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Neutrophil activity in chronic venous leg ulcers—A target for therapy?

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Abstract

Chronic venous leg ulcers (CVLUs) affect approximately 600,000 people annually in the United States and accrue yearly treatment costs of US$2.5–5 billion. As the population ages, demands on health care resources for CVLU treatments are predicted to drastically increase because the incidence of CVLUs is highest in those ≥65 years of age. Furthermore, regardless of current standards of care, healing complications and high recurrence rates prevail. Thus, it is critical that factors leading to or exacerbating CVLUs be discerned and more effective, adjuvant, evidence-based treatment strategies be utilized. Previous studies have suggested that CVLUs' pathogenesis is related to the prolonged presence of high numbers of activated neutrophils secreting proteases in the wound bed that destroy growth factors, receptors, and the extracellular matrix that are essential for healing. These events are believed to contribute to a chronically inflamed wound that fails to heal. Therefore, the purpose of this project was to review studies from the past 15 years (1996–2011) that characterized neutrophil activity in the microenvironment of human CVLUs for new evidence that could explicate the proposed relationship between excessive, sustained neutrophil activity and CVLUs. We also appraised the strength of evidence for current and potential therapeutics that target excessive neutrophil activity.

Chronic venous leg ulcers (CVLUs) pose a significant health and economic burden due to their high prevalence and recurrence rates. They comprise the largest single group of leg ulcers treated in wound care clinics in the United States.[1] Moreover, it has been estimated that up to 1 in 20 adults in all westernized countries are affected by venous ulcerations, either open or healed.[2–4] CVLUs can be traumatic for patients because of pain, reduced mobility, decreased quality of life, and health care costs related to protracted treatments. Current statistics show that approximately 15% of venous ulcers never heal and that recurrence occurs once or numerous times in up to 71% of cases,[5,6] which contributes to annual US treatment cost estimations of US$2.5–5 billion.[7–11] In addition, these costs are predicted to escalate because the incidence of CVLUs increases in persons aged ≥65 years, which is a US population segment expected to grow to approximately 71 million by 2030.[12,13] These dramatic health care statistics beg for a clearer understanding of the microprocesses that contribute to the development of CVLUs in patients with chronic venous insufficiency (CVI). Elucidating the pathobiology of CVLUs can inform the development of adjunct therapies to facilitate the healing of these recalcitrant wounds or help prevent their recurrence.

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Although the definitive link between CVI and CVLU is unclear, many acknowledge that a consistent feature of venous ulcer formation is chronic inflammation associated with the trapping of activated leukocytes in limbs with venous dysfunction.[14–18] Studies have shown increased neutrophil degranulation in all clinical stages of venous disease evident by enzyme-linked immunosorbent assay (ELISA) testing for plasma neutrophil elastase[19,20] and lactoferrin.[20] In addition, it has been showed that high numbers of activated neutrophils exist in the microenvironment of chronically inflamed ulcers secreting excessive amounts of proteases that can cause tissue destruction and persistent inflammation that delay advancement to subsequent healing stages.[2,21–23] Findings from collective studies suggest that both a sustained systemic and local inflammatory response involving prolonged neutrophil activation is occurring in patients with CVI. This article briefly reviews (1) the function of neutrophils in wound healing; (2) studies in the past 15 years (1996–2011) that have characterized neutrophil activity in the microenvironment of human CVLUs; and (3) therapeutics to target excessive neutrophil activity.

NEUTROPHIL FUNCTION IN WOUND HEALING

In the normal wound healing process, neutrophils are one of first cells to arrive at the site of tissue injury and have three primary actions: (1) sterilizing microbes; (2) producing molecular signals to limit the amassing of additional neutrophils; and (3) initiating an anti-inflammatory, tissue restorative process involving macrophages and epithelial cells. However, these actions require that neutrophils first migrate from the circulatory system to the wound site, a process that occurs within minutes of injury.[24] After moving into the tissues, there is a burst of transcriptional activity that results in the neutrophilic generation of chemokines and cytokines important for chemotaxis of additional neutrophils, macrophages and T cells to the injured site, and for the control of their inflammatory responses.[24,25] Interestingly, findings have differed among some studies examining the role of neutrophils in acute wound repair using animal models. For example, Simpson and Ross tested an antineutrophil serum in guinea pigs and reported findings suggesting that a neutrophil response is not necessary for normal wound healing.[26] Conversely, Dovi et al. reported accelerated wound closure in neutrophil-depleted mice and suggested that neutrophils may delay normal wound closure.[27] In studies of human wounds that fail to heal, such as CVLUs, it has been proposed that when signals from the inflammatory microenvironment are aberrant, and neutrophil influx continues unabated, neutrophils become resistant to apoptosis, the normal tightly regulated system of inflammation resolution fails, and tissue destruction occurs (Figure 1).[28]

Neutrophils are phagocytes that engulf microorganisms present in the wound and kill them using a variety of antimicrobial substances such as oxidants and proteases. These substances are meant to be protective; however, they can contribute to the development of CVLUs by leading to further tissue damage when produced at high levels (Figure 1). During phagocytosis, neutrophils generate copious amounts of reactive oxygen species (ROS) by way of respiratory burst to kill pathogens.[29] Apart from killing pathogens, ROS in the wound fluid help drive redox signaling.[30] Excessive ROS are deleterious for the regenerating host tissue. This is particularly apparent in chronic wound situations, and might as well underlie the persistent tissue-destroying nature of such wounds. Neutrophils in patients with chronic venous disease inappropriately produce more oxygen free radicals (ROS) as a result of amplification of a calcium-dependent signal pathway.[31] In addition, increasing evidence of high iron levels in venous leg ulcer tissue suggests that iron is playing an active role in perpetuating tissue damage in venous ulceration by augmenting local inflammation.[31,32] The iron-mediated Fenton reaction may be another mechanism of excessive tissue damage in CVLU.

In addition to producing high levels of ROS, neutrophils also release many types of proteases during the repair process. Although the primary function of the proteases is to help kill and degrade microbes, they can also be used by the neutrophil to degrade components of the endothelial basement membrane. This can aid in neutrophil movement from the circulation, into the tissue, and to the site of injury. The proteases also contribute to the debridement of necrotic tissue at the wound site. There are two main classes of proteases released by neutrophils that play a role in wound healing: serine proteases and matrix metalloproteinases (MMPs). Neutrophil-derived serine proteases include cathepsin G, elastase, and proteinase 3. Of the MMPs present in neutrophil granules, MMP-2, MMP-8, and MMP-9 have been studied the most frequently in the context of wound repair. Clinically, these proteases are important because they are responsible for the added damage to host tissues that can be caused by neutrophils when released into the extracellular space at high levels. While neutrophil-derived proteases have important functions during the normal repair process, there is a great deal of evidence suggesting that high levels of proteases can be detrimental to the repair process.

STUDIES PROFILING NEUTROPHILS, PROTEASES, AND PROTEASE INHIBITORS IN HUMAN CVLUs

Over the years, numerous studies have evaluated the microenvironment of human chronic wounds of various origins in an effort to characterize the molecular and cellular components associated with poor healing. A vigorous neutrophil response and high levels of neutrophil-derived enzymes were frequently reported.[21,33–41] However, to provide a more succinct, current analysis focusing specifically on CVLUs, this brief review will include observational or intervention studies within the past 15 years that have analyzed human CVLU wound fluid or tissue for neutrophil activity and/or other proteolytic enzymes. A search was conducted with PubMed, Medline, and Cochrane databases using combinations of the terms “neutrophils,” “enzymes,” “leukocytes,” “matrix metalloproteinases,” “proteases,” “proteinases,” “proteolytic activity,” “MMPs,” “microenvironment,” “human chronic wounds,” and “chronic venous leg ulcers.” Review articles were also searched.

Of the 24 studies identified using the chosen criteria, several evaluated the expression and activity of various MMP classes, including gelatinases (MMP-2, MMP-9), collagenases (MMP-1, MMP-8), stromelysins (MMP-3, MMP-10, and MMP-11) and membrane type MMPs (MT1-MMP, MT2-MMP) (Table 1). Elevated MMP levels have been associated with chronic wound types in previous studies.[33,41–43] In general agreement, some researchers included in this review reported that select MMPs were up-regulated in the microenvironment of CVLUs.[22,38,44–51] For example, levels of MMP-9, which is thought to be either neutrophil- or macrophage-derived in open non-healing ulcers,[52] were found to be significantly higher in CVLUs than acute wounds and higher levels were associated with a clinically worse wound.[53,54] Other studies reported similar data regarding MMP-9 activity[45,55] and that diminishing MMP-9 expression coincided with healing.[41,54,56] Mirastschijski et al. found that although MMP-9 activity did not differ significantly between acute and chronic wounds, MMP-9 was concentrated in inflammatory cells of the chronic ulcer bed, while in acute wounds, it was predominantly expressed by the advancing epithelium.[40] They posit that a persistence of MMP-9 in the ulcer bed may be degrading the extracellular matrix (ECM) and depriving keratinocytes at the wounds edges from stimulatory cell-matrix interactions.

Neutrophils contain large amounts of MMP-8 and MMP-9 in their granules[57] and are hypothesized to be principal contributors of MMP-2 to the wound environment.[41,42,58] Although MMP-9 and MMP-2 are believed to be essential for wound healing to occur when inflammation has subsided,[59] some suggest that if significantly increased levels of MMP-2

and MMP-9 are present before inflammation has subsided, the excessive proteolytic milieu will continue to degrade key elements for normal healing to ensue, such as protein compounds of the ECM.[53,60]

Studies in this review that evaluated MMP-8 reported increased levels and expression in tissues biopsied from CVLUs,[45] significantly higher levels in fluid from chronic ulcers compared with healing wounds,[39,54] and that MMP-8 was strongly expressed in all chronic wounds evaluated, but not in acute wounds.[61] Somewhat to the contrary, Nwomeh et al.[39] found a preponderance of MMP-8 in both healing wounds and nonhealing ulcers.

Four studies quantified total MMPs and found higher levels in ulcerated tissue compared with adjacent tissue,[45] higher levels in chronic wound fluid compare with acute wound fluid,[38] higher levels in tissue from ulcers that failed to heal when compared with those that healed,[62] and higher total MMP activity in ulcers than in normal skin.[62]

In addition to quantifying MMPs, three studies assessed tissue inhibitors of MMPs (TIMPs) because an imbalance between MMPs and TIMPs has been associated with ECM breakdown and thus, the pathogenesis of chronic wounds.[60,63] These studies reported lower levels of TIMP-1 in chronic wound fluid compared with acute wound fluid,[38] significantly lower levels in chronic ulcers compared with healing wounds[39] and that CVLU fluid reduced TIMP-1 levels in vitro significantly more than acute wound fluid.[64]

Along with MMPs, human neutrophil elastase (HNE) was measured in several of the studies reviewed. Neutrophil elastase has broad specificity and has been found to be present in several chronic wound types.[65–67] If uncontrolled, HNE can have damaging effects on a number of ECM proteins, cytokines and growth factors, and cell surface receptors.[66,68] Studies included in this review evaluating HNE reported high levels in CVLU fluid,[37,46,54] an up-regulation of HNE associated with ECM degradation in acute wounds of aged subjects and CVLUs,[69] and higher levels in chronic wounds compared with acute wounds[54,70] that were associated with degradation of growth factors[68] and ECM glycoproteins.[71] Furthermore, lower levels of HNE, along with MMP2, MMP12, and the proinflammatory cytokines IL-1β and IL-8 were associated with initiation of CVLU healing.[70] One study by Weckroth et al.,[48] however, reported low activity of HNE and cathepsin G of neutrophil origin in CVLU fluid.

Two of the studies reviewed quantifying neutrophil activity in wound fluid, reported strikingly elevated levels of a neutrophil marker of infiltration, myeloperoxidase (MPO), in CVLU fluid,[22] and markedly increased MPO levels in CVLU fluid compared with acute wound fluid.[55] Moreover, Lundqvist et al. determined that there were significantly higher absolute numbers of neutrophils and higher levels of α-defensins, an antimicrobial peptide in the azurophil granules of neutrophils in CVLU fluid and tissues when compared with acute wound fluid.[72] Although defensins are secreted into the phagolysosome following phagocytosis of bacteria, they may be released extracellularly from neutrophils. Importantly, recent findings reveal that excessive levels of defensins initiate proinflammatory,[73] and possibly cytotoxic actions[74] because of their participation in chemotaxis and activation of antigen-presenting cells.[75]

One of the studies included in the present review used multiplexed antibody microarray profiling to compare 48 different proteins in wound fluid from CVLUs and acute wounds.[76] No significant differences in levels of proteinases and antiproteinases, growth factors, angiogenic factors, or inflammatory cytokines were detected, contradicting the majority of earlier studies, but the injury-induced protein S100A8/A9 was lower in CVLU wound fluid compared with healing wounds. Although the implications of this finding are unclear, the

authors suggest that it may reflect a difference in inflammatory cell composition between CVLUs and healing wounds.[76]

Determining if there are parallel findings across studies examining neutrophil activity in the microenvironment of human CVLUs can be challenging because of variations in design and methodology. For example, different wound fluid collection methods may not produce comparable outcomes.[77] Similarly, variations in inclusion/exclusion criteria and CVLU diagnostic criteria complicate comparisons. Additionally, attaining a sample size adequate enough to generate statistically sound results continues to be a challenge for all human wound studies. However, the collective findings from the majority of studies reviewed for this paper provide evidence that imbalances in the expression and control of proteolytic enzymes are involved in the pathogenesis of CVLUs and that high levels of invading neutrophils are primary sources of several key proteases identified in these problematic wounds. So, how should these data be translated to clinical practice, or should they?

DIAGNOSTIC PROTEASE TOOLS

Several advisory panels assigned the task of creating evidence-based guidelines for treating chronic wounds such as CVLUs have recognized that a growing body of evidence supports a relationship between high wound fluid protease levels and nonhealing wounds.[78–80] Some have endorsed using protease levels as biochemical markers to guide treatment.[78,79,81,82] However, because clinical observations alone cannot detect high protease activity in the microenvironment of CVLUs, a simple diagnostic tool to quantify protease levels within the wound would help clinicians determine if appropriate management strategies designed to dampen excessive protease activity are indicated. One such tool (WoundChek Protease Status—Systagenix) has recently been brought to the market. It is a visually read immunochromatographic test that quantifies neutrophil-derived protease (MMPs and elastase) activity within 15 minutes using wound fluid swab samples. In addition, the World Union of Wound Healing Societies consensus document regarding the role of proteases in wound diagnostics has proposed an algorithm to determine when to use the point-of-care protease test in clinical practice.[79] Together, the point-of-care diagnostic tool and the proposed algorithm are cost-effective, personalized treatment approaches to CVLU care because they help select the patients who are most likely to benefit from protease inhibiting therapies and they help determine when to start and stop their use.

PERSPECTIVES FOR THERAPY

The mounting data showing high neutrophil concentrations and excessive levels of active neutrophil-derived proteases in the microenvironment of CVLUs are guiding the development of novel therapeutics for treatment. (Table 2) Interestingly, the benefits of compression therapy, the gold standard for CVLU treatment, have been linked to its ability to reduce excessive levels of MMP-3, MMP-8, and MMP-9 in CVLU tissue.[45] Additionally, an effect of negative pressure therapy to stimulate wound healing may be to diminish protease and proinflammatory mediator activity.[83–85] However, adjunct therapies to conventional care are needed to improve healing rates, especially for the many patients who cannot tolerate or do not respond well to compression therapy.

The testing of therapies to quell the damaging effects of high proteolytic activity has included topical doxycycline, which has been shown to be an inhibitor of MMPs.[86–89] The inhibitory action of doxycycline on these zinc-dependent proteases is likely due to its ability to disrupt the active site, and not through degradation of the proteases.[89] Studies of human and animal disease models characterized by high MMP activity such as osteoarthritis and periodontitis report that doxycycline and other tetracyclines not only reduce MMP catalytic activity, but suppress MMP synthesis when administered topically or systemically.[87,90–94]

Although there are only a few studies that have examined the effect of tetracycline derivatives on elevated levels of neutrophil-derived MMPs in chronic wounds, the findings are compelling. For example, when a topical nonantimicrobial tetracycline (chemically modified tetracycline) was applied to the wounds of diabetic rats, a dose-dependent effect was noted; there was decreased activity of MMP-9 and MMP-8 and increased granulation tissue when compared with untreated diabetic rat wounds.[94] In a more recent study, Chin et al.[95] reported that doxycycline reduced MMPs (collagenases: MMP-1, MMP-8, and MMP-13; gelatinases: MMP-2 and MMP-9) in fluid from human diabetic foot ulcers in a dose-dependent manner. The data also revealed that topical doxycycline therapy (1% gel) decreased levels of HNE and significantly increased ulcer healing when compared to ulcers treated with hydrogel with no adverse effects. Topical therapies containing tetracycline derivatives are associated with fewer complications when compared with oral forms, which can elicit allergic reactions, systemic reactions, and photosensitivity.[96] Therefore, topical doxycycline may be indicated for chronic wounds such as CVLUs to inhibit select MMP activity, but its effectiveness for this purpose must first be tested in larger randomized clinical trials.

Other topical approaches for sequestering, removing, or inactivating excessive proteases involve mechanism-based wound dressings that have showed variable degrees of effectiveness.[97,98] A few of the current designs include oxidized regenerated cellulose and collagen acting as a competitive substrate for wound fluid proteases,[52] nonocrystalline silver-coated high density polyethylene,[99] peptide,[100] and ionically derivatized dressings of cotton,[101] bacterial cellulose and collagen[70] and oleic acid, a potent, nontoxic selective inhibitor of HNE, bound to cotton.[102] Additionally, wound dressings composed of novel sulfonated hydrogel composites have been shown to be successful at appropriating and/or reducing excessive neutrophil-derived proteases. Sulfonated polymer is capable of binding proteases through electrostatic interactions.[22] In a 2005 study, sulfonated styrene-ethylene-butylenes-styrene triblock polymer (S-SEBS) was exposed to several cations such as silver (Ag+), sodium (Na+), and doxycycline H+. The S-SEBS material containing docycycline H + was found to be superior to a commercial dressing at inhibiting neutrophil-derived MMP-8 and HNE in chronic wound fluids when tested under the same conditions.[103] As expected, the dressing also inhibited bacteria growth. This study showed that the S-SEBS polymer could be tailored to sequester specific proteases. In a more recent study, another sulfonated polymer dressing was also associated with a reduction in the activity of both MMP-8 and HNE.[22] Novel wound dressings that sequester proteases or deliver inhibitors of neutrophil-derived proteases directly in the biochemical environment of CVLUs may be costly, but are reasonable approaches to consider.

In addition to applying specialized dressings to chronic wounds such as CVLUs to modulate neutrophil activity, there are systemic approaches. Two systemic agents that are included in the Guidelines for the Treatment of Venous Ulcers (Level I evidence) supported by the Wound Healing Society and recommended to be used in conjunction with compression therapy are pentoxifylline and micronized purified flavonoid fraction (MPFF).[80] The beneficial action of pentoxifylline in the treatment of CVLUs is associated with improvement to the microcirculation of the legs.[104–106] Specifically, studies have shown a diminishing of leukocyte adhesion to the endothelium, inhibition of synthesis of proinflammatory cytokines, decreasing platelet aggregation,[107] and the attenuation of oxygen free radical formation by leukocytes.[108] In a systematic review to answer the clinical question, “Does pentoxifylline aid the healing of venous ulcers?” Jull et al. concluded that “pentoxifylline is an effective adjunct to compression bandaging for treating venous ulcers and may be effective in the absence of compression.”[107] All six randomized controlled trials reviewed showed increased healing in the pentoxifylline group (400 mg) with no benefit shown for higher doses.

MPFF is a semisynthetic micronized preparation of the γ-benzopyrone family that has been found to have several beneficial protective actions in vein tissue including inhibiting leukocyte trapping and activation, decreasing microvascular leakage and impeding the synthesis of prostaglandins and free oxygen radicals without causing neutropenia.[109–112] The MPFF that has increased the most rapidly in popularity for CVLU treatment is Daflon. However, in spite of the various clinical trials already completed testing Daflon, some have suggested that additional redesigned trials should be implemented that include diabetes and infection as part of the exclusion criteria and larger samples sizes.[113]

Another promising systemic approach that is being explored to target excessive neutrophil activity is n-3 polyunsaturated fatty acid supplementation with eicosapentanoic acid (EPA) and docosahexanoic acid (DHA). Scientific evidence involving cell cultures and animal models of inflammation indicates that metabolism of EPA + DHA generates lipid mediators of inflammation, such as resolvins and protectins, that inhibit transendothelial and transepithelial migration of neutrophils to inflamed tissue sites and reduce cell synthesis and secretion of proinflammatory cytokines that are involved in recruiting and activating neutrophils.[28,114–116] Importantly, resolvins and protectins have also been shown to be key players in the active process of inflammation resolution by enhancing clearance of apoptotic neutrophils by macrophages.[28,114,116,117] Relative to wound healing, new evidence has emerged that oral EPA + DHA supplementation can push an EPA + DHA lipid mediator profile in the acute human wound microenvironment that is associated with decreased levels of MPO, a biomarker of neutrophil activity.[118] However, there have been no studies examining the effects of EPA + DHA-derived lipid mediators such as the resolvins and protectins on excessive neutrophil activity associated with human CVLUs. Therefore, additional studies are needed before EPA + DHA supplementation or molecular mimetics of resolvins and protectins[119] can be considered as adjunct therapeutics to current standard care regimens for CVLUs.

CONCLUSION

Although neutrophil-derived proteases such as HNE and select MMPs are important regulators of efficient wound healing,[120] findings from numerous studies continue to suggest that excessive, prolonged activity is associated with degradation of the ECM, receptors and growth factors that can impede CVLU healing by thwarting cellular migration and attachment. Adjunct treatment strategies that target excessive neutrophil activity may assist with resolving the persistent inflammation associated with CVLUs and improve healing outcomes for patients with these tenacious wounds.

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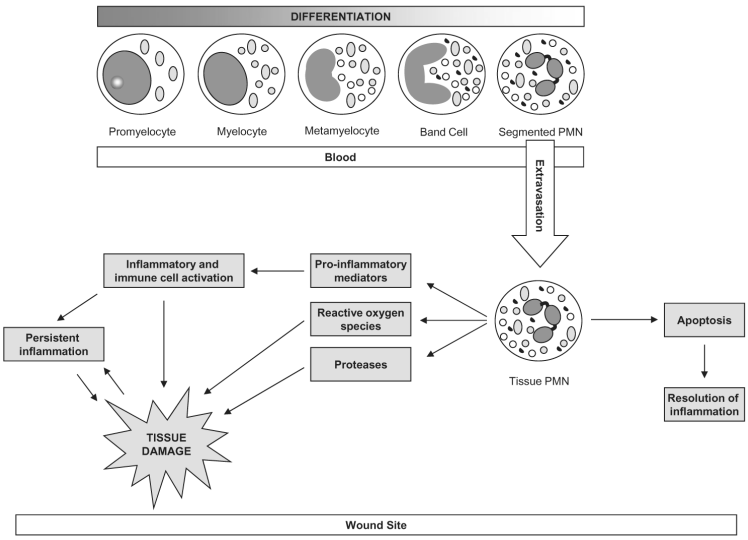

Figure 1.
Unregulated neutrophil activation associated with chronic venous leg ulcers.

Table 1

Trials characterizing proteolytic environment of human chronic venous leg ulcers from 1996–2011

Reference, year	Number of patients	Wound types	Samples	Sample collection method	Results
Grinnell and Zhu, 1996[37]	3	CVLUs—3 Acute—? (mastectomy)	Fluid	CVLUs—occlusive film (Tegaderm)—aspiration Acute—surgical drainages	↑ Levels of HNE associated with cleavage of α2-M and α1-PI and fibronectin degradation in CVLUs ↑ Levels of MMP-9 in CVLUs
Weckroth et al., 1996[48]	16	CVLUs—10 Acute—6 (donor sites: skin grafting)	Fluid	Blunt-end glass microcapillaries	↑ Activity of gelatinase and MMP-1 Low activity of elastase and capthepsin G (neutrophil origin)
Herrick et al., 1997[69]	28	CVLUs—5 Acute—72 (created)	Tissue	CVLU—excisional biopsies Acute—punch biopsies	Up-regulation of HNE in acute wounds of aged subjects and CVLUs associated with ECM degradation
Vaalamo et al., 1997[51]	22	CVLUs—11 Acute—11	Tissue	CVLUs—excisional biopsies Acute—biopsies of healing donor areas	↑ MMP-13 expression in CVLUs, but not in normally healing wounds
Yager et al., 1996[33]	20	CVLUs—3 Pressure ulcers-7 Acute—10 (mastectomy)	Fluid	CVLUs—occlusive film (Tegaderm)—aspiration Acute—surgical drainages	Levels of HNE > in chronic wounds than acute wounds—associated with degradation of growth factors
Latijnhouwers et al., 1998[71]	6	CVLUs—6	Fluid	CVLUs—occlusive film—aspiration	Degradation of tenascin-C (ECM glycoprotein) correlated with elastase and MMP activity
Nwomeh et al., 1999[39]	37	CVLUs—3 Pressure ulcers-10 Acute—4 (mastectomy) Controls—20	Fluid Tissue	CVLUs—occlusive film (Tegaderm)—aspiration Acute—surgical drainages Controls—biopsy, occlusive dressing—aspiration	↑ Levels of MMP-1 and MMP-8 and ↓ levels of TIMP-1 in chronic ulcers compared to healing wounds MMP-8 predominant collagenase in healing and nonhealing ulcers, but almost exclusively in inactive form in healing wounds
Trengove et al., 1999[38]	33	CVLUs—5 Mixed chronic—20 Acute—8 (mastectomy)	Fluid	CVLUs—occlusive film (Opsite)—aspiration Acute—surgical drainages	MMPs ↑ ×30-fold in chronic wounds compared to acute wounds Lower levels of TIMP-1 in chronic wounds; ↑ Levels of MMPs ↓ with healing of CVLUs
Tarlton et al., 1999[54]	35	CVLUs-25 Acute—10	Fluid	CVLUs—collection filters Acute—surgical drainages	Higher expression of pro- and activated MMP-2 and 9 and HNE in CVLUS than acute wounds Higher expression of MMP-2 and 9 and HNE with increased severity of wound site ↑ MMP-9 at ulcer edge associated with least evidence of healing ↓ Expression of MMP-9 where ulcers showed evidence of healing

Reference, year	Number of patients	Wound types	Samples	Sample collection method	Results
Mirastschijski et al., 2002[40]	52	CVLUs-10 Mixed chronic—24 Acute—18	Fluid Tissue	Foam disks CVLUs—excisional biopsies Acute—dermatome wounds or punch biopsies	MMP-9 activity did not differ significantly between acute and chronic wounds, but localization did. MMP-9 concentrated in inflammatory cells of ulcer bed in chronic wounds, but predominantly in advancing epithelium of acute wounds.[40]
Norgauer et al., 2002[44]	23	CVLUs—12 Controls—11	Tissue	Punch biopsies	Higher expression of EMMPRIN, MMP-2, MT1-MMP, and MT2-MMP in CVLUs than controls
Ulrich et al., 2005[50]	40	CVLUs—20 Acute—20 (donor sites: skin grafting)	Fluid	Absorption to release dressing	CVLU fluid inhibited angiogenesis significantly thought to be effect of MMP-2 and MMP-9 MMP-2/9 inhibition associated with angiogenesis stimulation
Mwaura et al., 2006[47]	40	CVLUs—40 (20 healing, 20 nonhealing)	Fluid Tissue	Aspirated from dressing with micropipettes Punch biopsies	↑ MMP-2 levels and ↓ TIMP-2 levels in nonhealing ulcers compared with healing ulcers Expression of EMMPRIN in all CVLUs
Pirila et al., 2007[61]	16	CVLUs—4 Mixed chronic—8 Acute—4	Tissue	Biopsies	MMP-8 predominantly expressed in all chronic wounds, but not detected in acute wounds MMP-26 expressed in most chronic wounds except for two most prolonged ulcers
Beidler et al., 2008[45]	29	CVLUs	Tissue—ulcers compared to healthy tissue	Punch biopsies	↑ MMP-1, 2, 3, 8, 9, 12, and 13 levels in ulcerated tissue MMP-8 and MMP-9 most highly expressed in ulcer tissue prior to compression treatment ↓ Levels of MMP-1, 2, and 3 associated with significantly improved healing rates
Lundqvist et al., 2008[72]	24	CVLUs—15 Acute—9 (mastectomy)	Fluid Tissue	CVLUs—occlusive film (Opsite)—aspiration Acute—surgical drainages 4 mm tissue biopsies	↑ Levels neutrophil α-defensins in CVLU fluid and tissue than in acute wounds Neutrophil counts significantly higher in CVLUs than in acute wounds
Meyer et al., 2008[62]	27	CVLUs—27 Acute—15	Tissue	Punch biopsies	MMP-1 > in healing ulcers than nonhealing ulcers Total MMPs significantly > in CVLUs than acute wounds

Reference, year	Number of patients	Wound types	Samples	Sample collection method	Results
Rayment et al., 2008[53]	12	CVLUs—9 Acute—3 (subepidermal blisters on feet)	Fluid	CVLUs—occlusive film—aspiration Acute—aspiration	Significantly > levels of MMP-9 in CVLUs than acute wounds Higher levels of MMP-9 in CVLUs associated with clinically worse wound
Smeets et al., 2008[46]	27	CVLUs—27	Fluid	Absorption onto Release dressing	↑ Activity of HNE, gelatinase, plasmin and MMP-2
Subramaniam et al., 2008[64]	17	CVLUs—9 Acute—8 (mastectomy)	Fluid	CVLUs—occlusive film (Opsite)—aspiration Acute—surgical drainages	↑ MMP-1, ↑ MMP-3, ↓ TIMP-1 significantly induced by CVLU fluid
Moor et al., 2009[22]	4	CVLUs—4	Fluid Tissue	Capillary wicking (Tegapore, Whatman 54 filter papers) 4 mm tissue biopsies	MMP-9 > in fluid than tissue MMP-8 in both fluid and tissue ↑ MPO, HNE and pro-inflammatory cytokines IL-6, IL-8, IL-1β in fluid
Wiegand et al., 2010[70]	38	CVLUs—11 Mixed chronic—17 Acute—10 (ablation of seborrheic warts)	Fluid	Saline rinse—glass washing chamber	HNE 10× > in chronic than acute wounds MMP-2, MMP-13 significantly > in CVLUs than acute wounds; IL-1β, IL-6, IL-8 significantly > in chronic than acute wounds ↓ Levels of HNE, MMP-2, MMP-13, IL-1β, IL-8 associated with initiation of CVLU healing
Eming et al., 2010[55]	28	CVLUs—19 Acute—9 (cutaneous wounds)	Fluid	Occlusive film (Hyalofilm)- aspiration	MMP-9, HNE, proteinase 3 exclusively detected in CVLUs MMP-9 22× > in CVLUs than acute wounds MPO markedly ↑ in CVLUs
Trostrup et al., 2011[76]	33	CVLUs—8 Acute—25	Fluid Tissue	CVLUs—Polyurethane foam covered with occlusive film (Tegaderm)– aspiration Acute—occlusive film-aspiration CVLUs—excisional biopsies Acute—punch biopsies	No differences in levels of proteinases/inhibitors between chronic and acute fluid

α1-PI, alpha1-proteinase inhibitor; α2-M, alpha2-macroglobulin; CVI, chronic venous insufficiency; CVLU, chronic venous leg ulcer; ECM, extracellular matrix; EMMPRIN, extracellular MMP inducer; HNE, human neutrophil elastase; IL, interleukin; MMP, matrix metalloproteinases; MPO, myeloperoxidase; MT, membrane type; TIMP, tissue inhibitor of metalloproteinases.

Table 2

Nonexhaustive list of therapeutics to reduce excessive neutrophil activity

Therapeutics	Principle	Level strength of evidence*	References
Topical			
Lower extremity compression	↓ Edema and venous hypertension ↓ Protease levels in CVLU tissue	Level I	(43)
Negative pressure therapy	Removes exudates containing excessive proteases and proinflammatory mediators	Level II	(81–83)
Mechanism-based dressings	To sequester, remove or inactivate excessive proteases	Level I	(22,49,68,97–101)
Doxycycline	Inhibits MMP	Level II	(84–87,92,93)
Systemic			
Pentoxifylline	Improves microcirculation of leg ↓ Neutrophil adhesion to endothelium ↓ Proinflammatory cytokine synthesis ↓ Free oxygen radical formation by neutrophils	Level I	(102–106)
MPFF	↓ Synthesis of prostaglandins, free oxygen radicals, and inhibits leukocyte trapping and activation	Level I	(107–110)
EPA + DHA supplementation	↑ Endogenous production of lipid mediators that reduce neutrophil influx ↓ Free oxygen radicals by leukocytes ↑ Macrophage clearance of apoptotic neutrophils	Level II	(26,112–117)

* Level I: Meta-analysis of multiple randomized clinical trials (RCTs) or at least two RCTs support the intervention. Level II: Less than level I, but at least one RCT and at least two significant clinical series or expert opinion papers with literature reviews supporting the intervention. Experimental evidence convincing, but not yet supported by adequate human experience is included. Level III: Suggestive data of proof of principle, but lacking sufficient data such as meta-analysis, RCT, or multiple clinical series. MMP, matrix metalloproteinase; MPFF, micronized purified flavonoid fraction; EPA + DHA, eicosapentanoic acid and docosahexanoic acid—polyunsaturated fatty acids.