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Wound Fluid Diagnostics in Diabetic Foot Ulcers

Markus Löffler[1,2], Michael Schmohl[3],
Nicole Schneiderhan-Marra[3] and Stefan Beckert[2]
[1]Department of Immunology, University of Tuebingen,
[2]Department of General, Visceral and Transplant Surgery, University of Tuebingen,
[3]NMI-Natural and Medical sciences Institute at the University of Tuebingen,
Reutlingen,
Germany

1. Introduction

Wound fluid seems -at least theoretically- easily accessible and might open a new window to the local wound microenvironment that cannot be evaluated by the analysis of serum or plasma markers. Recently, this strategy has been supported by a first time wound fluid proteome analysis comparing acute and chronic wounds (Eming et al. 2010). Interestingly, there seem to be essential differences with respect to wound fluid protein composition when comparing acute and chronic wounds. The wound fluid proteome of healing tissue is characterised by proteins involved in tissue growth and protection from inflammatory activity, whereas non-healing wounds are characterised by a chronic inflammatory environment primarily consisting of leukocyte proteases and inflammatory mediators (Eming et al. 2010). This is particularly striking since the non-healing state in diabetic foot ulcers has previously been linked to persistent inflammatory activity (Acosta et al. 2008). Thus, the wound fluid of chronic wounds seems to be characterised by an altered wound micro-milieu and may, therefore, provide deeper insights into the causes of delayed wound healing.

Tissue repair can be clearly characterised as an ubiquitous process and a wound is more precisely described as a transitional tissue rather than a static tissue. Physiological wound healing can be recognised as continuous change, which might be seen as a dynamic and interactive process resulting in remodelling and tissue restitution. This nature of transitional tissues comes down to the difficulty of obtaining appropriate sample materials. Nevertheless, the classical phases of healing are useful as a broad roadmap of the course of healing, since more precise models and clear landmarks are not available. The historical view on wound healing categorises the healing process in separate phases: starting with acute injury > I. coagulation > II. an early inflammatory phase > III. a late inflammatory phase > IV. a proliferative phase, and > V. remodelling, which ends with tissue restitution (Velnar, Bailey, and Smrkolj 2009).

Changes in wound fluid markers, cytokines or cell populations are more suitable for describing the local wound microenvironment than are alterations of systemic markers. The nature of systemic serum-based markers is likely the sum of different effects all over the body. Of course distinct and rather exceptional markers obtained from blood -such as PSA (prostate specific antigen) (Balk, Ko, and Bubley 2003) or troponin- have been proven to be tissue specific (Apple 1999) and can therefore reveal insights into local processes. However, most of the markers found in the bloodstream instead result from systemic alterations rather than from local changes. Wound healing takes place simultaneously at a variety of locations in the body. Therefore, the impact of serum markers on the assessment of the wound microenvironment is questionable so long as there is no systemic affection by septicaemia. In addition, many systemic inflammatory markers are altered in response to a variety of immune mediators which in turn are triggered by inflammatory events somewhere in the body. In this context, the secretion of CRP by the liver upon stimulation by circulatory cytokines such as IL-6 (Pepys and Hirschfield 2003) can be exemplified. In line with this concept, several studies evaluating CRP as a valuable marker of infection in diabetic foot ulcers were judged to be unable to underline its sole effectiveness (Dinh, Snyder, and Veves 2010).

Since most systemic markers do not exhibit clear tissue specificity, it might be of special interest to gain insights into the local wound microenvironment by assessing biochemical markers in wound fluid, which in turn might give us a clue about the local processes reflecting the current status of wound healing.

In the diabetic foot in particular -which is characterised by delayed healing- local biomarkers facilitating the evaluation of the wound may be of great clinical value. Being able to determine the probability of healing and the risk of infection by assessing markers in wound fluid might have a great impact on clinical practice.

2. Wound fluid

Wound fluid is an interesting object of investigation because its composition is influenced by the state of the wound and the phase of healing (Mani 1999; Trengove, Langton, and Stacey 1996). Wound fluid generally consists of an inhomogeneous mixture of different endogenous and exogenous sources. It is constituted by a provisional matrix of exudates or transudates that originate from the blood, which in turn has already been altered by multiple independent pathologic and physiological factors (such as cardiac failure or hydration status as well as vasoconstriction or diabetes). In addition, wound fluid composition is further modified by factors which are secreted by local cells in the wound or by cells that migrate into the wound, as well as by supplementary factors derived from microbial contamination. Since the wound cellular population is constantly changing during the physiological course of healing, wound fluid should exhibit altered characteristics over time (especially regarding its proteome).

Ideally, wound fluid should be collected non-invasively and with little effort, resulting in a better patient as well as greater physician acceptance. This is in contrast to tissue biopsies or techniques -which are defined as the gold standard in animal models- such as subcutaneously implanted wire mesh cylinders (Hunt Schilling Cylinders), which are inadequate for monitoring wound healing in humans (Hunt et al. 1967; Schilling, Joel, and

Shurley 1959). Thus, non-invasive techniques hold a relevant advantage for the future, allowing the assessment of longitudinal profiles and changes in wound fluid composition.

2.1 Sampling techniques

2.1.1 Sampling of chronic wound fluids

Covering wounds with occlusive dressings and harvesting the accumulated exudate underneath is probably the most common technique for obtaining wound fluid in chronic wounds. Various modifications of this technique have been described with respect to used materials, patient preparation and time scales, as well as with respect to wound bed preparation (Trengove et al. 1999; Trengove, Bielefeldt-Ohmann, and Stacey 2000; Yager et al. 1996; Wysocki and Grinnell 1990; James et al. 2000; Wallace and Stacey 1998; Chen et al. 1997; Wysocki et al. 1999; Gohel et al. 2008). Even very comprehensive protocols aiming for standardisation have been proposed, including the prior fasting of patients for eight hours as well as grading their hydration status by drinking one litre of water while hanging down the leg (Trengove, Langton, and Stacey 1996). However, this is not suitable for daily clinical routines. In addition, there is also major variability with respect to the time frames defined for exudate accumulation, ranging from about one hour (Trengove et al. 1999) to five days (Ono et al. 1994). Therefore, the technique of wound fluid collection may indeed impact upon subsequent analytic results.

For chronic wounds in particular, techniques for harvesting even small amounts of wound fluid directly from the wound surface have been described. For this purpose, blunt-end glass micro-capillaries have been used to collect fluid directly from the wound surface (Weckroth et al. 1996). In venous leg ulcers, even the manual expression of wound fluid from absorptive dressings has been proposed (Hoffman, Starkey, and Coad 1998).

Another method for obtaining wound fluid is to cover the wound surface with different types of dressings from which it is then mechanically extracted. In the past, this particular technique has frequently been modified. Protocols describing the extraction of fluids directly from dressings have been reported multiple times (Fivenson et al. 1997; Seah et al. 2005). For example, wound exudate trapped in a foam dressing has been obtained by squeezing the dressing material through a syringe (Seah et al. 2005).

Another option is placing a sterile mesh onto the wound which absorbs the wound fluid into a sterilised pre-weighed whatman paper, which was described as allowing the recovery of 50-200µl of sample material. Subsequently, wound fluid was extracted from the filters by washouts employing a suitable buffer (Moor, Vachon, and Gould 2009). Similarly, glass microfibre circle filters were inserted into wound centres in order to saturate for 2 hours with wound exudate. Wound fluid was then extracted by phosphate buffered saline (Schmidtchen 2000).

A further technique described for the acquisition of wound fluid is a modification of the methodology used by the Schirmer´s test, which was originally invented to measure tear secretion in an ophthalmologic setting (Schirmer 1903, cited by Kallarackal et al. 2002). This attempt consists of absorbent paper strips which are placed on the wound surface thereby soaking with fluid for 5 minutes. Subsequently, the sample material is recovered by stirring the paper strips in a buffer for 2 hours (Muller et al. 2008).

Also, hydrophilic dextranomer beads layered on wounds have been used. In preliminary experiments with defined amounts of proteins, a recovery of 88-98% was described when extracting the beads with equal volumes of phosphate buffered saline for 12 hours (Cooper et al. 1994).

One potential drawback to these approaches is the issue of sample dilution, thus altering the analyte containing matrix as well as leaving the precise amount of fluid enclosed and remaining in the dressing material undetermined. Furthermore, huge inter-patient variability in cytokine concentrations -possibly due to technical problems during wound fluid aspiration from under occlusive Tegaderm® dressings- was observed in one study (Gohel et al. 2008).

To overcome the obstacles of methodological limitations in sample material and sampling difficulties, a technique using washouts of wound areas by the application of 5ml of sterile saline to wounds and subsequent collection of the fluid again by needle aspiration has been proposed (Ambrosch et al. 2008). However, a relevant dilution of the sample may again occur and so exert undetermined effects on the original fluid matrix.

Microdialysis has also been proposed for collecting wound fluid. However, this is an invasive technique and it is not undisputed since the insertion of foreign material may affect the wound microenvironment and induce confounding effects on the expression of signalling molecules (Stenken et al. 2010; Mellergard et al. 2008). Nevertheless, this technique is well established for sampling the fluid of the interstitial space and the samples obtained are usually of high quality (Simonsen et al. 1998; Clough and Noble 2003).

A possible major issue in all these techniques -covering wounds with foreign material to a minor or major extent- is the fact that the collection materials may influence the biochemical or immunological properties of the wound (Schmidtchen 2000; Hoffman, Noble, and Eagle 1999) or else interfere with the analytes that are to be measured.

Given these findings, we have considered implementing a novel way of obtaining wound fluid which might overcome some of the shortcomings of the earlier proposed techniques. Being convinced that wound fluid is a complex substrate (displaying the recent condition of the wound healing process), the sample material should be rapidly harvested within a short processing time so as to remain unaltered. The new protocol should be neither time-consuming nor cause much logistical effort, and thereby be suitable for daily clinical routines.

The issue of limited sample volumes is diminishing in importance since novel analytic techniques and devices still provide good and reproducible measurements, even when using very little amounts of fluid (see section 4). However, the issue of acquiring suitable and high-quality material in a reproducible, standardised manner is increasingly relevant and has been sought for years (Yager, Kulina, and Gilman 2007).

Therefore, we performed wound swabs without pressure using micro-flocked swabs (Minitip Flocked Swabs; nylon flocked swabs with moulded breakpoint, MicroRheologics, Brescia, Italy) that adsorb the wound exudate through capillary action, coating nylon fibres without entrapment. In our setting, wound fluid was collected from diabetic foot ulcers by swabbing -after sharp debridement and haemostasis- using the Levine technique (Wound

infection in clinical practice. An international consensus 2008). Subsequently, the wound fluid was recovered by immediate centrifugation of swab tips at 10 000 rpm for 3 minutes at room temperature, using eppendorf cups equipped with a filter to hold the swab tip in place (Oxy Fill centrifugal filters without RoTrac capillary pore membrane, Carl Roth GmbH, Karlsruhe, Germany). The resulting cell free supernatant could then be used for subsequent analysis (Löffler et al. 2011).

2.1.2 Sampling of acute wound fluids

Techniques for harvesting acute wound fluid usually differ considerably from the methods described above for chronic wounds. This can be attributed to the different availability of wound exudates in the acute setting, as well as the different time frame until tissue restitution is reached. In several studies, wound fluid was obtained from suction drains, which have been inserted intra-operatively (Yager et al. 1996; Trengove et al. 1999; Baker and Leaper 2000). The collection of blister fluid is another opportunity for acquiring sample material (Oono et al. 1997; Ortega, Ganz, and Milner 2000).

Negative pressure therapy (Vacuum Assisted Closure, V.A.C.) permits additional access to wound fluid (Duckworth et al. 2004), which is also a suitable technique in diabetic foot ulcers (Noble-Bell and Forbes 2008). However, in this setting the disparity between methods seems quite obvious, so long as rather passive methods are compared to negative pressure therapy (Yager, Kulina, and Gilman 2007). Wound fluid obtained by V.A.C. therapy is characterised by an increased protein concentration. In a study of Dealey et al., the attempt to establish a suitable and valid technique for comparison failed due to technical reasons, causing the cessation of a second study arm (Dealey, Cameron, and Arrowsmith 2006).

Nevertheless, when interpreting the results with some caution, comparisons of the values from acute and chronic wound fluid can be worthwhile and can give important hints as to the differences between non-healing and healing wounds. Furthermore, the opportunity of analysing wound fluid over time will surely facilitate the discovery of relevant healing factors.

3. Sample requirements and challenges dependent on the choice of analytes

Besides all the obstacles that have been experienced in harvesting wound fluid and the interconnected high logistical effort to some extent also with respect to patient preparation, it is reasonable to assume that the sampling technique itself is likely to influence subsequent analysis.

So far, there is no gold standard to which a new sampling technique could be referenced to, nor any overview of the different methods that could elucidate the advantages and disadvantages of each particular technique. This lack of knowledge, along with the inherent variations in wound fluid composition -particularly in chronic wounds- complicates the picture and renders wound fluid a fairly complex analytic material. The missing standardisation in sampling wound fluid is likely to result in wound fluid being seen as an *undefined soup,* as termed decades ago by Trengove et al. (Trengove, Langton, and Stacey 1996). However, wound fluid might also be recognised as part of the problem in recalcitrant non-healing wounds and it might reflect the wound healing physiology better than any other sample material (Widgerow 2011).

Consequently, there are several inherent challenges in assaying chronic wound fluid. Very early comparisons between acute and chronic wound fluids showed a relevant difference in protease levels (Tarnuzzer and Schultz 1996) that has been confirmed by various authors subsequently (Chen et al. 1997; Moor, Vachon, and Gould 2009; Schmidtchen 2000). The issue of a proteolytic microenvironment in chronic wounds may be an important factor contributing to the non-healing phenotype, as growth factors and matrix components are subject to degradation (Widgerow 2011). This is not only true for growth factors and cytokines, but may also apply to receptors and signalling targets, which may thereby result in considerable loss of function. Therefore, merely quantifying analytes probably rather overestimates their protein bioactivity rather than reflecting the actual situation in the wound.

The composition and texture of wound fluid is influenced by the amount of exudate produced over a defined period of time as well as by its evaporation and absorption. Similarly, general factors such as leg positioning and the hydration status of the patient significantly alter the quality of wound fluid (Trengove, Langton, and Stacey 1996). Years ago, it had already been shown that the wound fluid of healing chronic leg ulcers showed relevant differences in global components -such as albumin and total protein content- when compared to the wound fluid of non-healing ulcers (James et al. 2000). Albumin reflects the plasma compartment of wound exudate and is, therefore, influenced by vascular leakage or acute vascular injury as well as by intravascular pressure.

When measuring protein biomarkers in wound fluid, the technical approach for how to obtain sample material must not be underestimated. It is still under discussion, whether values should be normalised to volume measures or total protein content for instance. Obtaining meaningful results is especially problematic when protease activity is high. Therefore, it is highly relevant to harvest immediate and unaltered sample material which is likely to reflect the original matrix of the wound microenvironment. In any cases, when intending to assess the amounts of peptide growth factors or other proteins in wound fluid, it should be recommended to determine the proteolytic activity of the environment in order to complete the picture. In this context, inert metabolites such as lactate might be considered advantageous since many of the limitations that apply to proteins do not apply here (Löffler et al. 2011).

There has been rapid progress in analytic techniques and devices, which allow for the accurate and sensitive determination of wound fluid composition. On the other hand, the techniques and methods used to obtain wound fluid need to catch up with this pace and should be thoroughly re-evaluated with regard to the requirements that have to be met for producing sound and meaningful results.

4. Protein arrays for the analysis of minute sample amounts

The analysis of cytokines and matrix metalloproteinases in complex biological fluids requires sensitive, accurate and reliable methods. This is usually accomplished using classical solid phase sandwich immunoassays, such as ELISA (de Jager and Rijkers 2006). However these methods are characterised by high sample consumption and the single analysis of only one mediator per experiment. Due to the frequently found low exudate levels in chronic wounds along with the inherent complexity of wound fluid, appropriate technologies are needed to accurately and reliably determine multiple parameters with

minimal sample consumption. In this context, protein microarrays are important tools for the simultaneous detection and quantification of multiple parameters in complex biological mixtures, such as plasma (Cassatella et al. 1997) or tissue lysates (Beidler et al. 2008). This technology has been shown to exhibit high sensitivity and optimal signal to noise ratios even in the case of low target concentrations (Ekins 1989, 1998).

There are two different types of microarray systems -planar and bead based arrays can be employed for multiplexed immunoassays. Whereas planar microarrays can be generated with hundreds and thousands of different capture spots, which are distinguished by their corresponding xy-coordinates, bead-based systems (e.g. Luminex) represent an interesting and flexible alternative, especially when the number of parameters to be determined in parallel is rather low. All bead-based array systems employ colour or size coded microspheres as solid support for the capture molecules. Beads are identified in a flow cytometer and the amount of captured target molecules is quantified on each individual bead using an appropriate reporter system. Sensitivity, reliability and accuracy are similar to those observed with standard ELISA procedures. Using bead-based technology, thousands of samples can be screened within a short time. Throughput neither seems to be a problem anymore, nor does sample volume (Stoll 2004; Templin et al. 2004).

Inflammatory processes such as wound healing involve a complex network of immunoregulatory molecules such as chemokines, cytokines, proteases and their respective counter-regulatory mediators (Hahm, Glaser, and Elster 2011). The analysis of a complete set of mediators can therefore be considered to be of more value than the single analysis of only one single analyte (Gardy et al. 2009; de Jager et al. 2003). Although protein microarray technology has been successfully applied for the analysis of multiple analytes in complex biological mixtures -such as plasma, serum, urine and cerebrospinal fluid- publications using protein arrays for the analysis of wound status are rare (Hsu et al. 2008; Weigelt et al. 2009; Craig-Schapiro et al. 2011; Swain et al. 2011). For instance, Grimstad et al. measured the concentration of 27 different cytokines in wound fluid derived from acute wounds using a bead-based system (Grimstad et al. 2011). Beidler et al. applied this technology to measure the concentrations of matrix metalloproteinases and cytokines in wound tissue lysate (Beidler et al. 2008, 2009). In combination with appropriate wound sampling procedures, the complex protein-composition of wound exudate together with limitations in the anticipated sample volume renders protein array technology -be it planar or bead-based- as an interesting tool for the analysis of wound-associated biomarkers (see section 6.1). Due to the inherent heterogeneity of wound exudate samples from chronic leg ulcers, the normalisation of the data needs to be discussed. In this context, the levels of albumin and total protein in wound fluid might represent an interesting reference value.

5. Infection in the diabetic foot

Foot pathologies are among the most frequent causes of hospitalisation in diabetic patients (Tecilazich, Dinh, and Veves 2011), with infection being the most dangerous complication. However, local processes triggered by microbial activity in the wound microenvironment have not gathered much interest until now. Apart from the classical means of diagnosing infection by clinical examination and microbiological swabbing, analysing wound fluid might provide additional important information. Furthermore, it is crucial to understand that microbiological sampling should be considered as a tool for proving the clinical

suspicion of infection rather than as a diagnostic instrument itself (Richard, Sotto, and Lavigne 2011).

Diagnosing infection in diabetic foot ulcers is challenging. This is especially true when the clinical assessment is not conclusive. The clinical signs for the diagnosis of infection may even be diminished by comorbidities -such as peripheral arterial disease, impaired leukocyte function and neuropathy- thereby reducing local signs of inflammation (Edmonds 2005; Williams, Hilton, and Harding 2004). However, since infection is a major cause of diabetes-related morbidity and mortality (Prompers et al. 2008) and as it paves the way towards limb amputation (Armstrong and Lipsky 2004), novel diagnostic tools that support prompt decision-making are urgently needed.

Currently there is ample room for the misclassification and delayed treatment of infection in the diabetic foot, and many issues in diagnosing diabetic foot infections are left unsolved (Jeffcoate et al. 2008). Furthermore, infection can show rapid onset and progression in diabetic foot ulcers.

In daily clinical practise, systemic markers of infection are the most established way of supporting the clinical suspicion of infection at present. The classical markers in this context include leukocyte count and C-reactive protein (CRP) as well as the erythrocyte sedimentation rate. Additionally, systemic markers specific to bacterial infection -such as procalcitonin, a peptide precursor of calcitonin, orosomucoid and haptoglobin- have been evaluated in serum of diabetic foot ulcers patients for the determination of infection (Jeandrot et al. 2008). Consecutively, a variety of immune mediators -including acute phase proteins, cytokines and chemokines- were examined in the plasma of patients with diabetic foot syndrome. These markers included CRP and fibrinogen as well as IL-6, IL-8, IL-18, macrophage inflammatory protein 1α (MIP1α), interferon γ-inducible protein-10 (IP10), macrophage migration inhibitory factor (MIF), macrophage chemoattractant protein 1 (MCP1) and RANTES (Weigelt et al. 2009). Both of the studies just cited were able to connect the occurrence of certain systemic inflammatory markers with the presence of infection in the diabetic foot.

In the first of these studies, serum CRP and procalcitonin were found to be significantly elevated during infection. CRP was discovered to be of a higher performance than procalcitonin, probably triggered by TNFα, IL-6 and IL-1 derived from the site of inflammation (Jeandrot et al. 2008). Still, the diagnosis of infection was principally determined by clinical assessment and the treatment was chosen accordingly.

Although various studies have been investigating CRP as a possible marker of infection in diabetic foot ulcers, sufficient support is not available for underlining its sole effectiveness (Dinh, Snyder, and Veves 2010) but it does add relevant supplementary information to the clinical assessment.

In the study conducted by Weigelt et al., besides CRP both IL-6 and fibrinogen -as acute phase reactants- were observed to be associated with the severity of foot ulcerations (88% type 2 diabetics) but not with the grade of infection. The overall findings in this fairly large study population can be interpreted to underline a specific alteration of the immune status of in diabetic patients, opposed to a rather general immune activation (IL-6 ↑, CRP ↑, fibrinogen ↑, MIP1α ↑, MIF↑, IP-10↑, RANTES↓, MCP-1 →, IL-8→ upon infection) (Weigelt

et al. 2009). This is a further important hint towards suspecting determined alterations in the immune response in diabetes, which may prove relevant to the future detection and assessment of diabetic foot ulcers. Anyhow, it should be noted that even low levels of most of the cytokines in circulation would reach toxic levels relatively fast, whereas -in principle- at their site of action in the local environment their levels can rise manifold higher.

When analysing serological markers and cytokine patterns in wounds together with wound microbiology, local IL-6 concentrations are rather likely to support the clinical suspicion of infection than serological markers can. The analysis of local IL-6 and TNFα was shown to be able to discriminate between monomicrobial and polymicrobial infections in contrast to serum CRP and liposaccharide binding protein (LBP). Furthermore, local IL-6 was reported to reflect higher bacterial loads and infection, especially with pseudomonas (Ambrosch et al. 2008). The determination of CRP as well as LBP as serological parameters, together with the semi-quantitative analysis of bacterial load and microbial identification, was performed. In this setting, the assessment of wounds was shifted to a first primary appraisal of microbiological rather than clinical examination. Although critical levels of bacterial loads in wounds have been proposed (Bowler, Duerden, and Armstrong 2001), the types of pathogen species are equally important. Nevertheless, in neuropathic diabetic foot ulcers the healing rate was also found to be inversely correlated with high bacterial loads (Xu et al. 2007). Furthermore, in a study by Ambrosch et al., a good correlation between local CRP and TNFα levels in wound washouts and tissue punch biopsies could be shown (Ambrosch et al. 2008).

In addition to the controversial findings on CRP, the upstream marker IL-6 seems to become even more meaningful in the local context. Overall, local alterations are likely to precede systemic changes. On the basis of this rationale, we advocate the investigation of the local microenvironment and possibly the correlation of these findings with circulatory markers. We are convinced that this local approach is more suitable for discovering useful markers for the assessment of infection in diabetic foot ulcer patients than the opposite approach of searching for alterations in the complicated circulatory proteome.

Of course, this local approach is not always suitable as -for instance- in the acute Charcot foot, which is a widely unresolved problem in diabetic foot care. Notably in this context, it would be of great interest to find circulatory markers disclosing the disease state and having possible therapeutic implications on hand. Initial attempts have already been made in this direction (Mabilleau et al. 2011).

6. Wound fluid - a window reflecting the wound microenvironment?

The question of whether wound fluid could serve as a valuable *window into the wound environment* has already been asked -years ago- by Dorne R. Yager et al. (Yager, Kulina, and Gilman 2007). Therefore, it is not a novel aspect but it is indeed an exciting option for having a closer look into the wound microenvironment. Recently, it has even been proposed that the corrosive nature of the chronic wound environment and the continuous breakdown of the extracellular matrix mediated by proteases may link wound fluid directly to the phenotype of chronic non-healing wounds (Widgerow 2011). If this were the case, the science of assessing wound fluid should be an important stepping stone in obtaining access to the problem of chronic non-healing wounds. Wound fluid -which has been investigated

in a variety of studies- has added much to our understanding of wound healing. Nevertheless, to date there are neither techniques nor markers that have found their way into clinical practise.

6.1 Biomarkers

A biomarker can be defined as a substance that is used as an indicator of normal biological processes, pathologic conditions and therapeutic interventions (Hahm, Glaser, and Elster 2011). They are widely attributed as playing a key role in the diagnosis, prognosis and clinical management of a broad range of disease states (Mueller, Muller, and Perruchoud 2008). Prominent examples are prostate specific antigen (PSA) (Makarov et al. 2009), or Her-2. However, due to complex disease pathologies and aetiological heterogeneity, it turns out that standalone markers are rather unlikely to be specific or else applicable on a wide-scale and a whole set of candidate markers are considered to be more promising e.g. as demonstrated by Domenici et al. (Domenici et al. 2010). According to Yager et al. there are as yet no accepted biomarkers, which allow the evaluation of the wound status and this critically hampers the improvement of appropriate treatments and effective therapies. Among the most promising candidate biomarkers are matrix metalloproteinases, cytokines and chemokines (Yager, Kulina, and Gilman 2007).

6.2 Proteases and their inhibitors

Chronic ulcers are characterised by a highly proteolytic microenvironment. Therefore, the four major biochemical classes of proteases (serine proteases, metalloproteinases, cysteine proteases and aspartic proteases) have generated great interest in the context of wound healing. In particular, matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) are critically involved in wound healing. Using protein array technology, Beidler et al. (Beidler et al. 2008) demonstrated that MMPs are differentially expressed in ulcerative and healthy tissue lysates. They demonstrated that MMP-8 and -9 in particular are strongly increased in ulcer tissue and decreased after the onset of the healing process. Yager et al.showed that MMP-2 and MMP-9 are elevated in chronic wound fluid compared with fluid from acute surgical wounds (Yager et al. 1996). Ladwig et al. (Ladwig et al. 2002) and Liu et al. (Liu et al. 2009) reported an increased ratio of MMP-9 to TIMP-1 in chronic non-healing wound fluid compared with healing wounds. Together with the finding that the MMP2:TIMP-2 ratio is elevated in non-healing compared with healing leg ulcers (Mwaura et al. 2006), this suggests that a disturbed homeostasis between MMPs and TIMPs might be correlated with the non-healing status. Also, in chronic non-healing diabetic foot ulcers, MMPs and their inhibitors (TIMPs) have received much interest. In a study by Lobmann et al., higher concentrations of the MMPs -2, -8 and -9, in conjunction with lower concentrations of TIMP-2, were determined in tissue biopsies when comparing diabetic wounds to healthy controls (Lobmann et al. 2002). The study by Liu et al. extended these findings of elevated MMP-9 in diabetic foot ulcers to healing rates. Elevated MMP-9 -in these ulcers- was suggested to be linked to inflammatory processes and poor healing. Furthermore, in conjunction with low TIMP-1 and TGFβ, high MMP-9 was proposed as being indicative of poor wound healing (Liu et al. 2009). These findings are coincident with other previous findings - for instance, in pressure ulcers where diminished healing rates have been described in a high MMP-9/low TIMP-1 environment (Ladwig et al. 2002) and

with a slightly different setting regarding the MMP-9:TIMP-2 ratio in diabetic foot ulcers (Lobmann et al. 2006). The interconnection of elevated MMP-9/MMP-8 levels and poor healing has further been described in neuropathic diabetic foot ulcers, but furthermore here an association between high levels of MMP-1 and TIMP-1 and better healing rates has been shown (Muller et al. 2008). Recently, Widgerow proposed a theory focusing on neutrophil-derived proteases -especially elevated MMP-9 in chronic wound fluid- as being involved in a vicious circle and stating that the corrosive nature of wound fluid was not only actively involved in matrix breakdown but also causative for non-healing a state probably further aggravated by bacterial factors (Widgerow 2011). According to this view, continued inflammation and leukocyte infiltration is triggered in chronic non-healing wounds, thereby perpetuating tissue destruction (Rayment, Upton, and Shooter 2008; cited by Widgerow 2011). Together with an imbalance towards a proteolytic microenvironment and in conjunction with bacterial colonisation causing the continuous breakdown of extracellular matrix and growth factors, this milieu keeps wounds in a prolonged destructive state and so averts healing (Rayment, Upton, and Shooter 2008).

In conclusion, we can see various overlapping motifs concerning an imbalance of MMPs and their inhibitory factors (TIMPs) in different types of wounds, which is also true for diabetic foot ulcers. MMPs seem to constitute some of the key players in this microenvironment and are probably linked to inflammatory activity and bacterial load, as well as the critical degradation of growth factors and extracellular matrix components. Nevertheless, as there are various MMPs involved in healing, such as MMP-1 to MMP-10, MMP-12 and MMP-13 and several of their inhibitors (Armstrong and Jude 2002; Gill and Parks 2008); they are also important for physiological wound healing, although in a timely and ordinary fashion. As MMPs also have physiological roles and intimate links with inflammatory processes, an overview investigation of their roles with regard to inflammatory activity and wound healing rates would be desirable for coming to novel conclusions notably in non-healing wounds. For this endeavour, wound fluid might constitute a nearly ideal sample material.

6.3 Cytokines and growth factors

Besides MMPs, cytokines and chemokines are frequently discussed as potential indicators for the status of wound healing. In general, there is an ample variety of factors which can be found in the wound microenvironment and that play a role in wound healing. Nevertheless, the cytokines found in the local microenvironment are usually produced by local cells (Holzheimer and Steinmetz 2000) and may, therefore, provide us with interesting information on the local environment. The issue of mediators being found in wounds is already complex in physiological terms, and confounding factors -such as the chronic wound environment and exogenous factors from bacterial colonisation and the endogenous reactions to this scenario- make it even more complicated. Besides this, it is a fact that the pathogenesis of wound healing on the molecular level is poorly understood and may be diverse according to various different wound entities. Nevertheless, we will attempt a concise digression on the topic without the intent to give a comprehensive impression of chemokines, cytokines and growth factors in wound fluids.

To our knowledge, there are no broad analyses available of these mediators in conjunction with clinical endpoints from chronic ulcers. Therefore, it may be prudent to begin with a glance at acute wound healing.

In a study by Grimstad et al. (already mentioned above) an attempt to characterise acute wound fluid from surgical drains at the first postoperative day after mastectomy was undertaken. This aspect therefore best describes the initial phase of the acute inflammatory response, which is particularly interesting in this relatively broad investigation assessing the levels of 27 different cytokines, chemokines and growth factors (Grimstad et al. 2011). Information as to this amount is generally scarce and will therefore be looked at in more detail here. In this context, the cytokines IL-6 and IL-8 -such as TNFα and IFNγ- were found in high abundance in acute wound fluid. In contrast with this pro-inflammatory pattern, the IL-1 receptor antagonist (IL-1RA) was also detected in high concentrations here. Interestingly, at this context the amounts of IL-1β detected and the anti-inflammatory cytokine IL-10 were found to be relatively low.

This pattern just as observed in wound fluid at the onset of inflammation suggests a distinct but contained local inflammatory environment. The picture witnessed in this phase of wound healing can be interpreted -in our view- as an inflammatory response with strong inhibitory and restorative stimuli. This might be the case, as IL-8 constitutes a chemotactic factor for neutrophil recruitment (Baggiolini and Clark-Lewis 1992) as well as a factor promoting epidermal cell proliferation (Tuschil et al. 1992); moreover, IL-6 plays an ambivalent role with pro- as well as anti-inflammatory properties, such as inhibiting TNFα and repressing IL-1β as well as inducing IL-1RA and IL-10 (Tilg et al. 1994). The low levels of the key pro-inflammatory marker IL-1β, which is -for instance- associated with inflammasome activity and the control of infection (Schroder and Tschopp 2010) is also notable in this early postoperative setting.

With regard to chronic non-healing wounds, a connection with persistent inflammatory activity is a guiding principle, which is observed in various entities of impaired healing (Acosta et al. 2008; Pukstad et al. 2010). In a longitudinal study in chronic venous leg ulcers, decreases in IL-1α and IL-1β were observed in healing ulcers, whereas increases in IL-8 and MIP-1α were associated with non-healing. This was observed in conjunction with decreased levels of toll-like receptor (TLR) activities in healing ulcers (Pukstad et al. 2010). The persistent activation of innate immune responses, in dividing the healing and non-healing entities of wounds by TLR activation and distinct cytokine patterns (IL-1α ↑, IL1-β↑, IL-8↓ and MIP-1α↓), may even be interpreted in favour of a constant bacterial challenge in non-healers with relevant effects on the wound microenvironment.

The elevated expression of IL-1β and excessive TNFα in non-healing wounds (Wallace and Stacey 1998) may, therefore, be linked to the distinct properties observed in this microenvironment. This may be the case, as high TNFα has been described not only as inducing IL-1β and its own synthesis but also their synergistic effects resulting in the suppression of extracellular matrix synthesis, whereas MMP synthesis is induced and TIMP synthesis is inhibited (Mast and Schultz 1996), thus contributing to persistent inflammatory activity and tissue destruction in diabetes (Nwomeh, Yager, and Cohen 1998; Naguib et al. 2004). This link may, therefore, constitute a part of the vicious circle that we know of, linking alterations observed in the microenvironment of non-healing wounds to persistent inflammatory activity.

For instance, levels of TNFα have been found to be elevated in non-healing compared to healing leg ulcers (Wallace and Stacey 1998). Trengove et al. found elevated levels of the

pro-inflammatory cytokines IL-1, IL-6 and TNFα in non-healing compared with healing ulcers and that these mediators decreased over the course of healing (Trengove, Bielefeldt-Ohmann, and Stacey 2000). Beidler and co-workers measured 22 mediators in healthy and ulcerative tissue lysates and found that IL-1α, IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12p40, G-CSF, GM-CSF, MCP-1, IFNγ, TNFα, MIP-1α, MIP-1β and TGFβ1 were expressed at significantly higher levels in ulcer tissue compared with healthy controls. Moreover, they found the analytes IL-1α, IL-1β, IFNγ, IL-12p40, GM-CSF and IL-1RA to be differentially expressed in rapid versus delayed healing ulcers (Beidler et al. 2009).

In summary, there are promising candidates that might be used as indicators of the healing status of chronic and acute wounds. However, additional efforts are needed to further validate the existing candidates and to discover new potential biomarkers. In this context, the use of entire biomarker panels instead of standalone molecules should be taken into account.

Especially in diabetes, it is established that a local over-secretion of pro-inflammatory cytokines (notably TNFα, IL-6 and IL-1) is observed, impacting the wound healing microenvironment and opposing healing (Acosta et al. 2008). These factors have been multiply observed in the context of failure to heal and are linked with other processes observed in the wound environment, such as elevated proteolysis and the degradation of growth factors and their receptors.

When it comes to growth factors, it is conceivable that beneath the mere abundance of these factors in wound fluid the aspect of bioactivity is of primary relevance. This may be best demonstrated by the reactions that have been described for ages, when comparing the diverse effects of wound fluids on cells in culture. The most important aspect of this may be the positive effect evoked by acute wound fluid on cellular proliferation. When acute wound fluid is added to cells in culture, increased proliferation of vascular endothelial cells, fibroblasts and endothelial cells was observed (Greenburg and Hunt 1978; Katz et al. 1991). Furthermore, the deposition of extracellular matrix and migration of cells are enhanced by acute wound fluid supplemented to cell cultures. In opposition to this, chronic wound fluid has been observed numerous times as inhibiting proliferation *in vitro* (Trengove, Bielefeldt-Ohmann, and Stacey 2000; Bucalo, Eaglstein, and Falanga 1993; Phillips et al. 1998), thus hampering tissue restoration. Of course, the converse argument that the lack of proliferative stimulus in chronic wound fluid may be attributed to a mere lack of growth factors is invalid. To underline this thesis, Trengove et al. were not able to detect significant changes within the levels of platelet-derived growth factor (PDGF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) or transforming growth factor beta (TGFβ) from healing and non-healing ulcers. However, in mitogenic assays in fibroblasts, wound fluid from healing ulcers was able to provoke a significantly enhanced proliferative response (Trengove, Bielefeldt-Ohmann, and Stacey 2000). Furthermore, additional growth factors have been implicated as playing an important role in wound healing, such as insulin-like growth factor (IGF) (Wagner et al. 2003) or keratinocyte growth factor (KGF) (Gibbs et al. 2000).

In a study by He et al., it was demonstrated that the addition of platelet derived growth factor (PDGF) to chronic wound fluid was able to synergistically enhance proliferation in fibroblasts, whereas chronic wound fluid alone also enhanced fibroblast proliferation at

lower levels (He et al. 1999). Nevertheless, the original matrix in this study was not preserved and the results suggest that essential factors can be found in wound fluid enhancing proliferation that can be stimulated by the addition of growth factors from external sources, whereas the original fluid matrix is suited to suppressing proliferative effects. To sum up the findings, a mere lack of growth factors does not seem to be responsible for impaired wound healing. In fact, other factors that characterise the wound environment seem to be of relevance in impairing local proliferation. For instance, it has been shown that high glucose levels are suited to inhibit fibroblast proliferation and to induce resistance to growth factors (Hehenberger and Hansson 1997). Furthermore, in fibroblasts from chronic diabetic wounds, lactate levels above 7 mM have been shown to abrogate cell proliferation as a specific effect of elevated L-lactate levels (Hehenberger et al. 1998). Another aspect that may be of relevance within this context is the enhanced degradation of growth factors by proteases which is observed in chronic wound fluid when compared with its acute counterpart (Trengove et al. 1999).

Eventually, cellular factors (such as reaching the end of their replicative life span, which was suggested for fibroblasts in chronic ulcers that had not healed for 3 years, and were non-responding to PDGF while exhibiting a normal expression of their respective receptors compared to their normal counterparts) are another possibility that may explain the lack of proliferation (Agren et al. 1999) together with the demand for antecedent therapeutic intervention. Interestingly, this condition of environmentally-driven cellular aging has been shown not to be correlated with telomere shortening but to be induced by other factors; possibly decreased resistance to oxidative stress (Wall et al. 2008), including the possibility of reversing this induced phenotype. Nevertheless, fibroblast dysfunction in late non-healing ulcers seems to constitute an important factor propagating this condition that cannot be overcome anymore by mere basic growth factor supplementation.

6.4 Role of lactate

Tissue lactate accumulates during the physiological course of healing to concentrations of ~ 10-12 mM as opposed to 1–3 mM normally found in blood and most uninjured, resting tissues (Ghani et al. 2004). Even though it has been shown that these high lactate concentrations persist irrespective of tissue oxygenation (Ghani et al. 2004), the concept of lactate as *a dead end product* in hypoxia has survived for many years still. Nowadays, it is widely accepted that tissue lactate is mainly derived from aerobic glycolysis.

Lactate is understood to have many fundamental metabolic and signalling functions (Gladden 2004). Thus, lactate enhances wound healing by the stimulation of extracellular matrix synthesis (Hussain, Ghani, and Hunt 1989; Green and Goldberg 1964) and angiogenesis (Beckert et al. 2006; Hunt et al. 2007). However, at higher concentrations these positive effects seem to deteriorate. In fibroblast experiments, a threshold for cell proliferation could be determined (Hehenberger et al. 1998). Cellular proliferation was enhanced so long as lactate levels were lower than this particular threshold but inhibited when raised above.

In chronic wounds, tissue lactate concentrations reach far higher levels compared with acute wounds. Inflammatory cells that are ubiquitously present in chronic wounds cover their energy demand mainly through aerobic glycolysis and they generate lactate as a by-product

and in huge amounts. Next, lactate leaves the cytosol to the extracellular space by monocarboxylic transferases (MCT) (Gladden 2004).

Almost every chronic wound is more or less subject to bacterial contamination. Even though bacterial contamination does not necessarily lead to immediate subsequent infection, wound healing is impaired when the bacterial load reaches a certain level. In addition, many bacterial strains that have been detected in chronic wounds -and in the diabetic foot in particular- cover their energy demand by fermentation, thus producing additional lactate.

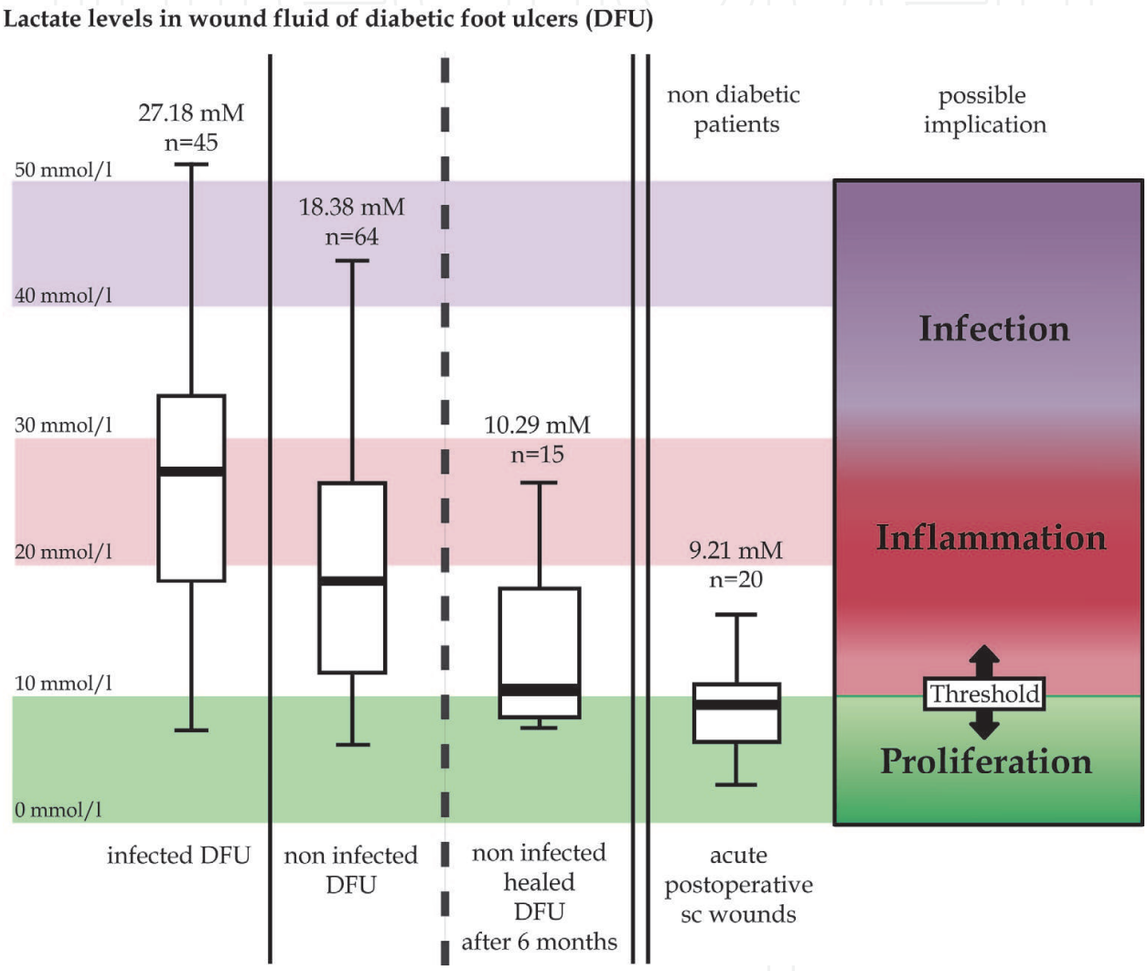

Fig. 1. Lactate levels in diabetic foot ulcers (DFU) with clinical signs of soft-infection are shown on the left side of the continuous line as opposed to non-infected DFU on the right. In addition, lactate concentrations of DFU that healed within a follow-up period of 6 months are given to the right of the dotted line. Further right, wound fluid lactate concentrations in surgical wounds are shown. Wound fluid lactate concentrations are given as box plots (median, minimum - maximum as well as the 25th and 75th percentiles). The respective median lactate concentrations are given above the corresponding box plots. The possible clinical implications of lactate are presented in coloured boxes (modified according to Löffler et al. 2011).

In diabetic foot ulcers, a correlation between elevated wound fluid lactate levels and the clinical signs of soft-tissue infection could be demonstrated. Wounds with clinical signs of soft-tissue infection showed significantly higher wound fluid lactate concentrations as opposed to those without soft-tissue infection. Out of the wound sub-population, without clinical signs of soft-tissue infection, wounds that healed within a 6 months follow-up period were characterised by wound fluid lactate concentrations comparable with those found in acute healing wounds (Löffler et al. 2011). In summary, elevated lactate levels seem to be attributed both to inflammatory and bacterial activity. Nevertheless, lactate is likely to favour healing unless its concentration exceeds a certain threshold. On the other hand, lactate in wound fluid might be a diagnostic marker both for impaired healing and soft-tissue-infection (Figure 1).

7. Conclusions

Sampling and analysing wound fluid is an inherently challenging task, since a variety of local and systemic factors influence its composition. This is particularly true for chronic wounds and for diabetic foot ulcers. In addition, there is no clear consensus yet about how to obtain adequate sample material. It is important nevertheless, depending on the analysis intended to be undertaken to choose the appropriate technique for obtaining sample material. Hence, to obtain meaningful results an adequate and suitable technique for harvesting wound fluid is a major pre-requisite when considering using sophisticated analytic methods. The technical developments in this field have brought novel analytical opportunities that require only very little amounts of sample material in order to get meaningful results. A variety of techniques have been proposed for obtaining sample materials and a multitude of possible wound fluid markers have been investigated. Finding meaningful biomarkers or biomarker panels allowing for the instant detection of infection or the determination of wound healing tendencies are urgently needed (Hahm, Glaser, and Elster 2011). We are convinced that it does make sense to analyse potential biomarkers in wound fluid, which is both easily accessible and can be harvested in a non-invasive way. The obstacle to overcome here is to implement techniques suitable for daily clinical routines, allowing for fast and reliable diagnostic procedures. In this setting, there are less confounding factors to deal with as opposed to systemic markers. No systemic marker has been proven to be capable of serving as a standalone diagnostic tool yet. To date, however, no wound fluid marker has been established that might fill this gap neither. We are at the beginning of an era which might appreciate wound fluid analysis as a novel opportunity to get deeper insights into a complicated and widely unknown micro-environment. There is still substantial room for improvement and plenty of work has to be done before we are able to understand the elementary mechanisms of non-healing.

8. Perspective

Valuable markers for clinical wound assessment are urgently needed. Wound fluid biomarkers are probably the best option for reaching this aim. For this purpose, wound fluid components -such as proteases and their inhibitors as well as cytokines and growth factors and other elements associated with the local inflammatory response- are worthwhile investigating. The wound fluid degradation products of the extracellular matrix represent another interesting aspect in assessing the pathophysiology of non-healing wounds

(Moseley et al. 2004). This is by virtue of the fact that the course of chronic wound healing may be directly reflected in matrix turnover due to a highly proteolytic and pro-oxidative environment. In addition, markers of oxidative stress and simple metabolites such as lactate may also give us important information on the state of wound healing. However, there is still ample room for the discovery of both valuable and inert wound fluid biomarkers. It can be anticipated that novel technical opportunities along with reproducible sampling methods will bring forward new biomarkers, which may aid in future wound assessment. The need is there, especially in the diabetic foot, and hopefully meaningful studies will soon be under way to bridge this gap, thereby substantiating the valour of wound fluid diagnostics.

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10. References

Acosta, J. B., D. G. del Barco, D. C. Vera, W. Savigne, P. Lopez-Saura, G. Guillen Nieto, and G. S. Schultz. 2008. The pro-inflammatory environment in recalcitrant diabetic foot wounds. *Int Wound J* 5 (4):530-9.

Agren, M. S., H. H. Steenfos, S. Dabelsteen, J. B. Hansen, and E. Dabelsteen. 1999. Proliferation and mitogenic response to PDGF-BB of fibroblasts isolated from chronic venous leg ulcers is ulcer-age dependent. *J Invest Dermatol* 112 (4):463-9.

Ambrosch, A., R. Lobmann, A. Pott, and J. Preissler. 2008. Interleukin-6 concentrations in wound fluids rather than serological markers are useful in assessing bacterial triggers of ulcer inflammation. *Int Wound J* 5 (1):99-106.

Apple, F. S. 1999. Tissue specificity of cardiac troponin I, cardiac troponin T and creatine kinase-MB. *Clin Chim Acta* 284 (2):151-9.

Armstrong, D. G., and E. B. Jude. 2002. The role of matrix metalloproteinases in wound healing. *J Am Podiatr Med Assoc* 92 (1):12-8.

Armstrong, D. G., and B. A. Lipsky. 2004. Diabetic foot infections: stepwise medical and surgical management. *Int Wound J* 1 (2):123-32.

Baggiolini, M., and I. Clark-Lewis. 1992. Interleukin-8, a chemotactic and inflammatory cytokine. *FEBS Lett* 307 (1):97-101.

Baker, E. A., and D. J. Leaper. 2000. Proteinases, their inhibitors, and cytokine profiles in acute wound fluid. *Wound Repair Regen* 8 (5):392-8.

Balk, S. P., Y. J. Ko, and G. J. Bubley. 2003. Biology of prostate-specific antigen. *J Clin Oncol* 21 (2):383-91.

Beckert, S., F. Farrahi, R. S. Aslam, H. Scheuenstuhl, A. Konigsrainer, M. Z. Hussain, and T. K. Hunt. 2006. Lactate stimulates endothelial cell migration. *Wound Repair Regen* 14 (3):321-4.

Beidler, S. K., C. D. Douillet, D. F. Berndt, B. A. Keagy, P. B. Rich, and W. A. Marston. 2008. Multiplexed analysis of matrix metalloproteinases in leg ulcer tissue of patients with chronic venous insufficiency before and after compression therapy. *Wound Repair Regen* 16 (5):642-8.

Beidler, S. K., C. D. Douillet, D. F. Berndt, B. A. Keagy, P. B. Rich, and W. A. Marston. 2009. Inflammatory cytokine levels in chronic venous insufficiency ulcer tissue before and after compression therapy. *J Vasc Surg* 49 (4):1013-20.

Bowler, P. G., B. I. Duerden, and D. G. Armstrong. 2001. Wound microbiology and associated approaches to wound management. *Clin Microbiol Rev* 14 (2):244-69.

Bucalo, B., W. H. Eaglstein, and V. Falanga. 1993. Inhibition of cell proliferation by chronic wound fluid. *Wound Repair Regen* 1 (3):181-6.

Cassatella, M. A., S. Gasperini, F. Calzetti, A. Bertagnin, A. D. Luster, and P. P. McDonald. 1997. Regulated production of the interferon-gamma-inducible protein-10 (IP-10) chemokine by human neutrophils. *Eur J Immunol* 27 (1):111-5.

Chen, S. M., S. I. Ward, O. O. Olutoye, R. F. Diegelmann, and I. Kelman Cohen. 1997. Ability of chronic wound fluids to degrade peptide growth factors is associated with increased levels of elastase activity and diminished levels of proteinase inhibitors. *Wound Repair Regen* 5 (1):23-32.

Clough, G., and M. Noble. 2003. Microdialysis--a model for studying chronic wounds. *Int J Low Extrem Wounds* 2 (4):233-9.

Cooper, D. M., E. Z. Yu, P. Hennessey, F. Ko, and M. C. Robson. 1994. Determination of endogenous cytokines in chronic wounds. *Ann Surg* 219 (6):688-91; discussion 691-2.

Craig-Schapiro, R., M. Kuhn, C. Xiong, E. H. Pickering, J. Liu, T. P. Misko, R. J. Perrin, K. R. Bales, H. Soares, A. M. Fagan, and D. M. Holtzman. 2011. Multiplexed immunoassay panel identifies novel CSF biomarkers for Alzheimer's disease diagnosis and prognosis. *PLoS One* 6 (4):e18850.

de Jager, W., and G. T. Rijkers. 2006. Solid-phase and bead-based cytokine immunoassay: a comparison. *Methods* 38 (4):294-303.

de Jager, W., H. te Velthuis, B. J. Prakken, W. Kuis, and G. T. Rijkers. 2003. Simultaneous detection of 15 human cytokines in a single sample of stimulated peripheral blood mononuclear cells. *Clin Diagn Lab Immunol* 10 (1):133-9.

Dealey, C., J. Cameron, and M. Arrowsmith. 2006. A study comparing two objective methods of quantifying the production of wound exudate. *J Wound Care* 15 (4):149-53.

Dinh, T., G. Snyder, and A. Veves. 2010. Current techniques to detect foot infection in the diabetic patient. *Int J Low Extrem Wounds* 9 (1):24-30.

Domenici, E., D. R. Wille, F. Tozzi, I. Prokopenko, S. Miller, A. McKeown, C. Brittain, D. Rujescu, I. Giegling, C. W. Turck, F. Holsboer, E. T. Bullmore, L. Middleton, E. Merlo-Pich, R. C. Alexander, and P. Muglia. 2010. Plasma protein biomarkers for depression and schizophrenia by multi analyte profiling of case-control collections. *PLoS One* 5 (2):e9166.

Duckworth, W. C., J. Fawcett, S. Reddy, and J. C. Page. 2004. Insulin-degrading activity in wound fluid. *J Clin Endocrinol Metab* 89 (2):847-51.

Edmonds, M. 2005. Infection in the neuroischemic foot. *Int J Low Extrem Wounds* 4 (3):145-53.

Ekins, R. P. 1989. Multi-analyte immunoassay. *J Pharm Biomed Anal* 7 (2):155-68.

Ekins, R. P. 1998. Ligand assays: from electrophoresis to miniaturized microarrays. *Clin Chem* 44 (9):2015-30.

Eming, S. A., M. Koch, A. Krieger, B. Brachvogel, S. Kreft, L. Bruckner-Tuderman, T. Krieg, J. D. Shannon, and J. W. Fox. 2010. Differential proteomic analysis distinguishes

tissue repair biomarker signatures in wound exudates obtained from normal healing and chronic wounds. *J Proteome Res* 9 (9):4758-66.

Ertugrul, B. M., O. Savk, B. Ozturk, M. Cobanoglu, S. Oncu, and S. Sakarya. 2009. The diagnosis of diabetic foot osteomyelitis: examination findings and laboratory values. *Med Sci Monit* 15 (6):CR307-12.

Fivenson, D. P., D. T. Faria, B. J. Nickoloff, P. J. Poverini, S. Kunkel, M. Burdick, and R. M. Strieter. 1997. Chemokine and inflammatory cytokine changes during chronic wound healing. *Wound Repair Regen* 5 (4):310-22.

Gardy, J. L., D. J. Lynn, F. S. Brinkman, and R. E. Hancock. 2009. Enabling a systems biology approach to immunology: focus on innate immunity. *Trends Immunol* 30 (6):249-62.

Ghani, Q. P., S. Wagner, H. D. Becker, T. K. Hunt, and M. Z. Hussain. 2004. Regulatory role of lactate in wound repair. *Methods Enzymol* 381:565-75.

Gibbs, S., A. N. Silva Pinto, S. Murli, M. Huber, D. Hohl, and M. Ponec. 2000. Epidermal growth factor and keratinocyte growth factor differentially regulate epidermal migration, growth, and differentiation. *Wound Repair Regen* 8 (3):192-203.

Gill, S. E., and W. C. Parks. 2008. Metalloproteinases and their inhibitors: regulators of wound healing. *Int J Biochem Cell Biol* 40 (6-7):1334-47.

Gladden, L. B. 2004. Lactate metabolism: a new paradigm for the third millennium. *J Physiol* 558 (Pt 1):5-30.

Gohel, M. S., R. A. Windhaber, J. F. Tarlton, M. R. Whyman, and K. R. Poskitt. 2008. The relationship between cytokine concentrations and wound healing in chronic venous ulceration. *J Vasc Surg* 48 (5):1272-7.

Green, H., and B. Goldberg. 1964. Collagen and Cell Protein Synthesis by an Established Mammalian Fibroblast Line. *Nature* 204:347-9.

Greenburg, G. B., and T. K. Hunt. 1978. The proliferative response in vitro of vascular endothelial and smooth muscle cells exposed to wound fluids and macrophages. *J Cell Physiol* 97 (3 Pt 1):353-60.

Grimstad, O., O. Sandanger, L. Ryan, K. Otterdal, J. K. Damaas, B. Pukstad, and T. Espevik. 2011. Cellular sources and inducers of cytokines present in acute wound fluid. *Wound Repair Regen* 19 (3):337-47.

Hahm, G., J. J. Glaser, and E. A. Elster. 2011. Biomarkers to predict wound healing: the future of complex war wound management. *Plast Reconstr Surg* 127 Suppl 1:21S-26S.

He, C., M. A. Hughes, G. W. Cherry, and F. Arnold. 1999. Effects of chronic wound fluid on the bioactivity of platelet-derived growth factor in serum-free medium and its direct effect on fibroblast growth. *Wound Repair Regen* 7 (2):97-105.

Hehenberger, K., and A. Hansson. 1997. High glucose-induced growth factor resistance in human fibroblasts can be reversed by antioxidants and protein kinase C-inhibitors. *Cell Biochem Funct* 15 (3):197-201.

Hehenberger, K., J. D. Heilborn, K. Brismar, and A. Hansson. 1998. Inhibited proliferation of fibroblasts derived from chronic diabetic wounds and normal dermal fibroblasts treated with high glucose is associated with increased formation of l-lactate. *Wound Repair Regen* 6 (2):135-41.

Hoffman, R., J. Noble, and M. Eagle. 1999. The use of proteases as prognostic markers for the healing of venous leg ulcers. *J Wound Care* 8 (6):273-6.

Hoffman, R., S. Starkey, and J. Coad. 1998. Wound fluid from venous leg ulcers degrades plasminogen and reduces plasmin generation by keratinocytes. *J Invest Dermatol* 111 (6):1140-4.

Holzheimer, R. G., and W. Steinmetz. 2000. Local and systemic concentrations of pro- and anti-inflammatory cytokines in human wounds. *Eur J Med Res* 5 (8):347-55.

Hsu, H. Y., S. Wittemann, E. M. Schneider, M. Weiss, and T. O. Joos. 2008. Suspension microarrays for the identification of the response patterns in hyperinflammatory diseases. *Med Eng Phys* 30 (8):976-83.

Hunt, T. K., R. S. Aslam, S. Beckert, S. Wagner, Q. P. Ghani, M. Z. Hussain, S. Roy, and C. K. Sen. 2007. Aerobically derived lactate stimulates revascularization and tissue repair via redox mechanisms. *Antioxid Redox Signal* 9 (8):1115-24.

Hunt, T. K., P. Twomey, B. Zederfeldt, and J. E. Dunphy. 1967. Respiratory gas tensions and pH in healing wounds. *Am J Surg* 114 (2):302-7.

Hussain, M. Z., Q. P. Ghani, and T. K. Hunt. 1989. Inhibition of prolyl hydroxylase by poly(ADP-ribose) and phosphoribosyl-AMP. Possible role of ADP-ribosylation in intracellular prolyl hydroxylase regulation. *J Biol Chem* 264 (14):7850-5.

James, T. J., M. A. Hughes, G. W. Cherry, and R. P. Taylor. 2000. Simple biochemical markers to assess chronic wounds. *Wound Repair Regen* 8 (4):264-9.

Jeandrot, A., J. L. Richard, C. Combescure, N. Jourdan, S. Finge, M. Rodier, P. Corbeau, A. Sotto, and J. P. Lavigne. 2008. Serum procalcitonin and C-reactive protein concentrations to distinguish mildly infected from non-infected diabetic foot ulcers: a pilot study. *Diabetologia* 51 (2):347-52.

Jeffcoate, W. J., B. A. Lipsky, A. R. Berendt, P. R. Cavanagh, S. A. Bus, E. J. Peters, W. H. van Houtum, G. D. Valk, and K. Bakker. 2008. Unresolved issues in the management of ulcers of the foot in diabetes. *Diabet Med* 25 (12):1380-9.

Kallarackal, G. U., E. A. Ansari, N. Amos, J. C. Martin, C. Lane, and J. P. Camilleri. 2002. A comparative study to assess the clinical use of Fluorescein Meniscus Time (FMT) with Tear Break up Time (TBUT) and Schirmer's tests (ST) in the diagnosis of dry eyes. *Eye (Lond)* 16 (5):594-600.

Katz, M. H., A. F. Alvarez, R. S. Kirsner, W. H. Eaglstein, and V. Falanga. 1991. Human wound fluid from acute wounds stimulates fibroblast and endothelial cell growth. *J Am Acad Dermatol* 25 (6 Pt 1):1054-8.

Ladwig, G. P., M. C. Robson, R. Liu, M. A. Kuhn, D. F. Muir, and G. S. Schultz. 2002. Ratios of activated matrix metalloproteinase-9 to tissue inhibitor of matrix metalloproteinase-1 in wound fluids are inversely correlated with healing of pressure ulcers. *Wound Repair Regen* 10 (1):26-37.

Liu, Y., D. Min, T. Bolton, V. Nube, S. M. Twigg, D. K. Yue, and S. V. McLennan. 2009. Increased matrix metalloproteinase-9 predicts poor wound healing in diabetic foot ulcers. *Diabetes Care* 32 (1):117-9.

Lobmann, R., A. Ambrosch, G. Schultz, K. Waldmann, S. Schiweck, and H. Lehnert. 2002. Expression of matrix-metalloproteinases and their inhibitors in the wounds of diabetic and non-diabetic patients. *Diabetologia* 45 (7):1011-6.

Lobmann, R., C. Zemlin, M. Motzkau, K. Reschke, and H. Lehnert. 2006. Expression of matrix metalloproteinases and growth factors in diabetic foot wounds treated with a protease absorbent dressing. *J Diabetes Complications* 20 (5):329-35.

Löffler, M., D. Zieker, J. Weinreich, S. Lob, I. Königsrainer, S. Symons, S. Bühler, A. Königsrainer, H. Northoff, and S. Beckert. 2011. Wound fluid lactate concentration: a helpful marker for diagnosing soft-tissue infection in diabetic foot ulcers? Preliminary findings. *Diabet Med* 28 (2):175-8.

Mabilleau, G., N. Petrova, M. E. Edmonds, and A. Sabokbar. 2011. Number of circulating CD14-positive cells and the serum levels of TNF-alpha are raised in acute charcot foot. *Diabetes Care* 34 (3):e33.

Makarov, D. V., S. Loeb, R. H. Getzenberg, and A. W. Partin. 2009. Biomarkers for prostate cancer. *Annu Rev Med* 60:139-51.

Mani, R. 1999. Science of measurements in wound healing. *Wound Repair Regen* 7 (5):330-4.

Mast, B. A., and G. S. Schultz. 1996. Interactions of cytokines, growth factors, and proteases in acute and chronic wounds. *Wound Repair Regen* 4 (4):411-20.

Mellergard, P., O. Aneman, F. Sjogren, P. Pettersson, and J. Hillman. 2008. Changes in extracellular concentrations of some cytokines, chemokines, and neurotrophic factors after insertion of intracerebral microdialysis catheters in neurosurgical patients. *Neurosurgery* 62 (1):151-7; discussion 157-8.

Moor, A. N., D. J. Vachon, and L. J. Gould. 2009. Proteolytic activity in wound fluids and tissues derived from chronic venous leg ulcers. *Wound Repair Regen* 17 (6):832-9.

Moseley, R., J. E. Stewart, P. Stephens, R. J. Waddington, and D. W. Thomas. 2004. Extracellular matrix metabolites as potential biomarkers of disease activity in wound fluid: lessons learned from other inflammatory diseases? *Br J Dermatol* 150 (3):401-13.

Mueller, C., B. Muller, and A. P. Perruchoud. 2008. Biomarkers: past, present, and future. *Swiss Med Wkly* 138 (15-16):225-9.

Muller, M., C. Trocme, B. Lardy, F. Morel, S. Halimi, and P. Y. Benhamou. 2008. Matrix metalloproteinases and diabetic foot ulcers: the ratio of MMP-1 to TIMP-1 is a predictor of wound healing. *Diabet Med* 25 (4):419-26.

Mwaura, B., B. Mahendran, N. Hynes, D. Defreitas, G. Avalos, T. Adegbola, M. Adham, C. E. Connolly, and S. Sultan. 2006. 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* 31 (3):306-10.

Naguib, G., H. Al-Mashat, T. Desta, and D. T. Graves. 2004. Diabetes prolongs the inflammatory response to a bacterial stimulus through cytokine dysregulation. *J Invest Dermatol* 123 (1):87-92.

Noble-Bell, G., and A. Forbes. 2008. A systematic review of the effectiveness of negative pressure wound therapy in the management of diabetes foot ulcers. *Int Wound J* 5 (2):233-42.

Nwomeh, B. C., D. R. Yager, and I. K. Cohen. 1998. Physiology of the chronic wound. *Clin Plast Surg* 25 (3):341-56.

Ono, I., H. Gunji, K. Suda, K. Iwatsuki, and F. Kaneko. 1994. Evaluation of cytokines in donor site wound fluids. *Scand J Plast Reconstr Surg Hand Surg* 28 (4):269-73.

Oono, T., Y. Fujiwara, T. Yoshioka, and J. Arata. 1997. Prolidase activity in chronic wound and blister fluids. *J Dermatol* 24 (10):626-9.

Ortega, M. R., T. Ganz, and S. M. Milner. 2000. Human beta defensin is absent in burn blister fluid. *Burns* 26 (8):724-6.

Pepys, M. B., and G. M. Hirschfield. 2003. C-reactive protein: a critical update. *J Clin Invest* 111 (12):1805-12.

Phillips, T. J., H. O. al-Amoudi, M. Leverkus, and H. Y. Park. 1998. Effect of chronic wound fluid on fibroblasts. *J Wound Care* 7 (10):527-32.

Prompers, L., M. Huijberts, N. Schaper, J. Apelqvist, K. Bakker, M. Edmonds, P. Holstein, E. Jude, A. Jirkovska, D. Mauricio, A. Piaggesi, H. Reike, M. Spraul, K. Van Acker, S. Van Baal, F. Van Merode, L. Uccioli, V. Urbancic, and G. Ragnarson Tennvall. 2008. Resource utilisation and costs associated with the treatment of diabetic foot ulcers. Prospective data from the Eurodiale Study. *Diabetologia* 51 (10):1826-34.

Pukstad, B. S., L. Ryan, T. H. Flo, J. Stenvik, R. Moseley, K. Harding, D. W. Thomas, and T. Espevik. 2010. Non-healing is associated with persistent stimulation of the innate immune response in chronic venous leg ulcers. *J Dermatol Sci* 59 (2):115-22.

Rayment, E. A., Z. Upton, and G. K. Shooter. 2008. Increased matrix metalloproteinase-9 (MMP-9) activity observed in chronic wound fluid is related to the clinical severity of the ulcer. *Br J Dermatol* 158 (5):951-61.

Richard, J. L., A. Sotto, and J. P. Lavigne. 2011. New insights in diabetic foot infection. *World J Diabetes* 2 (2):24-32.

Schilling, J. A., W. Joel, and H. M. Shurley. 1959. Wound healing: a comparative study of the histochemical changes in granulation tissue contained in stainless steel wire mesh and polyvinyl sponge cylinders. *Surgery* 46:702-10.

Schirmer, O. 1903. Studien zur Physiologie und Pathologie der Traenenabsonderung und Traenenabfuhr. *Graefes Arch Klin Exp Ophthalmol* 56 (2):197-291.

Schmidtchen, A. 2000. Degradation of antiproteinases, complement and fibronectin in chronic leg ulcers. *Acta Derm Venereol* 80 (3):179-84.

Schroder, K., and J. Tschopp. 2010. The inflammasomes. *Cell* 140 (6):821-32.

Seah, C. C., T. J. Phillips, C. E. Howard, I. P. Panova, C. M. Hayes, A. S. Asandra, and H. Y. Park. 2005. Chronic wound fluid suppresses proliferation of dermal fibroblasts through a Ras-mediated signaling pathway. *J Invest Dermatol* 124 (2):466-74.

Simonsen, L., P. Holstein, K. Larsen, and J. Bulow. 1998. Glucose metabolism in chronic diabetic foot ulcers measured in vivo using microdialysis. *Clin Physiol* 18 (4):355-9.

Stenken, J. A., M. K. Church, C. A. Gill, and G. F. Clough. 2010. How minimally invasive is microdialysis sampling? A cautionary note for cytokine collection in human skin and other clinical studies. *AAPS J* 12 (1):73-8.

Stoll, D. 2004. Microarray technology: an increasing variety of screening tools for proteomic research. *Drug Discovery Today: TARGETS* 3 (1):24-31.

Swain, A., J. Turton, C. L. Scudamore, I. Pereira, N. Viswanathan, R. Smyth, M. Munday, F. McClure, M. Gandhi, S. Sondh, and M. York. 2011. Urinary biomarkers in hexachloro-1:3-butadiene-induced acute kidney injury in the female Hanover Wistar rat; correlation of alpha-glutathione S-transferase, albumin and kidney injury molecule-1 with histopathology and gene expression. *J Appl Toxicol* 31 (4):366-77.

Tarnuzzer, R. W., and G. S. Schultz. 1996. Biochemical analysis of acute and chronic wound environments. *Wound Repair Regen* 4 (3):321-5.

Tecilazich, F., T. Dinh, and A. Veves. 2011. Treating diabetic ulcers. *Expert Opin Pharmacother* 12 (4):593-606.

Templin, M. F., D. Stoll, J. Bachmann, and T. O. Joos. 2004. Protein microarrays and multiplexed sandwich immunoassays: what beats the beads? *Comb Chem High Throughput Screen* 7 (3):223-9.

Tilg, H., E. Trehu, M. B. Atkins, C. A. Dinarello, and J. W. Mier. 1994. Interleukin-6 (IL-6) as an anti-inflammatory cytokine: induction of circulating IL-1 receptor antagonist and soluble tumor necrosis factor receptor p55. *Blood* 83 (1):113-8.

Trengove, N. J., H. Bielefeldt-Ohmann, and M. C. Stacey. 2000. Mitogenic activity and cytokine levels in non-healing and healing chronic leg ulcers. *Wound Repair Regen* 8 (1):13-25.

Trengove, N. J., S. R. Langton, and M. C. Stacey. 1996. Biochemical analysis of wound fluid from nonhealing and healing chronic leg ulcers. *Wound Repair Regen* 4 (2):234-9.

Trengove, N. J., M. C. Stacey, S. MacAuley, N. Bennett, J. Gibson, F. Burslem, G. Murphy, and G. Schultz. 1999. Analysis of the acute and chronic wound environments: the role of proteases and their inhibitors. *Wound Repair Regen* 7 (6):442-52.

Tuschil, A., C. Lam, A. Haslberger, and I. Lindley. 1992. Interleukin-8 stimulates calcium transients and promotes epidermal cell proliferation. *J Invest Dermatol* 99 (3):294-8.

Uzun, G., E. Solmazgul, H. Curuksulu, V. Turhan, N. Ardic, C. Top, S. Yildiz, and M. Cimsit. 2007. Procalcitonin as a diagnostic aid in diabetic foot infections. *Tohoku J Exp Med* 213 (4):305-12.

Velnar, T., T. Bailey, and V. Smrkolj. 2009. The wound healing process: an overview of the cellular and molecular mechanisms. *J Int Med Res* 37 (5):1528-42.

Wagner, S., S. Coerper, J. Fricke, T. K. Hunt, Z. Hussain, M. W. Elmlinger, J. E. Mueller, and H. D. Becker. 2003. Comparison of inflammatory and systemic sources of growth factors in acute and chronic human wounds. *Wound Repair Regen* 11 (4):253-60.

Wall, I. B., R. Moseley, D. M. Baird, D. Kipling, P. Giles, I. Laffafian, P. E. Price, D. W. Thomas, and P. Stephens. 2008. Fibroblast dysfunction is a key factor in the non-healing of chronic venous leg ulcers. *J Invest Dermatol* 128 (10):2526-40.

Wallace, H. J., and M. C. Stacey. 1998. Levels of tumor necrosis factor-alpha (TNF-alpha) and soluble TNF receptors in chronic venous leg ulcers--correlations to healing status. *J Invest Dermatol* 110 (3):292-6.

Weckroth, M., A. Vaheri, J. Lauharanta, T. Sorsa, and Y. T. Konttinen. 1996. Matrix metalloproteinases, gelatinase and collagenase, in chronic leg ulcers. *J Invest Dermatol* 106 (5):1119-24.

Weigelt, C., B. Rose, U. Poschen, D. Ziegler, G. Friese, K. Kempf, W. Koenig, S. Martin, and C. Herder. 2009. Immune mediators in patients with acute diabetic foot syndrome. *Diabetes Care* 32 (8):1491-6.

Widgerow, A. D. 2011. Chronic wound fluid--thinking outside the box. *Wound Repair Regen* 19 (3):287-91.

Williams, D. T., J. R. Hilton, and K. G. Harding. 2004. Diagnosing foot infection in diabetes. *Clin Infect Dis* 39 Suppl 2:S83-6.

Wound infection in clinical practice. An international consensus. 2008. *Int Wound J* 5 Suppl 3:iii-11.

Wysocki, A. B., and F. Grinnell. 1990. Fibronectin profiles in normal and chronic wound fluid. *Lab Invest* 63 (6):825-31.

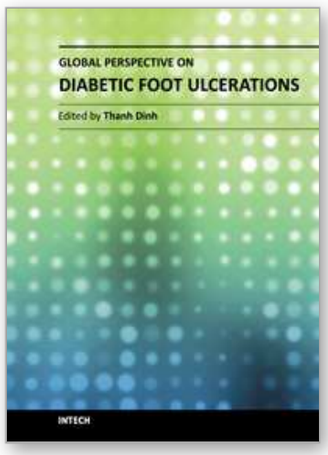
Wysocki, A. B., A. O. Kusakabe, S. Chang, and T. L. Tuan. 1999. Temporal expression of urokinase plasminogen activator, plasminogen activator inhibitor and gelatinase-B

in chronic wound fluid switches from a chronic to acute wound profile with progression to healing. *Wound Repair Regen* 7 (3):154-65.

Xu, L., S. V. McLennan, L. Lo, A. Natfaji, T. Bolton, Y. Liu, S. M. Twigg, and D. K. Yue. 2007. Bacterial load predicts healing rate in neuropathic diabetic foot ulcers. *Diabetes Care* 30 (2):378-80.

Yager, D. R., R. A. Kulina, and L. A. Gilman. 2007. Wound fluids: a window into the wound environment? *Int J Low Extrem Wounds* 6 (4):262-72.

Yager, D. R., L. Y. Zhang, H. X. Liang, R. F. Diegelmann, and I. K. Cohen. 1996. Wound fluids from human pressure ulcers contain elevated matrix metalloproteinase levels and activity compared to surgical wound fluids. *J Invest Dermatol* 107 (5):743-8.



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Over the last decade, it is becoming increasingly clear that diabetes mellitus is a global epidemic. The influence of diabetes is most readily apparent in its manifestation in foot complications across cultures and continents. In this unique collaboration of global specialists, we examine the explosion of foot disease in locations that must quickly grapple with both mobilizing medical expertise and shaping public policy to best prevent and treat these serious complications. In other areas of the world where diabetic foot complications have unfortunately been all too common, diagnostic testing and advanced treatments have been developed in response. The bulk of this book is devoted to examining the newest developments in basic and clinical research on the diabetic foot. It is hoped that as our understanding of the pathophysiologic process expands, the devastating impact of diabetic foot complications can be minimized on a global scale.

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InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

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