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ORIGINAL ARTICLE

Wound samples: moving towards a standardised method of collection and analysis

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Abstract

Chronic wounds, including diabetic foot ulcers, pressure ulcers and venous leg ulcers, impact the lives of millions of people worldwide. These types of wounds represent a significant physical, social and financial burden to both patients and health care systems. Wound care has made great progress in recent years as a result of the critical research performed in academic, clinical and industrial settings. However, there has been relatively little translation of basic research discoveries into novel and effective treatments. One underlying reason for this paucity may be inconsistency in the methods of wound analysis and sample collection, resulting in the inability of researchers to accurately characterise the healing process and compare results from different studies. This review examines the various types of analytical methods being used in wound research today with emphasis on sampling techniques, processing and storage, and the findings call forth the wound care research community to standardise its approach to wound analysis in order to yield more robust and comparable data sets.

Introduction

Chronic, complex and recalcitrant wounds impact the lives of an estimated 34·5 million people worldwide, and this number is growing at an alarming rate (1). Approximately 1–2% of individuals will develop a difficult-to-heal leg wound during their lifetime, and this number is likely to increase as the population ages and the incidences of obesity, diabetes and peripheral artery disease continue to surge. It is projected that 25% of the elderly population will suffer chronic limb ulceration by the year 2050 (2). Presently, between 10% and 15% of diabetics are expected to develop a chronic lower extremity wound. Diabetics account for more than 60% of non-traumatic limb amputations. Approximately 84% of those amputations are preceded by an ulcer (3). The number of slow-healing or complicated surgical, trauma and burn wounds is also increasing worldwide. Disease-related consequences of such wounds include local infection (including cellulitis and abscesses), bacteraemia and sepsis, pain, osteomyelitis, dermatitis, possible malignancy, amputation and death (4,5).

Key Messages

- this review examines the various types of analytical methods being used in wound research today with emphasis on sampling techniques, processing and storage
- a call to action for the wound care research community to standardise its approach to wound research in order to yield more robust and comparable data sets
- researchers and clinicians need evidence-based, standardised methods of sample collection that can be matched to robust analytical techniques in order to discover and accurately evaluate key wound biomarkers; once significant biomarkers are discovered, it is reasonable to imagine a set of valid tests that could be routinely performed on wound samples

These complications often result in an increased need for health care and hospitalisation and a reduced productivity and quality

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of life (6). Hence, it is clear that chronic wounds pose an enormous personal and global economic burden. Estimates of direct cost expenditures for chronic wound care are as high as $50 billion annually in the USA alone (7–9). Indirect costs including reduced productivity, lost income and decreased quality of life are also significant. It is estimated that 25% of those afflicted with chronic wounds report feelings of depression and/or anxiety (10).

Classic healing of full-thickness wounds is a well-documented dynamic process consisting of four overlapping yet distinct phases: (i) coagulation and haemostasis, occurring immediately after injury; (ii) inflammation, usually lasting between 2 and 4 days; (iii) proliferation (with new tissue formation); and (iv) remodelling/maturation, which may continue for a year or more (11–15). Chronic wounds are the result of aberrations in this process (16). Methods of measuring wound outcomes vary between researchers and are often subjective, providing little information as to whether and how a wound is progressing towards healing (17,18). The microenvironment of the wound, where the true biochemical status is found, is often overlooked (19). Evaluation of this microenvironment using new molecular techniques holds promise for the development of more effective treatment protocols tailored to wound status, and for the discovery and validation of novel therapeutics.

Wound specimens that are commonly obtained to assess outcome measures in research studies include biopsies, surface swabs, non-viable tissue slough or eschar removed by selective sharp debridement and wound fluids. For the evaluation of potential biomarkers and ease of analysis, wound fluid/exudate appears to be superior to other types of samples (20). Full-thickness biopsies of the wound bed or wound edge are typically obtained by the use of 3- to 6-mm punch biopsies. Such biopsies are considered the sample of choice for gene expression analysis (21,22). Biopsies are also considered the gold standard for analysis of wound bioburden, although superficial cotton swabs are used most often (23–25). Curette scrapings of the surface of the wound bed and fibrin, eschar and slough collections have also been used for the analysis of wound bioburden (26). Different types of samples, collection techniques and preservation methods may be preferred for various types of analysis; however, there is no ideal sample or method that can be used for all kinds of wound bioburden analysis. This review takes a closer look at the various types of analytical methods being used in research today with emphasis on sampling techniques, processing and storage, including the pros and cons of each method, standardisation, and their usefulness in wound evaluation.

Bioburden

Inflammation both incites and sustains lower limb ulceration. Chronic venous hypertension results in an inflammatory response from leucocytes leading to an increased expression of inflammatory cytokines and matrix metalloproteinases (27). Diabetes impacts leucocyte activity and impairs endothelium-regulated vascular function resulting in inflammation and subsequent ulcer formation (28). Inflammation is also often exacerbated and prolonged by leg ulcer infection or the presence of excessive levels of bacteria (16). The quality of granulation tissue, the new connective tissue formed in the wound bed during the proliferative or third phase of wound healing, is impaired by high levels of bacteria (29). Scarring, which occurs during the remodelling or last phase of wound healing, also tends to be more disfiguring after prolonged excessive inflammation exacerbated by wound infection (30). Lastly, wound closure is often delayed by levels of bacteria in the wound, which exceed the host's immune functions (16,31,32). In order to understand the continuum from contamination, through acute and critical colonisation, to infection, a brief review is in order.

Contamination is the presence of replicating bacteria attached to the wound surface but not causing systemic injury to the host. Contamination generally does not inhibit wound healing (33). Host immune functions are typically able to keep the contaminating bacteria from interfering with the patient's intrinsic wound healing capacity. However, infection does impair wound healing. Numerous studies have postulated that a microbial load of >10 (5) colony-forming units per gram of tissue (CFU/g) is deleterious to healing (34–38). One exception is the observation that any amount of certain extremely virulent bacteria, such as β-haemolytic streptococci, *Mycobateria* and *Clostridium perfringens*, is considered to negatively impact wound healing (37).

Several studies have examined the types and amounts of bacteria found in various types of wounds. Culture-dependent methods primarily detect numerically dominant organisms, or organisms that grow readily in the selected culture media of the testing laboratory, whereas culture-independent methods such as quantitative polymerase chain reaction (qPCR) and PCR-denatured gradient gel electrophoresis (DGGE), which allow for better microbial identification, tend to detect a far greater range of bacteria although they do not provide information on antibiotic susceptibility or virulence (26,39,40). It is unclear whether certain taxa play greater roles in impairing wound healing (31,41). However, it is clear is that PCR-based techniques show that the range of bacterial species found in wounds is far greater than what was once thought. From both culture-dependent and -independent studies, it appears that the most prevalent species in non-healing wounds are, in order of prevalence: *Staphylococcus aureus* (36), *Pseudomonas aeruginosa* (37), *Enterococcus* species (38) and *Escherichia coli* (35). *S. aureus* is by far the most prevalent strain, being found in 43–88% of non-healing wounds (35). Methicillin-resistant *S. aureus* (MRSA) has been implicated in many hospital-associated infections over the past two decades, and it has more recently become responsible for an emergence of community-associated skin and wound infections (42,43).

Unfortunately, most of the studies evaluating the polymicrobial diversity found in different types of wounds have used different analytical techniques, making the comparison of study results problematic. For example, strict anaerobic isolation and culture techniques are often neglected even though these techniques have shown that 60% of chronic venous leg ulcers harbour anaerobic bacteria including *Peptostreptococcus* and *Bacteroides* spp. (44,45). Also, anaerobic bacteria have been found in higher percentages in pressure ulcers than in diabetic foot

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ulcers, and in higher percentages in diabetic foot ulcers than in venous ulcers, indicating specific bacterial signatures for different wound types (26).

An ideal and agreed upon method of sampling wound microflora has yet to be established. Generally, wound tissue or wound fluid is used for qualitative and/or quantitative measurement of wound bioburden. The quantitative wound biopsy taken after debridement and cleaning of the wound surface is seen by many as the gold standard. However, biopsies are invasive and can be painful, and they are thus avoided in most primary care, home health care, long-term care and outpatient clinic settings because of the risk of introduction of additional contaminants and lack of appropriate skills, licenses and supplies (46). Furthermore, punch biopsy methods may be difficult to perform in the base of a wound bed with moist granulation tissue. Swab specimens are more commonly collected; however, the usefulness of the information provided by culturing such specimens is controversial (23). For instance, the techniques and materials used in swabbing vary greatly (24,36,47–49). Sterile culture swabs may be made of rolled cotton, flocked cotton, rayon or foam, and may be plain or embedded with nutrients to enhance microbial growth during transport. Clinicians may moisten the swab with sterile saline, water or culture media before collection of a specimen or they may collect the specimen with a dry swab. Clinicians may also roll the swab across one portion of the wound bed once or use a variety of strokes, rolls or patterns across the wound bed to collect a specimen (24,50–55). When performed correctly, some studies have found that the culture results obtained using quantitative swab cultures show concordance with those obtained using deep tissue biopsies (54,55), while others have found little correlation between the two (47,51,56,57).

The quantitative swab culture, or Levine technique, was first described for the evaluation of bioburden in burn patients. It involves rotating a wound swab over a 1 cm (2) area of the wound (36). The Z technique is an alternative to the Levine technique and involves rotating a swab between the fingers in a zigzag fashion across the wound without touching the wound edge. Results from Gardner *et al.* (53) and Angel *et al.* (50) suggest that the Levine technique, when performed correctly after appropriate wound cleansing with a simple sterile saline rinse, is superior to the Z technique and is a safe and valuable alternative to tissue biopsy. Another option for collecting surface level bacterial specimens is curettage. This gentle scraping of the wound surface, including the inner wound edges, with a sterile surgical curette (sharp, round, stainless steel loop at the end of a handle) is useful for retrieving biofilm, slough and non-viable tissue to which bacteria may adhere. Curette loops vary in size with the most frequently used curette sizes for wound sample collection being 3 and 4 mm. In one study comparing curette scrapings with swab and fine needle aspiration biopsy cultures, curettage was able to detect the same or greater amounts of bacteria as the other techniques (58). Regardless of the collection technique used, care must be taken to ensure that samples are collected and transferred in appropriate culture media as quickly as possible to the testing facility and are stored in a manner that preserves all types of bacterial species (59).

Biomarkers

Sampling of wounds for analysis of biomarkers has become more prevalent in recent years with the advent of advanced molecular techniques. Although this type of sampling and analysis is primarily performed in research settings, health care companies foresee bringing such analytical techniques to the general health care practitioner as evidenced by the filing of numerous patents in this domain (60–62). Although the test methodologies themselves are important, the sample collected for use with these proposed tests is critical to obtaining accurate and informative results. In most reported studies involving biomarker analysis, wound fluid is the sample analysed (see Table 1).

The collection and analysis of wound fluid is fraught with complications including: (i) obtaining adequate amounts of fluid; (ii) proper cleansing of the wound in a manner that does not destroy target analytes; (iii) proper preservation of the sample so as to avoid degradation; (iv) choice of the material to collect the fluid that easily releases the target analytes into extraction fluids; (v) interpretation of results; and (vi) the use of appropriate controls.

Table 2 outlines representative studies evaluating wound fluids for various biomarkers and the methods used. Methods were taken directly from the methods section of the papers, and the data show the wide range of methodology and the differences in degree of detail given. From these examples and other reports, three general methodologies for the collection of wound fluid appear most common. The first approach simply uses fluids collected from either negative pressure wound therapy (NPWT) canisters or wound drains (63–65). The second approach involves the placement of a semi-occlusive or occlusive dressing over the wound for varying amounts of time followed by the removal of fluid from beneath the dressing via syringe aspiration (66–75). The third approach involves the use of absorbent materials of varying types including Dextranomer beads, wound dressings and filter paper (64,76–83). Microcapillary collection of wound fluid (84) and microdialysis of wounds (85) have also been used. Wound fluids collected using these methods were typically stored at temperatures ranging from −20°C to −80°C. Some researchers added protease inhibitors to the collected fluid, others did not. The speeds, temperatures, and times at which the fluids were centrifuged varied from 800 to 14 000 *g*, 4°C to room temperature and 4 to 15 minutes, respectively. Some fluids appear not to have been centrifuged at all. When extracting analytes from collection materials, there was even greater disparity in the methods used, with varying types of extraction buffers and times ranging from 1 hour to overnight. Normalisation was also inconsistent, with some researchers normalising to total protein and others to volume.

Large volumes of fluid can be collected from acute surgical drains or NPWT reservoirs over time, allowing analysis of wound fluid content over the course of wound healing. However, it has been shown that wound fluid obtained from NPWT reservoirs differs from that collected through passive absorption (86), raising the question of whether fluid collected in this manner adequately mirrors normal wound healing physiology (87). Adding another layer of complexity, the highly

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Table 1 Pertinent publications on wound exudate collection and analysis

Reference	Wound type	Wound fluid collection method	Absorbent material	Processing	Storage	Analysis
Wysocki and Grinnell (72)	Chronic leg ulcers	Fluid collected from under vapour-permeable film Tegaderm™ (3M) in place for 4–12 hours with a tuberculin syringe and 20-gauge needle		Centrifugation for 4 minutes at 11 600 *g*	−70°C	Western blot
Dvonch *et al.* (63)	Surgical wounds	Fluid collected from negative pressure drainage system reservoir		Centrifugation for 10 minutes at 5000 *g*	−70°C	Western blot
Bucalo *et al.* (66)	Chronic wounds	Fluid collected from under polyurethane membrane (Hollister) or vapour-permeable film (Tegaderm™, 3M) in place for 24 hours		Centrifuged at 800 *g* and sterile filtered through 0·2-μm filters	−20°C	Effects on cell proliferation and viability
Cooper *et al.* (83)	Pressure ulcers	Dextranomer beads in place for 24 hours	Dextranomer beads	1 g of saturated beads mixed with an equal volume of PBS for 12 hours in a vertical shaker at 4°C. Centrifuged at 1000 *g* for 5 minutes at 4°C	−80°C	Total protein (Bradford assay) cytokine analysis (ELISA)
Harris *et al.* (67)	Venous leg ulcers	Ulcer bed wiped clean with sterile saline. Fluid collected from under vapour-permeable film Tegaderm™ (3M) in place for 4–6 hours with needle and syringe		Centrifuged at 12 000 *g* for 3 minutes. Diluted to 10% with sterile saline and filtered through 0·2-μm filters	−60°C	Total protein (Biuret method). Collagenase activity. Fibronectin degradation. IL-1 and IL-6 bioassays
Trengrove *et al.* (71)	Chronic lower leg ulcers	Patient fasted beginning at midnight. Transparent occlusive dressing (Opsite™, Smith & Nephew) applied at 8 am. Patient's leg placed in a dependent position and patient encouraged to drink 1 l of water. Fluid aspirated from under the dressing at 1 hour and transferred into Greiner Vacuette vacuum serum collection tubes				Osmolarity. General biochemical entities
Fivenson *et al.* (77)	Chronic venous leg ulcers	Each wound was dressed with a layer of non-adherent dressing, covered by a hydrofoam pad (Allevyn®, Smith & Nephew). The central 2 cm diameter of the hydrofoam dressing directly overlying the ulcer centre was collected and stored at −70°C	Allevyn®	Allevyn® dressing was homogenised in 2 ml of sample buffer (sterile PBS containing protease inhibitors) followed by sonication	−70°C	ELISA

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Table 1 Continued

Reference	Wound type	Wound fluid collection method	Absorbent material	Processing	Storage	Analysis
Yager *et al.* (73)	Venous Stasis and pressure ulcers, surgical wounds	Fluid collected from under vapour-permeable film Tegaderm™ (3M) in place for 4–8 hours with a tuberculin syringe		Centrifuged at 14 000 *g* for 15 minutes at 4°C	−20°C	ELISA, Western blot
Nissen *et al.* (65)	Surgical wounds	Fluid collected from closed negative pressure (69) drains		Centrifuged at 1300 *g* for 10 minutes	−70°C	VEGF FGF-2 ELISA. Endothelial cell chemotaxis
Hoffman *et al.* (64)	Venous leg ulcers	Wound fluid was manually extracted from an absorptive dressing soaked in ice-cold PBS (10–20 ml) containing 0·02% sodium azide. Mastectomy wound fluid was collected over a 24-hour period into a Bellova drainage unit	Absorptive dressing	Centrifuged at 13 000 *g* for 10 minutes	−70°C	Plasminogen degradation and plasmin generation
Simonsen *et al.* (85)	Diabetic foot ulcers	Microdialysis using probes made of 3 cm long sections of artificial dialysis kidney (Gambro GSF+12) and nylon tubing placed in situ via a G18 cannula				Glucose and lactate (YSI 2300 glucose-lactate analyser; Yellow Springs Instruments, Yellow Springs, OH)
Mendez *et al.* (78)	Venous ulcers	Foam wafer occlusive dressing (Allevyn®, Smith & Nephew), which was placed over the ulcer and covered by a paste bandage (Unna's boot) and a compression wrap. Dressings were changed weekly, and wound fluid was extracted from the foam wafer with a sterile syringe with a 20-gauge needle	Foam wafer occlusive dressing (Allevyn®, Smith& Nephew)	Diluted 1:10 with DMEM and filtered through 0·2-μm filter	−70°C	Fibroblast proliferation. TNF-α concentration (ELISA)
Tarlton *et al.* (81)	Venous leg ulcers	Absorptive filters of 1 cm (2) dimensions were prepared from Whatman 54 paper sterilised in ethanol, oven-dried at 60°C and pre-weighed in sterile 2 ml Apex tubes. Sterile Tegapore™ mesh (3M) was cut into 4 cm^2 segments. The mesh was placed on the ulcer and wound fluid absorbed through it into the collection filter excluding solid material, which would otherwise compromise subsequent quantification	Whatman 54 paper	Filters were incubated in extraction buffer (0·1% Brij 35 (BDH, Poole, UK) in 20 mM triethanolamine) added 50:1 (v/w) for 4 hours with agitation	−20°C	Zymography. Type I collagen C propeptide content (ELISA)

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Table 1 Continued

Reference	Wound type	Wound fluid collection method	Absorbent material	Processing	Storage	Analysis
James *et al.* (68)	Chronic leg ulcer	Leg kept dependent for 30–40 minutes. Fluid collected from under a transparent occlusive dressing (Opsite™, Smith & Nephew) in place for 4–8 hours with syringe and needle		Centrifuged at 8000 *g* for 5 minutes	−70°C	Total protein (Biuret method). Biochemical analysis
Cullen *et al.* (76)	Diabetic foot ulcer	RELEASE® (Johnson & Johnson Ltd.) dressing was cut to the size of the wound, placed in contact with the ulcer bed for 24 hours and covered with BIOCLUSIVE® (Johnson & Johnson Ltd.), an occlusive film. The dressing was then removed and frozen at −70°C until elution of wound fluid		Wound fluid was eluted from the RELEASE dressing by incubating the sample in 1 ml of wash buffer/cm^2 dressing (0·1 M Tris–HCL pH 7·4 containing 0·1% Triton X-100) for 2 hours at 4°C. Dressing compressed against the side of a container and eluent removed	−70°C	Total protein (Bradford). Protease activity. Zymography
Lauer *et al.* (69)	Venous leg ulcers	Ulcers were covered with a semipermeable polyurethane film (Hyalofilm, Hartmann, Heidenhein, Germany) for a maximum of 8 hours. Fluid was assumed to be collected from beneath the dressing although not stated		Centrifuged at 13 000 *g* for 10 minutes at 4°C	−80°C	VEGF levels (ELISA), Western blot). Plasminogen presence and plasmin activity
Moseley *et al.* (80)	Venous leg ulcers. Chronic wounds	Absorptive filters (1 cm^2) were prepared from Whatman 54 paper. Sterile Tegapore™ mesh (3M) was cut into 4 cm^2 segments; absorptive filters placed inside and the mesh thermally sealed. Each filter paper mesh was autoclave-sterilised and oven-dried. A filter paper mesh was placed onto the surface of each wound until filter paper was saturated	Whatman 54 paper	Wound fluid was recovered by removing the filter papers from the mesh and eluting with 1 ml PBS at 4°C for 1 hour	−20°C	Total protein assay (Bio-Rad). Total protein carbonyl and malondialdehyde content. Western blot. Total antioxidant capacity
Fernandez *et al.* (75)	Chronic venous leg ulcers	Ulcers were washed with sterile water and covered with an occlusive dressing. Exudate was collected from under the dressing after 30 minutes to 1 hour by washing with 1 ml of saline		Centrifuged at 14 000 *g* for 10 minutes. Filtered through 0·45-μm cellulose acetate filters. Samples from five patients were pooled and aliquoted. Immunodepletion	−80°C	Total protein, Western blot, 2D gel electrophoresis and proteome fractionation, liquid chromatography/mass spectrometry

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Table 1 Continued

Reference	Wound type	Wound fluid collection method	Absorbent material	Processing	Storage	Analysis
Moues *et al.* (87)		Wound fluid was collected daily for up to 10 days using sterile polyvinylidene fluoride filters (Durapore membrane filters, Millipore®, Amsterdam, The Netherlands, 47 mm area and 0·1 μm thick). Four filters per wound per dressing change were collected after full saturation (never exceeding 20 minutes)		Filters were extracted in 1 ml of cold (4°C PBS (pH 7·4) for 10 minutes with gentle rocking followed by centrifugation at 4000 rpm for 10 minutes at 16°C. Supernatant was aliquoted and stored	−20°C	ELISA, Biotrack Activity Assay system
Rayment *et al.* (70)	Chronic venous ulcers	Wounds washed with saline. Wound fluid collected from under occlusive dressing in place for 30 minutes to 1 hour by washing with 1 ml of saline. Wound fluid collected from blisters with 26-gauge needles and syringes		Centrifuged at 14 000 *g* for 10 minutes. Filtered using cellulose acetate filters	−80°C	Total protein (BCA). Zymography. MMP-9 levels (ELISA)
Moor *et al.* (79)	Venous leg ulcers	Tegapore™ mesh (3M) placed on wound bed and wound fluid collected by absorption through mesh onto 1 cm^2 Whatman 54 paper	Whatman 54 paper	Filters were extracted with 50 :1 (v/w) CAB buffer, pH 7·5 (25 mM Tris–HCl pH 7·5, 200 mM NaCl, 3 mM $CaCl_2$ and 0·03% Brij-35) overnight at 4°C. The extracts were centrifuged at 10 000 *g* for 15 minutes at 4°C	−80°C	MMP-13,1 activity assays (Sensolyte Plus assay kit Anaspec, San Jose, CA). MMP-8 levels (ELISA). MMP-2, P levels and activity. Zymography. Western blotting. Multiplex assay
Eming *et al.* (74)	Venous leg ulcers, acute wounds	Wounds were covered with a semipermeable polyurethane film (Hyalofilm, Hartmann, Heidelberg, Germany) for a maximum of 8 hours. It is assumed that fluid was collected from under the dressing		Centrifuged at 13 000 *g* for 10 minutes at 4°C and supernatants were frozen at −80°C	−80°C	SDS-PAGE, mass spectrometry, ELISA, dot plots
Wyffels *et al.* (82)	Pressure ulcers	Wound proteins were collected using sterile polyester tipped applicators gently rolled over the wound surface until saturated. The tip of the swab was broken off and placed in a 2 ml vial prefilled with 150 μl PBS (10 mM, pH 7·4)	Polyester tipped applicators	Proteins eluted from the polyester tip by the addition of 350 μl dH_2O and vortexed for 30 seconds. The swabs were inverted and all liquid removed from the polyester tip via centrifugation for 10 minutes at 6000 *g*, swabs were removed and debris pelleted by repeating the centrifugation. Supernatant filtered using spin columns (3-kDa cutoff) (Millipore, Amicon Microcon® UltracelYM-3, Billerica, MA)	−80°C	Two-dimensional gel electrophoresis

ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; VEGF, vascular endothelial growth factor, FGF, Fibroblast Growth Factor, DMEM, Dulbecco's Modified Eagle Medium, TNF, Tumor necrosis factor, SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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Table 2 Strengths and weaknesses of sample type and recommendations for optimisation

+++ Best, ++ better, + acceptable	Biopsy	Swab	Canister/Drain fluid	Absorption and extraction
Ease of sample collection		++ Technique critical	+++	+++
Cost		+++	+++ Not considering the cost of the therapy itself	+++ Depends on collection and preservation supplies
Patient comfort		++	+++	++
Sample quality and consistency	+++ Procedure most likely performed by highly trained personnel	+++ When using Z technique and correct preservation methods	+++ When comparing samples from the same patient over time	+++ Collection and processing technique critical. Well matched to point-of-care diagnostics
Gene expression/PCR	+++ Samples snap frozen with RNase inhibitors			
Bioburden	+++ Samples stored, processed and analysed for both aerobic and anaerobic organisms	++ Samples stored, processed and analysed for both aerobic and anaerobic organisms		
Biomarker analysis		+ Samples frozen with protease inhibitors	+++ Samples frozen with protease inhibitors. Take into consideration dilution of protease inhibitors over time. Samples should be compared only with other samples collected in the same manner (NPWT, surgical drain)	++ Samples frozen with protease inhibitors. Whatman paper discs, Periopaper. Would be useful to develop collection materials specific to this purpose

PCR, polymerase chain reaction; NPWT, negative pressure wound therapy.

proteolytic nature of wound fluid in the drainage reservoir leads to the degradation of the majority of biomarkers within a relatively brief period of time in the absence of protease inhibitors (73,88). However, the inclusion of protease inhibitors in collection reservoirs adds another level of complexity. As the rate and volume of wound fluid collected in this manner is highly variable, it is difficult to maintain a consistent and effective concentration of protease inhibitor.

Collection of fluid from beneath semi-occlusive and occlusive dressings eliminates the need for extraction from absorbent materials. Thus, this collection method is well suited for studies where the type of wound is highly exudative. In contrast, the collection of adequate volumes from non-exudative wounds is often difficult and requires patients to remain at the collection site for extended time periods.

The use of absorbent dressings as a collection material, although popular and relatively easy, is not ideal, as the binding properties of such materials are not well characterised. Several different types of absorbent materials have been used to collect wound fluid. The release characteristics of analytical grade filter paper are usually characterised, but care must be taken to adequately investigate such characteristics to choose the material that best matches the analyte(s) being studied. The method of collecting blood on cellulose filter paper has been used for over 50 years for neonatal screening of infants for assessing elevation in blood phenylalanine associated with phenylketonuria (89). Cellulose filter paper is also widely used in home test kits for measuring hormone levels and drug screening. Recent studies performed by the authors of this study have used Whatman paper discs (Cat# 2017-009) with success for the collection and analysis of proteins in chronic wound fluids. After testing 30 of the 9-mm size discs, it was found that they absorbed an average of $89 \pm 6{\cdot}5$ µl of fluid per disc when incubated at 37°C in vitro. Incubation of the same type of discs saturated with wound fluid in phosphate-buffered saline (PBS) containing protease inhibitors followed by high-speed centrifugation was sufficient for extraction of numerous proteins at concentrations that are adequate for use in multiplex assays (publication in process).

Measurement of gingival crevicular fluid and salivary secretions is often carried out in periodontal research using a Periotron and Periopaper, Sialopaper or PerioCol paper strips (Oraflow, Inc, Smithtown, NY). The Periotron and collection papers have replaced some of the cumbersome methods used in the past, including pipetting and blotting (90–92). In these studies, collection paper strips are placed in the area of interest for a predetermined period of time and then transferred to

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the Periotron, which measures fluid volumes based on electrical capacitance of the wet strip. Over the past three decades, researchers using this technique have investigated and reported on the methodological considerations including paper type, sampling technique, sampling time and environmental factors (93,94). These experimentations have resulted in a group of semi-standardised methods and materials that can be used by those investigating oral fluids, allowing for comparison of results from different studies and laboratories. Although this technique is primarily used to measure the volume of secretion, researchers have also extracted proteins from the strips after volume measurement, for further analysis (95). It would be beneficial to have a similar set of parameters for the collection and evaluation of wound fluid.

Proteases

Numerous researchers have evaluated the importance of gelatinases, collagenases and matrix metalloprotease (MMP) levels and their role in wound healing (79,84,96–102). Their work is beyond the scope of this review. However, as evidenced by the sheer volume of work in this area, proteolysis plays an important role in wound healing. The degradation of extracellular matrix (ECM) not only remodels wound tissue but also releases a variety of bioactive proteolytic cleavage products (103). The levels of proteases in the wound reflect only a portion of the overall story, as their activity is influenced by numerous factors. This interplay is best demonstrated by the dynamic equilibrium between inflammatory cytokines, MMPs and tissue inhibitor of matrix metalloproteases (TIMPs) (13). In 2009, Gibson and Schultz published preliminary testing results of a prototype point-of-care device that has the ability to rapidly measure MMP activity in wound fluids (104). This was followed by clinical assessment of the device, the results of which were presented at the 2010 spring meeting of the Wound Healing Society (105). In 2011, an international group of experts met to discuss and explore the importance of protease activity in wound healing. The result of this meeting was a consensus document, sponsored by an unrestricted education grant from Systagenix (Gatwick, UK), now a Kinetic Concepts Inc. company, titled 'The Role of Proteases in Wound Diagnostics' (106). The authors point to data suggesting a direct link between protease activity and wound healing, and how a point-of-care test to measure protease activity could be useful in clinical practice. However, the authors also point to the need for extensive data for demonstrating the validity of such a test across a spectrum of wound types. Subsequent to the publication of this consensus document, Systagenix released a point-of-care diagnostic device for the measurement of wound protease activity (107).

Storage, processing and analysis of samples

The basis of any reliable and accurate assay is the quality of the starting material. Sample processing and storage procedures greatly influence the stability of analyte. Samples stored at −80°C are more stable than those stored at −20°C; thus data obtained from samples stored at different temperatures cannot be accurately compared. Cytokines degrade over time even when stored at −80°C and multiple freeze-thaws also contribute to degradation (108–112). Furthermore, there is variation in the sensitivity of analytes to degradation. Kisand *et al.* (110) found that the biomarkers MMP-7 and vascular endothelial growth factor receptor 2 (VEGF-R2) were relatively stable at −75°C. However, VEGF and TIMP-1 degraded even when stored at ultra-low temperatures. Surface binding of storage vessels also needs consideration. Care must be taken when choosing between glass and plastic. For example, a recent study investigating peptide surface binding to common laboratory plastic and glassware found that ghrelin bound preferentially to flint glass over polypropylene. In contrast, insulin bound preferentially to polypropylene (113). Addition of bovine serum albumin reduced non-specific binding and significantly improved recovery of most peptides. Unfortunately, most researchers do not include vessel type, storage time or number of freeze-thaws in the materials and methods section of their publications. Recently, de Jager *et al.* (114) set forth the proposed prerequisites for cytokine measurements in clinical trial serum and plasma samples with multiplex assays. A similar study evaluating cytokine/biomarker stability in wound fluid would allow for the creation of standard processing and storage parameters.

Numerous substances are known to interfere with multiplex and enzyme-linked immunosorbent assays (ELISA) (115). As wounds are often treated with substances containing potentially interfering components, proper preparation of the wound site prior to sample collection is vital. At present, little is known about how various wound treatment components – including metal ions, iodine, collagen and alginate – impact cytokine stability and activity. Shi *et al.* (116) showed the influence of various wound washes on platelet-derived growth factor-BB (PDGF-BB) receptor binding activity. Solvent pH greatly influenced binding, with pH outside the 5·0–7·5 range being inhibitory. The results from this study also suggested that even low levels of hypochlorite salt, which is found in numerous washes, decreases receptor binding.

To further complicate matters, analytical techniques vary in complexity and sensitivity. The sensitivity of more modern methods of analysis has opened the door to the possibility of measuring quantities of analytes that were once impossible. However, the specifics of how any type of assay is performed can greatly influence the resulting data. Even when using commercially available kits, differences in operator technique can result in variable results. The inclusion of both negative and positive controls and the normalisation of samples are critical to obtaining accurate and reproducible data. Unfortunately, one or more of these requirements were not included in numerous reported wound fluid studies.

Antibody-based techniques including Western blotting, ELISA and multiplex analysis are widely used in the analysis of biomarkers. When analysing multiple biomarkers in low volume samples, it is impractical to use traditional 'singleplex' detection methods such as Western blotting and ELISA, as these assays require relatively large quantities of sample. With the advent of multiplex technology, researchers now have the ability to analyse numerous targets, including analysis of their phosphorylation states, simultaneously in comparably low volumes. Multiplex technologies provide the ideal combination of high density and high throughput analysis that is lacking in

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other methods. New machines and kits have made multiplexing a cost-effective and reliable choice for biomarker discovery.

Future directions/conclusion

The ability to objectively assess wound healing in a minimally invasive fashion is critical to effective wound care. Table 2 outlines the strengths and weaknesses of different types of wound samples taking into consideration the evidence presented in this review. However, a larger and more robust data set is required to optimise these guidelines. Thus, this review is also a call to action for the wound care research community to standardise its approach to wound research in order to yield more robust and comparable data sets. Researchers and clinicians need evidence-based, standardised methods of sample collection that can be matched to robust analytical techniques in order to discover and accurately evaluate key wound biomarkers. Once significant biomarkers are discovered, it is reasonable to imagine a set of valid tests that could be routinely performed on wound samples. This set would include a rapid bioburden test (including antibiotic susceptibility), a biomarker test and a protease activity test. The results of this set of tests would be used in concert with other methods of evaluation in order to more effectively identify and treat patients with non-healing wounds.

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