ORIGINAL ARTICLE

Longitudinal Evaluation of Biomarkers in Wound Fluids from Venous Leg Ulcers and Split-thickness Skin Graft Donor Site Wounds Treated with a Protease-modulating Wound Dressing

Jacek MIKOSIŃSKI[1#], Konstantinos KALOGEROPOULOS[2#], Louise BUNDGAARD[2,3], Cathrine Agnete LARSEN[2], Simonas SAVICKAS[2], Aleksander Moldt HAACK[2], Konrad PAŃCZAK[4], Katarzyna RYBOŁOWICZ[5], Tomasz GRZELA[6,7], Michał OLSZEWSKI[8], Piotr CISZEWSKI[9], Karina SITEK-ZIÓŁKOWSKA[10], Krystyna TWARDOWSKA-SAUCHA[11], Marek KARCZEWSKI[12,13], Daniel RABCZENKO[14], Agnieszka SEGIET[14], Patrycja BUCZAK-KULA[15], Erwin M. SCHOOF[2], Sabine A. EMING[16], Hans SMOLA[16,17§] and Ulrich AUF DEM KELLER[2]

[1]"MIKOMED", Clinic for Peripheral Vascular Diseases, Łódź, Poland, [2]DTU Bioengineering, Section for Protein Science and Biotherapeutics, Technical University of Denmark, Kgs. Lyngby, Denmark, [3]Department of Veterinary Clinical Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Taastrup, Denmark, [4]ECRAN – WOUND CARE CENTRE, Wrocław, Poland, [5]Professional Treatment and Research Centre, Ostróda, [6]Clinic of Phlebology, [7]Medical University of Warsaw, Warsaw, [8]Pratia Ostrołęka Embedded Hospital Clinical Research Site, Ostrołęka, [9]WILMED Specialist Medical Clinic Non-public Healthcare Centre, Warszaw, [10]SILMEDIC Medical Centre Sp. z o.o., Katowice, [11]Specialised Medical Centre "Medservice", Zabrze, [12]CSOLUMED Medical Centre, [13]Poland Department of General and Transplant Surgery, Poznan University of Medical Sciences, Poznan, [14]Instytut Edukacji i.e. sp.z o.o., [15]ClinMed Pharma, Warzawa, Poland, [16]Department of Dermatology, University of Cologne, Cologne, and [17]PAUL HARTMANN AG, Heidenheim, Germany
[#]Shared first authorship. [§]Shared last authorship.

Venous leg ulcers represent a clinical challenge and impair the quality of life of patients. This study examines impaired wound healing in venous leg ulcers at the molecular level. Protein expression patterns for biomarkers were analysed in venous leg ulcer wound fluids from 57 patients treated with a protease-modulating polyacrylate wound dressing for 12 weeks, and compared with exudates from 10 acute split-thickness wounds. Wound healing improved in the venous leg ulcer wounds: 61.4% of the 57 patients with venous leg ulcer achieved a relative wound area reduction of ≥40%, and 50.9% of the total 57 patients achieved a relative wound area reduction of ≥60%. Within the first 14 days, abundances of S100A8, S100A9, neutrophil elastase, matrix metalloproteinase-2, and fibronectin in venous leg ulcer exudates decreased significantly and remained stable, yet higher than in acute wounds. Interleukin-1β, tumour necrosis factor alpha, and matrix metalloproteinase-9 abundance ranges were similar in venous leg ulcers and acute wound fluids. Collagen (I) α1 abundance was higher in venous leg ulcer wound fluids and was not significantly regulated. Overall, significant biomarker changes occurred in the first 14 days before a clinically robust healing response in the venous leg ulcer cohort.

Key words: dressings; biomarker; proteomics; venous ulcer; exudate.

Accepted Oct 17, 2022; Epub ahead of print Oct 17, 2022

Acta Derm Venereol 2022; 102: adv00834.

DOI: 10.2340/actadv.v102.325

Corr: Hans Smola, Paul Hartmann AG, Paul Hartmann Str. 12, DE-89522 Heidenheim, Germany; and Ulrich auf dem Keller, PhD, DTU Bioengineering, Section for Protein Science and Biotherapeutics, Technical University of Denmark, Søltofts Plads, DK-2800 Kgs. Lyngby, Denmark. E-mails: hans.smola@hartmann.info; uadk@dtu.dk

SIGNIFICANCE

An increasing number of patients develop skin wounds that do not heal. These wounds are termed chronic wounds, are very painful, and strongly affect the patients in their daily lives. Exudates from non-healing wounds can add to the description of the wound status and whether wounds respond to therapies. This study analysed fluids from venous leg ulcers and compared the relative abundances of a set of proteins known to be associated with wound progression to normal healing wounds. This analysis helped to assess healing responses upon treatment with a polyacrylate dressing.

Venous leg ulcers (VLUs) are one of the most prevalent chronic wound types and have been studied in numerous clinical trials (1). Effective treatment of VLUs relies on the therapy of venous insufficiency through compression therapy (2). Several clinical parameters are associated with poor healing. The presence of ulcers for more than 6 or 12 months, a large wound surface area greater than 10 cm2, and reduced patient mobility have been identified as prognostic clinical markers (3, 4). Despite appropriate medical care 40–50% of VLUs remain unhealed after 12 months (5), and recurrence rates within 3 months of healing are high.

In the molecular pathology of VLUs, excessive protease activity contributes to degradation of newly formed extracellular matrix (6), as well as inactivation of growth factor activities in the wound bed (7–9). Apart from excessive levels of matrix metalloproteinases (MMPs), interleukin (IL)-1β and tumour necrosis factor alpha (TNF-α) cytokine abundances were elevated in chronic wounds (9). S100A8/S100A9 were measured in VLUs (10), as were fibronectin and collagen levels (10, 11). Moreover, proteomic approaches and multiplex enzyme-linked immunosorbent assay (ELISA) testing showed several additional differences between VLUs with good healing progression and poorly healing ulcers (12, 13). These results have been analysed regarding the potential to pre-

dict VLU ulcer healing rates. Some of these studies used patients with heterogeneous wound pathologies and the number of patients for each wound entity was rather low.

Few data are available that describe a larger patient population with more homogenous, clinically well-defined wound types and followed for a longer treatment period. This study analysed a set of biomarkers in a cohort of patients with VLU and examined whether and when these biomarkers revert to levels observed in normal, acute wound healing.

MATERIALS AND METHODS

Patients

The clinical part of this study was a 2-arm, non-comparative open-label prospective cohort study on the healing of VLU wounds (VLU cohort) and split-thickness donor site wounds (acute wound healing cohort). In both cohorts, the wounds were treated with a Ringer's solution pre-activated polyacrylate-containing wound dressing (HydroClean®, Paul Hartmann AG, Heidenheim, Germany), and all patients in the VLU cohort received additional compression therapy (PütterPro 2®, Paul Hartmann AG), key in venous leg ulcer treatment (2). This was a multicentre, prospective observational study, collecting clinical data of patients in VLU and acute wound healing cohorts receiving HydroClean® wound dressings from November 2018 to November 2019 across 9 specialized facilities in Poland. Detailed inclusion and exclusion criteria are listed in Table SI. After obtaining informed consent, patients were enrolled in the trial, and patients in the VLU wound cohort (group A) were followed for 12 weeks or until complete wound closure. Split-thickness donor site wounds (group B) were followed for 21 days in patients who underwent skin transplants to surgically close other wounds outside the scope of this study. The depth setting of the graft harvesting device was at the discretion of the clinician. Wound size was documented by planimetry (acetate tracings) every 2 weeks in the VLU cohort, and in the acute wound healing cohort, on days 0, 2, 4, 7, 10, 14, 17, and 21.

Clinical outcomes

Clinical endpoints included wound area reduction (absolute wound size (cm^2)) and relative wound area reduction (WAR in %; (wound size t_0 – wound size t_{last})/wound size t_0*100)), numbers of wounds with WAR ≥40 %, WAR ≥60%, number of wounds healed, physician estimated exudate levels, perilesional skin state, changes in wound pain, pain during the dressing change, and product-related adverse events. Details of the measurement variables are listed in Table SII. Granulation tissue development over time (as % of total wound surface area) was estimated by 2 independent investigators. In cases where estimation differed by more than 20%, the resolution was achieved by reviewing the wound pictures jointly. Wound fluid was extracted from spent dressings.

Wound exudate sampling and extraction from dressings

For all patients, baseline exudate was sampled by the application of a sterile non-woven compress (Medicomp® sterile, 70% cellulose fibre, 30% polyester fibre; Paul Hartmann AG) for 2 h before any debridement or wound cleansing occurred, followed by application of a HydroClean® dressing. The soaked Medicomp® compress was placed in a plastic bag and frozen at –20°C until the extraction steps. For all other time-points, the polyacrylate-containing dressings (HydroClean®) were removed at the visits, transferred into plastic bags, and frozen at –20°C until biochemical extraction.

The soaked Medicomp® (entire dressing) and Hydroclean® (inner polyacrylate layer) dressings were incubated in a buffer solution with cOmplete™ Mini EDTA-free protease inhibitor cocktail (50 mM HEPES pH 7.8, 150 mM NaCl, 10 mM EDTA) (Merck, Darmstadt, Germany) to extract the proteins. Polymer residues were removed by filtering the liquid phase through a 40 µm cell strainer (Fisherbrand™ Sterile Cell Strainers, 22-363-547, Thermo Fisher Scientific, Waltham, MA, USA), and cell debris removed by centrifugation (3×15 min, 4000 g, 4°C) followed by sample concentration (3 kDa Amicon® Ultra-15 Centrifugal Filter Unit, UFC9003, Millipore, Merck). Protein concentration was determined with Nanodrop One™ (Thermo Fisher Scientific).

Shotgun proteomics analysis of wound exudates

A total of 20 µg extracted protein were denatured in 2.5 M guanidine hydrochloride (GuHCl), 100 mM HEPES pH 7.8, cysteines reduced with tris(2-carboxyethyl)phosphine (TCEP) and alkylated with chloroacetamide, followed by tryptic digest (1:50 protease: protein (w:w)) overnight after adjusting the GuHCl concentration to 0.5 M. Samples were desalted using custom-packed StageTips. For liquid chromatography (LC)-mass spectrometry (MS) analysis, an EASY-nLC 1200 liquid chromatography system was used in line with a Q Exactive™ mass spectrometer (Thermo Fisher Scientific), which was operated in positive polarity at data-dependent acquisition (DDA) mode, with an active gradient of 140 min (starting at 6% of 80% acetonitrile in water, 23% at 85 min, 38% at 115 min, 60% at 125 min, 95% at 130 min until the end of the gradient). A sample volume corresponding to 500 ng of sample peptides determined with Nanodrop One™ was injected. MS1 resolution was set to 70,000, automatic gain control (AGC) target set to 3e6, maximum injection time set to 20 ms, scan range 300–1750 m/z, selecting the top 10 MS1 ions for MS2 analysis. MS2 scans used 17,500 resolution, AGC target of 1e6, maximum injection time 60 ms, and isolation window of 1.6 m/z at normalized collision energy of 25. Data analysis was performed using SpectroMine™ 3.0 (Biognosys, Schlieren, Switzerland). The raw MS spectra were searched against a reviewed *Homo sapiens* database downloaded from the UniProt Database (TaxID 9606, 42,252 SwissProt entries, 25 November 2017). Searches were run with tolerances of 10 ppm and 0.01 Da for precursor and fragment ions, respectively. Trypsin/P was set as the used protease, and carbamidomethylation of cysteine residues (+57.021 Da) was added as static modification, while oxidation (+15.995 Da) and protein N-terminal acetylation (+42.01 Da) were added as variable modifications. The length range of peptide candidates was 7–52 amino acid residues. Peptide-spectrum matches (PSMs) were set to not exceed 1% FDR. Filtered PSMs were further filtered for peptide and protein-level FDR of 1%. Quantification was performed using SpectroMine™'s label-free quantification (LFQ) algorithm.

SureQuant™ analysis of biochemical markers

Biochemical markers were quantified in the wound exudate by mass spectrometry-based proteomics. To assess pro-inflammatory activity, IL-1β, TNF-α, S100A8 and S100A9 were selected. IL-1β and TNF-α are key regulators of the inflammatory cascade, and S100A8 and S100A9 are cytokines highly expressed in neutrophil PMNs of up to 45% of their cytosolic protein (14). To characterize the proteolytic environment, the current study assayed neutrophil elastase, MMP-2, MMP-9, and to assess extracellular matrix constituents we selected collagen (I) α1 and fibronectin. For analysis of biomarkers, a MS-based targeted proteomics method was employed that is independent of antibodies and thus has demonstrated advantages over immunoassays such as Western blots and ELISAs in specificity of protein identification and accuracy of quantification. Specifically, the newly developed SureQuant™

method was used for parallel reaction monitoring-based targeted proteomics (15), optimized for the present project. Briefly, the detection of internal standard isotope-labelled heavy peptides triggers the acquisition of MS scans for the endogenous peptides of interest, allowing higher sensitivity and accuracy compared with previous targeted proteomics methods. Details of the optimized workflow will be published elsewhere.

In brief, a total of 100 µg extracted protein was denatured in 2.5 M guanidine hydrochloride (GuHCl), 100 mM HEPES pH 7.8, cysteines reduced with TCEP and alkylated with chloroacetamide, followed by tryptic digest (1:50 protease: protein (w:w)) overnight after adjusting the GuHCl concentration to 0.5 M. Samples (1,000 ng sample peptides and 0.8 pmol heavy peptides) were loaded on Evotip columns (Evosep, Odense, Denmark) and separated on an Evosep (Evosep). One chromatography system coupled in line with an Exploris 480 mass spectrometer (Thermo Fisher Scientific). Separation was achieved from the stationary phase over 11.5 min according to the manufacturer standard method "100 samples per day" (100SPD), and the SureQuant™ analysis mode containing inclusion lists of peptide precursors and their transitions was applied to record the spectra. Peak processing and filtering were performed in Skyline 20.1 (16). The processed report containing area under the curve (AUC) values for the precursor transitions selected was exported for further analysis. The total ion current chromatogram areas for all MS runs were extracted from the raw data and used to normalize the transition AUC values. Protein values were calculated by the sum of peptide transition values, with 2 peptides quantified for each protein. Protein values were rescaled for each protein separately, to a 0–100 scale, for comparison and visualization.

Statistical analysis

As this was an exploratory trial and no longitudinal data of acute and VLU wound fluid protein data were available for similar study populations, no sample size calculation was performed. The clinical data were analysed using descriptive statistical methods. Continuous variables were described by the number of observations (N), mean, standard deviation, median, first, and third quartiles, minimum, and maximum. Categorical and ordinal variables were described by their frequencies and percentages, where the percentage was calculated based on the observed values. The last observation was carried forward (LOCF) method was used for missing data imputation in the analysis of clinical wound parameters (Absolute wound area, WAR, WAR ≥40%, WAR ≥60%, granulation tissue on the wound surface, maximum wound pain during the last 24 hours (VAS), pain at dressing changes (VAS)). All outcomes were analysed for all patients who fulfilled the inclusion and exclusion criteria, whose wounds were treated at least once with the pre-activated polyacrylate-containing wound dressings, and who had a wound area value at the inclusion visit and at least 1 further wound area value. Only intention-to-treat (ITT) data are reported, unless otherwise indicated. To calculate *p*-values for significant differences for the biochemical marker values, pairwise comparison of all time-points used the Wilcoxon signed-rank test for dependent data and the Mann–Whitney *U* test for independent data, both with multiple testing corrections (FDR Benjamini Hochberg, alpha 0.05). Only significant *p*-values are mentioned in the results section.

Table I. Patient characteristics

	Venous leg ulcer wound patients (group A)	Split-thickness donor site wound patients (group B)
Age, years, mean (SD)	69.2 (11.9)	69.5 (11.7)
Median (min–max)	71.0 (34–90)	70.0 (44–85)
Sex, *n* (%)		
Male	26 (45.6)	4 (40.0)
Female	31 (54.4)	6 (60.0)
Height, cm, mean (SD)	167.3 (12.0)	165.3 (10.5)
Median (min–max)	166.0 (146–203)	166.5 (150–182)
Weight, kg, mean (SD)	85.5 (22.9)	90.1 (20.1)
Median (min–max)	82.0 (46.0–146.0)	94.5 (61.0–115.0)
Body mass index, kg/m^2, mean (SD)	30.3 (6.4)	32.8 (6.2)
Median (min–max)	29.4 (20.0–52.1)	30.9 (25.1–43.8)
Chronic venous insufficiency, *n* (%)	55 (96.5)	
ABPI, mean (SD)	0.957 (0.075)	
Median (min–max)	1.000 (0.81–1.05)	
Palpable pedal pulses, *n* (%)		
A. pedalis dorsalis	56 (98.2)	
A. tibialis posterior	56 (98.2)	
History of a deep vein thrombosis, *n* (%)	9 (15.8)	
History of venous surgery, *n* (%)	18 (31.6)	
Diabetes mellitus, *n* (%)	7 (12.3)	1 (10.0)
HbA1c, %, mean (SD)	6.37 (0.88)	7.30 (n.a.)
Median (min–max)	6.30 (5.2–7.8)	7.30 (7.30, 7.30)
Congestive heart insufficiency, *n* (%)	1 (1.8)	
Patient smokes currently, *n* (%)	8 (14.0)	0 (0.0)

SD: standard deviation; ABPI: ankle brachial pressure index; n.a.: not applicable.

Ethical considerations

This study was conducted in accordance with the Declaration of Helsinki (17) and laws and regulations in Poland. This study was approved by the Bioethics Committee at the Silesian Medical Chamber, Katowice, Poland (opinion number 35/2018), and registered with the German Clinical Trials Register (DRKS00015832).

Data availability

All mass spectrometry-based proteomics data discussed in this study have been deposited to PanoramaWeb (https://panoramaweb.org/RPD8Te.url) (e-mail: panorama+reviewer24@proteinms.net; password: ljLRyQCt) with the dataset identifier PXD025748 (http://proteomecentral.proteomexchange.org/cgi/GetDataset?ID=PXD025748).

RESULTS

Clinical outcomes in the venous leg ulcer cohort

A total of 74 patients were screened in the VLU cohort and 57 eligible patients were included in this prospective, multicentre observational trial and were treated for 12 weeks or until complete wound closure. Out of the 57 subjects included in the study (mean observation period 76.9 ± 18.7 days), 47 completed the study according to the protocol (mean observation period 84.5 ± 1.9 days). Ten subjects ended the study prematurely due to the complete wound closure before the final visit (6 patients), consent withdrawal (3 patients), and reason not provided (1 patient). To compare the biochemical markers in VLU exudates, the second cohort consisted of 10 patients with split-thickness donor site wounds after screening and inclusion in the study. Patients were followed for 21 days. Out of the 10 subjects included in the study (mean observation period 19.8 ± 3.3 days), 8 completed the study according to the protocol (mean observation period 21.2 ± 0.9 days). Two subjects ended the study prematurely due to the complete wound

Table II. Wound characteristics

	Venous leg ulcer wounds (group A)	Split-thickness donor site wounds (group B)
Wound size, cm^2, mean (SD)	18.7 (12.3)	40.3 (30.9)
Median (min–max)	14.0 (5.8–59.1)	33.5 (11.4–121.2)
Wounds >10 cm^2, *n* (%)	43 (75.4)	10
Wound age, months, mean (SD)	11.5 (8.4)	
Median (min–max)	8.0 (3–36)	
Wounds with ≥6 months, *n* (%)	32 (56.1)	
Relative wound area covered with		
Necrotic tissue, %, mean (SD)	0.3 (1.5)	
Fibrin deposits, %, mean (SD)	60,9 (30,7)	
Granulation tissue, %, mean (SD)	38.8 (30.8)	

SD: standard deviation.

closure before the final visit. The patient flow is shown in Fig. S1. **Table I** provides information about the patient's characteristics and medical history, and **Table II** lists a description of the patient's initial wound characteristics. All patients in the VLU cohort received compression therapy. Previous dressings, compression therapy use before enrolment in the study and concomitant drug use are listed in Tables SIII and SIV.

The absolute wound size reduction in the VLU cohort is shown in **Fig. 1**A. The relative WAR reached 38.5 ± 49.1% at week 8 and 48.9 ± 51.9% at week 12 (Fig. 1B). Clinicians recorded complete wound closure in 5 (8.8%) patients at week 8, and in 10 (17.5%) patients by week 12. The number of patients reaching a relative WAR of ≥40% was 32 (56.1%) and for the WAR of ≥60% 22 (38.6%) at week 8 and 35 (61.4%) for a WAR of ≥40% and 29 (50.9%) for a WAR of ≥60% at week 12 (Fig. 1C). After 4 weeks 35 out of the 57 patients did not achieve a WAR >30%, a predictor for healing at week 24 (17). Median and IQR values are reported in Table SV. This study did not collect data on recurrences after the 12-week observation period.

The description of local wound characteristics, perilesional skin, and pain reporting are listed in Tables SV and SVI. Granulation tissue reached a maximum at week 6 and robust epithelialization at week 10. Two adverse events were reported and are listed in Table SVII.

For the split-thickness donor site wounds, 2 out of 10 wounds healed within 21 days. The absolute decrease in the wound area and relative WAR development are reported in Fig. 1D and Table SV. Granulation tissue formed around day 4, as did epithelialization. Estimated levels of exudation reached their maximum on day 7 then declined steadily.

Analysis of biomarkers in wound exudates

Wound exudates were extracted from spent dressings and analysed for relative protein abundances of a set of known biomarkers relevant for wound healing (IL-1α, TNF-β, S100A8, S100A9, neutrophil elastase, MMP-2, and MMP-9, collagen (I) α1, fibronectin) (Table SVIII). For 43 out of the 57 wounds in the VLU cohort and for all of the acute wounds, high-quality, full datasets could be obtained and analysed. For 43 out of the 57 wounds in the VLU cohort and for all of the acute wounds, high-quality, full datasets could be obtained and analysed. For the VLU cohort, 301 wound fluids were available for analysis. For the split thickness group, 8 out of the 10 wounds and a total of 64 wound fluids were analyzed. In total, 337 wound fluids (full datasets for 39 VLU patients and 8 split thickness patients) were included in the final results after processing.

To ensure that both types of dressings (Medicomp® and HydroClean®) reliably absorb and release wound exudate proteins with no differences in binding, we first determined the global proteome extracted from either type of dressing from the same patient by shotgun proteomics. Thereby, we identified 1,150 unique proteins from Medicomp® (Table SIX) and 835 proteins from HydroClean® (Tables SX), respectively, including all selected wound markers except the least abundant TNF-α. Both proteomes showed high similarity with >80% identity of the top 100 most abundant proteins and no global shift in relative abundances as determined by label-free quantification (Fig. S2). This indicated that observed differences in protein abundances are indeed related to distinct wound time-points, but not to the type of dressing.

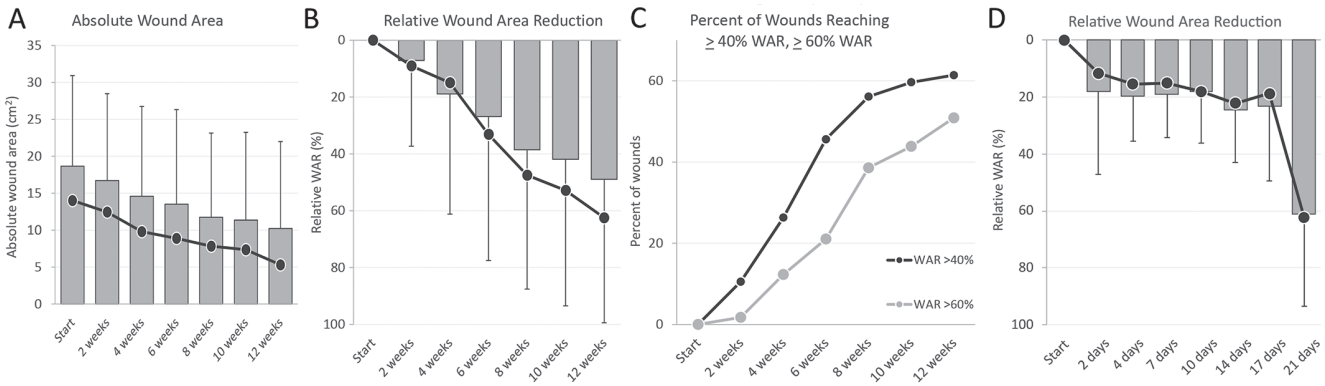

Fig. 1. Wound area development in the venous leg ulcer cohort. (A) Development of the absolute wound area (in cm^2). (B) Relative wound area reduction (WAR) (in %). (C) Percentage of wounds achieving a relative WAR of ≥40% (*dark dots*) and ≥60% (*grey dots*). (D) Relative wound area reduction (WAR) (in %) in the split-thickness donor site wounds. (A–D) Mean values are shown in *grey boxes* with the corresponding median values as *dark dots*.

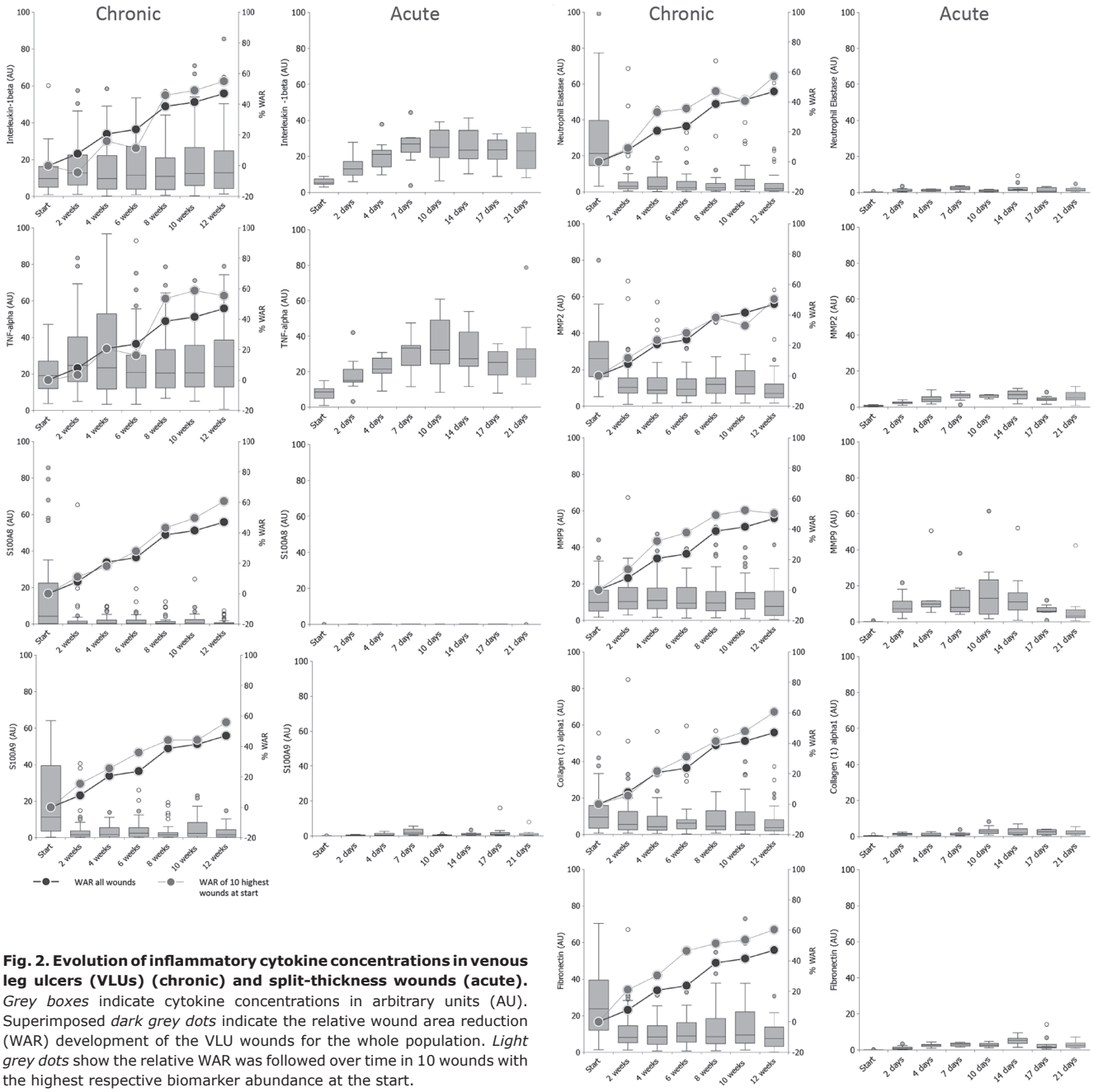

Fig. 2. Evolution of inflammatory cytokine concentrations in venous leg ulcers (VLUs) (chronic) and split-thickness wounds (acute). *Grey boxes* indicate cytokine concentrations in arbitrary units (AU). Superimposed *dark grey dots* indicate the relative wound area reduction (WAR) development of the VLU wounds for the whole population. *Light grey dots* show the relative WAR was followed over time in 10 wounds with the highest respective biomarker abundance at the start.

Between patients, large variations were observed in the abundance levels of inflammatory mediators in the VLU cohort. For IL-1β, the median values remained within a narrow band from 9.6 to 12.9 arbitrary units (AU) throughout the study (**Fig. 2**). There was a significant increase in TNF-α from the start, from 19.1 AU, to 24.7 AU at 2 weeks ($p=0.007$), remaining in this range for the whole study (Fig. 2). Both S100A8 and S100A9 showed markedly different regulations. S100A8 median values decreased significantly, from 4.3 AU at the start to 0.4 at 2 weeks ($p=0.002$), and remained between 0.3 and 0.6 AU (Fig. 2). S100A9 median values decreased from 11.2 AU at the start to 1.6 at 2 weeks ($p<0.001$), not exceeding 2.4 for the remainder of the time period (Fig. 2). The subgroup of wounds with the 10 highest values at the start showed comparable healing trajectories to the whole population (Fig. 2). For the proteases, we also noted a steep decrease in abundance within the first 2 weeks for neutrophil elastase and MMP-2. Starting median values for neutrophil elastase were 21.3 AU, decreasing to 3.0 AU ($p<0.001$)) (Fig. 2). For MMP-2, median values decreased from 26.2 AU at the start to 10.3 AU at 2 weeks ($p<0.001$) (Fig. 2). In contrast, MMP-9 remained almost unchanged throughout the study (Fig. 2). Collagen (I) α1, median values ranged between 9.5 AU and 3.7 AU at 12 weeks (Fig. 2). Finally, fibronectin median values started at 23.9 AU and decreased to 8.2 AU at 2 weeks ($p=0.002$) (Fig. 2). Again, comparing mean

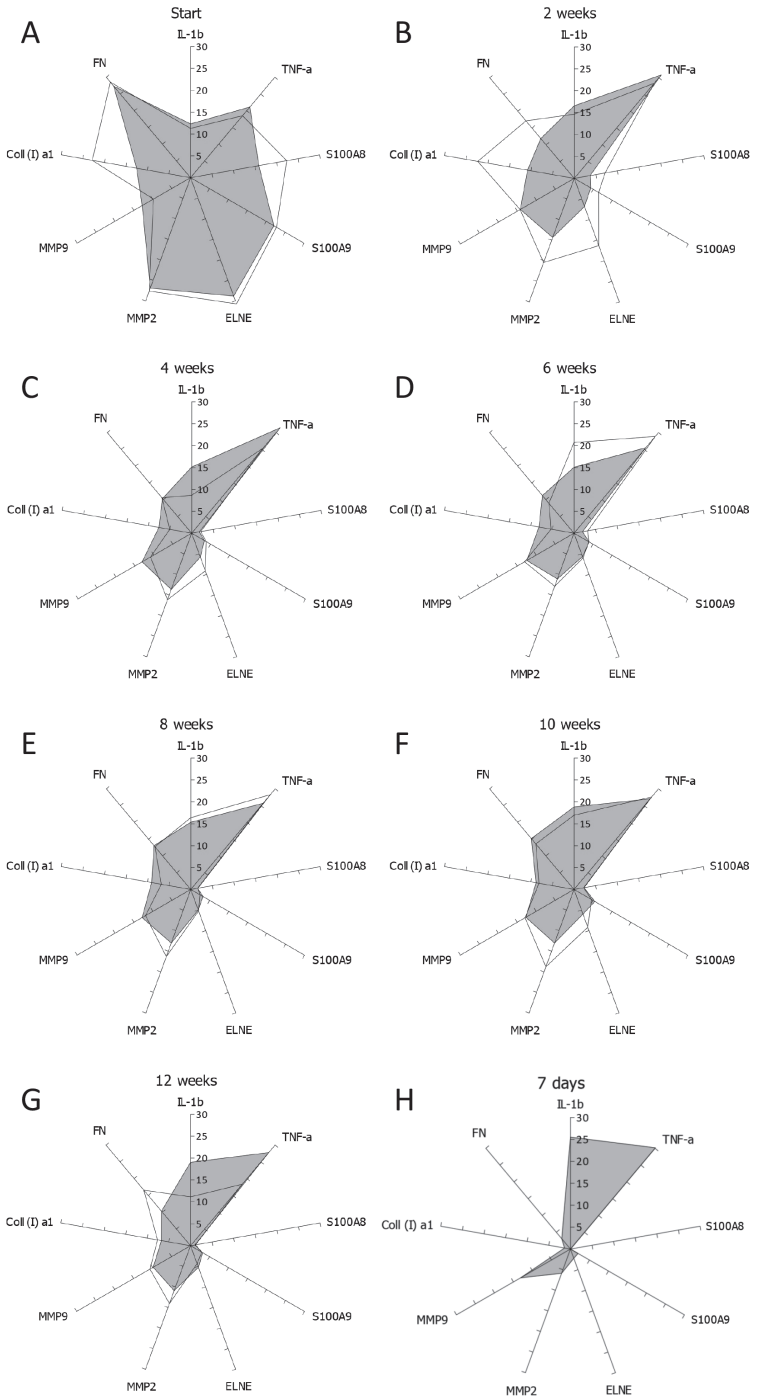

Fig. 3. Biomarker profiles of the venous leg ulcer wound fluids. *Grey areas* represent the mean values of the whole population; *dark lines* indicate the means of the biomarker of the 10 patients with the poorest performance or the relative wound area reduction (WAR) at the respective time-points. (A) At the start, (B) 2 weeks, (C) 4 weeks, (D) 6 weeks, (E) 8 weeks, (F) 10 weeks and (G) 12 weeks. As for the presentation of the 10 poorest responders, the calculation of the relative WAR development was not possible; the relative WAR values of the 2-week time-point were used to rank the biomarker responses at the start. (H) Expression profiles of the acute wound healing cohort at 7 days. IL-1b; interleukin 1b; TNF-a: tumour necrosis factor-alpha, ELNE: neutrophil elastase; MMP2: matrix metalloprotease-2; MMP9: matrix metalloprotease-9; Coll (I) a1: collagen (I) a1; FN: fibronectin.

relative wound area reduction values of the whole population with a subgroup of the 10 highest abundance values at the start did not show major differences (Fig. 2).

The pattern of relative biomarker abundance in VLU wounds shifted over the observation period towards a pattern reminiscent of acute wound healing (**Fig. 3**). Pronounced changes were evident between the beginning and the second week. It took until week 4 for the patterns to start to resemble each other. When comparing mean values of the 10 wounds with the poorest wound healing response, as reflected by WAR and at each time-point with the mean values of the whole cohort, it was noted that it took until week 4 until the patterns became similar. Before this time-point, the poorly responding wounds showed a pattern characterized by high neutrophil elastase, MMP-2, collagen (I) α1, and fibronectin values (Fig. 3). However, the differences did not reach significance (Mann–Whitney *U* test) as the number of patients was too small. Also, the cohort was too small to allow detailed statistical analyses of patient subgroups, such as patients with diabetes mellitus or local wound infections.

In the acute wound healing cohort, 64 wound fluid samples were available. IL-1β and TNF-α median values increased gradually up to day 7 and then decreased slightly until day 21 (Fig. 2). S100A8 values were barely detectable (Fig. 2), and S100A9 was very low (Fig. 2). Neutrophil elastase and MMP-2 values were low, reaching a maximum at 7 days and declining for the rest of the observation period (Fig. 2). MMP-9 median increased up to day 10 and then decreased (Fig. 2). Collagen (I) α1 and fibronectin were detectable, but remained low (Fig. 2). The abundance pattern in the acute wound fluids on day 7 corresponding to the highest expression levels is shown in Fig. 3H.

DISCUSSION

This study analysed the longitudinal pattern of biochemical markers in wound fluids of patients with VLU, and a second cohort of acute wounds illustrating the ranges of these markers in normal wound healing. The patient characteristics of the VLU patient

cohort were typical and comparable to other published venous leg ulcer trials (3, 18, 19). This study included a high proportion of wounds with poor healing tendencies, characterized as larger than 10 cm^2 and older than 6 months, and responding with less than 30% relative WAR at week 4, all indicators of reduced healing within 24 weeks (2, 3, 19–21). Also, fibrin deposits were predominant at the start, indicating that the formation of granulation tissue was still pending. The VLU healing results in the current study appear similar to published outcomes and a well-conducted randomized controlled trial (RCT) (22). The healing rates of the split-thickness donor site wounds in the current study were slightly slower compared with data from a large RCT on split-thickness donor site healing (23). This may have been a result of the harvesting technique with the preparation of thicker grafts, patient-specific parameters, or dressing-related factors, such as the binding of divalent ions and/or MMPs. Still, this group showed the anticipated regulation of the biochemical markers in the wound fluids, complementing earlier work in porcine excisional wound healing experiments (24).

When analysing the biomarker expression pattern in chronic wound exudates, the current study found significant differences in the first 2 weeks, and then the pattern remained relatively constant. When comparing the biomarker expression pattern with acute wounds, the levels of IL-1β or TNF-α showed comparable concentration ranges. For S100A8 and S100A9, however, the concentrations differed significantly between acute and chronic wounds at the start. A similar pattern was observed for neutrophil elastase and MMP-2, with high concentrations at the start in chronic wound fluids decreasing significantly within 14 days, but not reaching the low levels of acute wounds. MMP-9, on the other hand, was not regulated much in chronic wound fluids and the range was similar to that of acute wound fluids at 7 and 10 days. Notably, similar levels of MMP-9 in chronic and acute wounds have been reported before by Kirketerp-Møller et al. (25). Collagen (I) α1 and fibronectin abundances were elevated in chronic vs acute wound fluids, yet only fibronectin showed a decline in chronic wound fluids from the start to 2 weeks. It should be noted that, with the current method, it is not possible to distinguish between proteolytically activated, degraded, or inactive proforms, and thus this study cannot ascertain biological activities. Overall, the current data show complex patterns of biomarker regulation in acute and chronic wound fluids and during the treatment course of chronic wounds. It should be noted that the set of biomarkers does not aim to explain the wound healing cascade, as such, but rather provides a biomarker pattern to describe the wound state at a molecular level. The biomarkers were selected to represent key players in wound healing and which had been characterized in detail previously.

S100A8 and S100A9 were shown to be expressed in acute wounds (26). Neutrophil granulocytes contain large amounts of preformed S100A8/S100A9, which can be readily released and further stimulate neutrophil tissue influx (27), and which can further activate Toll-like receptor 4 (TLR4) (28). Apart from the release of preformed S100A8/S100A9, neutrophils were also shown to express mRNA in the wound bed (26) in addition to macrophages and keratinocytes of the wound borders. It is possible that in VLU wound fluids in the current study the excessively high levels of S100A8 and S100A9 originated predominantly from neutrophils at the start. Concomitantly high levels of neutrophil elastase at the start and the decrease paralleling S100A8 and S100A9 concentrations may support this hypothesis. For neutrophil elastase, wide variations in wound fluid concentrations for chronic wounds, as seen in this study, have been described here, with a trend to decrease with healing (29). Interestingly, our results regarding S100A8 and S100A9 contrast with those of Trøstrup et al. (10). These authors showed significantly lower abundances for both mediators in chronic wound fluids compared with acute wound fluids. These discrepancies cannot be explained with the available clinical data; however, differences in bacterial bioburden in the wounds might have influenced S100A8 and S100A9 levels in chronic wounds (28). Moreover, S100A8/A9 has been identified as a defence mediator in neuropathic diabetic foot ulcers (30). This will be very interesting to follow up in future studies, particularly regarding the correlation with bacterial bioburden.

MMP-2 has been mapped to the connective tissue fibroblasts and endothelial cells in acute wounds (30, 31). The epidermis was almost completely free of MMP-2 signals in *in-situ* hybridizations. MMP-2 mRNA and protein are constitutively present in normal skin, which might suggest a physiological role for MMP-2 in the connective tissue (32). In VLUs, MMP-2 staining was observed in the wound bed (33), and MMP-2 staining was more intense in non-healing ulcers (34). Moreover, MMP-2 among other MMPs was observed to respond to compression therapy alone (35). Better healing outcomes were noted for VLUs that showed a decrease in MMP-1, MMP-2, and MMP-3 after 4 weeks of treatment (35). The current data do not allow any speculation about whether the high neutrophil elastase and MMP-2 protein levels at the start translated into a microenvironment with excessive protease activities. We did not determine whether the proteases were activated and did not determine the abundances of protease inhibitors, such as the different TIMPs. From the literature, one might expect a high net protease activity (29).

Our data on collagen (I) α1 and fibronectin levels are difficult to interpret. Collagen (I) α1 protein in the wound fluids could reflect the degradation of connective tissue or new collagen production with poor deposition and cross-

linking in new fibrils. The higher levels in chronic wound fluids indicate that collagen (I) production and deposition in the granulation tissue is perturbed compared with the acute wound fluids around 10 days when granulation tissue formation was high. Measurement of telopeptides and collagen (I) propeptides in the wound exudates may help to differentiate whether new collagen is produced or if we detected breakdown collagen (I) α1 fragments in the chronic wound fluids. Moreover, for fibronectin, the source, circulation, and/or local production in the granulation tissue is unclear. It was not possible to determine whether the fibronectin we detected was produced as the Ectodomain A-fibronectin form, potentially stimulating TLR4 receptors. Earlier studies suggest local production of fibronectin in venous leg ulcers (10). It is possible that the early changes in the clinical response and biomarker regulation could be due to the biological effects of the polyacrylate superabsorbers in the wound dressings, such as protease inhibition (19).

Several other studies have analysed wound fluids from venous leg ulcers and the difference between acute and chronic wounds as well as between healing and non-healing chronic wounds (10, 12, 13, 24, 36, 37), with some aiming to provide predictive markers for healing/non-healing probabilities. The current study did not attempt to further identify predictive markers. The early clinical response might provide a robust and easily available parameter on the healing probability (3, 4).

This study suggests that there is a rapid biochemical response within 14 days, and then the pattern remained stable from weeks 2 to 12. The clinical parameters improved much later, with the peak of granulation tissue at 6 weeks and epithelialization at week 10. The biochemical signature points to an early diminishing of neutrophil activity. The biomarker signature in the chronic wound fluid at the start shares characteristics of chronic, non-productive inflammation. Clinically, this is evident. The changes during the first 2 weeks in the VLU wound fluids might suggest a decrease in STAT3 activity. S100A8, S100A9, and MMP-2 are regulated by numerous pathways, including STAT3 (38–41), and after the first 2 weeks, a more NF-κB-dominant signature continued and prevailed, reflected by elevated IL-1β, TNF-α, and MMP-9 levels. This hypothesis could unify our biomarker pattern in VLU wound fluids over the observation period. It will be interesting to test this hypothesis in future studies.

Study limitations

This prospective study has some limitations, especially in terms of generalization of the results. Mostly, the sample size was relatively small, especially in the acute wound healing cohort. The acute wound healing cohort was included to estimate the abundance levels of the biochemical parameters in normal wound healing and to provide a reference for the comparison of parameter abundance levels in the VLU cohort. Also, the wound area values between the VLU and acute wound healing cohort were different. Split-thickness wounds mostly epithelialize from skin appendages within the wounds so that overall healing is relatively short compared with the absolute wound size. Still, the expression pattern of the acute wound healing cohort reflected that of acute wound healing, as known from acute wound healing studies in animal models (26). Finally, wound fluid collection at baseline was shorter than for the subsequent time-points, and this may have an impact on the abundance of some of the parameters in the wound exudate (42).

ACKNOWLEDGEMENTS

The authors thank Astrid Filt Beyer, Caroline Uldahl Martensen, Kamille Mercedes Kranker, Karoline Skree-Lassen, and Line Damgaard Henriksen for excellent assistance in wound exudate extraction and sample preparation and the DTU Proteomics Core for maintenance of instruments.

This study was funded by Paul Hartmann AG. UadK acknowledges support by a Young Investigator Award from the Novo Nordisk Foundation (NNF16OC0020670) and PRO-MS: Danish National Mass Spectrometry Platform for Functional Proteomics (grant no. 5072-00007B).

Conflicts of interest: HS is a full-time employee of Hartmann. The other authors have no conflicts of interest to declare.

REFERENCES

1. Patton D, Avsar P, Sayeh A, Budri A, O'Connor T, Walsh S, et al. A meta-review of the impact of compression therapy on venous leg ulcer healing. Int Wound J 2022; Jul 18. [Online ahead of print].
2. O'Meara S, Cullum N, Nelson EA, Dumville JC. Compression for venous leg ulcers. Cochrane Database Syst Rev 2012; 11: CD000265.
3. Marston WA, Ennis WJ, Lantis JC, Kirsner RS, Galiano RD, Vanscheidt W, et al. Baseline factors affecting closure of venous leg ulcers. J Vasc Surg Venous Lymphat Disord 2017; 5: 829–835.e1.
4. Kantor J, Margolis DJ. A multicentre study of percentage change in venous leg ulcer area as a prognostic index of healing at 24 weeks. Br J Dermatol 2000; 142: 960–964.
5. Guest JF, Fuller GW, Vowden P. Venous leg ulcer management in clinical practice in the UK: costs and outcomes. Int Wound J 2018; 15: 29–37.
6. Herrick S, Ashcroft G, Ireland G, Horan M, McCollum C, Ferguson M. Up-regulation of elastase in acute wounds of healthy aged humans and chronic venous leg ulcers are associated with matrix degradation. Lab Invest 1997; 77: 281–288.
7. Lauer G, Sollberg S, Cole M, Flamme I, Stürzebecher J, Mann K, et al. Expression and proteolysis of vascular endothelial growth factor is increased in chronic wounds. J Invest Dermatol 2000; 115: 12–18.
8. Buchstein N, Hoffmann D, Smola H, Lang S, Paulsson M, Niemann C, et al. Alternative proteolytic processing of hepatocyte growth factor during wound repair. Am J Pathol 2009; 174: 2116–2128.
9. Tarnuzzer RW, Schultz GS. Biochemical analysis of acute and chronic wound environments. Wound Repair Regen 1996; 4: 321–325.
10. Trøstrup H, Lundquist R, Christensen LH, Jorgensen LN, Karlsmark T, Haab BB, et al. S100A8/A9 deficiency in non-healing venous leg ulcers uncovered by multiplexed antibody microarray profiling. Br J Dermatol 2011; 165: 292–301.

11. Harris IR, Yee KC, Walters CE, Cunliffe WJ, Kearney JN, Wood EJ, et al. Cytokine and protease levels in healing and non-healing chronic venous leg ulcers. Exp Dermatol 1995; 4: 342–349.
12. Stacey MC, Phillips SA, Farrokhyar F, Swaine JM. Evaluation of wound fluid biomarkers to determine healing in adults with venous leg ulcers: a prospective study. Wound Repair Regen 2019; 27: 509–518.
13. Eming SA, Koch M, Krieger A, Brachvogel B, Kreft S, Bruckner-Tuderman L, et al. Differential proteomic analysis distinguishes tissue repair biomarker signatures in wound exudates obtained from normal healing and chronic wounds. J Proteome Res 2010; 9: 4758–4766.
14. Hessian PA, Edgeworth J, Hogg N. MRP-8 and MRP-14, two abundant Ca(2+)-binding proteins of neutrophils and monocytes. J Leukoc Biol 1993; 53: 197–204.
15. Gallien S, Kim SY, Domon B. Large-scale targeted proteomics using Internal Standard Triggered-Parallel Reaction Monitoring (IS-PRM). Mol Cell Proteomics 2015; 14: 1630–1644.
16. MacLean B, Tomazela DM, Shulman N, Chambers M, Finney GL, Frewen B, et al. Skyline: an open source document editor for creating and analyzing targeted proteomics experiments. Bioinforma Oxf Engl 2010; 26: 966–968.
17. World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects. JAMA 2013; 310: 2191–2194.
18. Harding KG, Krieg T, Eming SA, Flour MLF, Jawien A, Cencora A, et al. Efficacy and safety of the freeze-dried cultured human keratinocyte lysate, LyphoDerm 0.9%, in the treatment of hard-to-heal venous leg ulcers. Wound Repair Regen 2005; 13: 138–147.
19. Humbert P, Faivre B, Véran Y, Debure C, Truchetet F, Bécherel PA, et al. Protease-modulating polyacrylate-based hydrogel stimulates wound bed preparation in venous leg ulcers – a randomized controlled trial. J Eur Acad Dermatol Venereol 2014; 28: 1742–1750.
20. Chaby G, Senet P, Ganry O, Caudron A, Thuillier D, Debure C, et al. Prognostic factors associated with healing of venous leg ulcers: a multicentre, prospective, cohort study. Br J Dermatol 2013; 169: 1106–1113.
21. Kurd SK, Hoffstad OJ, Bilker WB, Margolis DJ. Evaluation of the use of prognostic information for the care of individuals with venous leg ulcers or diabetic neuropathic foot ulcers. Wound Repair Regen 2009; 17: 318–325.
22. Meaume S, Truchetet F, Cambazard F, Lok C, Debure C, Dalac S, et al. A randomized, controlled, double-blind prospective trial with a Lipido-Colloid Technology-Nano-OligoSaccharide Factor wound dressing in the local management of venous leg ulcers. Wound Repair Regen 2012; 20: 500–511.
23. Brölmann FE, Eskes AM, Goslings JC, Niessen FB, de Bree R, Vahl AC, et al. Randomized clinical trial of donor-site wound dressings after split-skin grafting. Br J Surg 2013; 100: 619–627.
24. Sabino F, Egli FE, Savickas S, Holstein J, Kaspar D, Rollmann M, et al. Comparative degradomics of porcine and human wound exudates unravels biomarker candidates for assessment of wound healing progression in trauma patients. J Invest Dermatol 2018; 138: 413–422.
25. Kirketerp-Møller K, Doerfler P, Schoefmann N, Wolff-Winiski B, Niazi O, Pless V, et al. Biomarkers of skin graft healing in venous leg ulcers. Acta Derm Venereol 2022; 102: adv00749.
26. Thorey IS, Roth J, Regenbogen J, Halle JP, Bittner M, Vogl T, et al. The Ca2+-binding proteins S100A8 and S100A9 are encoded by novel injury-regulated genes. J Biol Chem 2001; 276: 35818–35825.
27. Ryckman C, Vandal K, Rouleau P, Talbot M, Tessier PA. Proinflammatory activities of S100: proteins S100A8, S100A9, and S100A8/A9 induce neutrophil chemotaxis and adhesion. J Immunol 2003; 170: 3233–3242.
28. Vogl T, Tenbrock K, Ludwig S, Leukert N, Ehrhardt C, van Zoelen MAD, et al. Mrp8 and Mrp14 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock. Nat Med 2007; 13: 1042–1049.
29. Trengove NJ, Stacey MC, MacAuley S, Bennett N, Gibson J, Burslem F, et al. Analysis of the acute and chronic wound environments: the role of proteases and their inhibitors. Wound Repair Regen 1999; 7: 442–452.
30. Trøstrup H, Holstein P, Christophersen L, Jørgensen B, Karlsmark T, Høiby N, et al. S100A8/A9 is an important host defence mediator in neuropathic foot ulcers in patients with type 2 diabetes mellitus. Arch Dermatol Res 2016; 308: 347–355.
31. Salo T, Mäkelä M, Kylmäniemi M, Autio-Harmainen H, Larjava H. Expression of matrix metalloproteinase-2 and -9 during early human wound healing. Lab Invest 1994; 70: 176–182.
32. Gill SE, Parks WC. Metalloproteinases and their inhibitors: regulators of wound healing. Int J Biochem Cell Biol 2008; 40: 1334–1347.
33. Norgauer J, Hildenbrand T, Idzko M, Panther E, Bandemir E, Hartmann M, et al. Elevated expression of extracellular matrix metalloproteinase inducer (CD147) and membrane-type matrix metalloproteinases in venous leg ulcers. Br J Dermatol 2002; 147: 1180–1186.
34. Mwaura B, Mahendran B, Hynes N, Defreitas D, Avalos G, Adegbola T, et al. The impact of differential expression of extracellular matrix metalloproteinase inducer, matrix metalloproteinase-2, tissue inhibitor of matrix metalloproteinase-2 and PDGF-AA on the chronicity of venous leg ulcers. Eur J Vasc Endovasc Surg 2006; 31: 306–310.
35. Beidler SK, Douillet CD, Berndt DF, Keagy BA, Rich PB, Marston WA. Multiplexed analysis of matrix metalloproteinases in leg ulcer tissue of patients with chronic venous insufficiency before and after compression therapy. Wound Repair Regen 2008; 16: 642–648.
36. Mast BA, Schultz GS. Interactions of cytokines, growth factors, and proteases in acute and chronic wounds. Wound Repair Regen 1996; 4: 411–420.
37. Kirketerp-Møller K, Doerfler P, Schoefmann N, Wolff-Winiski B, Niazi O, Pless V, et al. Biomarkers of skin graft healing in venous leg ulcers. Acta Derm Venereol 2022; 102: adv00749.
38. Hsu K, Chung YM, Endoh Y, Geczy CL. TLR9 ligands induce S100A8 in macrophages via a STAT3-dependent pathway which requires IL-10 and PGE2. PLoS One 2014; 9: e103629.
39. Lee MJ, Lee JK, Choi JW, Lee CS, Sim JH, Cho CH, et al. Interleukin-6 induces S100A9 expression in colonic epithelial cells through STAT3 activation in experimental ulcerative colitis. PLoS One 2012; 7: e38801.
40. Xuan X, Li S, Lou X, Zheng X, Li Y, Wang F, et al. Stat3 promotes invasion of esophageal squamous cell carcinoma through up-regulation of MMP2. Mol Biol Rep 2015; 42: 907–915.
41. Xie TX, Wei D, Liu M, Gao AC, Ali-Osman F, Sawaya R, et al. Stat3 activation regulates the expression of matrix metalloproteinase-2 and tumor invasion and metastasis. Oncogene 2004; 23: 3550–3560.
42. Zillmer R, Trøstrup H, Karlsmark T, Ifversen P, Ågren MS. Duration of wound fluid secretion from chronic venous leg ulcers is critical for interleukin-1α, interleukin-1β, interleukin-8 levels and fibroblast activation. Arch Dermatol Res 2011; 303: 601–606.