Contrasting host immuno-inflammatory responses to bacterial challenge within venous and diabetic ulcers

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ABSTRACT

Within chronic wounds, the relationship between the clinical diagnosis of infection and bacterial/immuno-inflammatory responses is imprecise. This study prospectively examined the interrelationship between clinical, microbiological, and proinflammatory biomarker levels between chronic venous leg ulcers (CVLUs) and diabetic foot ulcers (DFUs). Wound swabs and fluids were collected from CVLUs ($n = 18$) and DFUs ($n = 15$) and diagnosed clinically as noninfected or infected; and qualitative/quantitative microbiology was performed. CVLU and DFU fluids were also analyzed for cytokine, growth factor, receptor, proteinase/proteinase inhibitor; and oxidative stress biomarker (protein carbonyl, malondialdehyde, and antioxidant capacity) levels. While no correlations existed between clinical diagnosis, microbiology, or biomarker profiles, increasing bacterial bioburden ($\geq 10^7$ colony-forming unit/mL) was associated with significant alterations in cytokine, growth factor, and receptor levels. These responses contrasted between ulcer type, with elevated and decreased cytokine, growth factor, and receptor levels in CVLUs and DFUs with increasing bioburden, respectively. Despite proteinase biomarkers exhibiting few differences between CVLUs and DFUs, significant elevations in antioxidant capacities correlated with increased bioburden in CVLU fluids, but not in DFUs. Furthermore, oxidative stress biomarker levels were significantly elevated in all DFU fluids compared with CVLUs. This study provides further insight into the contrasting disease-specific host responses to bacterial challenge within infected CVLUs and DFUs.

Nonhealing chronic skin wounds represent an important source of morbidity in aging societies and are a significant financial burden to healthcare providers.[1] These wounds are characterized by prolonged inflammation, defective extracellular matrix turnover, and delayed reepithelialization, enhanced by increased proteolytic remodeling and oxidative stress.[2–5] Chronic wounds are colonized by a diverse bacterial microflora, with bacteria existing in complex polymicrobial biofilm communities of aerobic and anaerobic species.[6,7] Bacteria residing within the wound bed are capable of directly or indirectly perpetuating inflammation and impaired healing responses.[8–11] Interactions between wound bacteria and host immuno-inflammatory responses are complex and reflect both its induction (via pathogen pattern recognition and the innate immune system) and suppression by bacterial metabolites.[12,13] These interactions play an important role in determining clinical outcome and the subsequent development of clinical infection.[6,7,14] While bacterial burden has been an accepted means of diagnosing wound infection, with biopsy levels above 10^5 colony-forming units (CFUs) being the current quantification "gold standard,"[15] its validity has been disputed as it may be influenced by wound bacterial diversity and the underlying immuno-inflammatory host responses within chronic wounds, while tissue biopsy collection for quantitative wound infection analysis can cause granulation tissue trauma.[10,16,17] Indeed, seven log-fold differences have been shown in the bacterial bioburden of venous ulcers that are "clinically" noninfected.[17] These paradoxical observations have been attributed to the existence of symbiotic colonies of genotypically distinct, nonpathogenic species or functional equivalent pathogroups, acting synergistically to maintain biofilm infections and cause pathogenicity.[18] A subjective model of relating clinical outcome and treatment planning to bacterial challenge proposes a "shift" in colonization-critical colonization-infection with increasing bioburden and subsequent adverse effects on healing.[19]

Current clinical practice for the distinction of colonized and infected wounds involves initial noninvasive and subjective clinical evaluation, with associated microbiological sampling.[10] However, infection diagnosis is confounded by

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inflammation.[20,21] Therefore, a more scientific and objective rationale of noninvasively assessing wound microflora would be invaluable to the clinical management of patients. Workers, including ourselves, are developing techniques to rapidly characterize bacterial load in clinical samples.[22] While wound fluid represents an accessible source of sample material to indirectly monitor changes in the wound environment (via analysis of cytokines/growth factors, proteinases, and oxidative stress biomarkers[23,24]), few studies have correlated such wound fluid contents with infection.[25,26] As effecters of cell-mediated immunity within wounds, neutrophils, macrophages, and CD4+ T-cells instigate local innate immune responses to bacteria[2,5,27] and subsequently represent an alternative mechanism by which the bacterial host relationship can be assessed. Therefore, this prospective study characterized the relationship between proinflammatory biomarkers and bacterial bioburden/infection status in chronic venous leg ulcers (CVLUs) and diabetic foot ulcers (DFUs) in order to identify novel diagnostic indicator(s) of clinically or microbiologically defined infection. Despite molecular biology-based, diagnostic methods being increasingly used to characterize the wound microbiome,[6,7,17,18] as the use of such methodologies in clinical practice and patient management is currently limited, culture-based methodologies were adopted herein to analyze bacterial diversity within CVLUs and DFUs.

MATERIALS AND METHODS

Patient enrollment and microbiological assessment

Patients attending the University Hospital of Wales participated in the study, undertaken according to Declaration of Helsinki protocols with Local Research Ethics Committee approval and informed patient consent. Patients were included with a ≥1 cm^2 CVLU or DFU for ≥30 days, with exudate flow. Subjects were excluded if wounds were dry/predominantly healed, exhibited osteomyelitis or other conditions interfering with healing, or were receiving dialysis, corticosteroids, immunosuppressive agents, radiation therapy, or chemotherapy, within 30 days of enrollment. Wounds were clinically diagnosed as noninfected or infected, depending on whether wounds exhibited a minimum of four clinical signs/symptoms of infection, including cellulitis, pain, swelling, erythema, increased temperature, unhealthy granulation tissue, abnormal odor, pus, or increased exudate flow.[20] Wounds were swabbed for qualitative/quantitative microbiological analysis, using standard techniques.[17] Wounds were debrided if necessary, saline cleansed, and dried. Wound areas were traced and photographic records made of each wound. Based on these parameters, 10 CVLU and 10 DFU patients enrolled in the clinically noninfected groups. Eight CVLU and five DFU patients enrolled in the clinically infected groups. All enrolled patients received similar levels of wound care both prior to and following wound fluid/microbiological sampling, in line with standard clinical protocols. Wound infection status within the 10 clinically noninfected CVLU and DFU patients only were subsequently reassessed 1 week postobservation and microbiological/wound fluid sampling to ascertain whether any clinically noninfected patients exhibited clinical signs of infection at this time.

Wound fluid collection and protein quantification

CVLUs and DFUs were dressed with Release Non-Adherent Absorbent Dressing (nonwoven absorbent dressing consisting of ethylene-methyl acrylate enveloped in a viscose fiber polymeric perforated film) and covered with Bioclusive Transparent Dressing, consisting of a thin, transparent polyurethane film (both Systagenix Wound Management, Gargrave, United Kingdom). Dressings remained in situ for ≥4 hours (CVLUs) or 24 hours (DFUs), were removed, nonsaturated regions excised, and wound fluids buffer eluted (500 $\mu L/cm^2$ wound dressing).[28] Wound fluid protein concentrations were determined (Protein Assay Kit, Bio-Rad, Hemel Hempstead, United Kingdom) according to manufacturer's instructions.

Cytokine, growth factor, and cell surface receptor quantification

Analysis of wound fluid cytokine, growth factor and cell surface receptor levels was performed using Microarray technology (Whatman Schleicher & Schuell, Dassel, Germany). Microarrays were conducted using 16-pad FAST slides, "spotted" with monoclonal antibodies with specificity for human cytokines, growth factors and cell surface receptors of interest (Table S1, all purchased from R&D Systems, Abingdon, United Kingdom). These included interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p40, IL-12p70, and IL-13; tumor necrosis factor (TNF)-α and its receptor TNFr2; interferon (IFN)-γ; transforming growth factor (TGF)-β_1; vascular endothelial growth factor (VEGF); angiogenin; intracellular adhesion molecule (ICAM)-1; and the chemokine, IFN-γ-inducible-protein-10.

Following antibody "spotting," slides were blocked with protein array blocking solution (70 μL). Aliquots of each wound fluid and standard (70 μL) were added to duplicate pads, resulting in six replicates per sample/standard. Slides were incubated at room temperature (4 hours) and washed with Tris-buffered saline 0.05% Tween 20 (TBS-T, all Sigma-Aldrich, Poole, United Kingdom, 3×5 minutes), followed by incubation at room temperature (1 hour) in biotinylated detection antibody cocktail (70 μL). Slides were washed (TBS-T) and incubated at room temperature (1 hour) with streptavidin-Cy5 conjugate (0.125 μg/mL, 70 μL), washed (TBS-T and deionized water), and dried. Slides were imaged by Confocal Fluorescent Scanner (AXON GenePix 4000B Fluorescent Imaging System, Molecular Devices, Sunnyvale, CA) and analyzed using Imaging Research ArrayVision Software (Imaging Research Inc., St Catharines, Canada). Spot intensities were determined, average fluorescent units obtained, and the levels of each marker (pg/mL) calculated. Sample readings above standard curve limits were diluted within each standard curve range. Readings below standard curve limits were assigned values equivalent to half the limit of detection for each particular marker (Table S1). Microarray analysis was performed on three separate occasions.

Fluorometric proteinase activity quantification

Neutrophil-derived elastase- and collagenase-like activities were determined using fluorogenic substrate activity assays, as previously described.[28] Neutrophil-derived elastase-like activity was assessed using methoxy-alanine-alanine-proline-

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valine-7-amino 4-methylcoumarin (Bachem UK, St Helens, (Imaging Research Inc., St Catharines, Canada)), solubilized in methanol (Fisher Scientific, Loughborough, (Imaging Research Inc., St Catharines, Canada)). The assay buffer required for optimal enzyme activity was 0.1 M HEPES, pH 7.5, containing 0.5 M sodium chloride and 10% dimethyl sulphoxide (all Sigma-Aldrich). Collagenase-like activity was estimated using succinyl-glycine-proline-leucine-glycine-proline-7-amino-4-methylcoumarin (Bachem UK), solubilized in methanol. The assay buffer required for optimal enzyme activity was 40 mMTris-HCl buffer, pH 7.4, containing 200 mM sodium chloride and 10 mM calcium chloride (all Sigma-Aldrich). Wound fluid proteinase activities were correlated to neutrophil elastase (Merck Chemicals, Nottingham, (Imaging Research Inc., St Catharines, Canada)) and collagenase (*Clostridium histolyticum*, Sigma-Aldrich) standards.

Wound fluid aliquots (20 µL) were established in black, flat-bottomed microtiter plates (Corning Life Sciences, Amersham, (Imaging Research Inc., St Catharines, Canada)) with the respective substrate (20 µL, final concentration 50 µM) and assay buffer (160 µL). Absorbance values were read immediately and at 5 minutes intervals over a 1-hour period, using a fluorescent plate reader (Fluorolite 1000, Dynex Technologies, East Grinstead, (Imaging Research Inc., St Catharines, Canada)) at excitation (383 nm)/emission (455 nm). Between readings, microtiter plates were covered and incubated at 37 °C. Enzyme activities were determined via the release of the fluorogenic compound, 7-amino 4-methyl coumarin, with activities expressed as relative fluorescence units minute/mL wound fluid (RFUs/minute/mL) or corrected for wound fluid protein content (RFU/minute/mg protein). Six replicates/wound fluids were assayed on three separate occasions.

Zymographic quantification of proteinase activity

The caseinolytic and gelatinolytic activities of CVLU and DFU fluids were also assessed by casein and gelatin zymography, and compared against appropriate standards, neutrophil-derived elastase (above) and matrix metalloproteinase (MMP)-2/,MMP-9 (dermal fibroblast conditioned media). For caseinolytic activity assessment, 16 µg of each wound fluid protein was applied for the analysis based on equal wound fluid protein content, while 10 µL of each wound fluid was applied for equal wound fluid volume analysis. For gelatinolytic (MMP-2 and MMP-9) activity, 0.5 µg or 2 µg of each wound fluid protein was applied for equal wound fluid protein analysis in CVLU and DFU fluids, respectively, while 10 µL of each wound fluid was applied for equal wound fluid volume analysis.

Wound fluids and the appropriate standards were separated on preformed sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis gels, containing 12.5% casein or 10% gelatin (Bio-Rad), and run on a Mini-Protean 3 Gel Electrophoresis System (Bio-Rad) at 60 mA/1 hour, in 0.25 M Tris-HCl buffer, pH 8.3, containing 2 M glycine and 1% SDS (all Sigma-Aldrich). SDS was subsequently removed from each gel by washing in 2.5% Triton X-100 (2 × 30 minutes). Casein gels were activated overnight at 37 °C, in 0.1 M sodium phosphate, pH 7.4, containing 0.1% Triton X-100 and 10 mM ethylenediaminetetraacetic acid (EDTA) (all Sigma-Aldrich). Gelatin gels were activated overnight at 37 °C, in 40 mM Tris-HCl buffer, pH 7.5, containing 0.2 M sodium chloride and 10 mM calcium chloride. Gels were stained in 0.2% Coomassie Blue (Sigma-Aldrich) in 50% methanol/10% acetic acid and destained in 20% methanol/10% acetic acid. Clear zones of casein/gelatinlysis were visualized and captured, with SigmaGel Analysis Software (SPSS Inc, Chicago, IL) used to semi-quantify regions of caseinolytic/gelatinolytic activity. Each wound fluid sample was run on three separate occasions.

Proteinase inhibitor quantification

α_1-Anti-trypsin levels were quantified in wound fluids by enzyme-linked immunosorbent assay (ELISA) (Athens Research, Athens, GA), according to manufacturer's instructions. Wound fluids were initially diluted 1:10000 to fit α_1-anti-trypsin standard curves. Tissue inhibitor of metalloproteinase (TIMP)-1/TIMP-2 were quantified by ELISA (R&D Systems), according to manufacturer's instructions. Wound fluids were initially diluted 1:20 to fit TIMP-1/TIMP-2 standard curves. Absorbance values were read at 450 nm on a Dynatech MR5000 Microplate Reader (Dynex Technologies, Worthing, (Imaging Research Inc., St Catharines, Canada)). Six replicates/wound fluids were assayed for α_1-anti-trypsin and TIMP levels on three separate occasions.

Quantification of protein carbonyl levels

The protein carbonyl levels in CVLU and DFU fluids were quantified by commercial ELISA kit (Zentech PC Test, Zenith Technology Corporation, Dunedin, New Zealand) according to manufacturer's instructions, using aliquots of each wound fluid containing 20 µg protein. The protein carbonyl concentrations of each wound fluid were determined from the standard curve (0–1 nmol/mg protein, in Kit). Absorbance values were read spectrophotometrically at 450 nm on a MicroplateAutoreader (Labtech International, Ringmer, (Imaging Research Inc., St Catharines, Canada)). Protein carbonyl levels were expressed as nmol/mg wound fluid protein or nmol/mL wound fluid. Each wound fluid was assayed in triplicate on three separate occasions.

Quantification of malondialdehyde levels

Malondialdehyde levels in CVLU and DFU fluids were quantified, as previously described.[24] Aliquots (100 µL) of each wound fluid were added to a solution containing 10 mM N-methyl-2-phenylindole (Sigma-Aldrich)/10% methanol in acetonitrile (325 µL, Fisher Scientific), followed by the addition of 37% hydrochloric acid (75 µL, Fisher Scientific). Samples were incubated at 45 °C/1 hour, and the extent of chromogen formation measured spectrophotometrically at 586 nm. The malondialdehyde concentration in each wound fluid was determined against a malondialdehyde (Sigma-Aldrich) standard curve (1–10 nmol/mL). Malondialdehyde levels were expressed as nmol/mL wound fluid. Each wound fluid was analyzed in triplicate on three separate occasions.

Quantification of antioxidant capacity

The total antioxidant capacities of CVLU and DFU fluids were quantified, as previously described, via their ability to

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inhibit cytochrome C reduction by a superoxide radical ($O_2^{\cdot-}$) flux, generated via the oxidation of hypoxanthine by xanthine oxidase.[24] Reaction mixtures were established in a total volume of 1 mL of 100 mM potassium phosphate buffer, pH 7.8, comprising hypoxanthine (1 mM, Sigma-Aldrich), cytochrome C (10 μM, horse heart type III, Sigma-Aldrich), wound fluid (100 μL), and xanthine oxidase (5 mU/mL, grade III from buttermilk, Sigma-Aldrich) to initiate $O_2^{\cdot-}$ flux generation. Control reaction mixtures containing the $O_2^{\cdot-}$ scavenger superoxide dismutase (40 U/mL, bovine erythrocytes, Sigma-Aldrich) were also established. Following $O_2^{\cdot-}$ flux initiation, cytochrome C reduction was monitored spectrophotometrically at room temperature, at 550 nm. Absorbance values were read over a 120-second period, with each wound fluid assayed in triplicate on three separate occasions. The relative rates of O_2 production were calculated using a molar extinction coefficient of 21,000 mol/cm/L, followed by determination of the % inhibition of cytochrome C reduction by each wound fluid. Antioxidant capacities were expressed as % inhibition of cytochrome C reduction/mL wound fluid and corrected to account for variations in wound fluid protein contents (% inhibition/mg protein).

Statistical analysis

Statistical analyses were performed in conjunction with With Confidence (Woking, (Imaging Research Inc., St Catharines, Canada)). Biomarker levels were expressed as average ± standard deviation, from which the median values for each biomarker group were calculated and compared for each clinical and microbiological parameter of interest. Data were considered nonparametric and the Wilcoxon two-sided probability statistical analysis was used. When comparing ulcer types, data were considered parametric and the Student's *t*-test (two-tailed) was used, assuming unequal variance. Significance was considered at $p < 0.05$.

RESULTS

Patient information

CVLU and DFU patient information is detailed in Table S2, together with the full sets of corresponding microbiological and biomarker data in the Supplementary Excel spreadsheets. CVLU patients had an age range of 41–91 years, with 7/20 females. DFU patients had an age range of 39–85 years, with 5/15 females. Analysis of patient information between clinically noninfected and infected wounds showed no significant differences ($p > 0.05$) in gender, age (CVLU median 75.5 vs. 75.5 years, DFU median 61.5 vs. 55.0 years), or wound duration (CVLU range 6–542 months, median 60.0 vs. 48.0 months; DFU range 1–53 months, median 5.5 vs. 16.0 months). Furthermore, none of the 10 clinically noninfected CVLU and DFU patients were demonstrated to exhibit any clinical signs of infection when reassessed 1 week postobservation and microbiological/wound fluid sampling.

No significant correlations exist between clinical diagnosis and wound microbiology

Bacterial bioburden and the number of genera detected for each clinically noninfected/infected CVLU and DFU are presented in Figure 1 (more detailed microbiology data are provided in Tables S3 and S4). The data showed no significant differences in bioburden (Figure 1A) or genera (Figure 1B) between clinically noninfected and infected CVLUs or DFUs ($p > 0.05$), suggesting that clinical diagnosis alone is not completely reliable for determining infection status in chronic wounds. Bacterial counts in CVLUs ranged between 10^3–10^8 CFU/mL with one to four genera, while DFUs had 10^2–10^8 CFU/mL with one to eight genera. Most species detected were aerobes, with only three CVLUs and DFUs containing anaerobes. The most prevalent species in CVLUs were *Pseudomonas* (12/18), *Staphylococcus* (10/18), and *Corynebacterium* (9/18) spp. *Corynebacterium* (12/15) and *Staphylococcus* (8/15) spp. were most prevalent in DFUs, which were almost devoid of *Pseudomonas* spp. (Tables S3 and S4).

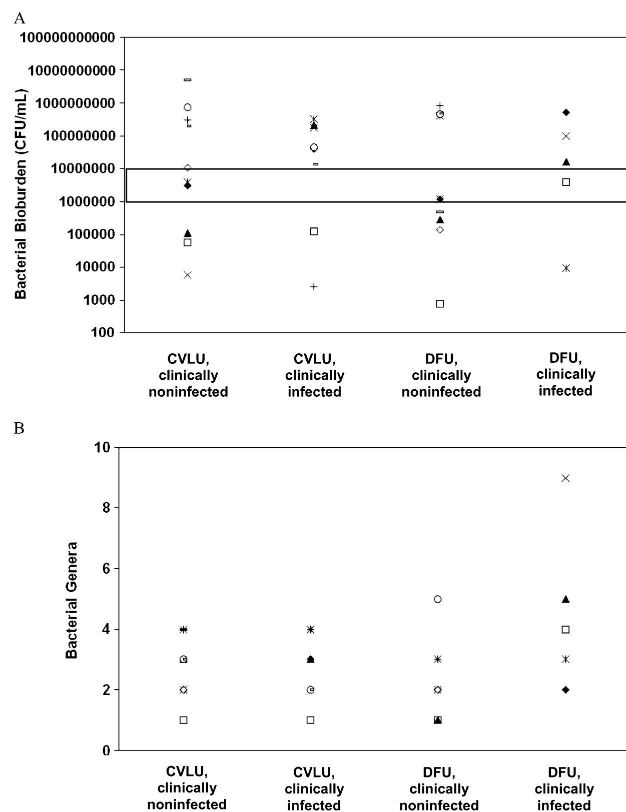

Figure 1. Comparison of bacterial numbers in clinically diagnosed noninfected and infected CVLUs and DFUs. No significant differences in (A) bacterial counts and (B) genera between clinically noninfected and infected CVLUs ($n = 10$ and $n = 8$, respectively) or DFUs ($n = 10$ and $n = 5$, respectively) ($p > 0.05$). Symbols are individual values for each wound. As microbiological comparisons with biomarker levels were made at $<10^6$ vs. $\geq 10^7$ CFU/mL, ulcers with bioburden levels between 10^6–10^7 CFU/mL (boxed area, A) were omitted from subsequent analyses. CFU, colony-forming unit; CVLU, chronic venous leg ulcer; DFU, diabetic foot ulcer.

The lack of significance based on "clinical classification" of wound infection[20,21] justified the more empirical approach of assessing proinflammatory biomarker levels vs. wound

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microflora. Wound microbiology criteria were based upon (i) bacterial bioburden; (ii) number of genera; and (iii) particular bacterial species. As the validity of 10^5 CFU as the infection "gold standard" has previously been queried[10,16] and to eliminate any inherent log-fold errors in bacterial counts, microbiological comparisons with biomarker levels were made at $<10^6$ vs. $\geq 10^7$ CFU/mL. However, this resulted in two CVLUs and three DFUs with bioburden levels between 10^6–10^7 CFU/mL being omitted from subsequent analyses (boxed area, Figure 1A). Comparisons were also made with genera number (≤3 vs. >3 genera) and to individual species at $<10^6$ vs. $\geq 10^7$ CFU/mL (*Pseudomonas*, *Staphylococcus* and *Corynebacterium* spp. for CVLUs; *Staphylococcus* and *Corynebacterium* spp. for DFUs), although individual species comparisons are less accepted than bioburden or genera.[17] Based on these definitions, 11/18 CVLUs and 7/15 DFUs were classed as infected at $\geq 10^7$ CFU/mL, reemphasizing inadequacies in diagnosing infection by clinical signs/symptoms alone. However, no significant correlations ($p > 0.05$) existed between patient gender, age or wound duration and CVLU/DFU bioburden, genera or the presence of *Pseudomonas*, *Staphylococcus*, or *Corynebacterium* spp.

Venous and diabetic ulcer cytokine, growth factor, and cell surface receptor levels exhibit distinct differences with bioburden and particular bacterial species

Microarray analysis showed significantly higher levels of angiogenin, ICAM-1, IL-1β, IL-4, IL-6, TNF-α, TNFr2; and VEGF in CVLUs at bioburden $\geq 10^7$ CFU/mL (Figure 2A), particularly IL-1β ($p = 0.004$) and IL-4 ($p = 0.005$). In contrast to CVLUs, DFUs exhibited significantly decreased IFN-γ, IL-2, IL-4, IL-5, IL-12p40, IL-12p70, IL-13; and TGF-β_1 at $\geq 10^7$ CFU/mL (Figure 2B), particularly IL-2 ($p = 0.002$) and TGF-β_1 ($p = 0.004$), with IL-12p70 being completely undetectable in DFUs at $\geq 10^7$ CFU/mL. Although no significant correlations were evident between genera and cytokine, growth factor and receptor levels ($p > 0.05$), significant increases with bacterial species were shown in CVLUs for IL-1β (*Pseudomonas* spp., Figure 2C), TNF-α, TNFr2, and VEGF (*Staphylococcus* spp., Figure 2C); and angiogenin, IL-4, IL-10, IL-12p70, IL-13, TGF-β_1, TNFr2, and VEGF (*Corynebacterium* spp., Figure 2D) at $\geq 10^7$ CFU/mL. Again, in contrast to CVLUs, significant decreases in IL-12p70 and TGF-β_1 were evident in DFUs with *Staphylococcus* spp. (Figure 2E), although no significant differences were identified with *Corynebacterium* spp. ($p > 0.05$). As a consequence of contrasting levels between CVLUs and DFUs, significantly higher IFN-γ ($p = 0.002$), IL-1β ($p = 0.002$), IL-2 ($p = 0.046$), IL-4 ($p = 0.002$), IL-13 ($p = 0.001$), TNF-α ($p = 0.026$); and VEGF ($p = 0.001$) levels were identified in CVLUs, compared with DFUs (Figure 2F). More detailed data are presented in Tables S5–S7.

Proteinase/proteinase inhibitor biomarkers exhibit no correlations with bioburden and few differences with genera/species in venous and diabetic ulcers

CVLU and DFU elastase activities did not correlate with bioburden (Figure 3A) or individual species (data not shown), based on wound fluid volume or protein analysis ($p > 0.05$). However, CVLU elastase activity was significantly increased with three or less genera, based on wound fluid volume analysis ($p = 0.009$, Figure 3B). Few differences were further identified when CVLU and DFU elastase activities were assessed by casein zymography, based on wound fluid volume or protein analysis (Figure 3C and D). However, significantly higher elastase activities were identified in DFU fluids, based on wound fluid protein analysis ($p = 0.002$, Figure 3E). CVLU and DFU collagenase activities showed no significant correlations with bioburden (Figure 4A), genera, or individual species (data not shown), based on wound fluid volume or protein analysis ($p > 0.05$). This was also true for CVLU and DFU gelatinase activity, based on wound fluid volume or protein analysis ($p > 0.05$, Figure 4B and C). However, significantly higher collagenase activities were identified in CVLU fluids, compared with DFU fluids, based on wound fluid volume analysis ($p < 0.001$, Figure 4D).

Proteinase inhibitors, α_1-antitrypsin, TIMP-1 and TIMP-2 showed no significant CVLU and DFU correlations with bioburden (Figure 5A, B, and C), genera, or individual species, based on wound fluid volume or protein analysis ($p > 0.05$). Consequently, no significant differences were shown between CVLUs and DFUs ($p > 0.05$, data not shown).

Antioxidant capacity correlates with venous bioburden, while oxidative stress biomarkers are significantly elevated in diabetic wound fluids

CVLU and DFU protein carbonyl and malondialdehyde levels showed no significant correlations with bioburden (Figure 6A and B), genera, or individual species (data not shown), based on wound fluid volume or protein analysis ($p > 0.05$). In contrast to protein carbonyl and malondialdehyde levels, CVLU antioxidant capacities significantly increased in association with bioburden, based on wound fluid volume analysis ($p = 0.035$, Figure 6C). However, CVLU and DFU antioxidant levels did not reflect genera or individual bacterial species, based on wound fluid volume or protein analysis ($p > 0.05$, data not shown). On comparison of CVLUs and DFUs, significantly higher protein carbonyl ($p = 0.002$, Figure 7A) and malondialdehyde levels ($p < 0.001$, Figure 7B) were determined in DFU fluids, based on wound fluid volume analysis. In line with elevated protein carbonyl and malondialdehyde levels in DFUs, these fluids also exhibited significantly greater antioxidant capacities than CVLUs, based on wound fluid volume analysis ($p < 0.001$, Figure 7C).

DISCUSSION

This study examined the relationship between pro-inflammatory biomarker levels and infection status in CVLUs and DFUs. Microbiological data emphasized the disparity between bacterial bioburden and the subjective clinical diagnosis of infection. The lack of correlation between conventional microbiological analyses and clinical diagnosis of infection was not completely unexpected, as chronic wound infection diagnosis currently relies upon rudimentary, nonspecific clinical signs/symptoms, which have been questioned because of the influence of chronic inflammation on these assessments.[20,21] While culture-based methodologies were

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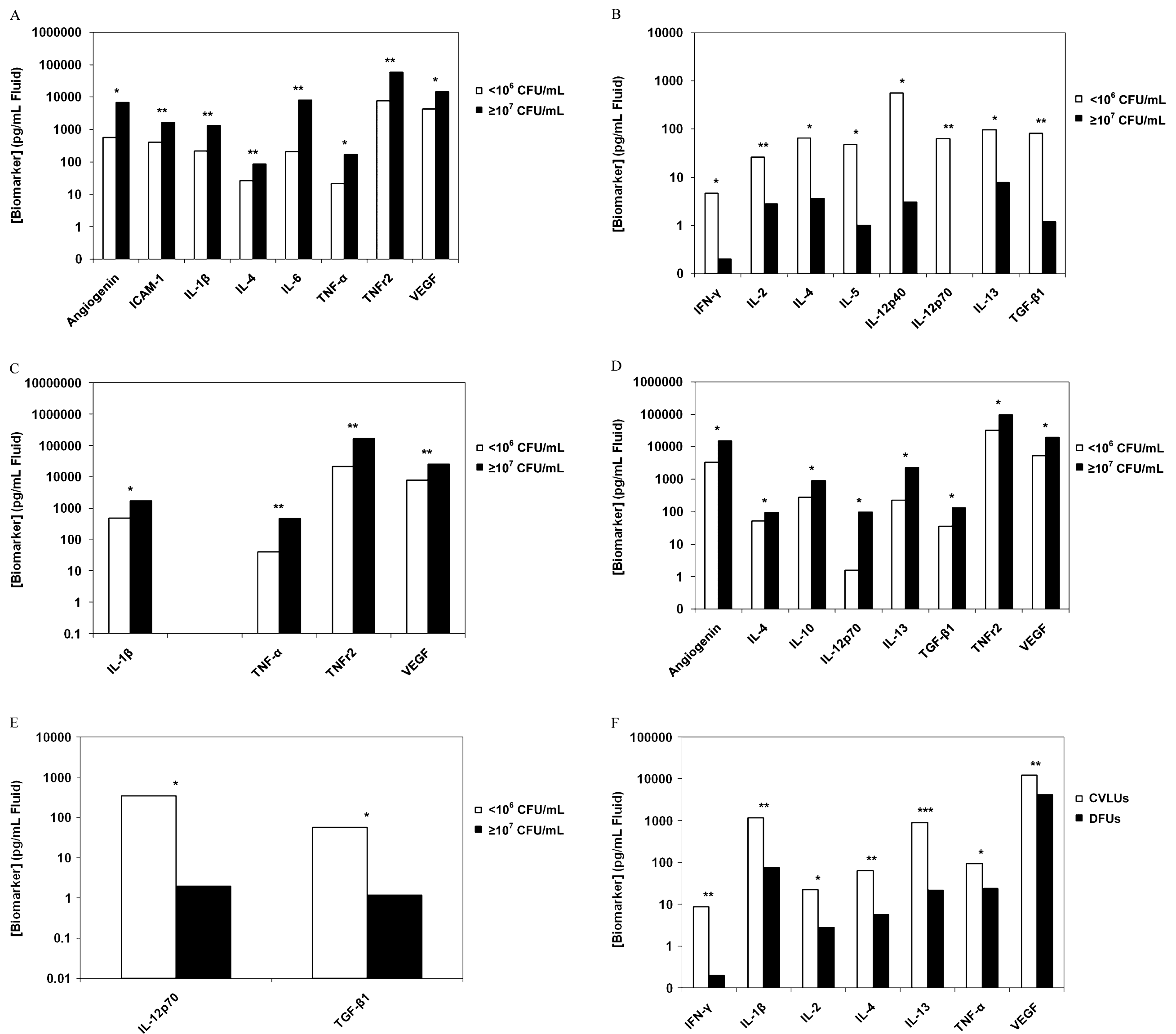

Figure 2. Median cytokine, growth factor, and cell surface receptor levels in CVLU and DFU wound fluids, based on microbiological analyses. (A) Significantly increased cytokine, growth factor and receptor levels in CVLU fluids with bioburden ≥10^7 CFU/mL (n = 11) vs. <10^6 CFU/mL (n = 5). (B) Significantly decreased cytokine and growth factor levels in DFU fluids with bioburden ≥10^7 CFU/mL (n = 7) vs. <10^6 CFU/mL (n = 5). (C) Significantly increased cytokine, growth factor and receptor levels in CVLU fluids with *Pseudomonas* or *Staphylococcus* spp. at ≥10^7 CFU/mL (n = 5 and n = 2, respectively) vs. <10^6 CFU/mL (n = 4 and n = 7, respectively). (D) Significantly increased cytokine, growth factor and receptor levels in CVLU fluids with *Corynebacterium* spp. at ≥10^7 CFU/mL (n = 5) vs. <10^6 CFU/mL (n = 3). (E) Significantly decreased cytokine and growth factor levels in DFU fluids with *Staphylococcus* spp. at ≥10^7 CFU/mL (n = 2) vs. <10^6 CFU/mL (n = 8). (F) Significantly increased cytokine and growth factor levels in CVLU (n = 18) vs. DFU (n = 15) fluids. All data derived from n = 3 independent experiments. Significance $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. CFU, colony-forming unit; CVLU, chronic venous leg ulcer; DFU, diabetic foot ulcer; ICAM, intracellular adhesion molecule; IFN, interferon; IL, interleukin; TGF, transforming growth factor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

adopted herein to characterize bacterial diversity, more accurate wound microbiome data would have been obtained by genomic analysis, although such techniques are currently limited in clinical practice and patient management.[6,7,17,18] Microfloral analysis was, however, consistent with previous culture-/molecular-based studies, showing the polymicrobial nature of CVLUs and DFUs with a wide range of bioburden and genera levels. In line with such studies, *Pseudomonas* and *Staphylococcus* spp. were highly prevalent in CVLUs and DFUs, while the importance of *Corynebacterium* spp. in

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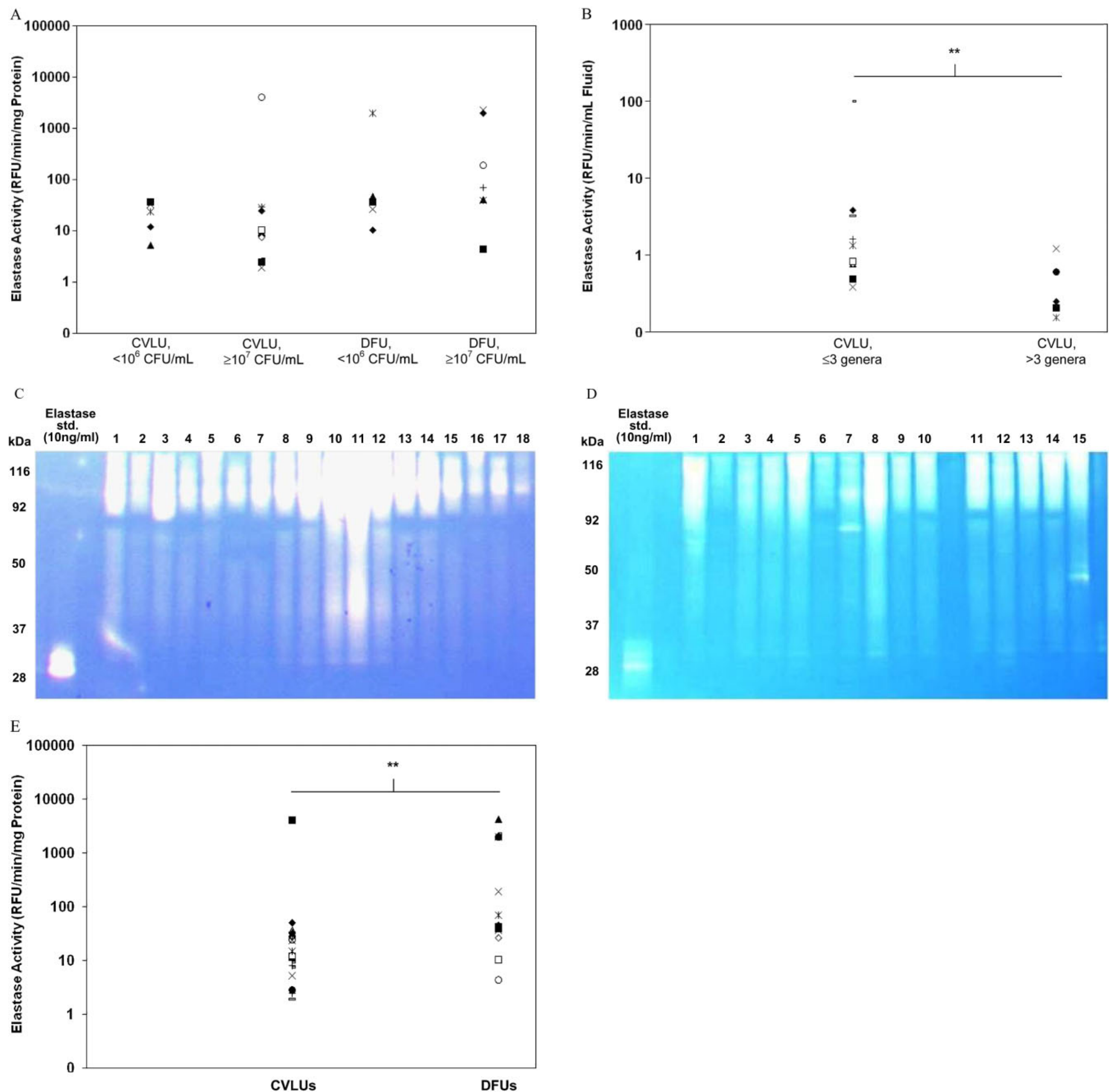

Figure 3. Comparison of elastase activities in CVLU and DFU fluids, based on microbiological analyses. (A) Nonsignificant differences in elastase activities with bacterial bioburden at $<10^6$ vs. $\geq 10^7$ CFU/mL in CVLU ($n = 5$ and $n = 11$, respectively) and DFU ($n = 5$ and $n = 7$, respectively) fluids, by fluorometric assay, based on wound fluid protein analysis ($p > 0.05$). (B) Significantly increased elastase activities in CVLU fluids in the presence of three or less genera ($n = 12$) vs. more than three genera ($n = 6$), based on wound fluid volume analysis. Nonsignificant differences in caseinolytic activity of (C) CVLU and (D) DFU fluids, based on wound fluid protein analysis ($p > 0.05$). (E) Significantly increased elastase activities in DFU fluids ($n = 15$) compared with CVLU fluids ($n = 18$), based on wound fluid protein analysis. Symbols are individual average values for each wound. All data derived from $n = 3$ independent experiments. Significance $^{**}p < 0.01$. CFU, colony-forming unit; CVLU, chronic venous leg ulcer; DFU, diabetic foot ulcer; MMP, matrix metalloproteinase; RFU, relative fluorescence unit.

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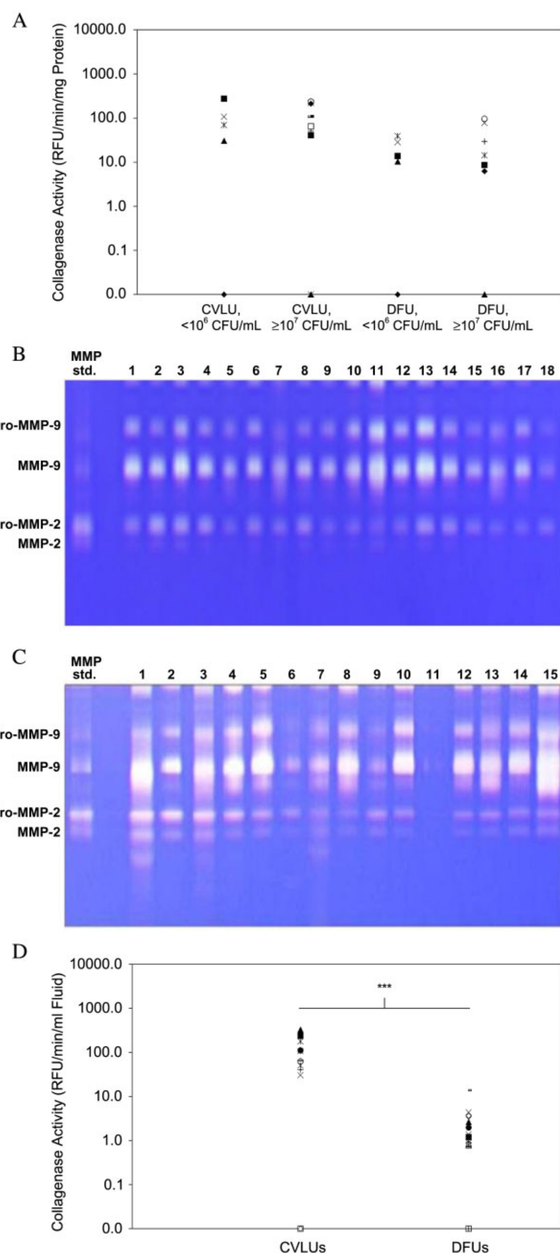

Figure 4. Comparison of collagenase/gelatinase activities in CVLU and DFU fluids, based on microbiological analyses. (A) Nonsignificant differences in collagenase activities with bacterial bioburden at $<10^6$ vs. $\geq 10^7$ CFU/mL in CVLU ($n = 5$ and $n = 11$, respectively) and DFU ($n = 5$ and $n = 7$, respectively) fluids, by fluorometric assay, based on wound fluid protein analysis ($p > 0.05$). Nonsignificant differences in gelatinolytic activity of (B) CVLU and (C) DFU fluids, based on wound fluid volume analysis ($p > 0.05$). (D) Significantly increased collagenase activities in CVLU fluids ($n = 18$) compared with DFU fluids ($n = 15$), based on wound fluid volume analysis. Symbols are individual average values for each wound. All data derived from $n = 3$ independent experiments. Significance $***p < 0.001$. CFU, colony-forming unit; CVLU, chronic venous leg ulcer; DFU, diabetic foot ulcer; TIMP, tissue inhibitor of metalloproteinase.

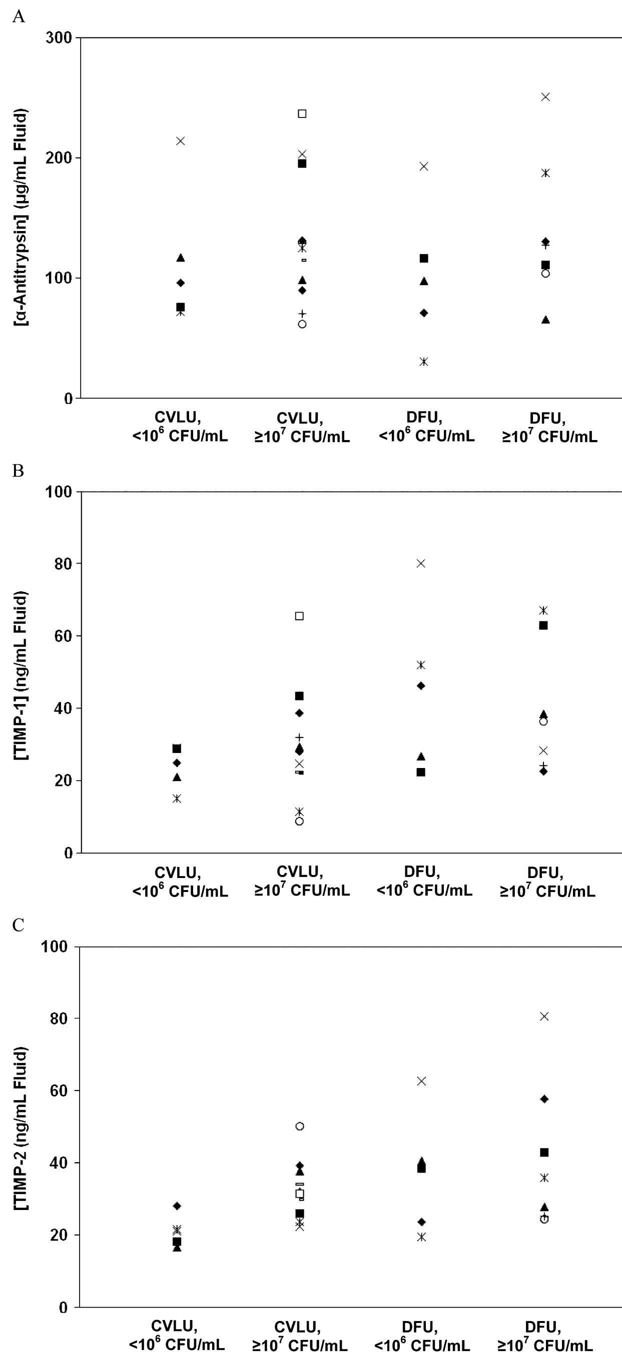

Figure 5. Comparison of proteinase inhibitor levels in CVLU and DFU fluids, based on microbiological analyses. Nonsignificant differences in (A) α_1-antitrypsin levels, (B) TIMP-1 levels and (C) TIMP-2 levels with bacterial bioburden at $<10^6$ vs. $\geq 10^7$ CFU/mL in CVLU ($n = 5$ and $n = 11$, respectively) and DFU ($n = 5$ and $n = 7$, respectively) fluids, by ELISA, based on wound fluid volume analysis ($p > 0.05$). Symbols are individual average values for each wound. All data derived from $n = 3$ independent experiments. CFU, colony-forming unit; CVLU, chronic venous leg ulcer; DFU, diabetic foot ulcer; ELISA, enzyme-linked immunosorbent assay; TIMP, tissue inhibitor of metalloproteinase.

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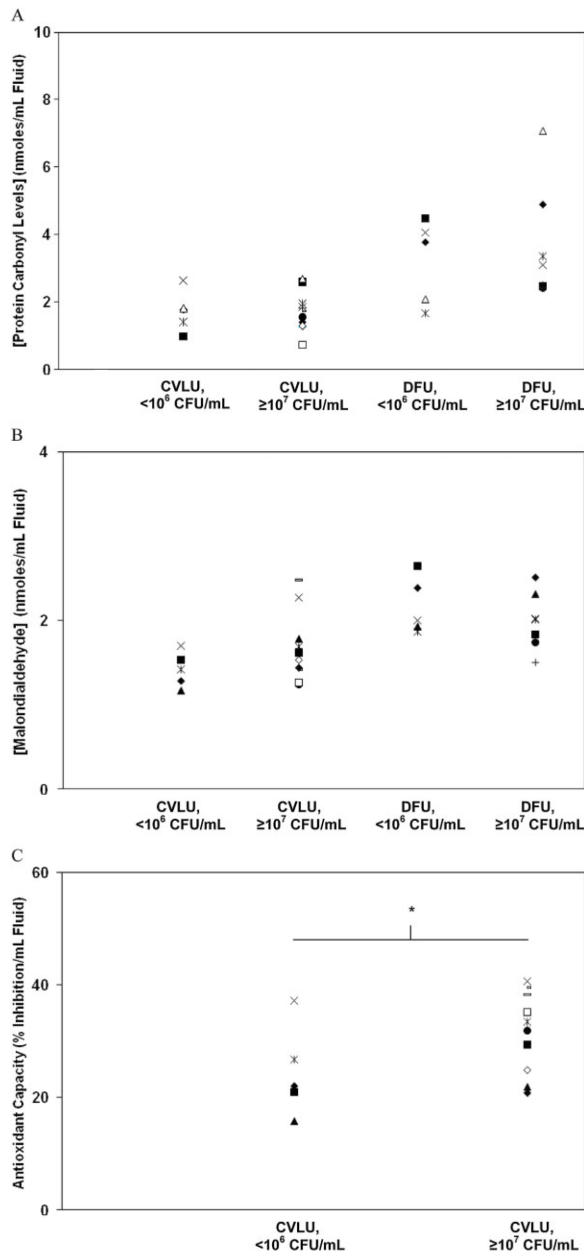

Figure 6. Comparison of oxidative stress biomarker levels in CVLU and DFU fluids, based on microbiological analyses. Nonsignificant differences in (A) protein carbonyl levels and (B) malondialdehyde levels with bacterial bioburden at $<10^6$ vs. $\geq 10^7$ CFU/mL in CVLU ($n = 5$ and $n = 11$, respectively) and DFU ($n = 5$ and $n = 7$, respectively) fluids, by ELISA and colorimetric assay, respectively, based on wound fluid volume analysis ($p > 0.05$). (C) Significantly increased antioxidant capacity in CVLU fluids with bioburden $\geq 10^7$ CFU/mL ($n = 11$) vs. $<10^6$ CFU/mL ($n = 5$), based on wound fluid volume analysis. Symbols are individual average values for each wound. All data derived from $n = 3$ independent experiments. Significance $*p < 0.05$. CFU, colony-forming unit; CVLU, chronic venous leg ulcer; DFU, diabetic foot ulcer; ELISA, enzyme-linked immunosorbent assay.

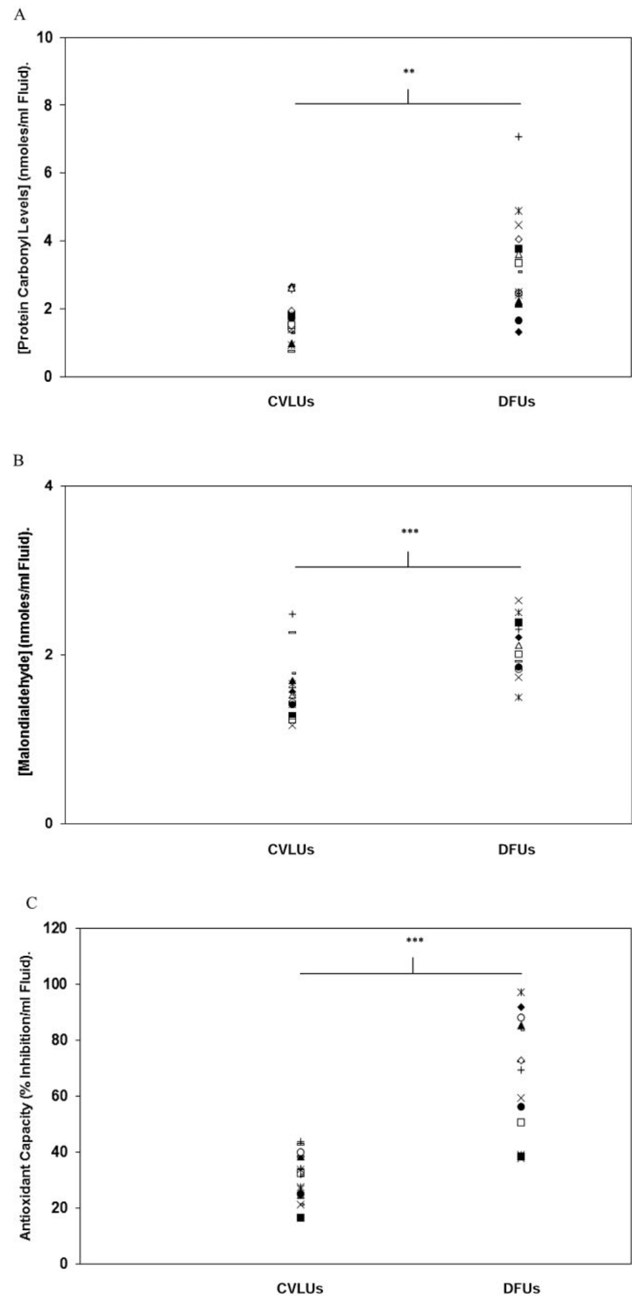

Figure 7. Comparison of oxidative stress biomarker levels between CVLU and DFU fluids. Significantly increased (A) protein carbonyl levels, (B) malondialdehyde levels and (C) antioxidant capacity in DFU fluids ($n = 15$) compared with CVLU fluids ($n = 18$), based on wound fluid volume analysis. Symbols are individual average values for each wound. All data derived from $n = 3$ independent experiments. Significance $**p < 0.01$ and $***p < 0.001$. CVLU, chronic venous leg ulcer; DFU, diabetic foot ulcer.

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CVLUs and DFUs is increasingly being recognized.[1,6,17,18] Other bacterial species were further identified in CVLUs and DFUs, consistent with previous findings.[1,6,17,18] The presence of *Staphylococcus* spp., *Pseudomonas* spp., and other species is significant, as many are implicated in impaired healing.[1,6,8,9,13,14]

Lack of correlation between conventional microbiological analyses and the clinical diagnosis of infection led to biomarker comparisons vs. bioburden, genera and bacterial species. This study identified significant increases in numerous cytokines, growth factors and receptors (IL-1β, IL-4, IL-6, TNF-α, angiogenin, VEGF, ICAM-1, TNFr2) in CVLU fluids compared with significant decreases in IFN-γ, IL-2, IL-4, IL-5, IL-12p40, IL-12p70, IL-13 ,and TGF-β_1 in DFU fluids with increasing bioburden. Indeed, many of these cytokines and growth factors (IFN-γ, IL-1β, IL-2, IL-4, IL-13, TNF-α, VEGF) exhibited significant differences between CVLUs and DFUs, irrespective of bioburden. DFU fluids from wounds containing *Staphylococcus* spp. also had lower IL-12p70 and TGF-β_1 levels. Such findings likely reflect inherent differences in the abilities of CVLUs and DFUs to orchestrate effective local cell-mediated immunological responses to resident bacteria and/or are the result of the distinct microflora within each ulcer type.

Many cytokines/growth factors (angiogenin, IL-1β, IL-4, IL-6, TNF-α, VEGF) and receptors (ICAM-1, TNFr2) were also significantly elevated in CVLUs with bioburden and *Pseudomonas* (IL-1β), *Staphylococcus* (TNF-α, TNFr2, VEGF), and *Corynebacterium* (angiogenin, IL-4, IL-10, IL-12p70, IL-13, TGF-β_1, TNFr2,VEGF) spp. This concurs with studies showing increased IL-6 and TNF-α levels in infected CVLUs, with IL-6 correlating with bioburden ($\geq 10^5$ CFU/mL) and *Pseudomonas* spp. infection.[25] Although IL-1β, IL-6, TNF-α, TNFr2, TGF-β_1, and VEGF have previously been quantified in acute and chronic wound fluids,[12,24,25] this study also provided data on previously unquantified cytokine, growth factor and receptor levels. IL-1β, IL-6 and TNF-α levels were not unexpected, as their increases are consequences of neutrophil/macrophage responses to bacterial challenge and well documented in nonhealing CVLUs.[12,24] However, our findings are significant, as these have previously been precluded as biomarkers of healing status, because of their multiple sources, interpatient variability and pleiotropic effects.[24]

Proinflammatory cytokines correlating with CVLU bacterial species are generally acute phase response activators. Their increased detection with bioburden is noteworthy, as *Pseudomonas* and *Staphylococcus* spp. induce inflammatory cell infiltration and cytokine release, while it has been suggested that cytokine production is deregulated in chronic wounds.[2,5,12,29] Increased IFN-γ, IL-1β, ICAM-1 and TNF-α may contribute to perpetual inflammation in CVLUs by facilitating further inflammatory cell migration/differentiation.[5,27] Early dermal healing is predominated by M1 macrophages activated by IFN-γ and IL-10, while IL-4 and IL-13 stimulate M2 macrophages during later healing.[27] IL-4 also regulates Th1/Th2 cell differentiation.[5] Although IL-10 suppresses innate immune responses in CVLUs, it may also impair healing.[30] Oxygen and nutrient requirements within infected CVLUs may reflect angiogenin and VEGF levels, as increased VEGF levels correlate with CVLU healing.[29] Therefore, increased bioburden and *Pseudomonas*, *Staphylococcus*, and *Corynebacterium* infection in CVLUs appear to be accompanied by enhanced neutrophil, M1 macrophage, and T-cell responses, particularly because of increased ILs and TNF-α.

DFUs are characterized by impaired cell migration/phagocytosis and inflammation resolution, leading to dysfunctional healing.[4,8,31] M1 macrophage depletion during early wound healing reduces M2 macrophage activation, granulation tissue formation and reepithelialization.[27] Such events may be confounded by decreased cytokines required for M1 (IFN-γ) and M2 (IL-4, IL-13) macrophage activation in DFUs, thereby reducing neutrophil, macrophage, and T-cell numbers.[5,27] This may explain the reduced cytokine and TGF-β_1 levels with increasing bioburden, as macrophages are major sources of these within wounds. Lower TGF-β_1 levels may also influence VEGF expression, disrupting angiogenesis.[32] Reduced Th1 (IFN-γ, IL-2) and Th2 (IL-4, IL-13) cytokine levels with bioburden may further indicate an inability to elicit appropriate immune responses to infection. Thus, DFU cytokine/growth factor levels may lead to increased infection susceptibility, dysfunctional immuno-inflammatory responses, angiogenesis and reepithelialization.[4,6,8,18,31]

While studies have shown increased wound fluid MMP levels and reduced TIMPs during impaired healing,[24,33] the present study suggests that these are of little use as specific biomarkers of infection. MMP biomarkers exhibited no significant differences between CVLUs and DFUs; and bioburden, genera, or species, although CVLUs possessed higher collagenase activities overall. Such findings contrast with studies reporting increased MMP-9 levels with bioburden[33] and elevated MMP-2/reduced TIMP-2 levels in DFUs.[34] Increased chronic wound fluid elastase activities are also suggested to reflect impaired healing, although no consistent correlations have been determined.[24,33] As elastase activity correlated with few microbiological parameters, this further questions its specificity as a biomarker. Interestingly, CVLU elastase activity was significantly increased with three or less genera, despite increased genera and elastase activity being implicated in impaired healing.[17,24] Ulcer comparisons further revealed higher elastase activities in DFUs. Elevated DFU elastase activities coincided with decreases in cytokines/growth factors that are susceptible to neutrophil-derived elastase inactivation,[35] suggesting that elastase may contribute to these decreased cytokine/growth factor levels. As most caesinolytic activity was at >30 kDa, this implies that other host/bacterial proteinases contribute to this activity.[36,37]

Inflammatory cell stimulation leads to excessive reactive oxygen species (ROS) production to counteract infection, which can also contribute to impaired healing.[2–5] Such ROS increases have led to oxidative stress biomarker correlations with impaired healing.[23,24] Protein carbonyl and malondialdehyde levels in CVLU and DFU fluids showed no significant differences with bioburden, genera, or individual species. Previous studies have shown that increased carbonyl levels in acute wound fluids simply reflect higher protein levels in these fluids, while malondialdehyde is a poor biomarker of healing.[23] However, significantly higher protein carbonyl and malondialdehyde levels were shown in DFU fluids. Despite DFUs and CVLUs sharing pathological similarities, higher glucose levels induce specific features in DFUs, including increased advanced glycation end product and ROS formation, contributing to elevated carbonyl and malondialdehyde levels.[4,38] In contrast, significantly higher

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CVLU antioxidant capacities were identified with increasing bioburden, possibly reflecting an effective adaptive response to bacterial challenge and chronic inflammation in CVLUs. Similar findings have been reported in sepsis and peptic ulceration, where antioxidant capacities only decline on eradication of infection.[39,40] However, DFU antioxidant adaptations to increasing bioburden may be effectively "overwhelmed" by the enhanced oxidative stress exposure, reflecting the higher antioxidant capacities in DFUs overall.

This study has characterized inflammatory responses in infected wounds of distinct etiologies and shown that bacterial numbers do not reflect the clinical diagnosis of infection. The data also show alterations in local cytokine/growth factor and oxidative stress profiles within these wounds associated with infection, providing insight into the distinct differences in CVLU and DFU responses to bacterial challenge. This study has further purported the potential of certain immuno-inflammatory mediators as diagnostic biomarkers of microbiologically defined infection; and the design and delivery of patient-/wound-specific therapies, which address the distinct host responses in these contrasting wound types. However, it is important to acknowledge that as this was a prospective study, further clinical studies are warranted with larger patient cohorts to validate these findings. Such verification may further aid wound infection diagnosis and the assessment of therapeutic intervention effectiveness.

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Conflicts of Interest: All other authors have no conflicts of interest to declare.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Microarray antibodies (R&D Systems) against the 18 cytokines, growth factors and cell surface receptors quantified in this study and their limits of detection (pg/mL). Samples with fluorescent units below the range of the standard curve were assigned values equivalent to half the limit of detection for each particular molecule

Table S2. CVLU and DFU patient details

Table S3. Qualitative/quantitative microbiological analysis of clinically diagnosed noninfected and infected CVLUs

Table S4. Qualitative/quantitative microbiological analysis of clinically diagnosed noninfected and infected DFUs

Table S5. Median cytokine, growth factor and cell surface receptor levels in microbiologically defined CVLUs and DFUs, on comparison with bioburden at $<10^6$ and $\geq 10^7$ CFU/mL. Significant differences ($p < 0.05$) are shaded (detailed further in Figure 2A and B).

Table S6. Median cytokine, growth factor, and receptor levels in microbiologically defined CVLUs, on comparison with particular bacterial species at $<10^6$ and $\geq 10^7$ CFU/mL. Significant differences ($p < 0.05$) are shaded (detailed further in Figure 2C and D).

Table S7. Nonsignificant median value differences ($p > 0.05$) in cytokine, growth factor and cell surface receptor levels in microbiologically defined DFUs, on comparison with particular bacterial species at $<10^6$ and $\geq 10^7$ CFU/mL. Significant differences ($p < 0.05$) are shaded (detailed further in Figure 2E).

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