonsteroidal anti-inflammatory drugs, like indomethacin (100 mg/day p.o. over 3 months) can be effective on the mucocutaneous lesions. Pentoxifylline (300 mg 1–3×/day p.o.) and oxypentifylline (400 mg 3×/day p.o.) treatment for 1 month induced a remission of oral ulcers. Pentoxifylline decreases superoxide production by neutrophils. High dosage of oral or pulse intravenous steroids may be indicated for large and refractory mouth ulcers larger than 10 mm or when the oropharynx is compromised. Severe mucocutaneous disease and arthritis may be treated with systemic corticosteroids in combination with azathioprine [35–39].

Colchicine (0.5–2 mg/day p.o.) can be used as a second-line alternative treatment. A recent randomized double-blind and placebo-controlled study has shown that colchicine reduces the occurence of genital ulcers among women. Colchicine inhibits the enhanced chemotactic activity of neutophils. Colchicine seldom eliminates oral ulcerations completely, but may reduce to an acceptable level the frequency and severity of oral ulcer [40, 41].

There is little evidence that antibacterials or antivirals are useful in the therapy of mucocutaneous ulcerations.There is some evidence that adjunctive penicillin treatment may enhance the clinical response to colchicine therapy for both orogenital ulcers. It has been proposed, although not proven, that an etiologic relationship exists between streptococcal infection and BD. In an uncontrolled study, benzathine penicillin improved the ulcerative manifestations of disease. Patients with mucocutaneous ulcerations had complete recovery in 5 to 20 days. In a retrospective study, benzathine penicillin had a beneficial effect on oro-genital ulcers. A prospective randomized study compared the efficacy of colchicine with colchicine and benzathine penicillin over 24 months. They reported the effectiveness of benzathine penicillin and colchicine on the mucocutaneous ulcerations, benefits not achieved with colchicine monotherapy. The result of an open study with minocycline treatment for 3 months were reported and it was observed that oro-genital ulcers improved at a rate of %10 to 100 [42–45].

Dapsone (100–150 mg/day p.o.) also inhibits the enhanced chemotactic activity of neutrophils and can be used as an alternative drug to colchicine. Quick relapses have been found after discontinuation of dapsone treatment. Intermittant ascorbic acid treatment (vitamin C; 500 mg/day) is advisable to prevent increased methaemoglobin serum levels. Its use is often complicated by haemolytic anemia, even in patients with normal glucose-6-phosphate-dehyrogenase activity [46].

Interferon-α has been successfully used in the treatment of BD. Its immunomodulatory effect, ability to augment the decreased activity of the patient's natural killer cells, capacity to intibit neovascular proliferation, and antiviral activity have been suggested to explain its action in BD. It was shown to markedly inhibit IL-8 synthesis and secretion form endothelial cells. Interferon-α-2a treatment at dose of 6 million IU/3× week s.c. for 3 months, is an effective alternative treatment, particularly for management of mucocutaneous ulcerations [47].

Azathiopurine (2.5 mg/kg body weight/day p.o.) has been found to be an effective choice in oral and genital ulcers in a randomized, double-blind and placebo controlled study [48].

Cyclosporin A (3 mg/kg/day p.o.) is capable of markedly ameliorating mucocutaneous ulcers. But, it should be reserved for the most severe patients because of its significant long-term adverse effects [49].

Methotrexat (7.5–20 mg/1× weekly p.o. over 1 month) is able to induce an improvement of a severe mucocutaneous ulcers [50].

Thalidomide (100–300 mg/day orally, optimal dose 100 mg/day in the evening for 8 weeks) has been approved for the treatment of male and sterilised or postmenapausal women with BD. Thalidomide was shown to selectively inhibit TNF-α synthesis by monocytes. In a randomized, double-blind placebo-controlled study with 63 BD patients, a remission of oral and genital ulcers was detected in %24 of the patients over 2 months. During the 6-month treatment, %30 of the patients with BD remained free of mucocutaneous ulcerations. Discontinuation of the treatment results in oro-genital ulcers recurrences; therefore, a maintenance treatment with 50 mg/day to 50 mg twice a week is recommended. Thalidomide is often highly effective at reducing the frequency and severity of mucocutaneous ulcerations resistant to colchicine. However, its widespread use is clearly limited teratogenic and neuropathic complications. The risk of developing irreversible peripheral neuropathy is thought to increase in a dose-dependant fashion, and so thalidomide should be recommended at the lowest dose possible to control symptoms, for example, 50 mg daily or 100 mg

3 times a week. Since thalidomide can be sedating, it is best taken at night [51–53].

Recent studies of anti-TNF agents such as infliximab (i.v. 0, 2, 4, 8 months), and etanercept (s.c. twice a week) have shown favorable results. Infliximab was also efficacious in extraocular manifestations, such as oral and genital ulcers in the majority of patients in three self-controlled studies. Almoznino et al. described a case of Behçet's syndrome in a 48-year-old woman whose oral ulcers were resistant to a wide range of topical and systemic treatments and remained unchanged for 7 weeks. Administration of a single dose of infliximab resulted in complete remission and recovery of the mouth aphtae within 7 days. Additional case series and case reports suggested that patients with severe mucocutaneous lesions exhibit rapid and good responses to infliximab administration, mostly using 5 mg/kg or 3 mg/kg. Some patients with orogenital ulcers unresponsive and/or intolerant to conventional treatments remained disease-free for the first time in years. Almost all patients were resistant to conventional treatments, and were treated with infliximab alone or as an add-on therapy. In a double-blind, placebo-controlled study of 40 male patients with BD, Melikoglu et al. reported that etanercept (25 mg twice/week, for 4 weeks) was effective in suppressing most mucocutaneous lesions. The drug had a clear effect on oral ulcers, and the response was evident as early as the first week. Almost half of the patients receiving etanercept were free of oral ulcers at the end of the study compared with 5% of the placebo group. Although, the drug decreased the number of genital ulcers and arthritis episodes during the treatment period, the difference was not significant [54, 55].

Lactobacilli, which have antiinflammatory activity, may be useful in some diseases, particularly in inflammatory bowel disease. In a study aimed at evaluating the efficacy of lactobacilli lozenges in the management of oral ulcers of BD, a significant decrease in the mean number of ulcers was found following treatment, especially among women [56].

5.1.2. Gis Ulcerations. The treatments used for inflammatory bowel disease including sulfasalazine and corticosteroids are also useful for the gastrointestinal lesions of BD. The dose of corticosteroids depends on the severity of lesions. Bowel rest is obligatory in patients with an acute abdomen and bleeding. Surgery is considered for patients with bowel perforation and persistant bleeding. Invasive surgical procedures often result in excessive infiltration of inflammatory cells into the treated tissues, with subsequent anastomotic leakage. To prevent this complication, undetermined doses of corticosteroids are given to the patients for several days after surgery. Even if the operation is successful, repeated operation because of recurrence is required in about half of the patients. There was a suggestion that azathiopurine use was helpful. The rate of reoperation can be lowered by using azathopurine in patients with entero-BD. Intra-arterial steroid injections into the mesenteric arteries were found to be effective in severe entero-BD unresponsive to conventional treatments [57] (Table 3).

Table 3

Manifestations	Treatments
Oral ulcers	1st line: Topical triamcinolone acetonide, prednisolone, amlexonax, anti-inflammatory rinses, topical anaesthetics 2nd line: Topical sucralfate, aminosalicylic acid, caustic solutions, oral tetracycline solutions, colchicine, levamisole, thalidomide, pulse methyl prednisolone, intralesional trimcinolone acetonide 3rd line: Cyclosporine, azathiopurine, methotrexate, chlorambucil, infliximab, etanercept, plasmapheresis-apheresis, zinc sulphate, penicilline, azithromycin, minocycline, dapsone, pentoxifylline, interferon-α
Genital ulcers	1st line: Topical triamcinolone acetonide, sucralfate, oral colchicine, azathiopurine, dapsone, prednisolone 2nd line: Levamisole, interferon-α, methotrexate, thalidomide, cyclosporine A
Vasculitic ulcerations	1st line: Topical bethametasone, oral colchicine, azathiopurine, dapsone, prednisolone 2nd line: Levamisole, dapson, interferon-α, thalidomide, azitromycin, pentoxifylline
Gastrointestinal involvement	1st line: Sulfasalazine, corticosteroids 2nd line: Cyclosporine A, azathiopurine, surgery 3rd line: İntraarterial corticosteroids injections

Behçet's disease is a multisystemic inflammatory disease of unknown etiology which usually occurs as ulcerative manifestations: aphthous stomatitis and genital ulcerations. At the beginning of the disease the diagnosis is uncertain because of various clinical manifestations and a long period up to the full clinical picture manifestation. Since neither the laboratory data nor the histopathological signs are truly pathognomonic in Behçet's disease, the differential diagnosis depends on a careful evaluation of the medical history and meticulous physical examination to detect concomitant systemic manifestations. Sometimes, some laboratory test may help establish the diagnosis. Subspecialty referral to ophthalmology, rheumatology, neurology, and gastroenterology should be considered when indicated.

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Research Article

Stratification of Highest-Risk Patients with Chronic Skin Ulcers in a Stanford Retrospective Cohort Includes Diabetes, Need for Systemic Antibiotics, and Albumin Levels

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Chronic nonsurgical skin wounds such as venous stasis and diabetic ulcers have been associated with a number of comorbid conditions; however, the strength of these associations has not been compared. We utilized the Stanford Translational Research Integrated Database Environment (STRIDE) system to identify a cohort of 637 patients with chronic skin ulcers. Preliminary analysis ($n = 300$) showed that 49.7% of the patients had a poor prognosis such as amputation or a nonhealing ulcer for at least a year. Factors significantly associated ($P < 0.05$) with these outcomes included diabetes mellitus, chronic kidney disease, peripheral neuropathy, peripheral arterial disease, and need for systemic antibiotics. Patients with poor outcomes also tended to have lower hemoglobin levels ($P = 0.01$), higher WBC levels ($P < 0.01$), and lower albumin levels ($P < 0.01$). On multivariate analysis, however, only diabetes mellitus (OR 5.87, 1.36–25.3), need for systemic antibiotics (OR 3.88, 1.06–14.2), and albumin levels (0.20 per unit, 0.07–0.60) remained significant independent predictors of poor wound-healing outcomes. These data identify patients at the highest risk for poor wound-healing and who may benefit the most from more aggressive wound care and treatment.

1. Introduction

Chronic wounds cause a significant morbidity and financial expense in the United States, affecting 6.5 million patients with estimated treatment costs of $25 billion per year [1, 2]. Venous leg ulcers, the most common type of chronic skin wound, alone affect more than 1 million US citizens per year with an associated annual cost of $2.5 billion [3, 4]. Of these patients, only 50% effectively heal, affecting both quality of life and requiring long-term care. Moreover, in the diabetic population, numbering approximately 17 million patients in the United States, nonhealing foot ulcers can become life threatening if infected and confer a 15% increased risk of amputation compared to the general population [5–8].

A number of factors have been documented in the medical literature which predispose patients to poor wound healing. These include underlying diseases such as diabetes mellitus, venous insufficiency, peripheral arterial disease, tobacco smoking, low serum albumin, and inflammatory conditions (such as pyoderma gangrenosum) among others [8–15]. According to a recent report, chronic kidney disease (CKD), hypertension, and myocardial ischemia may also be associated with increased risk of developing foot ulcers including severe ulcers that necessitate amputation [16, 17]. Additionally, there are reports of higher rates of malnutrition and deficiencies of vitamins and minerals such as zinc in patients with chronic venous leg ulcers compared to the general population [18–20].

Although risk factors for the development of skin ulcers have been identified, clinical indicators of poor wound healing are less well studied. There are no large, well-controlled studies on independent impact of multiple risk factors including demographic, clinical, and laboratory markers to prognosticate outcome. In this study, we seek to stratify the level of risk which comorbidities and laborators values may confer on poor wound healing. If these markers can be

identified, at-risk patients can be better identified and treated in a way that more aggressively addresses their comorbid medical condition, thus increasing the likelihood for effective wound healing.

2. Methods

2.1. Cohort Selection. Following Stanford Institutional Review Board approval, we employed a retrospective cohort study design using the Stanford Translational Research Integrated Database Environment (STRIDE) system. STRIDE includes data from Stanford University Hospital and Clinics and the Lucile Packard Children's Hospital (LPCH). It encompasses 13 years of clinical documents with information on medical diagnoses (including classification by ICD-9 codes), laboratory values, medications, radiology reports, pathology reports, and free text of progress notes, consultations, and discharge summaries. Previously, STRIDE has been successfully utilized to construct cohorts based on ICD-9 codes and laboratory values. In particular, the Stanford Dermatology Department used STRIDE to identify co-morbid medical conditions associated with transaminitis in psoriasis patients taking methotrexate.

The STRIDE Cohort Discovery Tool was used to select a cohort of patients with chronic skin ulcers. The following ICD-9 codes were used to select the cohort: 707.10–19, 785.4, 454.0, 454.2, and 440.23. These pertain to "unspecified ulcer of lower limb," "ulcer of thigh," "ulcer of calf, "ulcer of ankle," "ulcer of heel and midfoot," "ulcer of other part of foot," and "ulcer of other part of lower limb", respectively. The following text restrictions were used to further ensure that all patients included in the cohort had documentation of skin ulcer in physician-authored clinical notes: "ulcer," "wound," "erosion," "breakdown," and "gangrene." Patients aged 18 or older who had incident cases of skin ulcers between January 1, 2002 and January 1, 2005 were included. In the case of multiple ICD-9 codes meeting inclusion criteria only data on earliest ulcer within our date restrictions was used for the analysis.

2.2. Clinical Data Abstraction. Clinical data on exclusion criteria, outcomes, predictors, and covariates was extracted from STRIDE using its Data Review Tool. The Data Review Tool allows for optimized electronic chart review using string searches and filters based on prespecified criteria.

2.3. Exclusion Criteria. The following exclusion criteria were used based on manual review of clinical charts using STRIDE's data review tool: (1) no confirmation of ICD-9 code diagnosis with clinical documentation, (2) pressure ulcer, (3) oral or mucosal ulcer, (4) primary dermatitis rather than skin wound, (5) ulcer is a primary skin infection or cellulitis, (6) ulcer is actually a primary surgical wound, (7) thrombophlebitis without ulceration, (8) ulcer is actually not an ulcer but a deep vein thrombosis without ulceration, (9) ulcer is actually a fistula, and (10) malignancy within wound.

2.4. Outcome Assessment. The patients were followed for 1 year out from the date of diagnosis as documented in physician-authored clinical notes. They were assessed on chart review for wound outcomes. A healing wound or good wound-healing outcome was defined as a wound that was documented by a physician to have healed within 1 year of followup. A nonhealing-wound or poor wound-healing outcome was characterized as either (a) a wound that had not healed by 1 year of followup, (b) a wound that required amputation, or (c) a wound that required flap reconstruction over the followup time. Patients whose wound status was unknown after 1 year of followup were characterized as lost to followup. These were subdivided into those who died, those who returned to Stanford subsequently but lacked an update on wound status on clinical chart review, or those who were never again seen at SUH. All patients who were lost to followup were excluded from subsequent data analyses.

2.5. Data Collection. Using the STRIDE data review tool, we manually collected data on demographic and clinical variables including laboratory values. In the case of laboratory values with multiple entries over time, only the lab value which was closest to the date of diagnosis of the ulcer within a 3-month time window of date of diagnosis was used. We additionally collected data on age at ulcer diagnosis, sex, race, current smoking, maximum dimension of wound at diagnosis, and preexisting duration of wound at diagnosis. We also collected data on treatments administered for the wound at Stanford University Hospital and Clinics.

2.6. Data Analysis. We conducted bivariate analyses comparing mean (parametric variables) or median (nonparametric variables) values of continuous predictors among healing and non-healing wounds, using ANOVA or Kruskal-Wallis tests as appropriate. The association of dichotomous variables with the outcome was assessed using the χ^2 test or Fisher's exact test for cell counts <5. Finally multiple logistic regression models were used to assess the independent impact of predictors. Only those predictors that were significantly associated with the outcome on univariate analyses were selected for the multivariate regression models. A P value of 0.05 was used for all analyses.

3. Results and Discussion

As illustrated in Figure 1, using the STRIDE cohort discovery tool, a total of 637 patients with skin ulcers who were aged 18 or older were found to have been seen at Stanford University Hospital and Clinics between January 1, 2002 and January 1, 2005. Data for 300 of these patients was manually reviewed in the STRIDE data review tool in preliminary analysis. Out of these, 89 (29.7%) were excluded based on our prespecified exclusion criteria leaving 211 (70.3%) patients. From these, 76 patients (25.3%) were lost to followup before 1 year. The remaining 135 (45%) had complete data on wound outcomes over 1 year and were included in the final data analysis. Out of these 135 patients, 68 (50.3%) patients had wounds that healed within 1 year and 67 patients (49.7%) had wounds

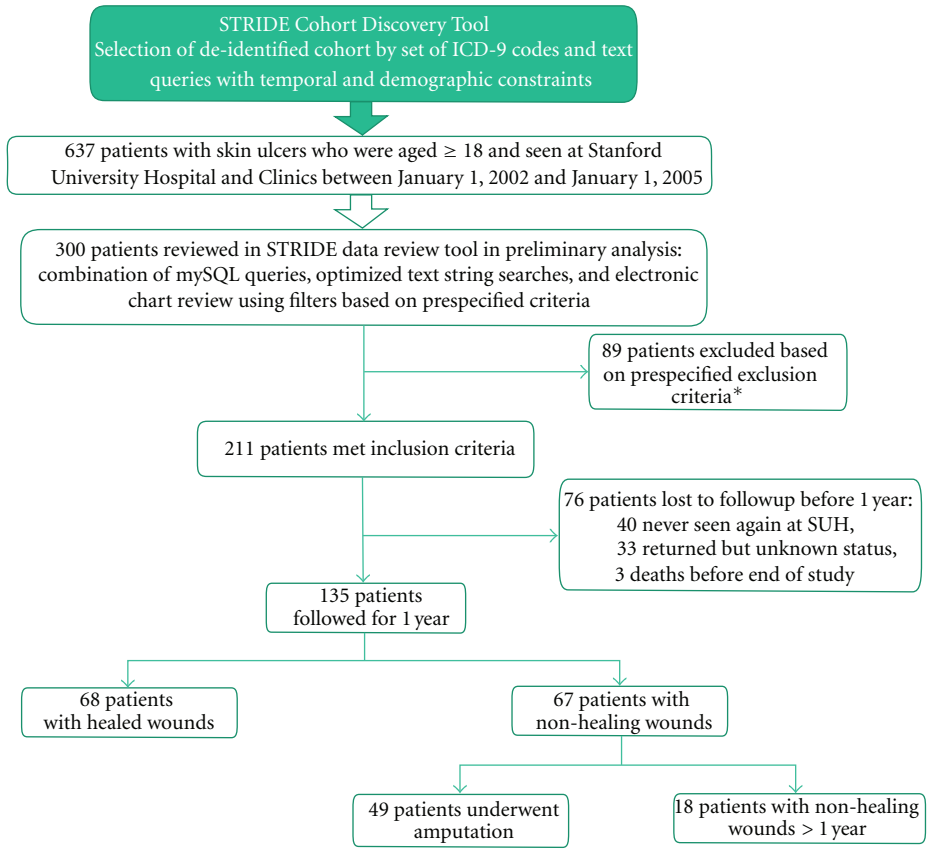

Figure 1: Cohort selection and refinement algorithm and results.

Table 1: Demographics and characteristics of patients with healing and non-healing wounds[1].

	Healing wound	Nonhealing wound	*P* value
Total number of patients	68	67	
Age[2] (years)	63.7 ± 15.6	67.5 ± 14.8	0.21
Gender (% male)	32/68 (47.7%)	34/67 (50.1%)	0.92
Race (% white)	49/68 (73.1%)	47/67 (69.1%)	0.61
Smoking[3] (% current smokers)	8/66 (12.1%)	7/57 (12.2%)	0.83
Size of wound[4,5] (cm)	1.5 (1.0–4.0)	1.7 (1.0–5.5)	0.78
Preexisting duration [6,7] of wound (months)	1.0 (0–2)	1.0 (0–3)	0.11
Location of wound			
Lower extremity (%)	63/68 (94.0%)	66/67 (98.5%)	
Upper extremity (%)	3/68 (4.4%)	1/67 (1.4%)	0.52
Other location (%)	1/68 (1.5%)	0/67 (0%)	

[1]Healing wounds were those that healed within 1 year of followup. Non-healing wounds were defined as wounds that required amputation or did not heal after 1 year of followup. [2]Data expressed as mean ± standard deviation. [3]Data based on smaller subsample of $n = 128$ due to missing data. [4]Data based on smaller subsample of $n = 72$ due to missing data. [5]Size expressed as median ± interquartile range of maximum dimension of wound at presentation. [6]Data based on smaller subsample of $n = 93$ due to missing data. [7]Wound duration expressed as median ± interquartile range.

that did not heal after 1 year. Among these 67 patients with non-healing wounds, 49 patients underwent amputation and 18 patients had wounds that were documented to be non-healing after 1 year. No patients underwent surgical flap reconstruction for the wound. For healing ulcers, median time to healing was 2 months with an interquartile range of 1–5 months (data not shown).

Table 1 shows the demographic and clinical characteristics of patients with healing and nonhealing wounds. On bivariate analyses, the distribution of demographic factors like age or gender did not vary significantly with the outcome. Current smoking did not significantly predict a poor wound-healing outcome. In addition, clinical characteristics of the wound such as preexisting wound duration and size

Table 2: Clinical risk factors[1,2] associated with non-healing wounds[3].

	Healing wound ($n = 68$)	Nonhealing wound ($n = 67$)	P value
Diabetes mellitus (%)	28/68 (41.8%)	55/67 (82.1%)	<0.001*
Peripheral neuropathy (%)	28/66 (42.4%)	51/67 (76.1%)	<0.001*
Renal insufficiency (%)	21/67 (31.3%)	36/67 (53.7%)	0.008*
Peripheral arterial disease (%)	35/67 (52.2%)	48/65 (73.8%)	0.02*
Venous stasis (%)	32/65 (49.2%)	21/63 (33.3%)	0.067
Congestive heart failure (%)	23/65 (35.3%)	24/61 (39.3%)	0.65
Immunosuppression[3] (%)	15/67 (22.4%)	13/68 (19.1%)	0.64
Nondermatologic malignancy[4] (%)	6/68 (8.9%)	3/67 (4.4%)	0.49

[1]Presence or absence of clinical risk factors was determined coincident with or prior to diagnosis of skin wound. They were assessed by text review of charts for physician documentation and query of specific tests such as echocardiogram. Where presence or absence of risk factors could not be determined the information was recorded as missing. Actual numbers of recorded data for each risk factor are presented in the table. [2]Healing wounds were those that healed within 1 year of followup. Non-healing wounds were defined as wounds that required amputation or did not heal after 1 year of followup. [3]Immunosuppression was defined as taking immunosuppressive medications like steroids or chemotherapy or the presence of conditions of immunocompromise such as HIV/AIDS.
[4]Only nondermatologic malignancies co-incident with skin wound were ascertained.
*indicates statistical significance at $\alpha = 0.05$.

Table 3: Laboratory biomarkers[1] in patients with healing and non-healing wounds.

	Healing wound	Non-healing wound	P value
Albumin[2] (g/dL)	3.4 ± 0.4	2.7 ± 0.8	<0.01*
Hb[3] (g/dL)	12.2 ± 1.6	11.3 ± 2.2	0.01*
WBC[4] (cells/mm^3)	8.2 ± 3.0	10.1 ± 3.4	<0.01*
Random glucose[5] (mg/dL)	148 ± 59	167 ± 56	0.13
HbA1c[6] (%)	7.2 ± 1.4	8.1 ± 2.2	0.17

[1]Laboratory measurements closest to diagnosis of ulcer but no more than 3 months prior to diagnosis were recorded.
[2]Based on $n = 88$; [3]Based on $n = 121$; [4]Based on $n = 121$; [5]Based on $n = 89$; [6]Based on $n = 35$.

of wound also were not significantly associated with poor wound healing. Nearly all the skin ulcers studied were located on the lower extremities.

As shown in Table 2, presence of the following comorbidities at the time of ulcer diagnosis was significantly associated with the outcome: diabetes mellitus (OR 6.38), peripheral neuropathy (OR 4.17), renal insufficiency (OR 2.54), and peripheral arterial disease (OR 2.31). While patients with venous stasis ulcers tended to be more likely to have healing wounds, this relationship was not statistically significant at $\alpha = 0.05$. Congestive heart failure, immunosuppression, and concurrent nondermatologic malignancy were not significantly associated with non-healing-wound outcomes.

Clinical lab data was obtained for a limited subset of the patients as detailed in Table 3. Albumin levels were significantly associated with the outcome such that patients with non-healing wounds had a mean albumin level of 2.7 compared to a mean albumin level of 3.4 for patients with healing wounds ($P < 0.01$). Hemoglobin levels were also significantly lower among patients with non-healing wounds ($P = 0.01$). WBC counts were significantly higher among patients with non-healing wounds ($P < 0.01$). Random glucose ($P = 0.13$) and HbA1c levels ($P = 0.17$) were not significantly different according to wound outcome.

Table 4 details the therapeutic interventions administered to patients with healing and non-healing wounds. Only the administration of systemic antibiotics and need for wound debridement were associated with poor wound-healing outcome. Clinical chart review of patients requiring systemic antibiotics confirmed that all these patients had developed clinical signs of secondary wound infection according to physician documentation with the exception of 2 cases. The need for surgical wound debridement likely reflected severe wounds that were at higher risk of poor healing.

Table 5 reports the independent association of clinical predictors and lab biomarkers with the outcome using multivariate logistic regression. Only the co-existing presence of diabetes (OR 5.87, 95% CI 1.36–25.3), albumin levels (OR 0.20 per unit, 95% CI 0.07–0.60) and need for systemic antibiotics (OR 3.88, 95% CI 1.06–14.2) remained independently predictive of the outcome after multivariate adjustment.

4. Conclusion

To our knowledge this is the first large study to quantify the independent impact of multiple clinical risk factors and lab biomarkers on wound outcomes in chronic skin ulcers.

Based on our preliminary analysis of 135 patients, we found that a high number of patients with chronic skin ulcers (49.7%) seen at a tertiary care center suffered extremely poor wound-healing outcomes (amputations or non-healing wound after 1 year of followup). 36.3% of these chronic

Table 4: Therapeutic interventions in patients with healing and non-healing wounds.

	Healing wound ($n = 68$)	Nonhealing wound ($n = 67$)	P value
Topical treatments (%)	66/68 (97.1%)	63/67 (94.0%)	0.44
Systemic antibiotics (%)	38/68 (55.9%)	55/67 (82.1%)	0.001*
Wound debridement (%)	15/68 (25.9%)	29/67 (43.3%)	0.008*
Peripheral revascularization (%)	16/68 (23.5%)	18/67 (26.9%)	0.65
Compression stockings (%)	4/68 (5.9%)	6/67 (8.9%)	0.53
Venous ablation or stripping (%)	5/68 (7.4%)	0/67 (0%)	0.058
Skin graft (%)	4/68 (5.9%)	6/67 (8.9%)	0.53
Wound vacuum (%)	4/68 (5.9%)	3/67 (4.5%)	0.99
Hyperbaric oxygen (%)	1/68 (1.4%)	1/67 (1.5%)	0.99

[1] Healing wounds were those that healed within 1 year of followup. Non-healing wounds were defined as wounds that required amputation or did not heal after 1 year of followup. [2]Topical treatments included but were not limited to wet-dry dressings, papain ointment, topical antibiotics, silvadene ointment, whirlpool treatment, accuzyme application, and Dakin's soaks. [3]Systemic antibiotics refer to antibiotics administered orally or intravenously. [4]Wound debridement refers to surgical debridement or debridement in physician's office. [5]Peripheral revascularization refers to angioplasty or bypass graft of peripheral arteries. [6]Skin graft referred to human skin autograft or synthetic skin graft/Dermagraft. [7]Hyperbaric oxygen refers to treatment in hyperbaric oxygen chamber.
*indicates statistical significance at $\alpha = 0.05$.

Table 5: Adjusted and unadjusted odds of having a poor wound-healing outcome[1] ($N = 135$).

Predictors	Unadjusted		Adjusted	
	OR[2]	95% CI[3]	OR[2]	95% CI[3]
Diabetes mellitus	6.38*	2.89–14.1	5.87*	1.36–25.3
Peripheral neuropathy	4.17*	1.99–8.76	0.97	0.24–3.91
Renal insufficiency	2.54*	1.26–5.15	1.32	0.42–4.1
Need for systemic antibiotics[4]	3.62*	1.65–7.95	3.88*	1.06–14.2
Peripheral arterial disease	2.31*	1.13–4.72	1.43	0.45–4.52
Albumin (per unit)	0.21*	0.09–0.48	0.20*	0.07–0.60
Hemoglobin (per unit)	0.79	0.65–0.95	1.05	0.73–1.4

[1] Poor wound-healing outcome was defined as wounds that required amputation or did not heal after 1 year of followup. [2]OR refers to odds ratio. [3]95% CI refers to 95% confidence interval. [4]All cases of systemic antibiotic administration were reviewed and found to reflect secondary infection of skin wound with the exception of 2 cases which were excluded from this analysis.
*indicates statistical significance at $\alpha = 0.05$.

wounds resulted in amputations. Among patients who had ulcers that ultimately healed within 1 year, median time to healing was 2 months with an interquartile range of 1–5 months. Demographic and behavioral factors such as age, sex, and current smoking status did not vary significantly for healing versus non-healing wounds. In addition, clinical features of the wound such as wound size and preexisting wound duration also did not significantly predict the outcome.

Of all the known clinical co-morbidities for poor wound outcomes such as diabetes, peripheral neuropathy, renal insufficiency, and peripheral arterial disease, the strongest association for poor outcome was diabetes. This was confirmed on multivariate adjustment indicating that diabetes confers additional risk for poor wound healing independent of peripheral neuropathy, macrovascular disease, or renal disease. This suggests that microvascular disease is very important and may indeed be the most critical factor in the pathogenesis of poor wound healing among diabetics, although it is difficult to draw causal conclusions based on this observational data. If validated by other clinical studies, this finding would indicate the need for further research to develop therapies targeted to diabetic microvascular disease.

Several lab biomarkers were significantly predictive of poor wound healing on the univariate analysis including hemoglobin, WBC count, and albumin levels. The association of WBC levels likely reflects underlying inflammation or infection while that of hemoglobin levels with wound healing suggests that local oxygen supply to a wound site through erythrocytes may be an important factor in wound healing [21–23]. However, these relationships did not remain significant on controlling for other factors.

Only low albumin levels remained significantly associated with poor wound healing upon multivariate adjustment. Hypoalbuminemia could be secondary to underlying malnutrition which would also cause poor wound healing [12]. Alternatively, the association of low albumin levels with wound healing may reflect a systemic inflammatory state in patients who go on to develop poor wound outcomes. Although there was insufficient data on ESR and CRP in this cohort, future work could focus on analyzing the role of systemic inflammation on wound healing using

these markers. Additionally, future analyses with a larger sample size could also help reveal significant independent associations between hemoglobin levels or WBC levels with wound outcomes since limited sample sizes make it difficult to ascertain their role in wound healing in our study.

In addition to lab biomarkers and clinical comorbidities coexistent with skin ulcers, the secondary infection of these wounds and need for systemic antibiotics were a significant prognosticator of poor outcome. This strongly emphasizes that the prevention of wound infection is critical to avoiding poor outcome such as amputation in skin ulcers. In this context, early or perhaps even prophylactic antibiotic use among high-risk patients (for instance those with diabetes) may be warranted.

There were several limitations of the present study including its observational design and use of ICD-9 codes to designate skin ulcers. However, rigorous chart review to confirm clinical documentation of a skin ulcer by a physician was done in order to ensure that all those included in the study had actual skin ulcers that met our rigorous inclusion and exclusion criteria. Regarding the observational design, while an attempt was made to control known demographic, clinical, and lab risk factors, there may still be unmeasured confounding. Additionally, there was a high loss to followup in our data largely due to the nature of retrospective review of patient charts. While some patients were true losses to followup in that they did not return to Stanford or died, there were several for whom no followup data on their skin ulcers was noted. The large losses to followup from these sources introduce the possibility of selection bias in our results. Future work may include a sensitivity analysis to examine the impact of that loss to followup on our study results. Another problem associated with electronic chart review is a high rate of missing data on lab and other covariates. Since we limited the selection of lab values to those within a clinically meaningful time window in relation to the diagnosis of skin ulcers, our analyses on lab biomarkers of wound healing were limited by small sample sizes.

In the future, this analysis will be extended to additional patients discovered through the cohort discovery tool. This will increase the power for a prognostication model that will incorporate a wide range of both clinical and lab biomarkers. This will allow us to comprehensively risk stratify patients to identify those who would most benefit from early and aggressive wound care therapies such as hyperbaric oxygen, wound vacuum, or skin grafting [24–26].

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