

# Biochemical profiling of proteins and metabolites in wound exudate from chronic wound environments

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## Abstract

The lack of fundamental knowledge on the biological processes associated with wound healing represents a significant challenge. Understanding the biochemical changes that occur within a chronic wound could provide insights into the wound environment and enable more effective wound management. We report on the stability of wound fluid samples under various conditions and describe a high-throughput approach to investigate the altered biochemical state within wound samples collected from various types of chronic, ulcerated wounds. Furthermore, we discuss the viability of this approach in the early stages of wound sample protein and metabolite profiling and subsequent biomarker discovery. This approach will facilitate the detection of factors that may correlate with wound severity and/or could be used to monitor the response to a particular treatment.

## Introduction

Wound care is a costly burden on society, accounting for 3% of the total health care expenditure in developed countries<sup>1</sup>. In terms of the Australian health budget, these rates equate to an annual cost of \$2.61 billion (3% of health spend in 2006)<sup>2</sup>. Indeed, wound care is the second most commonly billed Medicare item and consumes up to 22–50% of community nursing care time<sup>3,4</sup>. These figures are expected to increase in line with projected growth of the ageing population and an increasing burden of chronic disease, particularly peripheral vascular disease and diabetes. Currently, chronic wound management is guided by a few subjective tests but still relies heavily on the practitioner's expertise in the field. The current tests employed essentially identify the aetiology of these lesions but fail to assess wound chronicity and monitor the management of these wounds. In view of this, it is clear that improved diagnostic and prognostic tests are urgently required and this project is focused on addressing this problem.

Chronic wounds are a silent epidemic and despite many decades of research these wounds remain one of the greatest clinical and biochemical mysteries<sup>1</sup>. Chronic wounds are a heterogeneous group of disorders and are broadly classified into venous, diabetic, arterial and pressure wounds<sup>5</sup>. The

frequency of these chronic wounds has led current research to focus on the wound environment to provide an insight into the problem of non-healing wounds. The disruption to the normal healing process results in the modification of many biochemical factors and biochemical pathways. Therefore, attaining clinical samples from the site of injury will assist in the identification of a variety of biochemical factors that might be indicative of the state of healing. Wound fluid (WF) provides an insight into the local extracellular microenvironment of the wound. It contains a dynamic combination of proteins, peptides, metabolites and other breakdown products that reflect the current status of the wound<sup>6</sup>. Moreover, it is non-invasive, local to the site of disease, readily available for collection at frequent time points and can potentially be used for diagnostic and prognostic purposes<sup>7</sup>. However, it is important that any clinical investigation and/or biochemical analysis of WF takes into consideration the downstream implications associated with the sample collection and storage conditions. Of concern are variations in sample storage and handling conditions, the impact of multiple freeze–thaw cycles and the possible benefit of including protease inhibitors, each of which can impact on the information obtained during analysis. Thus, sample stability represents a critical consideration in the analytical pipeline.

The majority of wound research has focused on low-throughput ELISA and immunoblotting approaches that measure over-expressed proteins in WF from leg ulcers<sup>8-10</sup>. The recent advancement in technology has resulted in high-throughput identification delivered through proteomics and metabolomics. Proteomics is defined as the study of the full complement of proteins that are present at a given time and pathophysiological condition while metabolomics focuses on all the low molecular weight molecules below 1500 Da present in a particular physiological state. Whilst there have been numerous studies involving the application of metabolomics to investigate a number of disease states<sup>11-14</sup>, there have been no reported studies using this technology to

assess the chronic wound environment. On the other hand, there have been a limited number of proteomic investigations of WF but these have focused on method development<sup>15</sup> or, more recently, on the comparison of acute and chronic wounds<sup>16</sup>. Therefore, investigating the wound proteome and metabolome during healing and non-healing will give rise to new biomarkers that can be used as diagnostic indicators as well as identify new therapeutic targets.

Herein, we report on the stability of WF samples under various conditions and describe a high-throughput approach to investigate the altered biochemical state within wound samples collected from two separate cohorts of chronic ulcerated wounds (diabetic and non-diabetic aetiologies). Furthermore, we discuss the viability of this approach in the early stages of wound sample protein and metabolite profiling and subsequent biomarker discovery. Overall, the objective of this project was to assess the stability of the WF and develop a novel approach to allow identification of both proteins and metabolites in WF. This will lead to the detection of factors that may correlate with wound severity and/or could be used to monitor the response to a particular treatment.

## Methods

### Patient recruitment and clinical data

Ethics was obtained by the Queensland University of Technology Human Research Ethics Committee for the collection of wound samples from leg ulcers and Levine swabs from diabetic ulcers (ethics application numbers 1000001255 and HREC/11/QPCH/58, respectively). The study was conducted according to Declaration of Helsinki principles and written informed consent was obtained from all patients before enrolment. Patients referred to the Queensland University of Technology (QUT) Health Clinic's Wound Healing Community Outreach Service, Kelvin Grove, Queensland were recruited into the study. WF, swabs and clinical data were collected upon each visit. The pressure ulcer scale of healing (PUSH) tool was used to track healing of chronic leg ulcers. The score (between 1 and 16) incorporates wound area, measured by Visitrak™ (Smith and Nephew, Hull, UK), the amount of exudate and the type of tissue (that is, epithelial, granulating, slough or necrotic). For the diabetic foot ulcers the University of Texas Diabetic Wound Classification System was used to track healing<sup>17</sup>. Patients involved in the study were treated with evidence-based best practice depending on the wound type.

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## Wound sampling and processing

For patients with chronic leg ulcers, samples were collected sequentially until healing was achieved or up to 24 weeks, whichever came first. Following wound cleansing and debridement, a WF sample was collected from the ulcer. The WF was allowed to accumulate for up to 30 minutes underneath an occlusive dressing then aspirated using a sterile pasture pipette. Due to the nature of the ulcer, diabetic foot ulcers were sampled using a swab. The method employed for this collection procedure was the Levine swab technique. Samples were collected weekly from patients over a period of 12 weeks. Immediately following collection, both sample types were refrigerated prior to transport to the Institute of Health and Biomedical Innovation. WF samples were then clarified by centrifugation (14,000 relative centrifugal force (RCF) for 10 minutes), the supernatant sub-aliquoted and subsequently stabilised with 1:1 addition of 0.5 M sodium citrate, pH 4.5. Wound swabs were processed independently of aspirated WFs (Figure 1). Each swab tip was transferred to a sterile tube containing 500  $\mu$ L of 20% acetonitrile, 25 mM ammonium acetate and sonicated for 15 minutes. All wound samples were then stored at 80°C until further analysis.

## WF concentration

Protein concentration was determined using the Coomassie Plus protein assay kit (Thermo Fisher Scientific, Massachusetts, USA), a modification of the Bradford method. As per the manufacturer's instructions, a set of bovine serum albumin standards ranging in concentration from 0.03 to 1 mg/mL was prepared. In addition, wound samples were diluted 1:50 with phosphate buffered saline to fit within the standard curve. Then, 300  $\mu$ L of Coomassie Plus reagent was added to each well containing 10  $\mu$ L of sample or standard. The samples were mixed and then incubated for 10 minutes at RT. All samples were measured at 595 nm in a BioRad UV-Visible Benchmarkplus Microplate spectrophotometer (Bio-Rad, Hercules, USA). The protein concentration of each unknown sample was determined on the standard curve generated.

## WF protein stability studies

WF samples were subjected to four stability studies to determine if any protein degradation arises from sample handling. The degradation products of proteins can present confounding factors in the protein profiles of WF, making biomarker discovery difficult. Of particular interest are: the short-term and long-term storage temperatures of WF samples; the inclusion or exclusion of protease inhibitors; and

the number of freeze–thaw cycles. The literature indicates that these conditions can influence downstream results if not taken into account early within an investigation and if these conditions are not standardised across all samples collected within a study<sup>18</sup>.

## Protease inhibition

WF protein fractions were stored for one month at 24°C (room temperature; RT), 4°C and –80°C with and without Halt™ protease inhibitor cocktails (Thermo Fisher Scientific, Waltham, MA, USA). Frozen samples were simultaneously thawed on ice then all samples were analysed by SDS-PAGE. Pre-cast NuPAGE™ (Invitrogen, Carlsbad, CA, USA) Bis-Tris gels with a 4–12% gradient were used to separate proteins. Gels were run in MES running buffer at 200 V for 35 minutes and stained with silver stain (Pierce® Silver Stain Kit; Thermo Fisher Scientific) as per the manufacturer's instructions to visualise proteins.

## Long-term storage and freeze–thaw cycles

The effect of long-term storage temperature on the degradation of proteins in WF was determined by storing WF at 24°C (RT), 4°C, –20°C or –80°C for one month in the dark. After one month elapsed, frozen samples were simultaneously thawed on ice then all samples were analysed by SDS-PAGE and silver-stained as described previously. In addition, the effect of multiple freeze–thaw cycles on samples that are stored at –80°C was assessed by subjecting WF to 1, 2, 3, 5 or 10 freeze–thaw cycles. This procedure consisted of freezing WF at 80°C then thawing on ice followed by briefly vortexing and freezing again at 80°C. Proteins in the samples were separated by SDS-PAGE and silver stained as described previously.

## Short-term storage

WF was stored at 24°C (RT) or on ice for 24 h with aliquots removed at time points 0.5 h, 1 h, 2 h, 4 h, 7 h, 8 h and 24 h. After each collection time point, aliquots were snap frozen in liquid nitrogen and stored at –80°C. All samples were simultaneously thawed and analysed by SDS-PAGE and silver stained as described previously.

## Sample fractionation validation

Ultrafiltration was selected as the method to separate wound samples into metabolite and protein fractions, which allows two separate workflows of a single sample (Figure 1). In order to assess the robustness of the chosen sample fractionation method, WF samples from a previous library that used the same sample collection and processing technique were

used. Nanosep 3 kDa molecular weight cut off centrifugal filters (PALL, New York, USA) were used according to the manufacturer's instructions. Briefly, a standard volume of 5  $\mu$ L of WF was diluted in 295  $\mu$ L of buffer containing 25 mM ammonium acetate in 20% acetonitrile. Ammonium acetate was used in the buffering system as it is a volatile salt and was therefore compatible with downstream analysis. The ultrafiltration cartridges were washed with the dilution buffer prior to loading the diluted sample. The samples were centrifuged at 6000 RCF for 15 minutes at RT. Subsequently, the filtrates and retentates were dried using a vacuum concentrator (Labconco, Kansas USA). Following the drying of fractions, pellets were resuspended in loading buffer and the entire fractions were analysed using SDS-PAGE and visualised using silver stain (as described previously). A duplicate set of dried fractions were resuspended in appropriate buffers to allow analysis with proposed mass spectrometry approaches (Figure 1). Proteins were analysed using a surface enhanced laser desorption/ionisation time-of-flight mass spectrometer (SELDI-TOF MS) whilst metabolites were analysed using a quadrupole time-of-flight mass spectrometer (QTOF MS).

## Results

### Patient population

A total of 64 patients (34 male and 30 female), with 71 ulcer wounds, were recruited into the study over a 15-month period, with seven patients presenting with more than one ulcer. The cohort ranged from 29 to 98 years of age, with a median age of 72 years. For patients with non-diabetic ulcers ( $n=53$ ), over 71% ( $n=38$ ) of the patients' main income stream is from some form of pension, while only 17% ( $n=9$ ) are currently employed, highlighting the need for cheaper wound care options.

A total of 292 WF samples and 72 wound swab samples were collected from 71 ulcers of differing aetiologies. The most common types of ulcer recruited into the study was of mixed (arterial/venous) and venous aetiologies, accounting for 35% ( $n=25$ ) and 28% ( $n=20$ ) of the total ulcers, respectively (Figure 2). The remaining ulcers are made up of diabetic ( $n=11$ ), arterial ( $n=8$ ), pressure ( $n=4$ ) and surgical/amputation wounds ( $n=3$ ) (Figure 2). Twenty-eight ulcers had been present for over 24 weeks at the baseline date (the date the patient was recruited into the study). The size of the non-diabetic ulcers varied from 0.2  $\text{cm}^2$  to 35.5  $\text{cm}^2$  as measured by Visitrak<sup>TM</sup> with a median size of 2.2  $\text{cm}^2$ . The PUSH<sup>TM</sup> was used as a scoring method to grade the non-diabetic ulcers<sup>19</sup>.

In this population we found a PUSH score range of 5 to 16, with a median of 10. The University of Texas Diabetic Wound Classification System was used to measure the severity of a diabetic foot ulcer<sup>17</sup>. The depth of the wound, presence of infection and ischaemia in the wound environment were all taken into consideration when classifying this type of ulcer. Wounds that have no sign of infection and have epithelialised are scored Stage A, Grade 0 or A0 (least severe), while wounds penetrating to the bone and joints while exhibiting the presence of both infection and ischaemia are scored Stage D, Grade 3 or D3 (most severe). The diabetic foot ulcers in this cohort were predominantly neuro-ischaemic. An additional characteristic of these wounds is the protein concentration obtained from the exudates collected (Figure 3). There was high variation in the protein concentration obtained from each sample, regardless of the aetiology of the ulcer. Patient recruitment into this study is ongoing to expand the WF library for proteomic and metabolomic analyses.

### WF protein stability studies

#### *Protease inhibition*

Chronic wounds display exacerbated proteolytic activity which leads to enhanced degradation of the extracellular matrix, proteins and their receptors. Therefore, we assessed the stability of WF across 1 month at 4°C, 24°C and 80°C in the presence (+PI) or absence (-PI) of protease inhibitors. The results obtained demonstrated increased degradation and proteolytic fragmentation of proteins within WF in the absence of protease inhibitors at 4°C and 24°C (Figure 4). There was an increase in the number of protein bands visualised below 50 kDa when comparing WF stored at 24°C with (lane +24, +PI) and without (lane +24, -PI) the addition of protease inhibitors. In addition, a new band below 10 kDa was observed in WF stored at 24°C without protease inhibitors (lane +24, -PI). Similarly, the effects of protein degradation were also evident in WF stored at 4°C without protease inhibitor but to a much lower extent (lane +4, -PI). Specifically, an additional protein band at approximately 20 kDa was detected in WF stored at 4°C without protease inhibitors compared to the same sample treated with protease inhibitors (lane +4°C, -PI). In contrast, samples stored at 80°C show no difference in the protein profile with or without the addition of protease inhibitors.

#### *Long-term storage temperature and freeze-thaw cycles*

Sample storage and multiple freeze thawing are important factors that should be considered to prevent variability and

ensure consistent, reliable results. Long-term storage of WF at 24°C, 4°C, 20°C and -80°C was analysed using SDS-PAGE (Figure 5A). The results demonstrated that WF stored at 4°C and 24°C produced increased protein degradation patterns on SDS-PAGE as indicated by smearing below 50 kDa (lanes +24 and +4). In addition, there was a corresponding loss of protein bands above 250 kDa observed in Lane +24. There were new bands observed below 37 kDa in WF stored at 24 and 4°C when compared to samples stored at -20 and -80°C (lanes +24°C and +4°C). Storing WF samples at either 20°C or 80°C showed no obvious protein degradation in comparison to samples stored at 4°C or 24°C.

SDS-PAGE was also used to analyse WF subjected to 1, 2, 3, 5 and 10 freeze-thaw cycles when stored at 80°C. The results showed no additional bands or any protein smearing which is indicative of protein degradation (Figure 5B). However, a decrease in protein staining intensity was observed below 10 kDa after 10 freeze-thaw cycles. This suggests that up to five freeze-thaw cycles may not have an effect on the gross protein profile of WF.

### Short-term storage temperature

Short-term sample storage is especially important during sample processing as it could falsely introduce variation between samples. WF stored on ice and at 24°C over a period of 24 h were analysed to visualise sample integrity during these storage conditions. The results obtained showed no apparent changes in the gross protein profile of samples stored on ice for up to 24 h (Figure 6A). The SDS-PAGE analysis of WF samples stored at 24°C showed similar results except for an additional protein band at approximately 37 kDa after 24 h of storage (Figure 6B).

### Sample fractionation validation

Sample fractionation successfully separated the high molecular weight (> 3 kDa) proteins from the low molecular weight (< 3 kDa) peptides, metabolites and break-down products as indicated by SDS-PAGE (Figures 7A and 7B). This was evident in Figure 7B, which demonstrated no protein passed through the filter due to no protein staining in the metabolite fraction. In Figure 7A, the proteins in the retentate were separated by strong anionic chromatography

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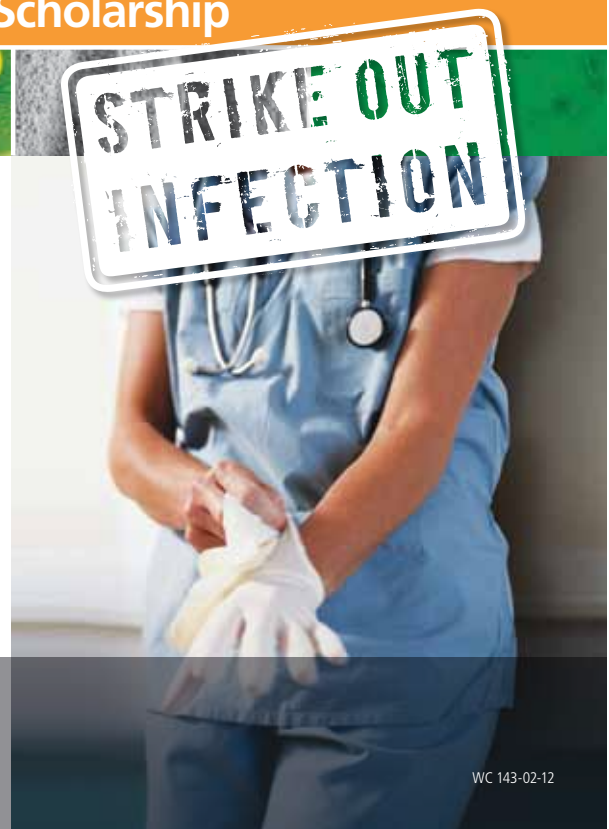
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on the surface of a ProteinChip array. The bound peptides and proteins were analysed by the SELDI-TOF MS and the spectral profile generated demonstrates the presence of these biomolecules within the retentate fraction (Figure 7A). Each peak within the spectrum can be considered a protein or peptide with the peak height as a measure of relative quantity. Similarly, the analysis of the filtrate fraction, by liquid chromatography mass spectrometry, demonstrated the presence of metabolites within this fraction (Figure 7B). This representative figure demonstrates the feasibility of using mass spectrometry to profile the metabolite molecules, which have been enriched by the fractionation approach.

## Discussion

The information presented in this paper is the preliminary data obtained from a larger study investigating changes in the biochemical profiles of longitudinal samples from chronic wounds. The burden of chronic wounds is significant and research within this area is underdeveloped. Through this work we have begun to address the current lack of information regarding biochemical profiling of chronic wounds. We have established a viable approach to profile the proteins, peptides and metabolites within chronic wounds and, furthermore, monitor the changes in these profiles as wounds heal or remain unhealed. Moreover, through this work we have determined a number of factors that need to be considered when conducting a biomolecular investigation of chronic wounds.

The variety of differences observed in patient demographics, ulcer aetiology and treatment regimes add a large amount of variation to the investigation of biomolecular changes that occur during wound healing. These pre-analytical variations can influence the conclusions made from biochemical data. Thus, any investigation of wounds should take into account the pre-analytical variables recorded in the clinic and within the laboratory. Furthermore, it is important under these circumstances to maintain a large sample population to reduce the confounding effects of pre-analytical variation. Our clinical data show a wide variation in the demographics of patients and, interestingly, our ulcer aetiology data does not reflect the cited 70% venous incidence within the literature<sup>20</sup>. We found the majority of patients present with ulcers of a mixed aetiology (37%), whereas venous ulcers comprised only 29% of aetiologies encountered. However, this may reflect the limited sample population and lack of multiple clinical sites. The clinical variables recorded at collection points (such as PUSH score) will be used during

the interrogation of wound sample profiles. Biochemical profiles can be correlated against these variables to discover indicators of healing or non-healing.

We normalised the amount of protein loaded onto the surface of ProteinChip arrays against the total protein of an individual sample collected from the wound site. Of importance is the variation in total protein that can be encountered in the collection of clinical samples (Figure 3). This can be attributed to the loss of wound exudate during wound closure. Moreover, this can limit some analytical approaches and impact on the amount of information obtained<sup>21</sup>. Therefore, the fractionation method allows for the analysis of one sample by two independent platforms. Through the analysis pipeline described herein we could successfully ascertain protein profiles from both swabs and aspirated WFs.

Our analytical approach incorporates an initial step to clarify and then stabilise the wound sample using sodium citrate. Previous studies indicate that 0.5 M sodium citrate, pH 4.5, is capable of stabilising low molecular weight molecules and inhibit the oxidation of free thiols<sup>22</sup>. Subsequently, we employed a sample fractionation method, which separated wound samples into a protein component and a metabolite component. We report an optimised method that enables clear separation of these two species from a single sample for independent downstream analyses. The use of a 20% acetonitrile, 25 mM ammonium acetate buffer permits dissociation of metabolites from proteins and, due to its high volatility, allows sample concentration with subsequent buffer exchange dependent on the profiling approach utilised<sup>23</sup>.

Irrespective of the collection method selected, any wound sample that is obtained should be collected in a non-variable and standardised fashion to ensure all samples are treated equally both at collection and then during subsequent analyses<sup>6</sup>. Once collected, however, the actions of proteases and spontaneous degradation within wound samples can continue and thus the biomolecular profile produced can include artefacts or degradation products that do not reflect the true biochemical situation of the wound at the collection time point. Thus, protein stability studies were performed to determine the amount of degradation that occurs as a result of sample handling. The inclusion of a protease inhibitor cocktail is not recommended when samples are stored at  $-80^{\circ}\text{C}$  as there is no apparent degradation (Figure 4). This is supported in the literature; however, controversy still surrounds this area particularly with the potential artefacts

produced by these inhibitors in mass spectrometry analyses [24]. Long-term sample storage should be maintained at a temperature of  $-80^{\circ}\text{C}$  (Figure 5A), this is consistent with our protease inhibitor study and supported by plasma protein storage conditions suggested in the literature<sup>18</sup>. In addition, every effort should be made to reduce the number of freeze–thaw cycles; however, we observed no changes in the protein profile above 10 kDa, when analysed by silver-stained SDS-PAGE, for up to 10 freeze–thaw cycles (Figure 5B). The number of freeze–thaw cycles should be reduced to five cycles when mass spectrometric examinations are undertaken<sup>24</sup>. Furthermore, results of the short-term temperature storage study indicate that samples should be kept at  $4^{\circ}\text{C}$  or on ice at all times if not frozen to reduce the incidence of protein degradation (Figure 6B). Ideally, these samples should be used within eight hours of defrosting.

Mass spectrometry is an analytical technique that provides a sensitive and accurate method to measure the relative amounts of metabolites and proteins present in wound samples. Spectral profiles generated by mass spectrometry contain information on the mass-to-charge ratio of biomolecules,

which are then used to identify specific biomolecules by comparing these to a spectral database of known molecules. In our approach, the complexity of biomolecules within a sample is reduced through our fractionation approach. The chemistry of the chromatography approach used determines the binding affinity and selectivity of molecules within a sample, allowing for richer datasets. Moreover, to monitor for variations and maintain high reproducibility in the spectral data, samples were analysed in triplicate with quality control samples or molecular standards. Weakly correlating replicate spectra can then be investigated further and discarded if necessary.

The use of a SELDI-TOF MS provides for a high-throughput discovery phase tool that can produce protein profiles of WFs. The selectivity of different chromatographic surfaces on ProteinChip arrays provides sample complexity reduction and sub-grouping of the WF proteome based on protein/peptide hydrophobicity, ionic charge or metal affinity. The spectral profiles, of bound peptides and proteins, generated by the SELDI-TOF MS (for example, Figure 7B) can be achieved for each wound sample collected and thus a library



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of wound protein profiles can be produced. This library can then be mined for patterns associated with clinical factors including healing outcomes. Thus, the SELDI-TOF MS is a suitable biomarker discovery tool and, although limited to this use, it is complemented by the utilisation of more sensitive mass spectrometers for targeting, analysing and validating any potential biomarkers. Likewise, the metabolites and breakdown products present in wound samples can be investigated in a similar fashion. Metabolites are considered to be breakdown products of metabolic reactions and are, therefore, the most predictive of phenotype<sup>25,26</sup>. As demonstrated in our work flow (Figure 1) we use a quadrupole time-of-flight mass spectrometer to profile the metabolites. Similar approaches have been reported on the analysis of cell or plant extracts using the QTOF MS as the mass analyser<sup>27</sup>. This provides accurate mass measurements with high-mass resolution which is important for downstream metabolite identification<sup>28</sup>. Therefore, a slight protein or enzyme imbalance could create an abundance of certain metabolites that may be indicative of a pathological state. We believe that the proposed workflow of splitting a single sample into two "omics" approaches will allow complementation of results and aid in the discovery of novel biomarkers indicative of wound status. Of note, patient recruitment and wound sample collection is ongoing to expand the current library to allow for a larger set of proteomic and metabolomics profiles to enable the identification of biomarkers. These biomarkers can then assist clinicians with treatment management and provide better outcomes for patients. This will reduce ulcer duration and consequently improve patient outcomes and, in turn, reduce the cost on the health care system.

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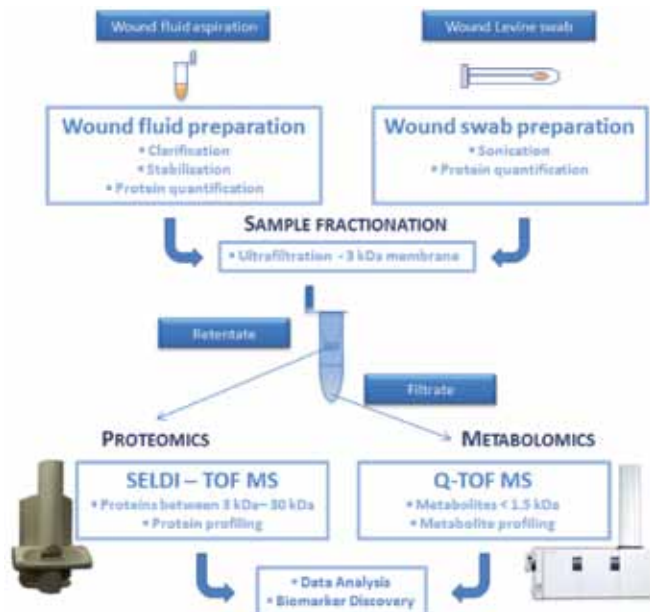


Figure 1.

A schematic representation of wound sample fractionation and analysis. The experimental approach developed to analyse the proteins, peptides and metabolites present within wound fluid and wound swab samples. Initial fractionation of sample into low and high molecular weight components is followed by sample concentration and buffer exchange prior to analysis using SELDI-TOF MS or QTOF MS.

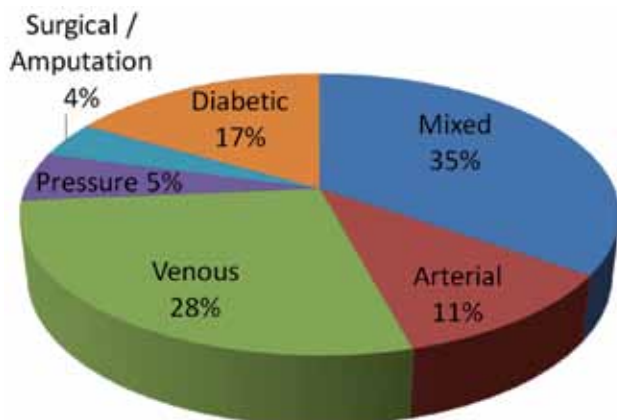


Figure 2.

**Distribution of wound types currently recruited in the study.** A total of 71 wounds with various aetiologies sampled from 64 patients. The majority of wounds encountered are of mixed aetiology, which is a combination of venous and arterial aetiologies.

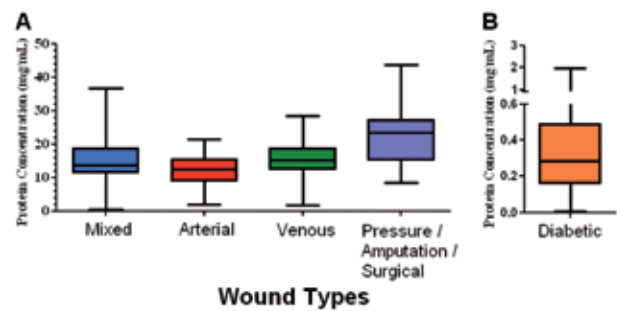


Figure 3.

**Protein concentration of wound samples.** A. Represents the median (Q2), minimum (lower bar) and maximum (upper bar) concentrations for the various wound fluid samples collected from non-diabetic wounds. B. Represents the median (Q2), minimum (lower bar) and maximum (upper bar) concentration of wound swabs collected from diabetic foot ulcers.

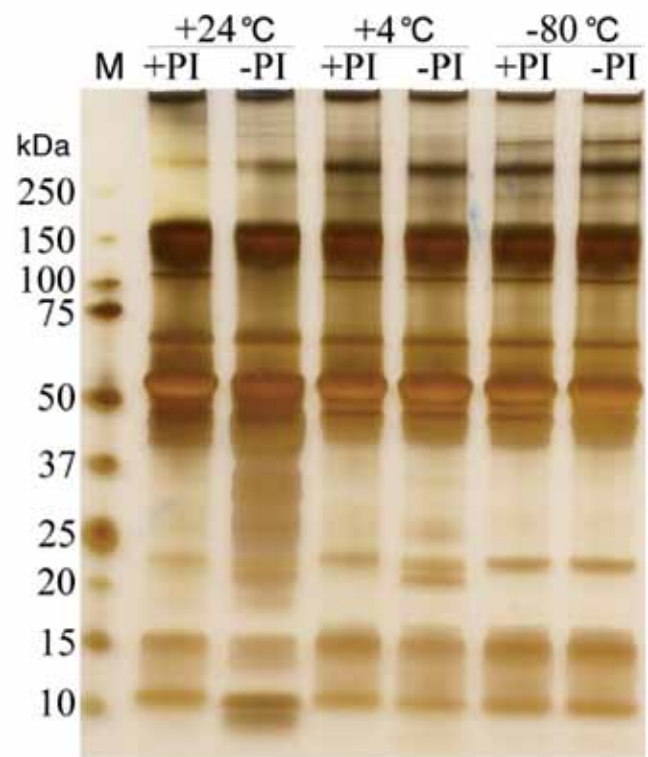


Figure 4.

**Effect of protease inhibitors on WF stability.** WF protein degradation is apparent, as smears or new bands, in samples stored for one month at +24°C and +4°C without the protease inhibitor cocktail. In lane 3 (+24°C -PI), a protein at approximately 100 kDa has been degraded and there are a number of fragments under 50 kDa present. There is no apparent difference in the samples stored at -80°C with or without protease inhibitor cocktail.

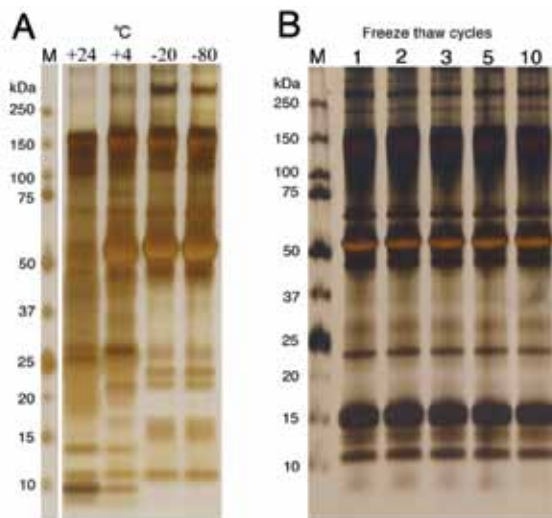


Figure 5.

**Effect of temperature and freeze–thaw cycles on WF stability.**

**A.** WF stored for one month at either 24°C, 4°C, –20°C or 80°C (without protease inhibitors). Degradation of protein through enzymatic and spontaneous events is apparent in samples stored at RT and at 4°C when compared to samples stored in the freezer. Protein smears are present below 50 kDa in lanes +24°C and +4°C and there is a new band at 75 kDa in lane +4°C. **B.** WF samples taken through repeat freeze thaw cycles show minor changes after five freeze–thaw cycles with the disappearance of protein bands under 10 kDa in lane 6 (10 freeze–thaw cycles).

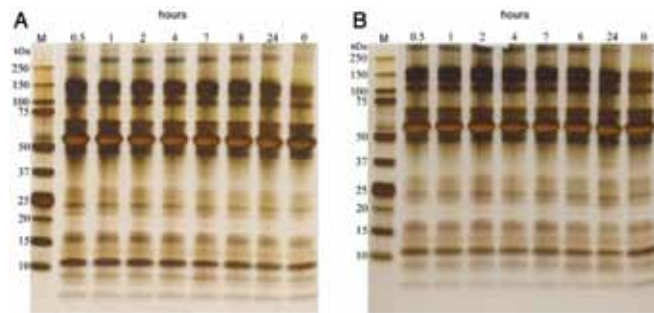


Figure 6.

**Short-term effect of temperature on WF stability.**

**A.** WF samples stored on ice with sampling time-points at 0.5 h, 1 h, 2 h, 4 h, 7 h, 8 h, 24 h and control kept at 80°C prior to SDS-PAGE analysis. No gross changes evident in WF samples stored on ice for up to 24 h as indicated by no new protein bands or smears. **B.** WF samples stored at 24°C with sampling time-points at 0.5 h, 1 h, 2 h, 4 h, 7 h, 8 h, 24 h and control kept at 80°C prior to PAGE analysis. New protein band at 37 kDa and smearing between 20 kDa and 25 kDa post 8 h time-point suggests protein degradation.

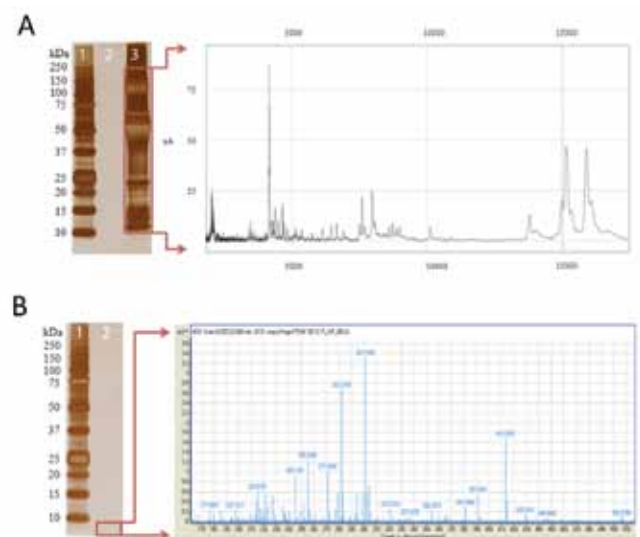


Figure 7.

**Representative profile analysis of WF.**

**A.** SDS-PAGE separation of the retentate fraction from a single WF sample (left). Proteins in the retentate were also loaded onto a ProteinChip array and profiled using the SELDI-TOF MS (right). Gel bands represent WF proteins, the red box and arrows indicate that the sample was then run on the SELDI-TOFMS and peak features in the exemplar spectrum represent proteins and peptides from WF. **B.** SDS-PAGE separation of filtrate fraction from a single WF sample (left), which has also been loaded onto a reverse phase column coupled to a QTOF MS to profile for metabolites (right). Lack of bands in the gel indicated the absence of proteins in the filtrate fraction of WF and peak features in the exemplar spectrum represent metabolites that are present in the filtrate fraction.

## Australian Wound Management Association

### Membership information

Membership of the Australian Wound Management Association may be achieved in two ways:

- Membership of a state wound care association – the annual subscription rate varies from state to state in the range \$20 to \$80.
- Direct membership of the Australian Wound Management Association – the fee is \$160.

With either form of membership, members will automatically receive copies of *Wound Practice and Research*, the Australian journal of wound management, which is published every three months.

Please direct enquiries regarding membership to:  
 Tabatha Rando  
 Email [tabatha.rando@rdns.org.au](mailto:tabatha.rando@rdns.org.au)

The membership secretary will send you the membership form appropriate for your state and include details of direct membership.