DIAGNOSTIC MARKERS OF INFECTION, IN CHRONIC WOUNDS

BY

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ABSTRACT

A complication associated with wound healing is wound infection. The diagnosis of infection in chronic wounds can be a difficult clinical decision. Signs and symptoms used to diagnose infection can often be masked by factors relating to the host, chronic inflammation and the tissue damage, associated with chronic wounds.
This Study aimed to determine whether there is a measurable biochemical host response that could serve as potential biomarkers of chronic wound infection, providing an alternative diagnostic tool to aid the Clinician. An *in vitro* model further investigated the expression of markers from neutrophils, in response to bacterial supernatant from *Pseudomonas aeruginosa* and *Staphylococcus aureus* commonly associated with chronic wounds and their infections.

Chronic venous leg ulcer and diabetic foot ulcer wound fluids were collected, wound microflora assessed, and a variety of host factors, including serine proteases, matrix metalloproteinase, their inhibitors and cytokines/growth factors, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, Il-12 p40, IL-12p70 and IL-13, angiogenin, IFN-γ, TGF-β_1, VEGF, TNF-α, TNF-r2, ICAM-1 and IP-10, analysed. No significant differences ($p>0.1$) in the activities/levels of host factors were observed between non-infected and infected wounds, based on clinical diagnosis. Significant differences ($p<0.05$) in a variety of these host factors were observed in these ulcer types, upon defining infection by wound microbial bioburden, the number of genera or bacterial species. Of these factors, cytokines were found to distinguish on two of the three defining parameters. Specifically, a number of cytokines were found to be significantly elevated in venous leg ulcer wounds, IL-1β, TNF-α, TNFr2 and ICAM-1, with an additional set of cytokines, IL-2, IL-5, IL-12p40, IL-12p70, IFN-γ and TGF-β_1, significantly decreased within diabetic foot ulcer wounds.

I*n vitro*, neutrophils were treated with *Pseudomonas aeruginosa* or *Staphylococcus aureus* 'acellular' supernatants, at mid-log or stationary growth phases, for 4 and 24h. Significant increases ($p<0.05$) in the levels of proteases and cytokines were observed from neutrophils, treated with both mid-log and stationary phase supernatants, from both species. These increases were both neutrophil donor- and growth phase-dependent. A larger cytokine response was induced from neutrophils stimulated with stationary phase, compared with mid-log phase supernatants. Combined with the increased expression of virulence factors, including bacterial enzymes, this Study suggests that bacterial growth is an important feature of chronic wound infection.

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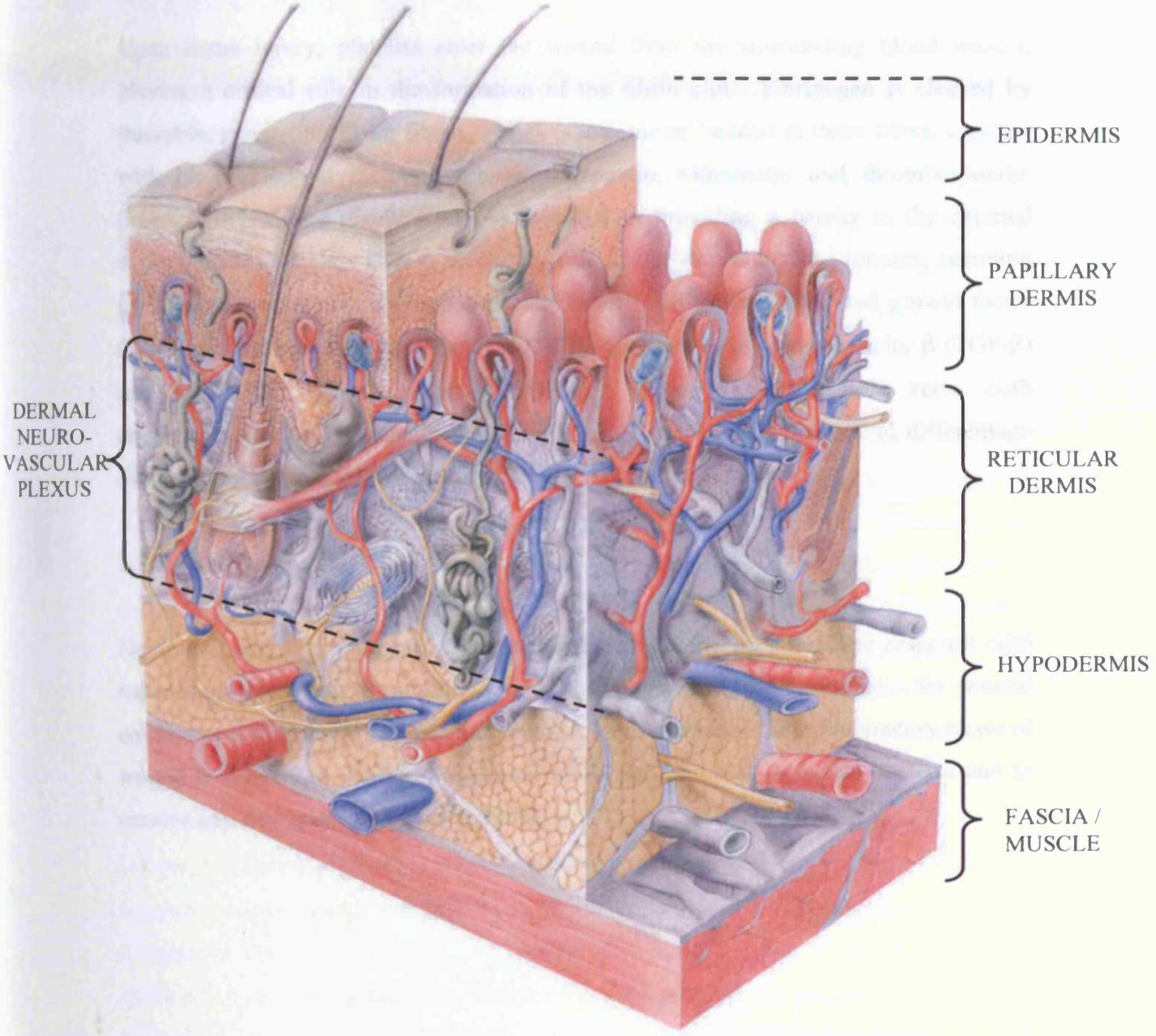
Introduction

1.1 Wound healing

The skin is an important functioning organ that maintains life through the regulation of water, electrolytes and temperature, and also provides a barrier to external substances. Thus the wound healing process is initiated upon tissue injury, in order to restore its functional integrity. The skin consists of three main components; the epidermis, the dermis and the skin appendages (Figure 1.1). Many cell types contribute to both structural and functional attributes of skin, including fibroblasts, monocytes/macrophages, neutrophils, endothelial cells and epidermal cells (Singer and Clark, 1999, Galkowska et al., 2005).

Upon tissue injury, the combined cellular responses within the local environment contribute to the wound healing process, which comprises of functionally distinct, although overlapping phases, encompassing haemostasis, inflammation, granulation tissue formation, extracellular matrix (ECM) formation, re-epithelialisation, remodelling and scar tissue formation (Moore, 1999). In the majority of cases, the wound healing process is rapid and occurs uneventfully in a sequential, timely fashion, with intervention unnecessary (Robson, 1997). However, with the onset of aging or the complications of underlying pathologies, this healing process can be delayed giving rise to chronic or non-healing ulcers. A chronic wound is defined as one which fails to heal in a timely and orderly manner, compromising overall anatomical and functional integrity (Stadelmann et al., 1998a). It is therefore important that we understand the normal wound healing process if we are to correct or promote healing in these 'chronic' conditions.

Figure 1.1: The structure of normal skin. Adapted from Wikipedia.org.



1.1.1 Haemostasis

Upon tissue injury, platelets enter the wound from the surrounding blood vessels, playing a critical role in the formation of the fibrin clot. Fibrinogen is cleaved by thrombin, generating fibrin fibres. Platelets become embedded in these fibres, together with plasma-derived factors, such as fibronectin, vitronectin and thrombospondin (Martin, 1997). The resulting clot is essential in providing a barrier to the external environment. Platelets have a secondary role in the wound healing process, secreting growth factors, such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), transforming growth factor α (TGF-α), transforming growth factor β (TGF-β) and insulin-like growth factor-1 (IGF-1). These growth factors serve both chemoattractant and mitogenic functions in order to recruit, activate and differentiate different cell types at the wound site (Blitstein-Willinger, 1991).

1.1.2 Inflammation

Neutrophil and macrophage infiltration characterises the inflammatory response with subsequent secretion of additional growth factors and proteases into the wound environment (Tarnuzzer and Shultz, 1996). A key function of the inflammatory phase of wound healing is to debride the wound of foreign material including bacteria and to remove any damaged tissue (Levy, 1996).

1.1.2.1 Inflammation and the neutrophil

The neutrophil is a small cell (9-10μm), being the most abundant leukocyte in the blood, with between 3-7 x 10^6 cells/ml (George-Gay and Parker, 2003). Originally thought to be a terminally differentiated cell, evidence now suggests the cell is capable of synthesising and secreting a number of cytokines, including platelet-activating factor (PAF), interleukins-1 (IL-1) and IL-8, tumour necrosis factor α (TNF-α), granulocyte-macrophage colony stimulating factor (GM-CSF) and TGF-α and TGF-β (Cassatella, 1995, Euler et al., 1998). The neutrophil (Figure 1.2) has a segmented nucleus and a cytoplasm that contains numerous storage granules (Miyasaki, 1991, Li et al., 2002, Mayer-Scholl et al., 2004).

Neutrophils are usually the first cell type to populate the wound site (Stadelmann et al., 1998b), due to their high abundance in the circulation (Clark, 1998), characterising the early inflammatory response. Neutrophils secrete pro-inflammatory cytokines, de-granulate releasing proteases, and engulf and destroy bacteria through the generation of reactive oxygen species (ROS) (Chapter 1, Section 1.5.3). Neutrophilic proteases, elastase and collagenases, remove damaged and denatured ECM components. Early bursts of proteolytic activity debrides the wound of damaged tissue and cellular proteins (Mast and Shultz, 1996).

The extent of neutrophil infiltration into the wound depends on the number of cells activated, which controls their ability to adhere to the endothelium (via both selectin and integrin receptors), and complete the process of diapedesis and migration (Figure 1.3) (Nussler et al., 1999). The β2 sub-family of integrins, appear to control acute and chronic inflammatory responses. These particular integrins are unable to interact with ligands on un-stimulated neutrophils, thus providing a safety mechanism for the inflammatory response (Witko-Sarsat et al., 2000).

Figure 1.2: (a) Structure of inflammatory cells, a neutrophil and a monocyte (b) Inflammatory cells, a monocyte and two neutrophils in a blood film. Adapted from Miyasaki, 2006.

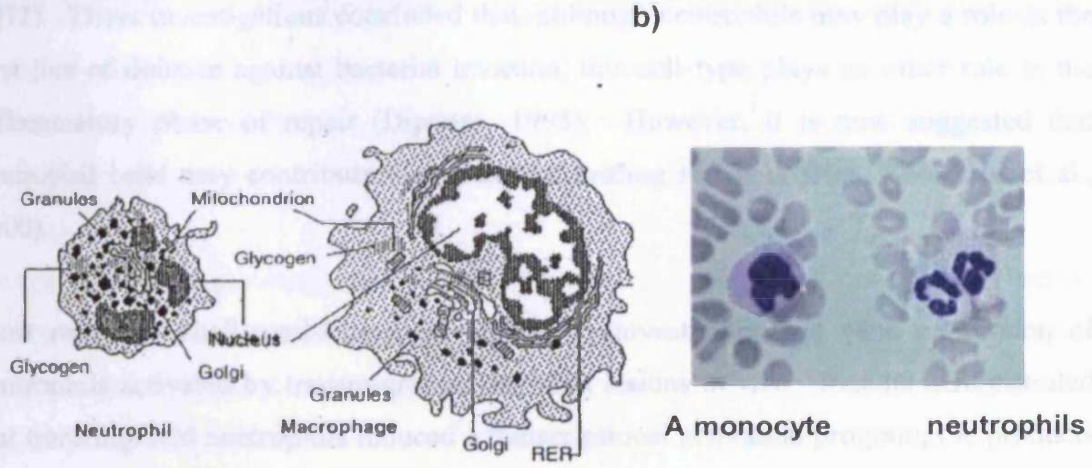
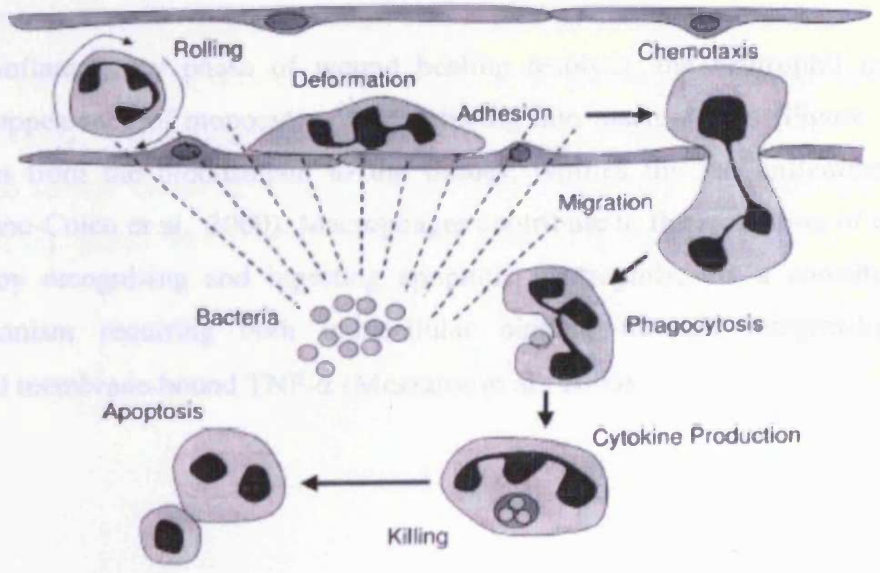


Figure 1.3: Inflammatory cell migration into wound sites (Van Eeden et al., 1999). The broken lines represent chemoattractants generated at the site of infection. Neutrophils flatten and adhere to the endothelium, migrate out of the vessel towards the infectious site, phagocytose and kill the bacteria. Additionally they secrete cytokines to recruit other inflammatory cells and eventually undergo programmed cell death (apoptosis).



The importance of the neutrophil cell in wound healing is a matter of debate. *In vivo* studies have demonstrated no difference in wound debridement, cellularity or connective tissue formation in the wounds of control and neutropenic animals (Simpson and Ross, 1972). These investigations concluded that, although neutrophils may play a role as the first line of defence against bacterial invasion, this cell type plays no other role in the inflammatory phase of repair (Dipietro, 1995). However, it is now suggested that neutrophil cells may contribute to the wound healing repair process (Meszaros et al., 2000).

Most recently, Theilgaard-Monch et al. (2004) investigated the gene expression of neutrophils activated by transmigration into skin lesions *in vivo*. Results demonstrated that transmigrated neutrophils induced a transcriptional activation program, the products of which regulate healing responses. Up-regulation of neutrophil cytokines in the promotion of angiogenesis, the proliferation of keratinocytes and fibroblasts are observed. Additional up-regulation occurs with genes involved in adhesion and migration of keratinocytes, fibroblasts and endothelial cells, and genes associated with tissue remodelling. The importance of neutrophil cells in host defence is not questioned, as highlighted in conditions, such as neutropenia and severe neutrophil dysfunction. The manifestations of such conditions include severe, recurrent and often life threatening bacterial and fungal infections (Steinbeck and Roth, 1989).

As the early inflammatory phase of wound healing resolves, the neutrophil influx subsides. The appearance of monocytes, differentiating into macrophages (Figure 1.2), upon migration from the bloodstream to the tissues, typifies the late inflammatory response (Staiano-Coico et al., 2000). Macrophages contribute to the resolution of early inflammation by recognising and ingesting apoptotic neutrophils, via a constitutive effector mechanism requiring both intracellular binding through integrin-ligand interactions and membrane-bound TNF-α (Meszaros et al., 2000).

1.1.2.2 Inflammation and the macrophage

The macrophage is larger than the neutrophil, has a segmented nucleus and a cytoplasm that contains granules (Figure 1.2). Macrophages can be divided into normal and inflammatory macrophages. Normal macrophages reside in tissues, such as the connective tissue, liver, lung, spleen, bone marrow, serous fluids, skin and other tissues. Normal skin contains resident macrophages at low density of approximately 1-2 per mm^2. During wound repair this density increases more than five fold (DiPietro, 1995).

Emigration of inflammatory cells initially depends on the activation of the endothelium within the wound and subsequent expression of adhesion molecules on the endothelial surface. These adhesion molecules are co-ligands for receptors on both neutrophils and mononuclear cells. Sustained infiltration of monocytes is believed to be due to a local chemo-attractant gradient in their favour, hence, inducing monocyte transformation into macrophages within the tissues (Engelhardt et al., 1998, Gillitzer and Goebeler, 2001).

Within the wound environment, neutrophils and macrophages can be activated by numerous stimuli;

- Release of factors from platelets, TGF-β and PAF (DiPietro, 1995).
- Phagocytosis of cellular debris, including ECM components, such as fibronectin and collagen (Witte and Barbul, 1997).
- Cell-derived factors, including interferon-γ (IFN-γ) and IL-4 (Kodelja et al., 1997), TNF-α, TGF-β, chemokines (e.g. MCP-1), arachadonic acid metabolites (DiPietro, 1995), and complement (Dennison and Van Dyke, 2000).
- Bacterial factors (Hauschildt and Kleine, 1995), including the Gram-negative bacterial cell component, lipopolysaccharide (LPS) (Fujihara et al., 2003).

- Local wound microenvironment conditions, such as hypoxia, lactate concentration, and pH, also influence the activation status of inflammatory cells (DiPietro, 1995).
- Specifically, macrophage stimulating protein (MSP) within wound exudates, stimulate macrophage cells via the recepteur d'origine nantais (RON) receptor. This RON receptor is upregulated upon tissue injury, as it is infrequently detected on normal skin (Nanney et al., 1998).

Macrophages serve an important role in the host defence against pathogens, but also have other functions including a role in wound healing processes. Within the wound, macrophages have a largely scavenging role, clearing cellular debris and senescent cells. However, when present in the wound they release numerous factors, such as growth factors, that allow endothelial cells to migrate and proliferate, thereby stimulating both angiogenesis and fibroblasts to synthesise collagen and fibronectin (DiPietro, 1995). On cessation of the inflammatory phase, macrophages are de-activated, by both host-derived molecules, such as TGF-β_1 and TGF-β_2 and microbial-derived molecules (Tsunawaki et al., 1988). Macrophage activity is limited, as the prolonged presence of macrophage-derived inflammatory mediators has proven to be detrimental to the endothelium, fibroblasts, smooth muscle and parenchymal cells (Tsunawaki et al., 1988).

1.1.3 Extracellular matrix and granulation tissue formation

A provisional ECM is created during the inflammatory phase of healing, consisting mainly of fibrin, fibronectin and glycosaminoglycans; which allows cells to migrate into the wound site (Mast and Shultz, 1996). Fibroblasts can then stimulate the production of collagen and overall ECM deposition. The collagen and fibronectin serve as an intermediatory matrix, restoring structure and function needed for repair, thus leading to re-establishing dermal integrity (Brem et al., 2001).

The matrix provides optimal conditions for cell growth and angiogenesis and is generally referred to as granulation tissue; composing of numerous capillaries and a matrix rich in fibroblasts, endothelial cells, pericytes and myofibroblasts. This initial highly vascular tissue is gradually replaced by a relatively avascular and acellular collagen matrix (Stadelmann et al., 1998b), as healing progresses and the wound matures.

1.1.4 Re-epithelialisation, remodelling and scar formation

The newly synthesised collagen matrix provides a medium/structure across which keratinocytes can migrate, as the process of re-epithelialisation is initiated. Keratinocytes produce a variety of matrix metalloproteinases (MMPs) and plasmin that allows the cells to detach from the basement membrane, facilitating migration (Parks, 1999). Cells migrate across the wound space, from either the leading edge of the wound, or alternatively from skin structures, for examples, hair follicles, that can contain an additional pool of keratinocytes (Martin, 1997, O'Toole, 2001). As these cells migrate, they continuously express proteases, and then re-attach to the basement membrane through collagen/fibronectin/vitronectin and keratinocyte integrin receptor interactions (Martin, 1997).

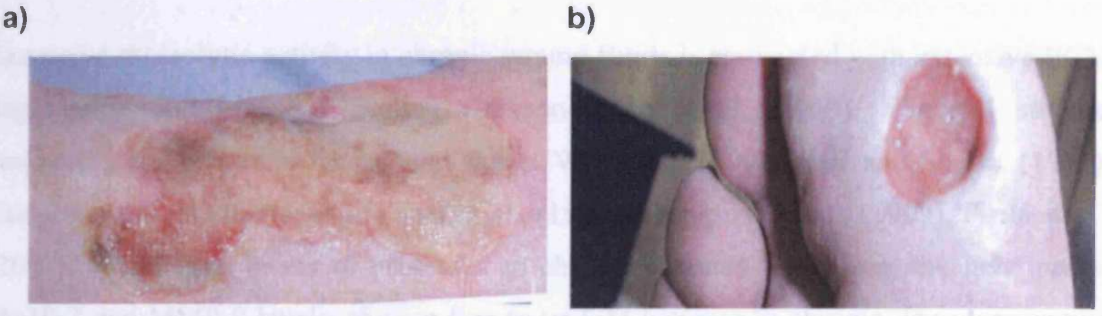
The mechanisms by which keratinocytes migrate and cover the wound space are still being elucidated. It is believed that keratinocytes can migrate over one another, the 'leap-frog' theory (Garrett, 1998) or alternatively that the cells act as a coherent sheet, migrating over the wound bed directly, termed the 'sliding' mechanism (Laplante et al., 2001) or 'Tractor tread' theory (Patel et al., 2006). Evidence suggests that the ability of the keratinocyte to migrate occurs through independent mechanisms to the cells ability to proliferate (Woodley et al., 1993). Keratinocytes undergoing migration activities limit their proliferative capacity, though conversely, keratinocytes farthest from the wound edge preferentially proliferate (Patel et al., 2006). Gradually, the granulation tissue is replaced with new dermis by remodelling and scar formation occurs. The process to re-epithelialisation typically takes up to 14 days, during which time the integrity of the skin is restored quickly to avoid wound infection, sepsis and other complications associated with open wounds. The process of remodelling and scar formation can last for up to a year or more (Staiano-Coico et al., 2000), involving controlled proteolytic ECM degradation and re-structuring.

1.2 The abnormal wound healing process, chronic wound healing

Aberrations in the wound healing process can result in pathologic healing. Examples of these include exuberant fibrotic distortions of appearance/function (e.g. keloids scars, scar contractures) or insufficient replacement of tissue, leading to a chronic non-healing wound (Mast and Shultz, 1996). Diabetic foot ulcers, venous stasis ulcers and pressure ulcers are all chronic wounds from the moment they occur. In this sense, these are time-dependent and intervention is always necessary to assure prevention of their further progression.

Venous leg ulcers, most commonly caused by long-standing venous hypertension (Palolahti et al., 1993); diabetic foot ulcers characterised by vascular and neurological abnormalities, with neuropathy playing a major role (Falanga, 2004) (Figure 1.4); and pressure sores, resulting from pressure damage, ischaemia and characterised by deep tissue necrosis (Heggars, 1998); are all examples of chronic wound healing.

Figure 1.4: Types of chronic wound. Photographs of (a) venous leg ulcer, and (b) diabetic foot ulcer wounds, taken as part of the present Study.



Many investigators have reported differences between non-healing and healing environments. Those observed include the following paramaters; lower protein and albumin levels (James et al., 2000), lower pO_2, lower pH and high lactate levels (Moore, 1999), total antioxidant capacity (Moseley et al., 2004a), defective keratinocyte migration, aberrant keratinocyte integrin expression (Medina et al., 2005), ECM defects (Loots et al., 1998), cytokine/growth factor defects (Trengrove et al., 2000), inappropriate lymphocyte, macrophage and fibroblast populations and functions (DiPietro, 1995, Hasan et al., 1997, Moore et al., 1997, Cook et al., 2000), inappropriate endothelial cell adhesion molecules (Topham et al., 1998, Galkowska et al., 2005) and differentially expressed (Pirila et al., 2007) and higher levels of proteases (Yager et al., 1996, Wysocki et al., 1993). The relative importance of each and their contribution to the non-healing state remain to be determined. The main biochemical, cellular and micro-vascular abnormalities associated with chronic wounds are discussed below.

1.2.1 Biochemical abnormalities

In order to understand why a chronic wound fails to heal, several investigators have examined the biochemistry of chronic wound pathology. Identification of certain components believed to contribute to the non-healing state of the chronic wound environment, are discussed. Common characteristics of chronic wounds include elevated pro-inflammatory cytokines, high protease levels and activity, and diminished growth factor activity (Mast and Shultz, 1996, Agren et al., 2000).

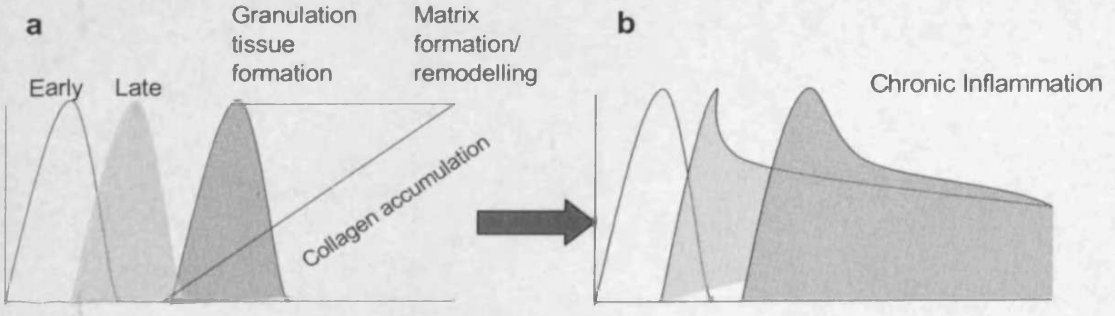
Excessive proteolytic activity in chronic wound fluids is associated with extensive ECM degradation and impaired healing (Staiano-Coico et al., 2000). Several studies, comparing acute and chronic wound fluids, Wysocki, (1999), Mast and Shultz, (1996), Tarnuzzer and Shultz, (1996), Yager et al., (1996), Trengrove et al., (1999), Pirila et al., (2007), report high levels of proteases in chronic wounds. Proteases, the gelatinases, MMP-2 and MMP-9 levels, show a five to tenfold increase in chronic, leg ulcer wound fluids compared to levels in acute, mastectomy fluids (Wysocki et al., 1999). Proteases and their inhibitors are discussed further in Chapter 1, Section 1.6. Reduced levels of tissue inhibitor of metalloproteinase (TIMP) and serine proteases, including α2-macroglobulin and α1-antitrypsin, or a diminished ability of these protease inhibitors to function, have also been observed in chronic wounds (Trengrove et al., 1999, Schmidtchen, 2000).

Consequences of high proteolytic activity within the chronic wound healing environment, include the degradation of wound healing factors, such as growth factors, necessary to stimulate the wound healing process. Degradation of EGF, PDGF and TGF-β_1 by chronic wound fluid has been demonstrated previously (Mast and Shultz, 1996, Yager et al., 1997, Trengrove et al., 1999). Vascular Endothelial Growth Factor (VEGF), whose expression is increased in chronic venous leg ulcers, is also extensively degraded in wound fluid (Lauer et al., 2000). This implies that it is not an inability of the tissue to produce positive wound healing factors, but the hostile chronic wound

environment that delays healing. Increased levels of elastase and the associated degradation of fibronectin and vitronectin, may contribute to the lack of adhesion molecules and cell attachment sites necessary for normal wound closure (Wysocki, 1996, Herrick et al., 1997).

Acute mastectomy wounds have a rapid burst of pro-inflammatory cytokines that progressively decreases, in contrast to chronic wounds, which appear to have an imbalance of pro-inflammatory cytokine (Tarnuzzer and Shultz, 1996). TNF-α, IL-1 and IL-6 have all been demonstrated to be higher in chronic wound fluids than in acute wound fluids (Harris et al., 1995, Trengrove et al., 2000). Decreased levels of TGF-β have also been reported in chronic, compared to acute wounds (Cooper et al., 1994). This imbalance in cytokines is believed to be the cause of chronic inflammation, which itself is perceived as a pre-requisite to wound chronicity (Figure 1.5).

Figure 1.5: Inflammation and wound chronicity (Moore, 1999). (a) The inflammatory response in the healing wound is initiated early and resolves as granulation tissue forms and ECM formation proceeds. (b) A chronic wound is characterised by a non-resolving chronic inflammatory response that persists in the presence of fibroblast proliferation and formation of an aberrant ECM.



Upon comparing cytokine/growth factor levels and the mitogenic ability of cells in healing and non-healing venous leg ulcer wounds, Trengrove et al. (2000) concluded that impaired healing of these ulcers could be due to the increased inflammatory mediators. Soo et al. (2000) suggested that chronic wounds do not progress from the inflammatory stage of healing to granulation tissue formation, as a consequence of excessive proteolytic activity. While Tarnuzzer and Schultz (1996) suggest that repeated trauma, ischaemia and infection are the leading causes of wound chronicity. Longer exposure to high levels of TNF-α may also reduce the tensile strength of wounds (Mast and Shultz, 1996).

1.2.2 Cellular abnormalities

The inflammatory infiltrate within chronic wound tissue is composed primarily of blood-derived lymphocytes and macrophages, with additional neutrophils in infected tissue (Moore, 1999). Literature suggests that the level of cellular infiltrate and cellular activation differs in chronic diabetic and venous leg ulcers from that of acute wounds (Loots et al., 1998).

The fibroblast cell of a diabetic, produce more gelatinase (MMP-2) and stromelysin (MMP-3), *in vitro*, than their non-diabetic counterparts (Wall et al., 2003). As such, this ability of the fibroblast may contribute to the increased proteolytic nature observed in chronic wounds. In addition, there appears to be decreased inflammatory leukocytes in diabetic foot ulcer tissue, despite the fact that the expression of endothelial adhesion molecules is increased (Galkowska et al., 2005).

Conversely, there is evidence to suggest that within venous leg ulcers, inflammatory cells, such as leukocytes, become trapped and activated within capillaries, contributing to the pathogenesis of this ulcer type (Li et al., 1998). These trapped cells may cause the blockage of blood vessels and/or tissue damage, due to the secretion of inflammatory cytokines or proteases.

A potential mis-regulation of the cytokine secretion profiles of venous leg ulcer leukocytes, specifically T-lymphocytes, have been proposed by Moore et al. (1997). T-lymphocytes are a key component to the immune system, essential for most antibody and cell-mediated responses (Playfair and Chain, 2001, Section 1.5). It is believed that the balance of T-cells in venous leg ulcers, in favour of the T-suppressor cells, down-regulates the immune response, and the subsequent cytokine secretion does not favour wound healing processes. Additionally, Li et al, (1998), concluded that the strong expression of IL-10 in venous leg ulcer tissue indicates suppression of the cell-mediated immune response, which would normally be present in acute wound healing. Additionally, a lack of activated macrophages (negative for the activation-associated markers CD16 (Fc gamma III receptor) and CD35 (C3b receptor), in the majority (60%) of venous leg ulcers (Moore et al., 1997), may decrease bacteria/foreign matter removal from the wound.

The concept of cellular senescence in chronic wound (diabetic and pressure ulcer) fibroblasts has also been postulated. Decreased proliferative capacity of fibroblasts exposed to chronic wound fluid has been observed (Mendez et al., 1998, 1999, Vande Berg et al., 1998). Epithelial cells and fibroblasts at the wound periphery also appear to show signs of senescence, with a failure of these cells to respond to growth factor signals, such as PDGF or TGF-β (Hasan et al., 1997). Subsequent findings of Stephens et al. (2003), however, suggest that the fibroblast phenotype in chronic wounds is due to the disease-specific cellular alterations of the cell itself, rather than a consequence of age-related senescence.

1.2.3 Micro-vascular abnormalities

The micro-vasculture within wounds ensures adequate perfusion of tissue with oxygen. Pressure sores demonstrate poor tissue perfusion, as a consequence of collapsed and weakened blood vessels. The resulting tissue ischemia leads to tissue breakdown, bacterial contamination and inflammation. The underlying pathogenic mechanism associated with venous leg ulcers is believed to be venous hypertension, whereby venous pressure is maintained between the deep and superficial circulatory system (Mast and Shultz, 1996).

Venous hypertension can result from the following; the presence of dysfunctional valves, venous outflow obstruction, calf muscle dysfunction and calf muscle failure (Stvrtinova et al., 2001, Valencia et al., 2001). Many theories exist surrounding the micro-vascular alterations resulting from venous hypertension, including the presence of fibrin cuffs, red blood cell aggregation, leukocyte 'plugging' of the capilleries with the release of toxic metabolites and proteolytic enzymes, and the increased presence of molecules able to sequester growth factors. The effects of these may cause further vascular permeability, with extravasation of molecules from the blood to the tissue. The availability of oxygen may be compromised due to the presence of the fibrin cuff or leukocyte 'plug', resulting in tissue ischemia, a potential contributing factor to the formation of a venous ulcer (Fernandes Abbade and Lastoria, 2005).

Microvasular disease associated with the diabetic foot results from a decreased nutrient and oxygen transport across the thickened basement membrane. Diabetic feet are also characterised by macro-vascular abnormalities resulting from atheroschlerosis, leading to absence of pulses, delay in capillary refill and muscle atrophy (Medina et al., 2005). Additionally, diabetic foot ulcers have deficits in angiogenic mediators including PDGF-BB, TGF-β, and angiopoietin-1, with glucose itself anti-proliferative for endothelial cells.

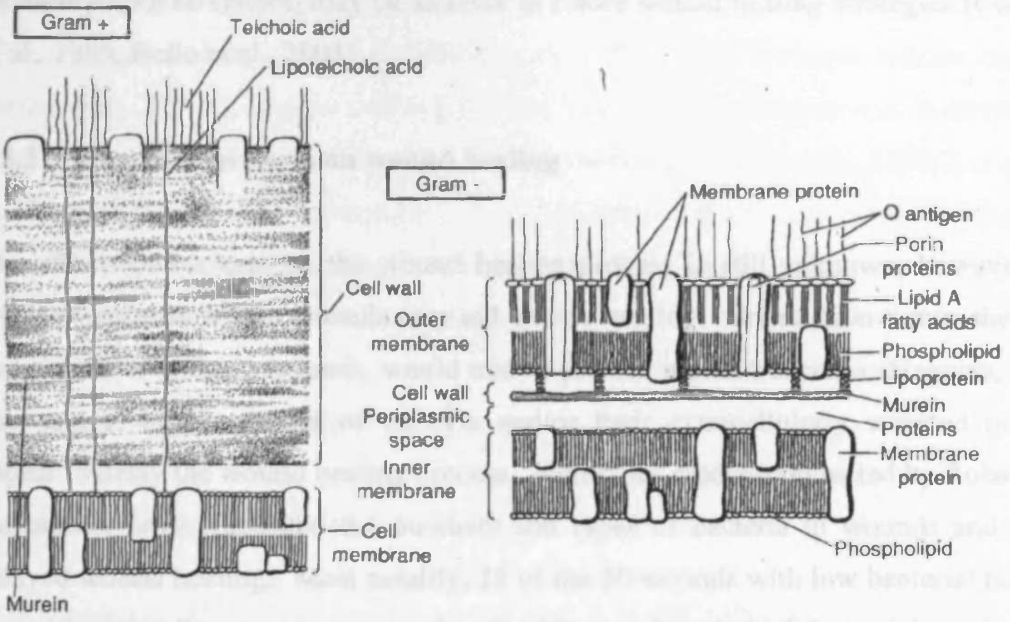
1.3 Microorganisms and wounds

The microbiology of acute and chronic wounds has been comprehensively reviewed by Robson (1997) and Bowler (1998), Bowler and Davies (1999), Bowler et al. (2001), Bowler (2003). Typically, microorganisms isolated and cultured from wounds are bacteria and occasionally fungi. It is generally accepted that wounds are polymicrobial in nature, sometimes containing both aerobic and anaerobic organisms. Wounds contain both non-pathogenic (commensal) and pathogenic (capable of damaging the host and causing disease) bacteria. Synergy may exist between bacterial species and whether the number or type of organism is important in the development of a wound infection or the impact of these microorganisms on wound healing processes, is still debated.

1.3.1 Bacteria and biofilms

Bacteria are single-celled organisms, typically 0.3-1.4μm in size, which are usually classified according to structure (Gram-negative or Gram-positive), shape (cocci, spiral or bacilli), their requirement for oxygen (aerobic or anaerobic), the presence and shape of endospore and its position in the cell, and their requirement for special growth media (Gillepsie and Bamford, 2000). Bacteria are grouped into a genus, e.g, *Staphylococcus*, and given a species name, that further identifies certain characteristics of the organism, e.g. *Staphylococcus aureus (S. aureus)*. The difference in the bacterial envelope structure differentiates between Gram-negative and Gram-positive, allowing Gram-positive bacteria to retain the crystal violet dye (Lee and Schneewind, 2001) (Figure 1.6).

Figure 1.6: The envelope structure of a Gram-negative and Gram-positive bacterial cell (Schaechter et al., 1993).
Capsules and appendages are not shown, nor are surface proteins like the M protein of Streptococci indicated. There is approximately a 20-fold greater amount of peptidoglycan (murein) constituting the Gram-positive bacterial cell wall than in the Gram-negative bacteria.



An emerging concept surrounding the presence of bacteria in acute and chronic wound healing is the presence of biofilms (Harrison-Balestra et al., 2003). These are polymicrobial colonies of bacteria encased in an extracellular polysaccharide matrix (EPS) or glycocalyx (Bello et al., 2001, Mertz, 2003, Percival and Bowler, 2004). Given the correct conditions, all bacteria can grow as a biofilm (Percival and Bowler, 2004). However, bacterial structure, function and susceptibility are typically studied *in vitro* in a liquid medium, where bacteria exist in a planktonic (free-floating) form.

Evidence of biofilms within acute wounds has been demonstrated in animal models with some evidence within the chronic wound environment (Bello et al., 2001). These observations include staining of the EPS from a chronic wound smear and bacteria isolated and grown from a chronic wound, can form a biofilm. Their presence in wounds, is gaining acceptance as the amount of indirect evidence increases.

Osteomyelitis (infection of the bone), as seen in diabetic foot ulcers, is characterised by a bacterial and fungal biofilm (Percival and Bowler, 2004). The impact of biofilms on wound healing processes and wound infection, however, remains to be determined. If biofilms are a causative factor of delayed wound healing, their resistance to antimicrobial penetration, by antimicrobial agents, antibiotics or mediators of the host immune response/system, may be an issue in future wound healing strategies (Costerton et al., 1999, Bello et al., 2001).

1.3.2 Impact of bacteria on wound healing

The effects of bacteria on the wound healing process is still unknown, however, low levels of bacteria within wounds may aid wound healing. Granulation tissue, the major component of chronic wounds, would not be present without bacteria (Heggers, 1998). Conversely, high numbers of bacteria and/or their extracellularly secreted products appear to delay the wound healing process. Numerous studies conducted by Robson and colleagues, have correlated the numbers and types of bacteria in wounds and shown delayed wound healing. Most notably, 28 of the 30 wounds with low bacterial numbers healed in a timely manner, compared to the 10 wounds with high bacterial numbers that did not heal. An additional study concluded that 89 of 93 wounds with 10^5 bacteria per gram of tissue of bacteria or fewer showed a rapid, uncomplicated healing profile.

The 10^5 bacteria per gram of tissue is a numerical number associated with successful or unsuccessful wound healing (Bowler, 2003). This amount of bacteria is often used as an indicator of infection in both acute surgical wounds and chronic wounds. A retrospective analysis of the clinical outcomes of skin grafts and the microbiological contamination levels, showed 94% survival of grafts in wounds with 10^5 fewer bacteria per gram tissue (Robson, 1997). Tarnuzzer and Shultz, (1996), suggest that the presence of bacteria induces inflammatory products, the consequences of which contribute to wound chronicity.

The significance of bacteria in non-healing wounds that do not exhibit clinical signs of infection, are still debated. Topics of debate include whether it is the number of microorganisms or the presence of particular pathogens that are important factors, or indeed whether bacteria contribute to delayed wound healing at all (Bowler et al., 2001). More recently the microflora in chronic wounds has been assessed using molecular based analyses, through amplification of 16S rDNA (Hill et al., 2003, Davies et al., 2004). This technique allows identification of 'unculturable' bacteria, and the results of Davies et al., (2004), suggest that the bacterial load for *Pseudomonas* spp. is increased in non-healing, rather than healing wounds. More recently, Davies et al., (2007), suggested that both microbial diversity and microbial load are correlated with non-healing chronic venous leg ulcer wounds. Cooper and Lawrence, (1996), have shown that the quantities of bacteria decreases as wound healing progresses. In 29 nonhealing wounds, a marked clinical improvement was seen in the majority of wounds with a reduction in superficial microorganisms (Bowler, 2003).

1.4 Wound infection

A consequence of bacteria in the wound and a complication associated with normal wound healing is wound infection. Wound tissue is susceptible to contamination by microorganisms (predominantly bacteria) from the external environment. Examples include normal resident skin flora and microorganisms introduced following traumatic injury (non-resident exogenous flora). However, the majority of wound contaminants are derived from the mucosal surfaces of the host (endogenous flora), particularly the oral cavity and gut (Bowler, 2003).

Contamination, colonisation or infection, are possible when a microorganism enters the wound. Contamination is the presence of microorganisms with no multiplication. Colonisation occurs when the local host defence strategies are overcome, but do not provoke a clinical or detectable immunological response. Infection is marked by a detectable immune response (Cooper et al, 1996).

The term critical colonisation has been adopted in the wound-healing arena. This is the mid-point between colonisation and infection, whereby the hosts' defences have been overwhelmed and signs of cellulitis are present (Davies, 1998). During severe infection, vesiculation, pustulation, ulceration and necrosis are observed (Finch, 1988). Cellulitis is the development of peri-wound erythema and pus formation, clinically observed as a hot, red and painful wound. Left untreated, it can delay wound healing and lead to septicaemia (Grey, 1998).

Simplistically, if the number or virulence of the microorganism, outweighs the ability of the host to mount an efficient/appropriate immune response, then infection arises and wound healing is retarded (Stadelmann et al., 1998a). This interaction, has been expressed as an equation, by Mertz and Ovington, (1993).

$$\text{Infection} = \frac{\text{dose of organism x virulence}}{\text{host resistance}}$$

The potential for wound infection depends on the microorganism in the wound and the person's resistance to that contamination (Kingsley, 1992). However, the exact mechanisms by which bacteria cause wound infection are still debated.

1.4.1 Predicting wound infection

The local environment of the bacteria dictates its response. There are likely to be multiple factors, both microbial- and host-derived, that contribute to developing a wound infection. These may include the general health and immune status of the host, the location, type, size, and depth of the wound, the microbial load, and level of virulence of the microorganism(s) (Woods et al., 1986, Cooper and Lawrence, 1996, Kingsley, 2001). Acute, surgical wounds, adequately perfused with oxygen and nutrients, rarely become infected (Bowler et al., 2001). Within acute wounds, the bacterial load and the ability to cause infection, is probably dependent on both microbial load and net pathologic effect.

The infecting dose is known to vary with the type of organism and the way it is presented (Bowler, 2003). Evidence suggests that if two bacteria act synergistically, their pathogenic effect is greater than if the same organisms worked independently (Bowler, 2003). The presence of foreign matter, e.g. suture material, reduces the number of bacteria needed to infect the wound. The infecting dose necessary to cause skin infections in human volunteers falls from $2\text{-}8x10^6$ to approximately 10^3 microorganisms per gram tissue (Elek, 1956). In addition, the abundance of necrotic tissue, commonly associated with chronic wounds, provides a suitable growth medium to bacteria (Stadelmann et al., 1998a).

Generally, the chronic wound environment may favour bacterial infection, specifically if wound tissue is ischemic and a dry environment. This environment limits neutrophil migration into the area and neutrophil impairment has been reported, as a consequence of diabetes and in venous leg ulcers (Rubinstein et al., 1983, Whiston et al., 1994). The study by Rubinstein et al. (1983), established that the probability of infection is five times greater in diabetic feet. Surgical infection rate are documented when surgical site infections are monitored. Post-operative infection of a 'clean' wound is 1-5% risk, increasing to 27% risk in a 'dirty' procedure, e.g. head, neck or large intestine surgery, where endogenous microorganisms behave opportunistically, relocating themselves to other, previous uncolonised areas (Bowler et al., 2001). However the monitoring of chronic wound infection, and the definition of chronic wound healing has proved more difficult, especially distinguishing the clinical signs and symptoms of chronic wound infection from chronic inflammation (Chaper 1, Section 1.8.3). Establishing the incidence of chronic wound infections is also a difficult task. Hutchinson and Lawrence, (1991), estimated a 5% incidence in leg ulcers, while Thompson, (2000), suggests the infection rate in chronic wounds is 6.5%.

Numerous studies have investigated the microflora of acute wounds. In one of the largest studies, of 676 surgical wounds, with patients showing signs and symptoms of wound infection, the common pathogens were found to be *S. aureus* (28.2%), *Pseudomonas aeruginosa (P. aeruginosa)*(25.2%) *Escherichia coli* (*E.coli)* (7.8%), *Staphylococcus epidermidis* (*S. epidermidis*) (7.1%) and *Enterococcus faecalis (E. faecalis)* (5.6%) (Giacometti et al., 2000). The Centers for Disease Control and Prevention guideline for surgical site infection have recognised *S. aureus*, coagulase-negative staphylococci, *Enterococcus* spp., *Escherichia coli, P. aeruginosa*, and *Enterobacter* spp. as the most frequently isolated pathogens (Bowler et al., 2001). Studies investigating the type of bacteria colonising chronic wounds is further discussed (Chapter 1, Section 1.8).

1.4.2 Invasion of host tissues

Bacteria adhere to host cells, usually epithelial cells. Once in the body, bacteria multiply and spread, causing local infection. Upon entering the bloodstream, these can cause systemic infection (Yoshimura et al., 1999). Bacteria rely upon many different strategies to successfully invade and multiple within the host. The three main mechanisms by which bacteria achieve this are; adhesion to host cells (via adhesins), the secretion of tissue damaging toxins and mechanisms by which bacteria are resistant to host antimicrobial defences, thereby ensuring successful multiplication and survival (Peschel, 2002).

Initial binding of bacteria to the host cell is through weak, reversible bonds, with recognition of receptors leading to an irreversible interaction (Heinzelmann et al., 2002). Pili and fimbriae are filamentous proteins on the surface of a bacterial cell that behave as adhesins of specific-adherence for particular bacteria. M proteins, glycocalyx, capsule, LPS, teichoic and lipoteichoic acids of Gram-positive and Gram-negative bacteria are all implicated as adhesins, by non-specific and specific interactions (Vercellotti et al., 1985).

The use of adhesins changes the surface hydrophobicity of bacteria, an important feature of invading bacteria. Both bacterial and host cells have prodominantly negative surface charges, which would normally repel one another. By changing their hydrophobicity, some strains of species are more adhesive than others (Wadstrom, 1987).

Bacteria secrete a range of molecules that damage host cells and tissues including, enzymes (Table 1.1), which cleave cellular components leading to tissue damage, and toxins. Exotoxins are released by bacterial cells and are capable of travelling through the body. These can be produced by Gram-positive and Gram-negative bacteria acting to generally disrupt normal cell function, e.g. botulism blocks the release of acetylcholine resulting in paralysis. Endotoxins, produced by Gram-negative bacteria, for example *P. aeruginosa* and *E. coli*, are typically cell bound and only released in large amounts when cells are lysed. Symptoms associated with endotoxin release include those of food poisoning and diarrhoea. The most commonly associated endotoxin is LPS, a component of the Gram-negative cell wall. The effects of an over-reaction to LPS include sepsis, septic shock or systemic inflammatory response syndrome. The consequences of which can include death of the host (Guha and Mackman, 2001).

Table 1.1: Bacterial enzymes.

Enzyme	**Effect**
Collagenase	Degrade collagen/connective tissue.
Coagulase	Coagulates plasma, provides fibrin and aids in the protection from phagocytosis.
Hyaluronidase	Connective tissue degradation.
Strepokinase/Staphylokinase	Kinase enzyme converts plasminogen to plasmin, which digests fibrin and prevents blood clotting.
Hemolysins and leukocidins	Cytolysins dissolve red blood cells (hemolysins) or kill tissue cells and leukocytes (leukocydins).
IgA1 protease	IgA secretory antibodies in mucosal surfaces. IgA1 protease allows pathogens to inactivate primary antibodies found on mucosal surfaces and eliminate protection of the host by the antibodies.

1.4.2.1 Lipopolysaccharide

LPS, the Gram-negative bacterial endotoxin, can be released from the cell surface when the cell multiplies, lyses or dies (Heinzlemann et al., 2002). It consists of a lipid A region attached to long chain fatty acids. The host response to LPS is mediated through LPS binding to lipopolysaccharide binding protein (LBP), and the LBP-LPS complex stimulating reactions from the hosts' immune system and tissue cells.

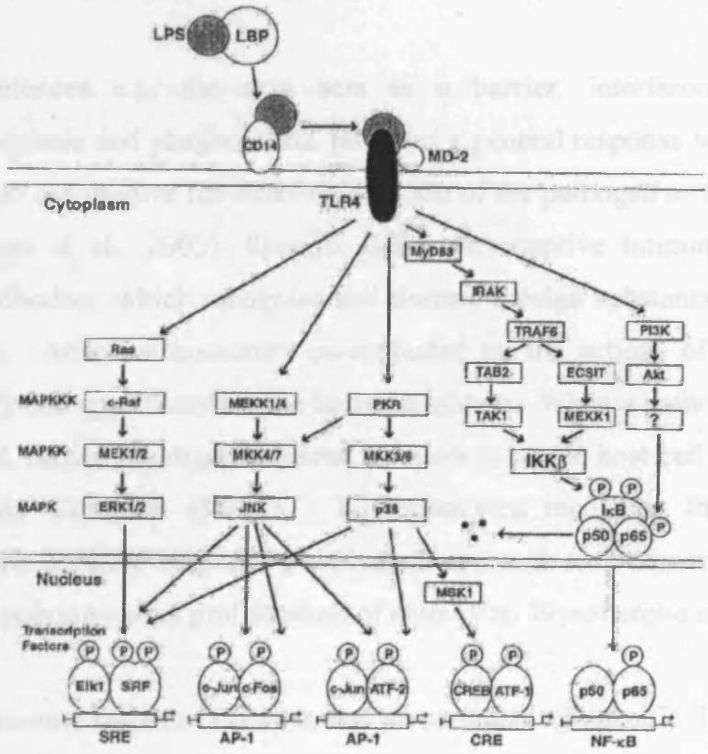
Generally, binding of the LPS-LBP complex to host cells occurs through the 55kDa membrane receptor CD14, glycosylphoshatidyl inositol (GPI) anchored glycoprotein (Ziegler-Heitbrock et al., 1992, Heumann and Roger, 2002), defined as a pattern recognition receptor (Chapter 1, Section 1.5). Binding can occur to either the membrane bound receptor (mCD14) and/or the soluble form (sCD14) of CD14, an enzymatically cleaved form of mCD14 (Kielian and Blecha, 1995, Heumann and Roger, 2002).

Binding of this complex, can induce host cells, such as neutrophils and lymphocytes, to initiate a cellular signalling cascade (Figure 1.7), via PRRs e.g TLR-4 (Remer et al., 2003), the activation of signalling pathways, e.g., ERK, JNK, p38 and IκB, activating transcription factors, e.g. NF-κB and AP-1, that encode various proteins (Guha and Mackman, 2001). B-cells are stimulated to proliferate and produce antibody, T-cells to secrete cytokines, and the down-regulation of T-suppressor cells. A number of pro-and anti-inflammatory cytokines are produced and secreted depending on the cell type LPS is bound to including IL-1, IL-6, IL-8, IL-10, and IL-12, macrophage migration inhibitory factor, and TNF-α (Yamamoto and Akira, 2005, Stavitsky, 2007).

In vivo, injection of endotoxin causes (i) the production of cytokines (mediators of inflammation) (ii) activation of the complement cascade, and (iii) the activation of the coagulation cascade. Clinically, exposure of the host to LPS is known as either 'sepsis', when the infection is detected (the causative pathogen can be isolated, cultured and identified) or systemic inflammatory response syndrome (SIRS), in the absence of documented infection (Guha and Mackman, 2001, Lopez-Bojorquez et al., 2004).

Preventing the binding of the LBP-LPS complex to CD14, blocks the activity of the endotoxin (Opal et al., 1994, Weiss, 2003). LPS can be neutralised naturally within the body by bactericidal/permeability-increasing protein (BPI), a cellular component of the azurophil granules of neutrophils (Chapter 1, Section 1.5.3). BPI itself is released from neutrophils stimulated *in vitro* with LPS, fMLP and cytokines (Marra et al., 1992: 1993).

Figure 1.7: LPS cell signalling pathway, via toll like receptor-4.
LPS binds LPS binding protein (LBP), the complex transfers to CD14 on the cell surface and then interacts with toll like receptor-4 (TLR-4). This interaction stimulates various signaling pathways and transcription factors (Guha and Mackman, 2001).



1.5 The immune response

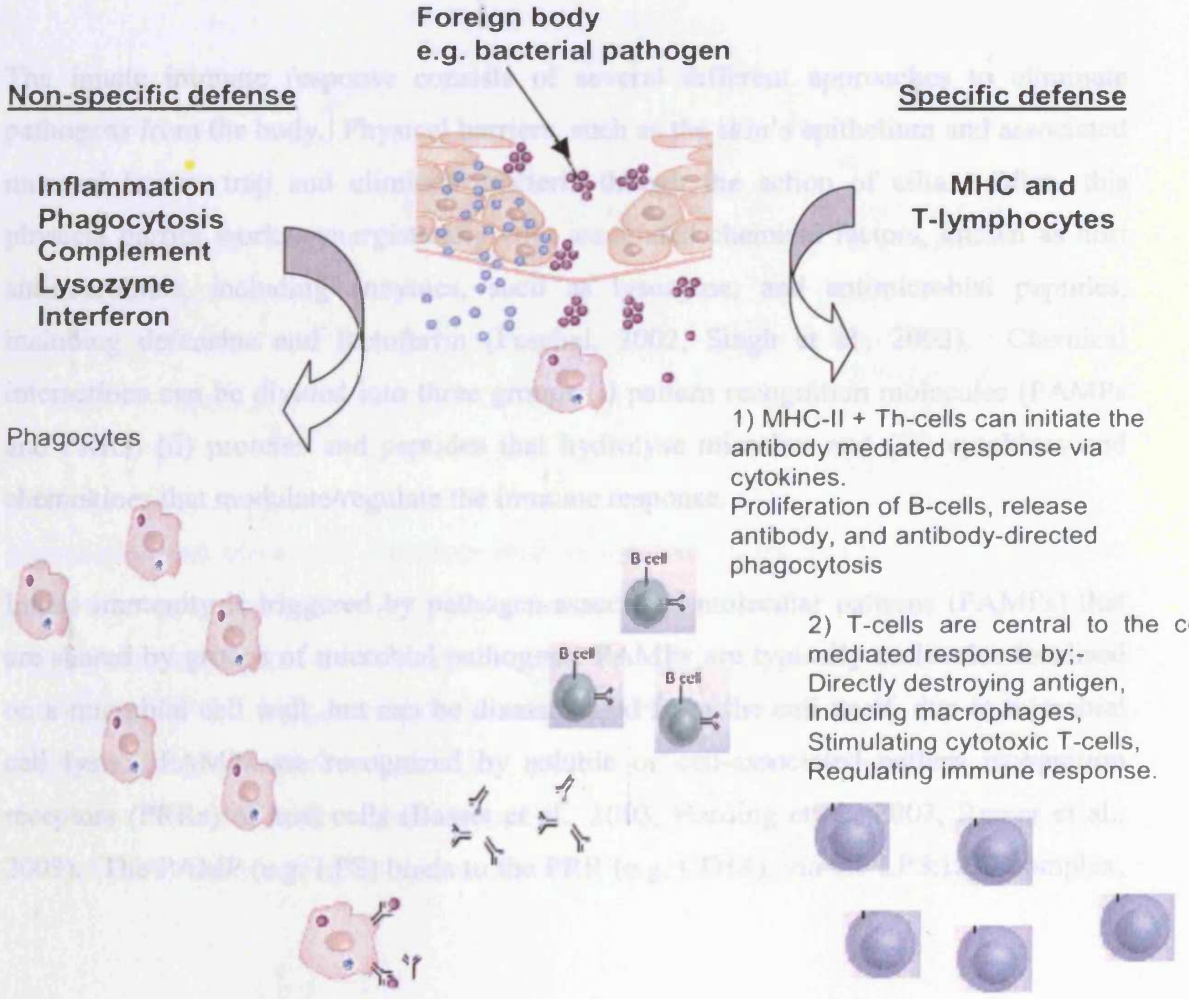
The immune systems primary function is to protect the body against infection and the development of disease (Playfair and Chain, 2001). It can differentiate between self and non-self and is composed of the lymphatic vessels and organs (thymus, spleen, tonsils/adenoids and lymph nodes), white blood cells (lymphocytes, neutrophils, basophils, eosinophils, monocytes, etc.), specialised cells residing in tissues (macrophages, mast cells, etc.) and specialised serum factors. The body has both non-specific (innate) and specific (adaptive, humoral and cellular), responses to protect itself against invading pathogens (Figure 1.8).

Non-specific defences e.g. the skin acts as a barrier, interferons, complement, inflammatory response and phagocytosis, provides a general response to destroy foreign substances, but do not involve the exact recognition of the pathogen or the production of antibodies (Basset et al., 2003). Specific defences, adaptive immunity, involve the formation of antibodies, which recognise and destroy foreign substances (Hancock and Diamond, 2000). Adaptive immunity co-ordinated by the actions of T-lymphocytes, provides memory and specificity for the immune system. When a pathogen invades and enters a host cell, bacterial antigens present themselves on the host cell wall with Major Histocompatibility Complex (MHC). T-lymphocytes recognise the antigen/MHC complex and two immune responses, cell mediated and antibody mediated, can be initiated, both involving clonal proliferation of cells (Van Wynsberghe et al., 1995).

Cell mediated immune response refers to the involvement of specific T-lymphocytes in co-ordinating the elimination of bacteria. If the antigen is presented with MHC I, cytotoxic T-cells cause cell death in grafts, tumour cells or cells infected with a virus. If the antigen is presented with MHC II, helper T-cells leads to the development of delayed hypersensitivity reactions and defence against intracellular agents, such as viruses.

In contrast, the antibody mediated, humoral response is initiated through recognition of the antigen presented along with MHC II. The T-cells allow the proliferation of B-cells, which produce antibodies capable directly neutralising bacteria and can present the antigen back to T-cells increasing the adaptive immune response (Clark and Kupper, 2005). Antibodies allow for the increased opsonisation of bacteria, which in turn leads to the bacteria's internalisation and destruction by phagocytes (Chapter 1, Section 1.5.1). The antibody response can be two-staged, the primary response which lasts for weeks/months dependent on antigen, and the secondary exposure, during which the response is much more rapid, due to the action of memory B-cells (Van Wynsberghe et al., 1995).

Figure 1.8: The immune response to a foreign body.
Adapted from Van Wynsberghe et al., 1995, Clark and Kupper, 2005.



The maturation of the helper T-lymphocytes (Th), divided into two groups, Th1 (CD4+) and Th2 (CD8+) populations, play an integral role in the enhancement of cell-mediated and humoral immune responses (Heinzelmann et al., 2002). Th1 cells preferentially secrete pro-inflammatory (IL-1, IL-2, IL-6, IFN-γ and TNF-α) and Th2 cells anti-inflammatory (IL-4, IL-5, IL-10 and IL-13) cytokines (Cassatella, 1995, Basset et al., 2003). There appears to be an overlap between the initial innate immune response and the adaptive immune response. These early induced (non-adaptive) responses bridge the time it takes the body to induce cellular or antibody mediated responses (Basset et al., 2003). For the purposes of this Introduction and relevance to chronic wound healing, the innate immune response will be discussed, with particular attention/reference to the inflammatory response.

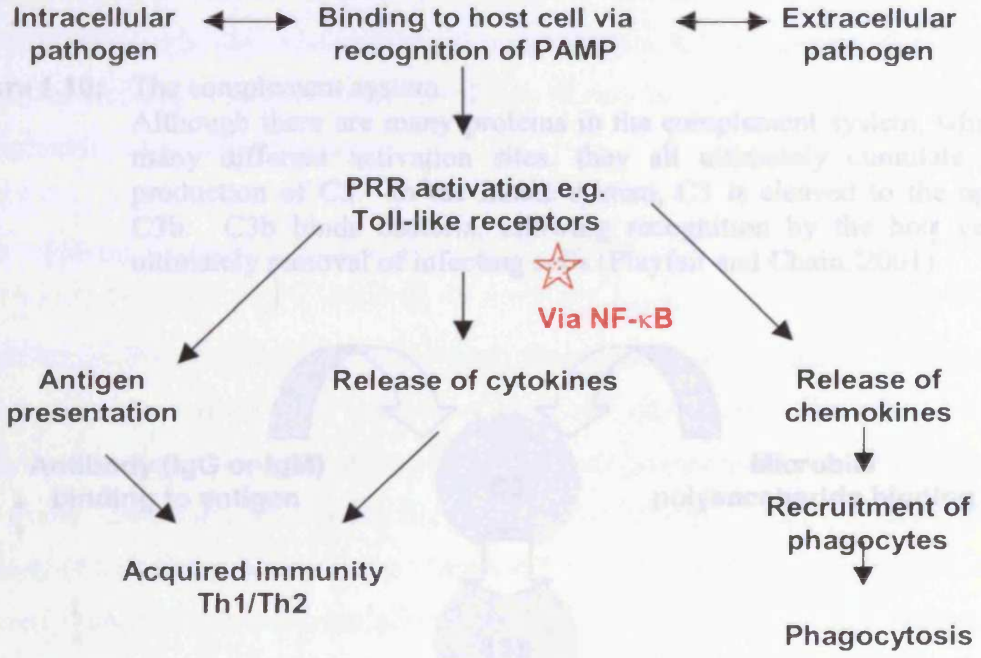
1.5.1 Host response to infection

The innate immune response consists of several different approaches to eliminate pathogens from the body. Physical barriers, such as the skin's epithelium and associated mucosal layers, trap and eliminate bacteria though the action of cilia. Often, this physical barrier works synergistically with associated chemical factors, known as host antimicrobials, including enzymes, such as lysozyme, and antimicrobial peptides, including defensins and lactoferrin (Peschel, 2002, Singh et al., 2002). Chemical interactions can be divided into three groups (i) pattern recognition molecules (PAMPs and PRRs) (ii) proteins and peptides that hydrolyse microbes and (iii) cytokines and chemokines that modulate/regulate the immune response.

Innate immunity is triggered by pathogen-associated molecular patterns (PAMPs) that are shared by groups of microbial pathogens. PAMPs are typically molecules localised on a microbial cell wall, but can be disassociated from the cell itself, due to microbial cell lysis. PAMPs are recognized by soluble or cell-associated pattern recognition receptors (PRRs) of host cells (Basset et al., 2003, Harding et al., 2003, Remer et al., 2003). The PAMP (e.g. LPS) binds to the PRR (e.g. CD14), via the LPS:LBP complex,

initiating an intra-cellular signaling cascade, commonly by activating the nuclear transcription factor, NF-κB (Tato and Hunter, 2002). This cascade ultimately triggers the synthesis and release of other molecules of the innate immune system, including inflammatory mediators, such as cytokines and chemokines (Figure 1.9).

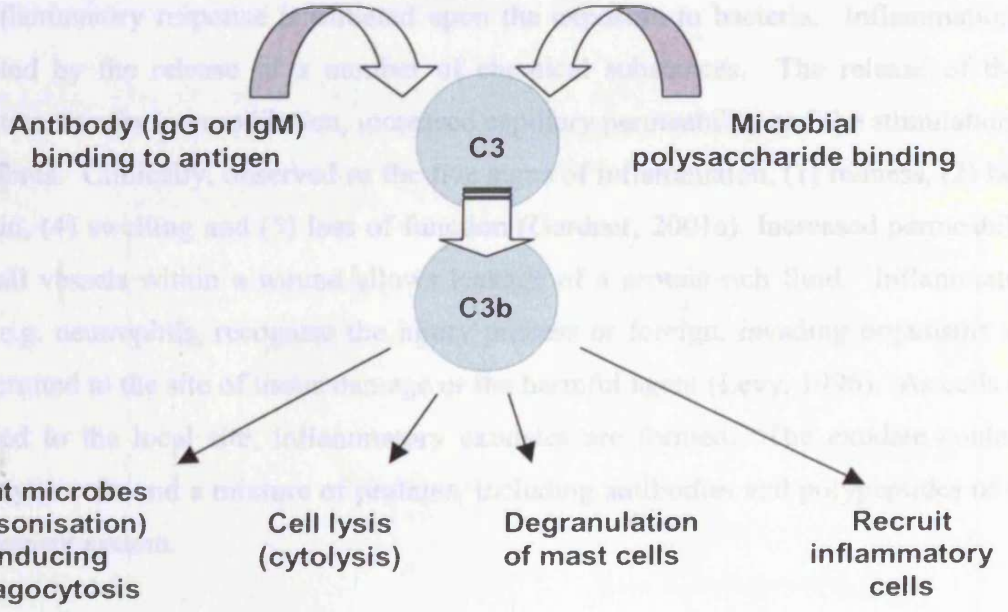
Figure 1.9: Mechanisms of innate immunity in pathogen-host interaction. Activation of the PRRs can lead to both moderators of the innate immune system, resulting in phagocytosis. Alternatively, this immune response can also mediate acquired immune response, via Th1 and Th2 cytokines, that result in the production of antibodies. Modified from an illustration by Basset et al. (2003).



Mammalian cell-associated receptors include toll-like receptors (TLR). There are 10 toll-like receptors in the human that respond to components of Gram-positive and Gram-negative bacteria to initiate an inflammatory response (Yoshimura et al., 1999, Heuman and Roger, 2002). It appears that each TLR has a different specificity and binding ability, and together with other PRRs, probably accounting for the immune systems ability to non-specifically eliminate a variety of microbes through phagocytosis or destruction by the complement system (Dumitru et al., 2000) (Figure 1.10).

Two pathways exist in the complement system; the classic and alternative pathways. The alternative, antibody independent, pathway is a component of the innate immune system. The classical pathway, is dependent on the production of antibodies, forming part of the adaptive immune system (Playfair and Chain, 2001). Complement is a series of thirty proteins that function to lyse cells (bacterial and tumour) and to stimulate the production of mediators that participate in inflammation and attract phagocytes. Complement proteins are synthesised by the liver and are heat labile molecules that can be inactivated at 56°C for 30 minutes. The recruitment of cells and proteins and fluid accumulation in sites of infection is mediated by the anaphylotoxins, complement components C3a, C4a, and C5a (Yao et al., 1998, Dennison and Van Dyke, 2000).

Figure 1.10: The complement system.
Although there are many proteins in the complement system, which has many different activation sites, they all ultimately cumulate in the production of C3. In the innate system, C3 is cleaved to the opsonin, C3b. C3b binds bacteria, allowing recognition by the host cell and ultimately removal of infecting cells (Playfair and Chain, 2001).



The biological effects of complement include;

- Opsonisation. The coating of cells, associated with an increased ability to phagocytose antibody:antigen complexes, via C3b receptors of phagocytes.
- Chemotaxis. Complement proteins, C5a and C567 complexes attract leukocytes.
- Anaphylatoxin. The degranulation of mast cells, leads to an increase in mediators that affect vascular permeability and smooth muscle contraction.
- Cytolysis. The insertion of the C56789 complex, results in the killing/lysis of bacteria, red blood cells and tumour cells.

Cells central to the innate immune system include epithelial cells, mast cells, dendritic cells and the phagocytes, neutrophils and macrophages. A consequence of the action of the innate immune system, through the release of various mediators, is recognised as an inflammatory response in the host.

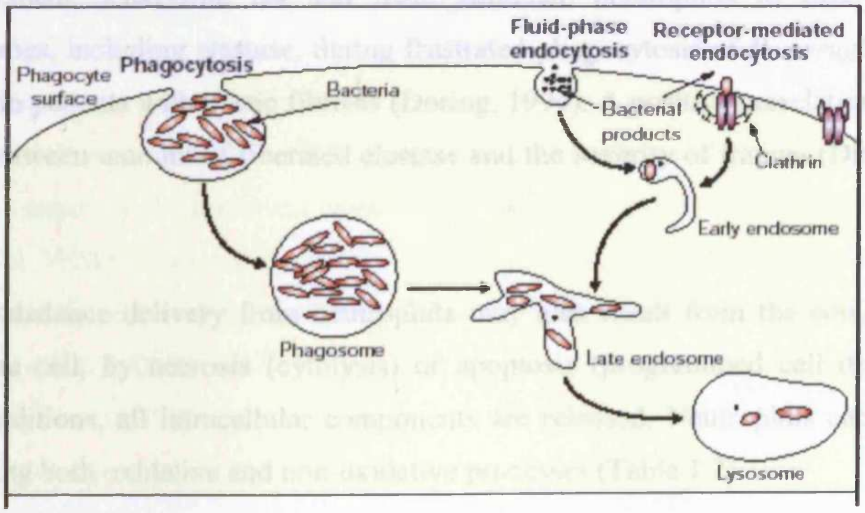
1.5.2 The inflammatory response

An inflammatory response is initiated upon the exposure to bacteria. Inflammation is mediated by the release of a number of chemical substances. The release of these mediators, results in vasodilation, increased capillary permeability and the stimulation of pain fibres. Clinically, observed as the five signs of inflammation, (1) redness, (2) heat, (3) pain, (4) swelling and (5) loss of function (Gardner, 2001a). Increased permeability of small vessels within a wound allows leakage of a protein-rich fluid. Inflammatory cells, e.g. neutrophils, recognise the injury process or foreign, invading organisms and are recruited to the site of tissue damage or the harmful agent (Levy, 1996). As cells are attracted to the local site, inflammatory exudates are formed. The exudate contains phagocytic cells and a mixture of proteins, including antibodies and polypeptides of the complement system.

1.5.3 The phagocyte and the process of phagocytosis

Neutrophils and macrophages are both phagocytic cells. These particular cells are capable of ingesting and killing microbes. Neutrophils and macrophages are attracted and move towards an area of infection through chemotactic gradients. Host molecules, such as the chemokine, IL-8, and bacterial components, mainly N-formylpeptides, are chemotactic to these cells (Normark et al., 2001, Henry and Garner, 2003). Once at the infected/inflamed site, microbes/foreign matter can be ingested and killed through the process of phagocytosis (Figure 1.8). Phagocytes can bind bacteria or foreign matter by both Fc (antibody receptors) and/or complement receptors (CR). Phagocytes recognise antibodies or complement bound to foreign matter or bacteria (opsonised bacteria), binding occurs though the Fc or CR of the phagocyte (Heinzlemann et al., 2002). Particles are ingested, and once within the phagocyte cell, are combined with the phagosome, where bacterial killing is mediated though the release of lysosomal enzymes (acid and serine proteinases) and potent ROS (Barrick et al., 1999). These enzymes in the resulting phagolysosome cell kills the microbe/degrades foreign matter, completing the process of phagocytosis is complete (Figure 1.11).

Figure 1.11: The process of phagocytosis (Pieters, 2001).
Phagocytosis results in the internalization of bacteria into phagosomes. Bacterial products can also be taken up by fluid-phase or receptor-mediated endocytosis.



1.5.3.1 The neutrophil

The neutrophil is an efficient, but short-lived (hours-days) cell that can ingest up to twenty bacteria before dying (Cassatella, 1995, Witko-Sarat et al., 2000, Segal, 2005). Neutrophil cells are granular in nature, with granules containing antimicrobial substances, including enzymes. The contents of the azurophil granules are primarily designed for killing microorganisms. There are four ways in which a neutrophil cell can deliver antimicrobial substances; (1) phagocytosis (as previously discussed, Chapter 1, Section 1.5.3), (2) secretion, (3) apoptosis/cytolysis, and (4) via the respiratory burst (Miyasaki, 1991).

Secretion (degranulation) is required from the granule into either the phagosome or extracellularly, upon contact of the granule with the cell surface (Owen and Campbell, 1995). This extracellular secretion does not normally occur until there is close proximity of the neutrophil itself with the target of interest. A review of neutrophil degranulation in the acute inflammatory response, is provided by Faurschou and Borregaard (2003). Extracellular secretion has been mimicked *in vitro*, stimulated by agents such as LPS (Section 1.4.2.1), phorbol-mysitrate-acetate (PMA) and formyl-methionyl-leucyl-phenylalanine (fMLP) (Xu and Hakansson, 2002, Gasser et al., 2003). Cytokines, IL-1, IL-6, IL-8 and TNF-α, can all stimulate neutrophil degranulation *in vitro* (Bank et al., 1995, Topham et al., 1998). In the lung, mediators of the immune system, complement, leukotrine B4 and IL-8, stimulate neutrophils to exocytose lysosomal enzymes, including elastase, during frustrated phagocytosis of *P. aeruginosa* micro-colonies in patients with cystic fibrosis (Doring, 1994). A positive correlation has been reported between amount of liberated elastase and the severity of trauma (Dittmer et al., 1985).

Antimicrobial substance delivery from neutrophils may also result from the complete disruption of the cell, by necrosis (cytolysis) or apoptosis (programmed cell death). Under these conditions, all intracellular components are released. Neutrophils can kill microbes, utilising both oxidative and non-oxidative processes (Table 1.2).

Non-oxidative mechanisms involve substances, such as cationic antimicrobial proteins (CAP), elastase, bacterial permeability-inducing protein (BPI), low molecular weight cationic antimicrobial proteins (defensins), cathepsin G, lysozyme and lactoferrin.

Table 1.2: The molecules/substances that make up the contents of the neutrophils granules (Miyasaki, 1991). The reliance of these molecules/substances to either the oxidative or non-oxidative mechanisms are shown.

	Antimicrobials		
Granule Type	**Non-oxidative ($O_2^{\cdot-}$ Independent)**	**Oxidative ($O_2^{\cdot-}$ Dependent)**	**Other**
Azurophil (lysosome)	CAP57, CAP37 BPI, Elastase, Cathepsin G Defensins, Lysozyme	Myeloperoxidase	None stated
Specific	Lactopherrin Lysozyme	NADPH oxidase cofactors	C5a receptors Bacterial chemotaxin receptors Collagenase/ Gelatinase

Oxidative antimicrobial mechanisms rely on the consumption of oxygen by the phagocyte. The respiratory burst, reduces oxygen to oxygen metabolites, using the NADPH oxidase system or myeloperoxidase (MPO) (Van Eeden et al., 1999, Dennison and Van Dyke, 2000). The killing of the bacterial cell is due to exposure to high concentrations of toxic ROS and the halogenation of H_2O_2 by neutrophil Myeloperoxidase (MPO), to hypochlorous acid (HOCl).

Additionally, a number of cytokines can by synthesised and released from neutrophils, which regulate neutrophil function, contributing to the inflammatory response (Cassatella, 1995, Witko-Sarsat et al., 2000). These pro-inflammatory cytokines and other mediators aid in the ability of neutrophils to control the growth and eliminate bacteria (Fujihara et al., 2003).

1.5.3.2 The macrophage

Within the wound site, TGF-β, IL-1, and TNF-α, can be chemoattractants, playing a role in monocyte infiltration. Upon entering the tissue, monocytes differentiate to macrophages (Henry and Garner, 2003). Macrophages lack some of the more potent destructive enzymes of the neutrophil, and generally survive longer. These cells also produce inflammatory cytokines and mediators that contribute to the efficient control of bacterial growth and destruction (Fujihara et al., 2003). Macrophages are able to phagocytose a number of times, capable of removing up to 100 bacteria, in addition to recognising and removing cell debris, and protozoan contamination.

Peripheral blood monocytes resemble neutrophils in that they contain elastase and cathepsin G in peroxidase positive granules. Upon differentiating into macrophages, monocytes lose their complement of serine proteinases, and matrilysin (MMP-7), but are capable of synthesising and secreting a variety of MMPs e.g. human alveolar macrophages can produce elastase (MMP-12) (Sharpiro, 1998). Macrophages contain different proteases to the neutrophil, and also do not exhibit the same oxygen-dependent antimicrobials systems. Macrophages, however, can display a range of functional and morphological phenotypes, which allows them to participate in both specific, antigen presentation and IL-1 production, and non-specific immunity, against bacterial, viral, fungal, and neoplastic pathogens (Harding et al., 2003).

Neutrophils and monocytes store proteinases for rapid release, although macrophages monitor and respond to their environment; properties that may allow for tissue remodelling and possibly control of other inflammatory events (Shapiro, 1998). Activation of macrophages leads to the production of signals of inflammation, but over-activation of macrophages is recognised as playing a part in chronic inflammatory conditions, such as chronic obstructive pulmonary disease (Sharpiro, 1998) and rheumatoid arthritis (Kodelja et al., 1997).

1.6 Proteases and their inhibitors

Proteases are a group of enzymes that catalyse the cleavage of peptide bonds (Owen and Campbell, 1995). There are two distinct proteolytic systems central to homeostatic mechanisms, degradative proteolysis and limited proteolysis. Limited proteolysis describes essential post-translational modifications, usually involved in the regulation of many biological processes, for example, cleavage of pro-MMP to active-MMP (Section 1.6.1). Proteinases are responsible for extracellular proteolysis, which is essential for many normal physiological processes, including tissue remodelling, coagulation, regulation of inflammatory response and host defence mechanisms, to name but a few. Proteinases themselves are classified into four groups, aspartic, cysteine, metallo and serine, based upon their catalytic mechanism and the structure of their active site (Barrett et al., 1998) (Table 1.3). The two classes of protease involved in and studied extensively in the wound healing process are the MMPs and serine proteinases, due to their optimal activity at neutral/physiological pH. The cysteine and aspartic proteases are acidic in nature and are therefore thought to have a limited role extracellularly (Barrick et al., 1999).

Table 1.3: Classification of proteases (Barrett et al., 1998).

Catalytic class	Mechanism	Common example
Aspartic	Water molecule attack polarised by enzyme aspartic side-chains	Pepsin, cathepsin D
Cysteine	Covalent attack of polarised cysteine side-chain	Papain, caspases, calpain, cathepsin B
Metallo	Water molecule attack polarised by enzyme co-ordinated Zinc	Thermolysin, matrix metalloproteinases (MMPs), TACE, CPA, TOP
Serine	Covalent attack of polarised serine side-chain	Trypsin, tissue plasminogen activator (tPA), elastase, plasmin

Protease inhibitors are molecules, both natural and synthetic, that inhibit the activity of proteases in complex solutions such as cell lysates, blood, or even within cells. During normal physiology, protease inhibitors play an important role preventing excessive ECM degradation. These properties have been exploited *in vitro*, where they have been used extensively in research, as they can preserve the native state of the proteinase and prevent hydrolysis of their natural substrates.

In addition, their potential therapeutic use in the treatment of conditions, such as cystic fibrosis, has been proposed (Doring, 1994). Some 100 naturally occurring proteinase inhibitors of bacterial, plant and animal origin have been studied. These vary in size from 50 to 500 amino acid residues and act by binding in a reversible or semi-irreversible manner to the target proteases (Bieth, 1996).

1.6.1 Matrix metalloproteinases

Many wound healing processes, including tissue remodelling, cellular migration, angiogenesis, re-epithelialisation, depend upon the controlled ECM degradation. The MMPs are a family of zinc-dependent enzymes that can degrade all major components of the ECM; collagen, proteoglycans, laminins and fibronectin (Xue et al., 2006), providing the rate-limiting step in collagen degradation (Soo et al., 2000).

MMPs are typically classified into groups; collagenases, gelatinases, stromelysins/matrilysins and membrane-type, based on their structure and preferred substrates. Helical collagen is the preferred substrate for the collagenases (MMP-1 and MMP-8), with the gelatinases (MMP-2 and MMP-9) also being susceptible to enzymatic cleavage by this group. MMP-2 and MMP-9 cleave de-natured collagen (gelatin) and elastin, with gelatinase A (MMP-2) capable of cleaving thereby activating pro-gelatinase B (pro-MMP- 9), resulting in activation. The stromelysins (MMP-3) demonstrate preferred specificity for the pro-forms of collagenases and gelatinase B, along with matrix components, such as fibronectin and proteoglycan core proteins (Murphy, 1995; Johnson et al., 1998).

Membrane-type MMP (MT-MMP), have a variety of preferred substrates, and are classified according to their related structure. This type of MMP contains a transmembrane domain that binds the extracellular side of the cell membrane (Johnson et al., 1998).

The structure of MMPs is an important feature regulating their ability to function (Figure 1.12). Generally, with the exception of MT-MMPs, MMPs are secreted as inactive zymogens, that require the removal of the propeptide domain to become biologically active (Xue et al., 2006). Typically, extracellular activation of MMPs is initiated by other MMPs or serine proteases. MMP-2, however, is activated through a process upon the cell surface, involving MT-MMPs and TIMP-2 (Xue et al., 2006).

Many types of cells produce MMPs, including keratinocytes, fibroblasts, macrophages, endothelial cells, mast cells, eosinophils and neutrophils. However, with the exception of MMP-8 and MMP-9 in neutrophils, and MMP-7 in epithelium of sweat glands, MMPs are not constituatively expressed in the skin, but induced by various external stimuli, including cytokines (Kahari and Saarialho-Kere, 1997).

Currently, there are upwards of 20 known MMPs in humans and 4 tissue inhibitors of metalloproteinases (TIMPs). MMPs affect numerous biological molecules, thereby altering biological processes. Pathological conditions, in which MMPs have been implicated include, cardiovascular disease, cancer and bullous pemphigold (Sharpiro, 1998). The role of MMPs in normal and chronic wound healing is summarised in Table 1.4. Kahari and Saarialho-Kere, (1997) and Xue et al., (2006) provide good reviews of MMPs in the skin, given their roles in normal and chronic wound healing.

Figure 1.12: Structure of human MMPs (Kahari and Saarialho-Kere, 1997).

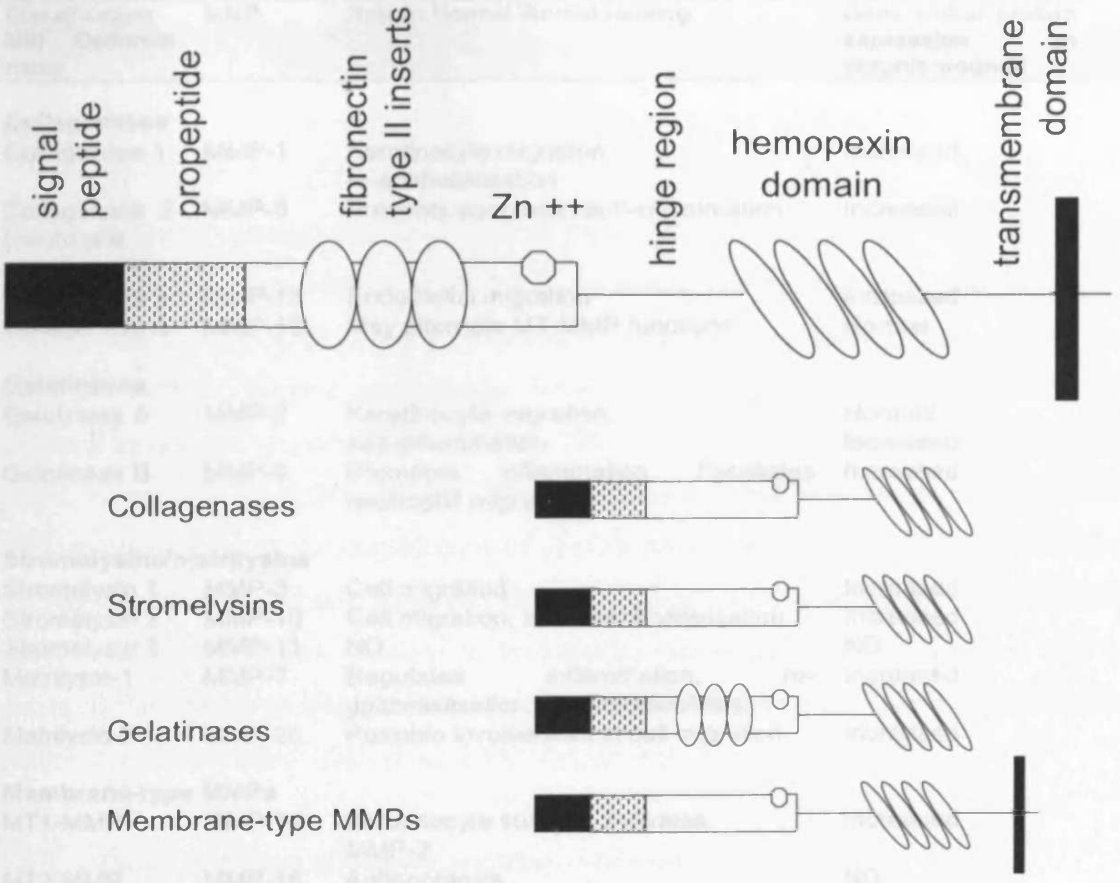


Table 1.4: Classes of MMPs, their expression and function in normal and chronic wound (Xue et al., 2006).

Classification and Common name	MMP	Role in Normal Wound Healing	Gene and/or protein expression in chronic wounds
Collagenases			
Collagenase 1	MMP-1	Keratinocyte migration, re-epithelialisation	Increased
Collagenase 2 (neutrophil collagenase)	MMP-8	Prevents apoptosis, anti-inflammation	Increased
Collagenase 3	MMP-13	Endothelial migration	Increased
Collagenase 4	MMP-18	May alternate MT-MMP functions	Normal
Gelatinases			
Gelatinase A	MMP-2	Keratinocyte migration, anti-inflammation	Normal/ Increased
Gelatinase B	MMP-9	Promotes inflammation, Facilitates neutrophil migration	Increased
Stromelysins/matrilysins			
Stromelysin 1	MMP-3	Cell migration	Increased
Stromelysin 2	MMP-10	Cell migration, and re-epithelialisation	Increased
Stromelysin 3	MMP-11	ND	ND
Matrilysin-1	MMP-7	Regulates inflammation, re-epithelialisation, inhibits apoptosis,	Increased
Matrilysin-2	MMP-26	Possible involvement in cell migration	Increased
Membrane-type MMPs			
MT1-MMP	MMP-14	Keratinocyte survival, activates MMP-2	Increased
MT2-MMP	MMP-15	Antiapoptosis	ND
MT3-MMP	MMP-16	Activates MMP-2	ND
MT4-MMP	MMP-24	Activates MMP-2	ND
MT5-MMP	MMP-17	ND	ND
MT6-MMP	MMP-25	Neutrophil migration, activates MMP-2	ND
Others			
Macrophage elastase	MMP-12	Promotes epithelial proliferation during corneal injury	Increased
-	MMP-19	Possibly involved in migration of macrophages in wound	Increased
Enamelysin	MMP-20	Regulates enamel formation	ND
XMMP (Xenopus)	MMP-21	ND	ND
CA-MMP	MMP-23	ND	ND
CMMP	MMP-27	ND	ND
Epilysin	MMP-28	Epithelial cell proliferation	ND

MMP: Matrix Metalloproteinase; MT: Membrane-type; ND: Not determined

In healthy, uninjured skin, MMPs are not detected at an mRNA level, although low levels of MMP-2 can be extracted from the skin (Parks, 1999). Upon injury and the normal wound healing process, MMPs are expressed and function to allow cell migration, regulation of the inflammatory response, re-epithelialisation and the wound to heal.

However, tissue damage due to excessive proteolytic activity is well documented in several pathological conditions of the skin, including chronic wounds. Herouy et al. (2000), and Herouy, (2001), have reviewed MMPs in skin pathology and venous leg ulcer ulceration. Several investigators have shown that MMP levels are markedly elevated in chronic wounds (Tarlton et al., 1997, Yager and Nwomeh, 1999, Wysocki, 1996, Wysocki et al., 1993, 1999). It is hypothesised that this increased proteolytic activity contributes to the degradation of growth factors and ECM components within the chronic wound. It is also possible that this excessive activity accounts for the limited success of growth factor therapy in stimulating the wound healing process, and a potential therapeutic approach may be to combine growth factor and proteinase inhibitor delivery into the wound (Soo et al., 2000, Krishnamoorthy et al., 2001).

1.6.2 Tissue inhibitor of matrix metalloproteinases

TIMPs are naturally occurring proteins, 21-34kDa, that are produced by many cell types, and are the major local inhibitors of MMP activity (Murphy, 1995, Xue et al., 2006). To date, four TIMPs, TIMP-1, -2, -3 and -4 have been described, that interact with MMPs forming 1:1 complexes (Wojtowicz-Praga et al., 1997). TIMP-1 and TIMP-2 can inhibit all known MMPs, and play a critical role in ECM deposition and degradation processes (Gomez et al., 1997). Other proteins capable of inhibiting MMP activity include, plasma α2-macroglobulin and tissue-factor pathway inhibitor-2, a serine proteinase (Xue et al., 2006).

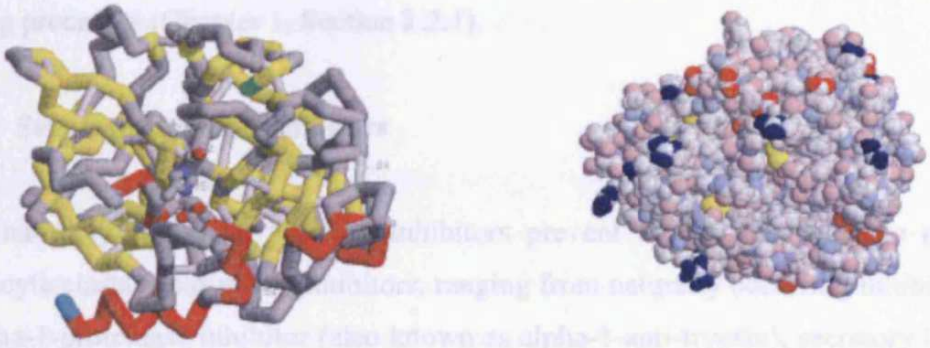
1.6.3 Serine proteinases

Serine proteinases are the largest group of proteases and can be classified by the chemical nature of their active site, the pH at which have greatest activity, and the predominant type of substrate(s) they prefer. This family of proteases include elastase, proteinase 3, trypsin, plasmin, thrombin, cathepsin G (Barrick et al., 1999, Medina et al., 2005). However, only the roles of elastase and proteinase 3 in wound healing will be discussed further.

Elastase, has its optimal activity at neutral pH (7-9), and has the ability to degrade insoluble, mature (cross-linked) elastin into soluble peptides (Owen and Campbell, 1995). However, in addition to elastin, elastase cleaves many other proteins including different types of collagen, fibronectin and laminin. Neutrophil or human leukocyte elastase (HLE) consists of a single peptide chain of 218 amino acid residues and four disulphide bridges. Its tertiary structure is similar to other serine proteases of the chymotrypsin family, and consists of two interacting anti-parallel β-barrel cylindrical domains and a small proportion of helices (Figure 1.13). The two domains form a crevice that contains the catalytic site. Three hydrogen bonds (His57, Asp102 and Ser192) play a pivotal role in peptide bond hydrolysis by charge-relay system that involved nucleophilic attack by the γ-oxygen of Ser195 (Owen and Campbell, 1995, Bieth, 1996).

The major physiological function of HLE is to digest bacteria and immune complexes phagocytosed by neutrophils. It is possible that elastase can act directly on bacterial cells, following extracellular release from the granule, independent of oxidative mechanisms (Reeves et al., 2002). Elastase has been implicated in many pathological conditions, including tissue destruction associated with pulmonary emphysema, rhuematoid artritis, cystic fibrosis, adult respiratory distress syndrome, chronic bronchitis and pancreatitis (McBride et al., 1999).

Figure 1.13: The 3D structure of elastase.
The models show (a) the alpha carbon backbone, with α-helices shown in red, β- sheets in yellow, loops in grey and the N- and C- terminus in green and cyan respectively. The space fill model (b) shows carbon (white), oxygen (pink/red), nitrogen (blue) and sulphur (yellow) atoms of the enzyme.The active site of serine proteases contain three critical amino acids, serine, histidine, and aspartate, referred to as the catalytic triad. This structure is common to elastase, trypsin and chymotrypsin. (Centre for BioMolecular Modeling, 2007).



Macrophage elastase (MMP-12) was first detected in 1975, is capable of degrading ECM components (as other MMP's), but is distinct in the MMP family, due to its predominantly macrophage specific pattern of expression (Shapiro, 1999).

Proteinase 3 is a 29kDa serine proteinase. It is biologically distinct from human neutrophil elastase, although as prominent in the neutrophil azurophil granule (Dolman et al., 1992). Proteinase 3 is synthesised as a pre-proenzyme, and cleaved to its enzymatically active form and stored (Specks, 2000). It has two distinct biological features compared to elastase, it aids myeloid differentiation and is the main target for Werners granulomatosis, a type of vasculitis characterised by high amounts of antineutrophil cytoplasmic antibodies (ANCA) (Witko-Sarat et al., 1999, Specks, 2000, Bartunkova et al., 2003). Proteinase 3 is also expressed by monocytes, basophils and mast cells.

Proteinase 3, like other serine proteases, is capable of cleaving elastin, degrading a number of macromoleules and activating cytokines (Halbwachs-Mecarelli et al., 1995, Campbell et al., 2000, Sugawara, 2005). It has been detected in inflammatory exudates, and chronic wound fluid (Dolman et al., 1992, He et al.,1998).

The increased levels of serine proteases, most notably elastase, in chronic wounds is believed to contribute to the continued inflammatory phase, thereby delaying wound healing processes (Chapter 1, Section 1.2.1).

1.6.4 Serine proteinase inhibitors

Most naturally occurring protease inhibitors prevent the action of serine proteases. Leukocyte elastase has many inhibitors, ranging from naturally occurring inhibitors such as alpha-1-proteinase inhibitor (also known as alpha-1-anti-trypsin), secretory leukocyte protease inhibitor (SLPI), elafin and alpha-2-macroglobulin, to commercially available non-specific irreversible (DFP and PMSF), specific irreversible (DCI and MeOSuc-Ala-Ala-Pro-Val-CH_2Cl) and reversible inhibitors (aprotinin, turkey ovomucoid and soybean trypsin inhibitor) (Chua and Laurent, 2006).

SLPI, a 11.7kDa protein and elafin, a 6kDa protein, are locally produced inhibitors of elastase. SLPI and elafin are produced by epithelial cells, immune cells, possibly, neutrophils, mast cells and macrophages, and keratinocytes. However, inhibitors are mainly produced by hepatocytes and circulate in the bloodstream. Elafin is structurally similar to SLPI, although it has different substrate specificities, inhibiting neutrophil elastase but additionally, proteinase 3 (Hlasta and Pagani, 1994, Hiemstra, 2002, Fitch et al., 2006).

SLPI inhibits a variety of proteinases, including neutrophil elastase, cathepsin G, trypsin, chymase and tryptase (Hiemstra, 2002). SLPI has multiple roles in the innate immune system, with anti-proteolytic, anti-microbial and anti-inflammatory profiles. Ashcroft et al. (2000), suggest that SLPI may provide an important regulatory role prior to cellular activation of inflammatory cells in the wound healing process, to inhibit the release of proteases and pro-inflammatory cytokines. Delayed wound healing is observed in SLPI null-mice, and SLPI is currently in clinical trials, indicated as a potential treatment for chronic wounds (website, www.clinicaltrials.gov).

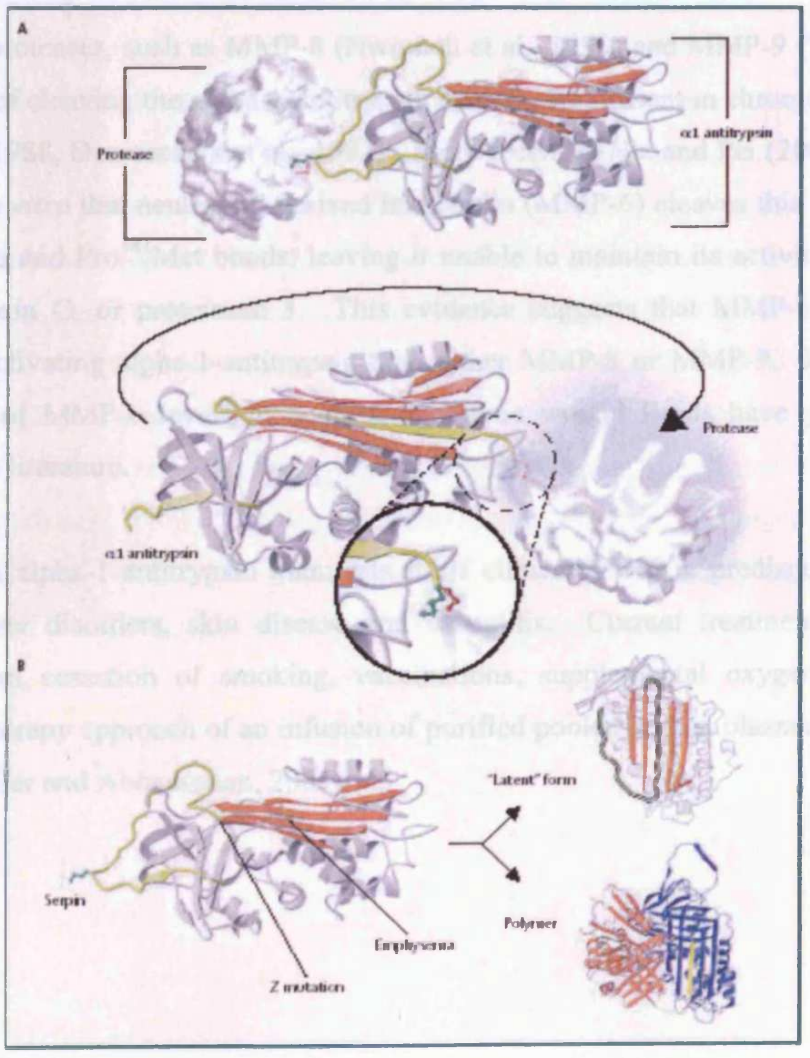
1.6.4.1 Alpha-1-antitrypsin

Alpha-1-antitrypsin is a globular plasma protein, first described in 1963 by Laurell and Eriksson. It is a 52kDa, single-chain glycoprotein comprised of 394-amino acids. The gene for the protein is located on chromosome 14 (Brantly et al., 1988). The protein has nine α-helices, three β-pleated sheets and a reactive centre loop. Upon binding to protease (Figure 1.14), this loop is able to translocate and form a more stable conformation within the main β-pleated sheet.

The human alpha-1-antitrypsin gene is transcribed by both hepatocytes (liver cells) and macrophages (Perlino et al., 1987). Synthesis and secretion of the protein from the liver, systemically, inhibits plasma proteins circulating in the blood. The protein can also be secreted locally, by macrophages, at sites of inflammation or infection. There it helps prevent tissue damage, by inhibiting the high concentration of proteases.

The main function of alpha-1-antitrypsin is to inhibit the activity of elastase, a serine protease. It is also capable of inhibiting trypsin (Beatty et al., 1980), cathepsin G and proteinase 3 (Coakley et al., 2001).

Figure 1.14: Inhibition of elastase by alpha-1-antitrypsin, and mechanism of serpin alpha-1-antitrypsin pathologies.
(a) Binding of protease occurs through an active site located at the amino acid side-chain, methionine 358. Translocation of the protein occurs from its high-energy unstable state, to a more stable conformation. The translocation causes inactivation of the protease and the process causes destruction of both molecules (b) mutations within the alpha-1-antitrypsin molecule can result in a latent form or an irreversible polymerization of the molecule, in both cases compromising alpha-1-antitrypsins ability to inhibit protease (Stoller and Aboussouan, 2005).



Excessive degradation of the ECM, by proteases, is usually prevented by the presence of naturally occurring protease inhibitors. A defect in this control system would ultimately compromise tissue integrity, impair healing, and is thought to be relevant to chronic wounds (Staiano-Coico et al., 2000). The powerful neutrophil oxidant, HOCl, will inactivate alpha-1-antitrypsin that, in turn will favour an uncontrolled proteolytic activity, contributing to tissue injury by activating free elastase (Nussler et al., 1999). There may be a localized deficiency, inactivity and degradation of alpha-1-antitrypsin in chronic wounds (Rao et al., 1995, Krishna et al., 1997, Yager et al., 1997).

High levels of proteases, such as MMP-8 (Nwomeh et al., 1999) and MMP-9 (Wysocki, 1996), capable of cleaving the alpha-1-antitrypsin protein, are present in chronic wounds (Vissers et al., 1988, Desroschers et al., 1992). More recently, Nie and Pei (2004), have demonstrated *in vitro* that neutrophil-derived leukocidin (MMP-6) cleaves this molecule at the $Phe^{376}Leu$ and $Pro^{381}Met$ bonds, leaving it unable to maintain its activity against elastase, cathepsin G, or proteinase 3. This evidence suggests that MMP-6 is more effective at inactivating alpha-1-antitrypsin than either MMP-8 or MMP-9. However, determinations of MMP-6 levels in acute and chronic wound fluids have yet to be published in the literature.

A deficiency in alpha-1-antitrypsin manifests itself clinically with a predisposition to emphysema, liver disorders, skin disease and vasculitis. Current treatment for the disorder includes cessation of smoking, vaccinations, supplemental oxygen, or the augmentation therapy approach of an infusion of purified pooled human plasma alpha-1-antitrypsin (Stoller and Aboussouan, 2005).

1.7 Cytokines

Cytokines orchestrate the cellular activities associated with inflammation and wound healing. Initially, cytokines are released from platelets, influencing tissue oedema and initating inflammation. All cytokines activate complement and coagulation processes. A cytokin is defined as a small, 5-30kDa, soluble (glyco) protein, non-immunoglobulin in nature, released by living cells of the host (Nathan and Sporn, 1991). An important feature of cytokines is the ability of an individual cytokine to elicit varying responses, acting at picomolar to nanomolar concentrations (Henry and Garner, 2003).

As many cytokines have been described, numerous terminologies are associated with this class of molecule, with many families having arisen. Such family classifications include the growth factors, interleukins, chemokines, interferons, lymphokines and monokines (Henry and Garner, 2003). Some cytokines belong to multiple families, reflecting their multiple actions. The cellular source and biological functions of the cytokines relevant to the present Study are summarized in Table 1.5. Of the particular cytokines investigated in the present Study, those that have been described in the wound healing process will be described further. Cytokines have been catergorised as (i), pro-inflammatory cytokines, (ii) IL-1 to -13), (iii) angiogenic cytokines and growth factors; angiogenin, TGF and VEGF, (iv) cell surface receptors; TNF-r2 and intra-cellular adhesion molecule (ICAM) and, (v) IFN-γ, and inducible protein-10.

Table 1.5: Cytokine cellular source and biological function.

Cytokine	Cellular source/s	Major biological effects
Pro-inflammatory cytokines		
IL-1β	Astocytes Adrenal cortical cells NK cells Monocytes/macrophages Endothelial cells Keratinocytes Megakaryocytes and platelets Neurons Neutrophils Oligodendroglia Osteoblasts Schwann cells Trophoblasts T-cells Fibroblasts	(i) Regulation of inflammatory response, pro-inflammatory. (ii) Bone formation and re-modeling. (iii) Insulin secretion. (iv) Appetite regulation. (v) Fever induction. (vi) Neutronal phenotype development. (vii) IGF/GH physiology. (viii) Required for efficient production of IFN-γ. IFN-γ activation of macrophages. (ix) Induces expression of MMPs from resident fibroblasts resulting in (1), ECM degradation to facilitate monocyte migration, (2), MMPs degrade IL-1β, causing down modulation of the response.
IL-6	Many cells express IL-6 including; Fibroblasts Mast cells Keratinocytes Monocytes Neutrophils Osteoblasts	(i) Il-6 production is generally correlated with cell activation. (ii) Serves as a pro-inflammatory and anti-inflammatory molecule. (iii) Modulator of hematopoiesis. (iii) Inducer of plasma cell development. (iv) Influences IL-4 production.
TNF-α	Neutrophils Activated B- and T-cells NK cells LAK cells Astrocytes Endothelial cells Smooth muscle cells Some transformed cells	(i) Mediators of host resistance to infections and tumour progression. (ii) Over-production can lead to pathologies like wasting, systemic toxicity and septic shock. (iii) Promotes inflammatory cell infiltration by up-regulating leukocyte adhesion molecules on endothelial cells. (iv) Chemotactic agent for monocytes. (v) Activate phagocyte killing mechanisms.

Cytokine	Cellular source/s	Major biological effects
Interleukins		
IL-2	Activated Th cells Thermocytes Gamma/delta T-cells B-cells $CD4^+$ /$CD8^+$ T-cells Neurons Astroctyes	(i) Directs synthesis and secretion of additional interleukins, mainly from Th cells. (ii) Clonal expansion of antigen-specific T-cells (iii) Mediation of the immune response.
IL-4	Activated T-cells Mast cells Basophils	(i) Regulator of isotype switching, inducing IgE production by B cells. (ii) Modulator in differentiation of precursor Th cells to the Th2 subset. (iii) Anti-tumour activity.
IL-5	Eosinophils NK cells $CD8^+$ T-cells Mast cells $CD45^+$ $CD4^+$ T-cells Il-1β activated B-cells	(i) Activity on B cells and eosinophils. (ii) Differentiation of B cells to Ig-secreting cells. (iii) Growth of B-1 progenitors and IgM production in B-cells. (iv) Multiple actions on eosinophils.
IL-8	Many cells including; Monocytes/macrophages T-cells Neutrophils Fibroblasts Endothelial cells Keratinocytes	(i) Potent neutrophil chemotactic and activating factor. (ii) Primary inflammatory cytokine.
IL-10	T- and B-cells Activated monocytes	(i) Cytokine synthesis inhibitory factor. (ii) Modulator of monocyte/macrophage function. (iii) Down-regulator of the cell-mediated immune response. (iv) Surpresses the production of prostaglandin E2, and numerous pro-inflammatory cytokines, including TNF-alpha, IL-1, Il-6, and IL-8 by monocytes following activation. (v) Macrophage activator and deactivator and exhibits potent anti-inflammatory activities. (vi) Marked effects on B-cells. (vii) Anti-inflammatory response in LPS activated neutrophil cells.
IL-12p40/p70	Monocytes/ macrophages Dendritic cells B lymphocytes	(i) Stimulate cytotoxicity. (ii) Proliferation. (iii) Cytokine production and Th1 subset development.

Cytokine	Cellular source/s	Major biological effects
Angiogenic cytokines and growth factors		
Angiogenin	Vascular endothelial cells Smooth muscle cells Fibroblasts Colonic epithelium Peripheral blood Lymphocytes Lung and colonic epithelial tumour cell lines Adrenocarconomas	Neovascularisation/ angiogenesis activities include; (i) Endothelial cell migration, proliferation, and differentiation. (ii) Bind cell-associated actin and the resulting complex can accelerate the generation of plasmin by tissue plasminogen activator (tPA) (iii) No impact on plasmin activity (degrades basement membrane) (iv) Initial stage of angiogenesis is impacted by angiogenin activity Non-angiogenic activities include; (i) Inhibition of neutrophil degranulation. (ii) Stimulation of cholesterol esterification. (ii) An un-defined role in liver.
TGF-b1	Synthesised by virtually all cells (with only a few exceptions). TGF receptors are expressed by all cells	Transforming growth factor, a 'factor' that transforms fibroblasts into a tumour-like phenotype. Many activites, three fundamental activities (i) Modulates cell proliferation (suppresses). (ii) Enhances deposition of ECM through promotion of synthesis and inhibition of degradation. (iii) Immunosurpressive.
VEGF	Most cell types, but usually not endothelial cells themselves.	(i) Induces angiogenesis and endothelial cell proliferation. (ii) Increase vascular permeability. (iii) Causes vasodilation, through stimulation of nitric oxide synthetase in endothelial cells. (iv) Stimulate cell migration and inhibit apoptosis.
Cell surface receptors		
ICAM	Leukocytes Endothelial cells	Intracellular adhesion molecules (ICAMs), Ig Superfamily of adhesion molecules. (i) Bind integrins on leukocytes and mediate flattening onto blood vessel wall and subsequent extravasation.
TNF-r2	All cells show the presence of one or both TNF receptor types.	(i) High affinity for both TNF-α, and TNF-β. But immunologically distinct.

Cytokine	Cellular source/s	Major biological effects
IFN-γ	T-cells Natural killer cells	(i) Inhibitor of viral replication and a regulator of immunological functions. (ii) Class of antibody production from B-cells. (iii) Up regulates MHCI and MHCII complex antigens. (iv) Increases killing of parasites by macrophage.
IP-10	Primarily leukocytes Also Epithelial cells Dendritic cells Neutrophils	Interferon inducible protein 10 (IP10), CXCL10, (i) Angiostatic chemokine. Genes encoding CXC chemokines are clustered on chromosome 4, homolog peptides that exhibit positive or negative activity on the control of angiogenesis. Block angiogenesis through interaction with the receptor, CXCR3-B, expressed mainly in micro-vascular endothelial cells. CXCR3 expression could be a self-regulatory mechanism to avoid excessive angiogenesis. Chemokines are multifunctional mediators mainly responsible for leukocyte recruitment to inflamed areas.

1.7.1 Pro-inflammatory cytokines

Although in general, the effects of cytokines are exerted locally at their site of production (autocrine and paracrine), TNF-α, TNF-β, IL-1 and IL-6, have major systemic (endocrine) effects. Cytokines contribute to both the inflammatory response and host defense. Infection can cause the acute, systemic release of cytokine, in large quantities, as in the case of bacterial sepsis, or the chronic release of cytokine, in lesser amounts, as in the case of chronic infections (Feghali and Wright, 1997).

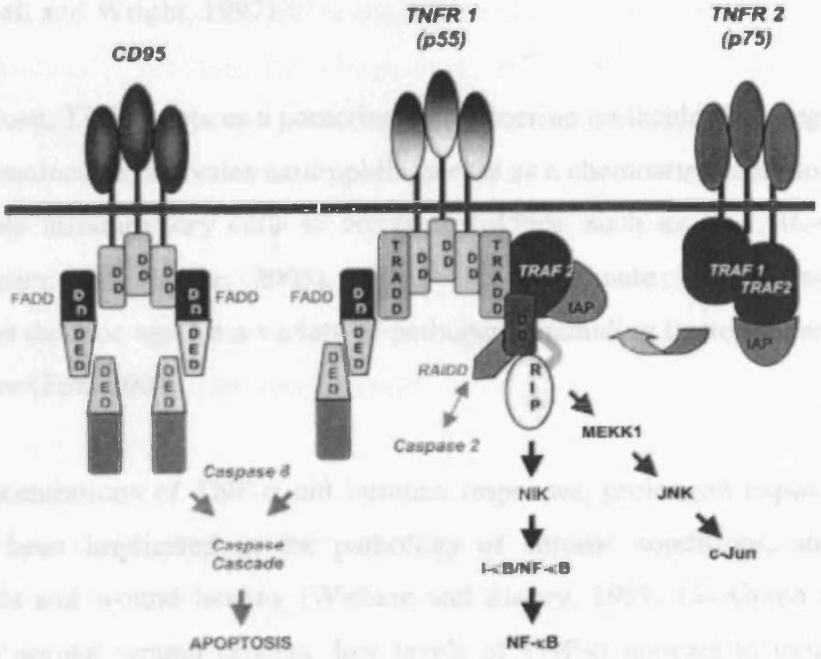
1.7.1.1 Tumour necrosis factor

TNF-α first appeared in serum, believed to be macrophage-derived, and caused regression of some transplanted tumours *in vivo*, and was cytostatic/cytocidal for tumour cells *in vitro*, but not normal fibroblasts (Steinbeck and Roth, 1989). It has since been determined that macrophages are the primary source of TNF, although many cells can produce this molecule (Fox, 2000). There are two forms of the molecule, TNF-α (produced by neutrophils, activated T- and B- cells, natural killer cells, LAK cells, astrocytes, endothelial cell, smooth muscle cells and some transformed cells) and TNF-β, produced by lymphocytes.

Soluble TNF-α is derived from its 26kDa membrane precursor, by enzymatic cleavage with the metalloprotease, termed TACE (TNF converting enzyme). Cleavage occurs at the extracellular domain between Ala76 and Val77 residues and releases the 17kDa soluble form of TNF (Coeshott et al., 1999, Liz-Grana et al., 2001).

Responses to both forms of TNF are mediated by two membrane receptors, TNF-R1 (p55) and TNF-R2 (p75), and soluble TNF receptors (sTNF-R), which are proteolytically cleaved from the membrane-associated TNFrs (Wallace and Stacey, 1998, Lin et al., 2000). Phorbol esters, IL-1, IL-6, TGF-β, retinoids and TNF itself reduce the synthesis of these receptors. Regulation of TNF receptors is stimulated mainly by IFN-γ and this may explain the synergism between these two cytokines. Ligation of membrane bound TNF-α receptors into dimers or trimers, results in the activation of cellular signaling pathways (Figure 1.15) (Liz-Grana et al., 2001). The two main transcription factors activated by TNF are NF-kB and AP-1. Like IL-1 and LPS, TNF-α signaling occurs through the activation of NF-kB, which in turn enhances the transcription of genes that mediate innate immune responses (Keane and Strieter, 2002). TNF is related to both the innate and specific immunity.

Figure 1.15: TNF signaling pathways.
Signaling pathway for TNFR-1 and TNFR-2. Optimal signaling activity requires receptor trimerisation. TNFR-1 initially recruits TRADD and induces apoptosis through the actions of proteolytic enzymes known as capases. RIP has a functional component that can initiate NF-κB and c-Jun activation, both favouring cell survival and pro-inflammatory functions. TNFR-2 recruits adaptor proteins, known as TRAF1 and TRAF2, that interact with RIP to mediate NF-κB and c-Jun activation. TRAF2 also recruits additional proteins that are anti-apoptotic, known as IAP (inhibitors of apoptosis protein) (Lin et al., 2000).



At high concentrations, TNF-α can enter the bloodstream and becomes an endocrine hormone, where it acts as a pyrogen. Although the half-life of TNF-α is less than 20 minutes, it can stimulate excessive cytokine liberation from mononuclear cells, activates the coagulation system and suppresses bone marrow stem cell maturation (Lin et al., 2000). The consequence of this endocrine activity is the induction of tissue damage, disseminated intravascular coagulation, fever, shock and ultimately death (Fox, 2000). Additionally, the systemic effects of TNF-β in response to antigens, such as staphyloccocal toxic shock syndrome toxin, are presented clinically as fever and hypotension (Feghali and Wright, 1997).

At low concentrations, TNF-α acts as a paracrine and autocrine molecule. It upregulates vascular adhesion molecules, activates neutrophils, serves as a chemoattractant molecule stimulating multiple inflammatory cells to secrete cytokines, such as IL-1, IL-6 and more TNF-α (Henry and Garner, 2003). This aids the innate immune system, contributing to host defence against a variety of pathogens, including bacteria, parasites and possibly viruses (Fox, 2000).

Although low concentrations of TNF-α aid immune responses, prolonged exposure to the cytokine has been implicated in the pathology of chronic conditions, such as rheumatoid arthritis and wound healing (Wallace and Stacey, 1998, Liz-Grana et al., 2001). Within the normal wound process, low levels of TNF-α appears to indirectly enhance collagen deposition, increases wound tensile strength, is mitogenic for wound fibroblasts and stimulates the synthesis of MMPs (Mast and Shultz, 1996). However, elevated levels of TNF-α are detected in chronic wounds, where the ratio of the cytokine to its soluble receptor favours the cytokine (Wallace and Stacey, 1998). This leads to increased secretion of MMPs from cells, such as fibroblasts and inflammatory cells, thereby contributing to excessive ECM degradation and the degradation of growth factors and their receptors (Mast and Shultz, 1996, Yager and Nwomeh, 1999, Baker and Leaper, 2000).

1.7.1.2 Interleukin-1

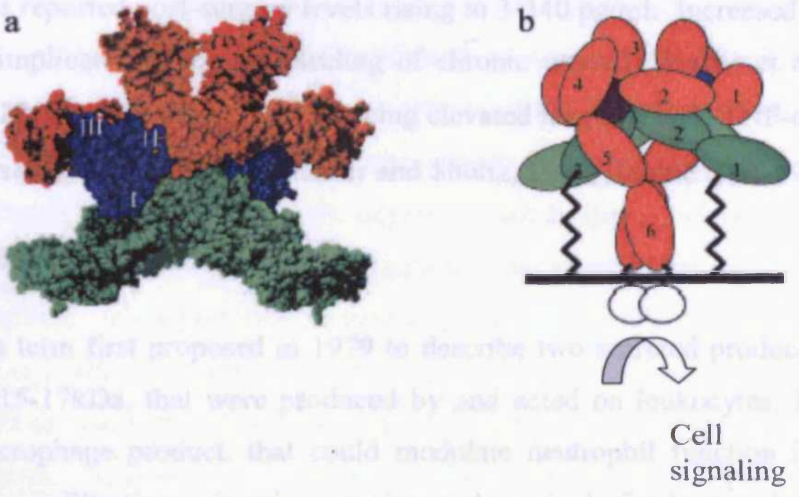
There are two forms of IL-1, IL-1α and IL-1β. IL-1α, an active 33kDa molecule, is located on cell membranes and relies upon cellular contact for biological activity (Lin et al., 2000). Conversely, IL-1β is synthesized as pro-IL-1β, which is biologically inactive, and is subsequently cleaved to its soluble, 17kDa biologically active peptide (Coeshott et al., 1999). IL-1β is readily detected in the circulation.

IL-1 is produced by mononuclear cells in response to LPS and other stimuli, similar to TNF-α. Although they are structurally different and bind different receptors, TNF-α and IL-1β share most biological functions (Liz-Grana et al., 2001). Previous nomenclature, endogenous pyrogen and mononuclear cell and lymphocyte activating factor (LAF), hint at the molecules importance in inflammatory responses (Feghali and Wright, 1997). The pro-inflammatory effects of IL-1, are inhibited by IL-1 receptor antagonist (IL-1Ra). The ratio of the two molecules favours the antagonist in acute wound healing and the pro-inflammatory cytokine in chronic wounds (Mast and Schultz, 1996), thus contributing to the non-healing wound environment.

1.7.1.3 Interleukin-6

Many cells produce IL-6, most notably fibroblasts, monocyte/macrophage and neutrophils. Much debate surrounded whether neutrophils produced IL-6 until evidence was provided, showing both the synthesis and secretion from the cell (Cassatella, 1995). IL-6 is a 26kDa molecule consisting of 184 amino acids. It is essential that IL-6 binds gp130, and this combined structure, binds the receptor IL-6R, causing activation and a signal (Figure 1.16). Interaction of IL-6 with its receptor induces homodimerisation of gp130 (Kishimoto et al., 1995). IL-6 causes the transcription of a variety of proteins through three major signal transduction pathways, protein kinase C, camp/protein kinase A and the calcium release pathway (Nakajima et al., 1995).

Figure 1.16: IL-6, IL-6R and gp130 binding, translating to an intracellular signal. (a) space filling model of two molecules of IL-6 (blue) binding to the IL-6R (green) dimer by means of site I, and two gp130 (red) molecules each binding through sites II and III. (b) Orientation of gp130 toward cell membrane, and signaling activated by dimerization and potential cross-linking (Varghese et al., 2002).



IL-6 has a wide range of immune and hematopoietic activities. It enhances the innate immune system, through the synthesis of acute phase proteins, c-reactive protein (CRP), that increases the rate of phagocytosis of bacteria, and serum amyloid A (SAA) (Lind, 2003). IL-6 induces neutrophil activation, but also may delay the phagocytic disposal of senescent or dysfunctional neturophils, thereby delaying the injurious nature of the cell and protecting against tissue damage. Additionally, IL-6 is reported to promote the release of sTNFRs and IL-1α, during tissue injury, limiting the effects of TNF and IL-1, respectively (Lin et al., 2000). IL-6 can also inhibit the production of TNF itself (Feghali and Wright, 1997), thereby serving a role as an anti-inflammatory molecule, limiting the acute inflammatory response, in addition to its pro-inflammatory properties (Lin et al., 2000).

TNF-α, IL-1β and IL-6 share several pro-inflammatory properties, such as inducing fever, stimulating the production of collagenase and the induction of acute phase protein production. Upregulation of IL-6 production has been observed in a variety of chronic inflammatory and autoimmune disorders such as type I diabetes and rheumatoid arthritis (Feghali and Wright, 1997). Circulating levels of IL-6 in the blood are typically 1 pg/ml, with levels reported post-surgery levels rising to 3-340 pg/ml. Increased levels of IL-6 have been implicated in the non-healing of chronic wounds (Harris et al., 1995, Trengrove et al., 2000), with studies also showing elevated levels of both TNF-α and IL-1β in chronic verses acute wounds (Tarnuzzer and Shultz, 1996, Barone et al.,1998).

1.7.2 Interleukins

Interleukin was a term first proposed in 1979 to describe two secreted products with a similar mass of 15-17kDa, that were produced by and acted on leukocytes. IL-1 was given to the macrophage product, that could modulate neutrophil function including their accumulation, infiltration, migration, number and survival after bacterial challenge (Steinbeck and Roth, 1989). The actions of IL-1 and IL-6 have been described (Chapter 1, Section 1.7.1), the other interleukins relevant to the present Study are discussed here.

IL-2 is produced and secreted by activated T-cells, the effects of which include the expansion of antigen specific cells and altering the activity of B cells, natural killer cells. IL-2 is a product of the Th1 subset of T-cells (Chapter 1, Section 1.5), that contribute to antibody-mediated cellular responses. Its study in the wound healing arena has been limited, but has been studied in relation to transplantation, cancer and infectious disease.

IL-4 is produced by Th2 cells being important in antibody-mediated responses and in antigen presentation. It serves an anti-inflammatory molecule in wound healing, where it can down regulate the activities of IL-1β, IL-6, IL-8 and TNF-α from activated macrophages and can also promote collagen synthesis (Lin et al., 2000).

IL-5 is a cytokine produced by many cells, whose functional activities include the co-ordination of the eosinophils inflammatory processes, promotes eosinophil growth and differentiation and B-cell responses (Takatsu et al., 2005, Simon and Simon, 2007). However, its role in wound healing has not been extensively studied.

IL-8 is an interleukin, but is also known as a chemokine. Chemokines are a group of small proteins (8-14kDa), divided into four subfamilies, based on structural properties and primary amino acid sequence (Onuffer and Horuk, 2002, Romagnani et al., 2004). Chemokines play a role in the migration of leukocytes to sites of inflammation with IL-8, known to be responsible for chemotactic migration and activation of neutrophils and other cell types (Feghali and Wright, 1997). Additionally, chemokines other biological roles include dendritic cell maturation, macrophage activation, B-cell antibody class switching and T-cell activation (Wong and Fish, 2003). Neutrophils are capable of producing IL-8, thus, once these cells are in the infected site, the presence of this cytokine (IL-8) attracts additional neutrophils into the area (Cassatella, 1995). The time course for the production of IL-8 coincides with increased angiogenesis in wounds, resulting in the formation of new blood vessels (Romangani et al., 2004). In addition to this early involvement within normal wound healing, IL-8 appears to stimulate keratinocyte proliferation and integrin expression, thereby possibly promoting re-epithelialisation (Rennekampff et al., 2000). However, it is possible that elevated levels of IL-8 may retard wound-healing processes (Iocono et al., 2000, Henry and Garner, 2003).

IL-10 is an anti-inflammatory cytokine, synthesised by monocytes, B- and Th cells, contributing to lymphocyte differentiation and proliferation (Cassatella, 1995). IL-10 has many roles, including its ability to inhibit the synthesis of pro-inflammatory cytokines, modulating the activity of TNF-α (Lin et al., 2000), inhibiting antigen presentation and stimulating Th2 cell differentiation (Boniface et al., 2005). Elevated levels have been reported in chronic venous leg ulcers by Lundberg et al. (1998), who suggest these levels are sufficient to delay wound healing, possibly by impairing cell mediated immunity responses (Li et al., 1998).

IL-12 may contribute to the host inflammatory response after injury or severe infection. It is capable of inducing an inflammatory response for 48 hours in nonhuman primates. Its major biological function in cell-mediated immunity and promoting the differentiation of Th1 cells. It also activates neutrophils and the expression of both pro- and anti-inflammatory cytokines (Lin et al., 2000).

IL-13 modulates macrophage function, inhibits nitric oxide production and the expression of pro-inflammatory cytokines. Like IL-4 and IL-10, its main purpose is an anti-inflammatory molecule.

1.7.3 Angiogenic cytokines and growth factors

There are two related, although functionally distinct forms of TGF, both serving roles in wound healing processes. TGF-α, belonging to the epidermal growth factor family of mitogens, and TGF-β, a superfamily encompassing a diverse range of proteins. TGF-α binds the EGF receptor, and may serve a role in re-epithelialisation. However, its importance within wound healing processes is still to be determined. It appears that in its absence, the effects of TGF-α can be substituted by other endogenous growth factors, with no detrimental impact on the healing process (Werner and Grose, 2003).

Three forms of TGF-β exist; TGF-β_1, TGF-β_2, and TGF-β_3. Active forms of TGF-β, are synthesized from their latent precursors, and they bind a heteromeric receptor complex, thereby initiating various signaling cascades. TGF-β is present throughout the whole wound healing process, and has been shown to influence angiogenesis, fibroblast proliferation, matrix deposition (stimulating the expression of ECM proteins), and re-epithelialisation. Yet within wounds, TGF-β is inhibitory to most cells including keratinocytes.

The application of exogenous TGF-β has been found to be beneficial to wound repair, with respect to the rate of wound healing and strength of healed wound (Werner and Grose, 2003). Although TGF-β seems to be an essential component to ensure normal wound healing, with decreased amounts reported in chronic wounds, it also appears to have a detrimental effect, with higher levels associated with excessive scar formation (Henry and Garner, 2003).

Angiogenin and vascular endothelial growth factor (VEGF) are known for their roles in neovascularisation/angiogenesis. New blood vessels can arise from existing vasculture induced by various factors including, hypoxia, nitric oxide, and growth factors such as, VEGF and fibroblast growth factor-2 (FGF-2) (Neal, 2001).

Angiogenin induces blood vessel formation, and its activity can be regulated by its activation or inhibition of its ribonucleolytic activity in addition to cellular mechanisms. Angiogenin can bind endothelial cells, transmigrate to the nucleus and induce neovascularisation. Angiogenin circulates in the plasma at 250-360ng/ml (Hu, 1997). Angiogenin can be cleaved by elastase, but also has the ability to inhibit neutrophil degranulation (Tschesche et al., 1994).

Different forms of VEGF exist, including diffusible proteins $VEGF_{121}$ and $VEGF_{165}$, in addition to proteoglycan-bound $VEGF_{189}$ and $VEGF_{206}$, that can bind three receptors, dictating the biological response (Folkman, 1997, Drinkwater et al., 2003). VEGF is involved with the regulation of angiogenesis during wound repair. Gene expression has been observed post-injury, with reduced angiogenesis, fluid accumulation and granulation tissue formation in an *in vivo* model, upon application of $VEGF_{121}$ neutralizing antibodies (Werner and Grose, 2003).

In chronic wounds, VEGF mRNA is strongly expressed, however its staining is weak within the epidermis. This decreased immuno-reactivity for VEGF was attributed to the presence of serine proteases, with chronic wound fluid, degrading rVEGF165 *in vitro* (Lauer et al., 2000). Another study has shown that non-healing venous leg ulcers have increased VEGF expression at both the gene and protein level, yet angiogenesis is inhibited (Drinkwater et al., 2002, 2003).

1.7.4 Cell surface receptors

TNF receptor-2 (TNF-r2) is one of the two membrane receptors that binds TNF (Chapter 1, Section 1.7.1).

Intracellular adhesion molecule-1 (ICAM-1) belongs to a family of molecules that allow leukocyte rolling and attachment to endothelial cells, enabling migration of these cells (Lind, 2003). Athough ICAM-1 is an essential mediator within normal wound healing processes (Pierce et al., 2001), increased expression of ICAM-1 has been observed histologically within venous leg ulcer tissue sections (Wilkinson et al., 1993, Hahn et al., 1997). This expression has been linked with the increased number of mast cells, macrophages and T-lymphocytes, that may contribute to the inflammatory nature of this wound type. IFN-γ appears to preferentially induce the expression of ICAM-1 and could possibly be a more important adhesion promoter than TNF-α (Wilkinson et al., 1993).

1.7.5 Interferon-γ and Inducible Protein-10

IFN-γ prevents viral replication in target cells. Dendritic cells demonstrate 10^3-fold higher production of IFN, compared with fibroblasts when virally challenged (Bonjardim, 2005). IFN-γ is also produced by T-cells and natural killer cells, and has multiple actions in inflammation and on many cell lineages (Cassatella, 1995). IFN-γ is an important positive regulator of inflammatory responses, priming neutrophils to secrete IL-1β, TNF-α, and IL-8, and activating circulating and tissue macrophages (Lin et al., 2000). IFN-γ can maintain neutrophil phagocytic action and is believed that, like TNF-α, it can prevent adherence and decrease migration, thus localising neutrophils to the site of infection (Steinbeck and Roth, 1989).

IFN-γ also has a number of inhibitory roles within wound healing, inhibiting fibroblast proliferation, matrix production, and in addition to IL-12 and IP-10, can inhibit the re-vascularisation process (Duncan and Berman, 1985, Ghosh et al., 2001).

In wound healing, chemokines provide a regulatory role in the angiogenic and inflammatory reactions. The chemokine, IP-10, has an angiostatic activity, mainly by binding VEGF directly, preventing the formation of VEGF homodimers, or competing with cell surface receptors (Romagnani et al., 2004). The prevention of unlimited angiogenesis within the wound is believed to be due to the action of IP-10, with IP-10 also appearing to co-ordinate the re-epitheliasation process (Romagnani et al., 2004). Additionally, controlled angiogenesis within the wound may be regulated by the balance of the pro-angiogenic molecule, IL-8, and the angiostatic molecule, IP-10 (Rosenkilde and Schwartz, 2004). IP-10's inhibitory action over endothelial cell differentiation may increase the inflammatory response, alter ECM deposition, with disorganized granulation tissue formation (Medina et al., 2005). IP-10 is believed to be involved in Th1 directed responses, thereby serving as an antagonist of Th2 driven responses (Onuffer and Horuk, 2002, Moser et al., 2004).

1.8 Chronic wound infection

1.8.1 Bacterial colonisation of chronic wounds

It is accepted that all chronic wounds are colonised with a wide diversity of bacteria (Bowler and Davies, 1999). It is thought that the bacteria within chronic wounds have a beneficial role, by increasing the inflammatory response and contributing to the formation of granulation tissue (Robson, 1997). Microorganisms exist within chronic wounds, and these wounds persist for months or years. However, these organisms are usually of little threat to the patient (Freidman et al., 1984). Yet, when the bacteria outweigh the host's ability to defend, infection ensues (Chapter 1, Section 1.4).

A literature review of microbiological studies by Bowler (1998), Bowler and Davies (1999) and Bowler (2003), has revealed that the most predominant bacteria of chronic wounds are *S. aureus* in diabetic foot ulcers, with other aerobes including *S. epidermidis*, *Streptococcus* spp., *P. aeruginosa*, *Enterococcus* spp., and coliform bacteria.

S. aureus appears to be the most prevalent bacteria in leg ulcers, but there is also a significantly greater frequency of anaerobes. Anaerobes are believed to be isolated from 30% of leg ulcers, but 95% of diabetic foot ulcers. In all studies that formed part of this review, the aerobic population was always greater then the anaerobic population. Of the anaerobes, the most frequently reported were *Bacteriodes* spp. and *Peptostreptococcus* spp. The low amount of anaerobes that were reported may be a consequence of the extent to which they were investigated. A mixed microflora is observed in pressure ulcers, with *S. aureus, Peptostreptococcus* spp., *Bacteriodes* spp., and *P. aeruginosa.*

The most commonly cultured bacteria from chronic wounds are represented in Table 1.6. The bacterial flora in the tissue of a chronic wound, is different to that found in acute wounds (Robson, 1997). The frequency at which *S. aureus* is observed in chronic wounds, is not surprising, as it is a bacteria of the skin, and previously reported to be present in 65% of all wounds (Colsky et al., 1998).

Table 1.6: Types of organisms commonly associated with chronic wounds.

Gram Positive		Gram Negative	
Aerobe	**Anaerobe**	**Aerobe**	**Anaerobe**
Staphylococcus	*Peptostreptococcus*	*Escherichia*	*Bacteriodes*
Streptococcus	*Clostridium*	*Pseudomonas*	*Prevotella*
Enterococci		*Klebsiella*	*Fusobacterium*
			Porphyromonas

The types of bacteria in acute and chronic ulcers have been well reported, and a greater frequency of anaerobes in infected than non-infected venous leg ulcers observed (Bowler, 2003). Of the aerobic population, *S. aureus* is the most commonly observed Gram-positive aerobe. A recent study by Gardner et al. (2004), has shown that nonarterial wounds containing *S. aureus*, have a higher bacterial bioburden than those without the organism, but the presence of this microorganism does not increase the microbial diversity in the wound. The significance of these findings on wound healing or wound infection, remains to be determined.

The negative impact of bacteria within the chronic wound has been reported in a recent study (Davies et al., 2007), whereby the presence of four or more bacterial genera within the wound correlated with a non-healing ulcer. A discrepancy was revealed in both of these studies, between the microbial load cultured and the clinical diagnosis of infection (Chapter 1, Section 1.8.3). *Staphylococcus* spp. and *Pseudomonas* spp. are aerobic pathogens, detectable in all wound types, and predominant in chronic wounds (Davies et al., 2004). Their general microbial characteristics and the mechanisms by which these can evade host defences, will be described further.

1.8.1.1 *Staphylcoccus* spp.

Staphylococcus spp. are Gram-positive cocci, typically 1μm in diameter, and are fermentative (capable of producing acid from glucose anaerobically). Staphylococci divide in two planes, and can be observed microscopically as clusters, often described as resembling bunches of grapes (Schaechter et al., 1993). *S. aureus* is a known pathogen. This species is coagulase, DNase and phosphatase positive, forms acid from mannitol, trehalose and sucrose and is sensitive to novobiocin. Depending on the age of the culture, the pigmentation of the colony can vary from none to golden yellow. Staphylococci are present in the nose, and on the skin in a large proportion of the population, with 20% persistently colonised, and 50% are intermittent carriers (Gillepsie and Bamford, 2000, Gerold et al., 2007). Staphyloccocci can be spread upon contact or by airborne routes. It is therefore possible to spread the bacteria during activities relevant to hospital care, such as bed making (Mayer-Scholl et al., 2004).
Subpopulations of *S. aureus* include antibiotic resistant strains, such as Methicillin-resistant *S. aureus* (MRSA) and small colony variants. MRSA is commonly associated with hospital infections. 54.4% of *S. aureus* isolates from surgical wounds were found to be methicillin resistant (Giacometti et al., 2000). Small colony variants (SCV) demonstrate characteristics atypical of *S. aureus* that it is believed affords them survival advantages. These SCVs manage to evade host defences by hiding within the host cell, and waiting until the host immune system is abated and antibiotic therapy completed. The organism then quickly reverts to a highly virulent form and lyses the host cell (Proctor and Peters, 1998).

S. aureus infections include boils, styes, pneumonia, phlebitis, urinary tract infection and deep-seated infections, such as osteomyelitis (Asensi et al., 2004) and endocarditis. It also causes food poisoning and toxic shock syndrome. *Staphylococcus* spp., have a number of approaches to allow binding, invasion and destruction of tissues, as summarised in Table 1.7. Virulence is multifactorial, including cell-associated and extracellular products (Todar, 2004), although no single virulence factor has been identified as responsible for tissue damage (Socransky and Haffajee, 1991).

Table 1.7: The mechanisms by which *Staphylococcus* spp. can bind, invade, evade host defences by destruction of tissues (Wadstrom, (1987), (*), Villavicencio and Wall, (1996) (+), Scholl et al., (2004) (#), Todar, (2004) (^)).

***Staphyloccoccus* spp. component or mechanism**	**Effects: Such as, binding, invasion and evasion of host defence mechanisms with biological/pathological consequences**
Surface Hydrophobicity	Binding of microorganism to host cell. Colonisation of various surgical aids, adjuncts. *
Surface proteins	Binding of microorganism to host cell. Ability to bind fibronectin, gelatin coated surfaces and laminin (components of the extracellular matrix). Presence of protein A interacts with host IgG antibodies, reducing opsonisation and causing local activation of complement. * +
Protein A	
Fibronectin binding protein	
Fibrinogen binding protein	
Collagen binding protein	
ExoToxins	Released and travel though body, systemic effects.
Cytotoxins (leukocidin	Necrosis, hemolysis, can lyse neutrophils. +
Exfoliatin	Scalded skin syndrome.
TSST-1	Aids invasion. Superantigen, capable of causing non-specific inflammatory response, resulting in toxic shock. Causative for toxic shock syndrome. +
Enterotoxins	Superantigen +, CNS effect, nausea.
Haemolysins	Lyse cell membranes.^
Epidermolytic toxin	
Capsule	Inhibit phagocytic engulfment.^ #
Cell Wall and membrane	Resistance to host defence proteins, defensins. #
Clumping factor	Induces cell clumping. Fibronectin, collagen and fibrinogen adhesions are known as clumping factor. Specific adherence factors associated with endocarditis and bacterial arthritis. * +
Tissue degrading enzymes	
Staphylokinase (fibrinolysin)	Aid invasion. Promote spread in tissues. *^
hyaluronidase	
lipase	

1.8.1.2 *Pseudomonas* spp.

Pseudomonas spp. are Gram negative rods, typically 3μm x 0.5μm, and are motile by flagella. They are oxidase positive, oxidise glucose (or produce no acid) and do not produce gas. *Pseudomonas* use a very wide range of carbon and energy sources and are able to grow over a wide temperature range. They do not usually grow anaerobically and some produce a fluorescent pigment (Collins and Lyne, 2004).

P. aeruginosa demonstrate a variety of colonial forms, with a greenish pigment, giving the characteristic green colour to a contaminated wound. Two pigments are formed, pyocyanin and (fluorescent) pyoverdin. The organism is very resistant to antibiotics, mainly due to its low outer membrane permeability and to active efflux of antibiotics (Stover et al., 2000). It is commonly found in the soil and in water, and is an opportunist pathogen for humans. *Pseudomonas* can be cultured from clinical material, for example, the sputum of patients with cystic fibrosis.

P. aeruginosa will infect any body site, given the correct conditions and spread anywhere there is a moist environment by either direct or indirect contact from those sites. *P. aeruginosa* can cause skin and burn infections, is the major lung pathogen in cystic fibrosis patients and can cause pneumonia in intubated patients. In addition, it has also been reported to cause urinary tract infections, septicaemia, osteomyelitis and endocarditis (Todar, 2002). The long-term colonisation of *P. aeruginosa* in the lungs of cystic fibrosis, is the predominant cause of diseases morbidity and mortality (Stover et al., 2000). As with *S. aureus*, virulence is multifactorial, with a number of multiple and diverse pathogenic factors (Table 1.8), contributing to virulence, which may explain the wide array of diseases caused by the organism.

Table 1.8: The mechanisms by which *Pseudomonas* spp. can bind, invade, evade host defenses by destruction of tissues (Wadstrom, (1987), (*),Heggars, (1998) (+), Todar, (2002), (^)).

***Pseudomonas* spp. component or mechanism**	**Effects: Such as, binding, invasion and evasion of host defence mechanisms with biological/pathological consequences**
Surface Hydrophobicity	Binding of microorganism to host cell. Colonisation of various surgical aids, adjuncts. *
Fimbriae	Adhesin. Binds galactose, mannose or sialic acid receptors. ^
Proteases	Help bacteria invade tissues.
Elastase	Cleaves collagen, IgG, IgA and complement. ^+
Alkaline phosphatase	Interferes with fibrin formation, and will lyse fibrin.*
Capsule (extracellular slime layer)	Protects cells from opsonisation by antibody, complement and phagocytic engulfment. ^ +
Haemolysins	Contribute to invasion. Break down lipids and lecithin.^
Exotoxins	
Cytotoxin (leukocidin)	Lyses neutrophils and other human cell types.^
Exotoxin A	Actions as diptheria toxin. Necrotising action at the site of colonisation, systemic role, lethal to host.+
Exoenzyme S	Particularly in burned tissue. May impair function of phagocytic cells, prepare organs for invasion. ^
Endotoxin, e.g. LPS	Associated with Gram negative septicemia. +
Pigment	
Pyocyanin	Impairs nasal cilia, induces pro-inflammatory effect on phagocytes.^
Pyochelin (a derivative of pyocyanin)	Is a siderophore, sequesters iron from the environment for growth.^

Both *S. aureus* (Gram-positive) and *P. aeruginosa* (Gram-negative) are capable of eliciting damage to the host by activation of toll-like receptors. Lysis of Gram-positive organisms causes the release of teichonic acid and peptidoglycan monomers. Gram-positive bacteria have more peptidoglycan than Gram-negative bacteria (Yoshimura et al., 1999). These bind CD14 on macrophages, promoting TLR-2 receptor responses to peptidoglycan. Macrophages release pro-inflammatory cytokines, such as TNF-α, with causative effects similar to LPS from Gram-negative organisms.

Minor local infections induce a moderate cytokine response that enhances neutrophil diapedesis. Neutrophils kill bacteria through the exposure to protease and the generation of various ROS. In severe systemic infection, the cytokine release increases and the shock cascade results from excessive amounts of TNF-α, IL-1, IL-6, IL-8 and PAF. Neutrophils further contribute to tissue damage. Fever indicates the systemic impact of the bacteria, blood septicemia can develop and ultimately death may occur. Gram-negative bacteria, are the causative pathogen in the majority of sepsis cases, however, the incidence of sepsis attributed to Gram-positive bacteria has increased over the years from 10% to 30% (Lopez-Bojorquez et al., 2004).

1.9 Diagnosing wound infection

1.9.1 Clinical diagnosis of infection

Deciding whether or not a wound is infected can be a difficult clinical decision (Cutting and Harding, 1994). Clinical signs and symptoms relating to infection are pain, erythema (redness), edema (fluid retention), heat and purulent exudate. The first four signs (pain, erythema, oedema, heat) are also known as the classical signs of the inflammatory response (Gardner, 2001a).

Increasing pain, is caused by the activation of plasma-derived mediators, especially bradykinin, near tiny unmyelinated nerve-fibre endings. Erythema, is observed as the capillaries fill with oxygenated blood (oxyhemoglobin) through the action of vasoactive mediators. Damage to mast cells in connective tissue, or exposure to microorganisms can cause the release of mediators, the effect of which causes the redness observed clinically. Oedema, is due to the extravasation of fluid from the intravascular to extravascular space. Initially, the ability of fluid to move from the intravascular to extravascular space is a result of permeability-increasing mediators, followed later by the action of cytokines (e.g. TNF-α and IL-1). Persistant inflammation leads to the increased permeability of the capilleries. Heat, is caused by increased blood flow to the area. Purulent exudate, rich in white blood cells, is more acidic than plasma, has a higher concentration of potassium and phosphate, and lower protein content. This exudate is common in infections produced by strongly chemotactic bacteria, such as *Staphylococcus* spp. (Gardner et al., 2001b).

Not all wound infections are 'typical'; some show all the classical clinical signs, others a combination of signs, while others may show none of these signs. Therefore, in addition to these classical signs and symptoms of infection, a variety of other signs and symptoms have also been recognised by Clinicians, and are used as diagnostic signs of infection.

These other signs, originally identified by Cutting and Harding (1994), and assigned signs 'specific to secondary wound healing' by Gardner et al. (2001b), include serous exudate with concurrent inflammation, delayed healing, discolouration of the granulation tissue, friable granulation tissue, pocketing at the base of the wound, foul odour and wound breakdown. Additionally, the patient themselves may be pyrexial and/or have an elevated neutrophil count (Kingsley, 2001).

When wound infection is suspected (due to host signs and symptoms), a microbiological assessment of wounds may be made in order to determine the amount of bacteria or fungi (bioburden) or the types of bacteria or fungi present. This microbial assessment is typically made using a surface swab of the wound, or taking a tissue biopsy, the information obtained from which and their limitations will be discussed later. Wound bioburden is used as a measure in wound infection as several studies have established a link between this level of bacteria, delayed wound healing and the clinical signs and symptoms of infection, initially within the acute wound healing setting and later applied to chronic wounds (Heggars, 1998). The 10^5 or greater colony forming units (CFU)/gram tissue is a quantifiable standard that seems to be held in regard by Clinicians as a useful figure in diagnosing wound infection (Bowler, 2003). However, exceptions to this rule, including a wound containing β-haemoltyic streptococci, which is considered infected independent of the number, as this particular microorganism can cause infection at a lower infecting dose (Heggars, 1998).

1.9.2 Diagnosing chronic wound infection

Diagnosing chronic wound infection remains a challenge for Clinicians, with visual observation of signs and symptoms being relied upon. Wound bed swabbing and tissue biopsy may be used to assess the level of bacterial bioburden. However, the difficulty presented to the Clinician in the diagnosis of chronic wound infection, is that the clinical signs and symptoms of infection can also be elicited by tissue damage, unrelated to the infection status of the patient (Gardner et al, 2001b). Furthermore, clinical signs of infection are often masked by factors relating to the host, such as immuno-suppression, diabetes and medication, as these factors may also influence whether any bacteria present will impair wound healing (Bello et al., 2001).

These reasons could account for the apparent lack of correlation between the clinical signs and symptoms to diagnose wound infection and the level of wound bacterial bioburden, currently used by both Clinicians (as a confirmatory tool to their clinical judgement) and Researchers, to define chronic wound infection. It has been reported that 21% of wound patients (all wound types, acute and chronic) with potential wound infections may go undiagnosed, if clinical signs and symptoms alone are utilised in the diagnosis (Bamberg et al., 2002). This has been further confirmed by studies of Gardner et al. (2004), where 18% of nonarterial chronic wounds appeared clinically infected, although 36% of wounds contained $>10^5$ microorganisms per gram tissue. High bacterial bioburden ($>10^5$), in venous leg and diabetic foot ulcer wounds clinically diagnosed as non-infected, have also been reported by Davies et al. (2004, 2007) and Ennis et al. (2005). Furthermore, analysis of 120 chronic wounds by six specialists in wound management, could not accurately diagnose infection upon visual cues, such as redness, hypergranulation and impression of infection (Lorentzen and Gottrup, 2006).

A study on the clinical diagnosis of chronic wound infection by Gardner et al. (2001b), showed that upon comparing the classic and those clinical signs and symptoms specific to secondary wound healing, the latter were better indicators of infection, defined as $>10^5$ CFU/gram tissue. The study design included a number of types of chronic wound,

the majority of which were pressure sores (53%), and venous leg ulcers (19%). The clinical signs and symptoms were recorded on a clinical signs and symptoms checklist (CSSC), designed and developed for the study. The CSSC included all signs and symptoms of infection and was tested by principle Investigators and trained Nurses. Two independent observations of the wound were made and the reliability of the sign was then possible by comparing the two independent assessments. Only four signs on the checklist had agreement values of less than 70%, indicating most signs can be readily detected in the same wound and agreement achieved between both sets of Clinicians.

The Author concluded that the adoption of a structured approach to monitoring wound infection may improve clinical skill and accuracy in identifying specific signs and symptoms of infection. Further analysis of results from the same study revealed that delayed healing and friable granulation tissue was observed in 80% of all infected wounds (n=11) and also in 80% of non-infected wounds (n=25), which did not show signs of increasing pain, heat and foul odour. When increasing pain was reported, all wounds showed 10^5 or greater bacterial cfu/gram tissue and were classified as infected. However, the study suggested that no singular sign or symptom is adequate to diagnose infection. None of the signs or symptoms were a necessary indicator of infection, but increasing pain and wound breakdown were both sufficient indicators, with 100% specificity. The Authors concluded that although there were several limitations regarding the design of the study, that all 11 signs and symptoms should be used as clinical assessments for wound infection. Clinicians responsible for wound monitoring should be as aware of the signs specific to secondary healing, as they are of the classical signs.

1.9.3 Microbiological assessment of wounds

Swabs and tissue biopsies are sampling techniques for collecting microorganisms from a wound. Routinely, microorganisms are cultured, over a period of day, with the quality of the sample being critical to the microbial analysis. Variability in the microbial results obtained can be due to factors, including the sampling technique itself, the type of collection medium, time to laboratory, the standard nature of laboratory procedures, the ability to select the appropriate culture media and ability to grow anaerobic microorganisms (Lawrence and Ameen, 1998, Bowler et al., 2001, Gardner et al., 2006).

1.9.3.1 Superficial verses deep tissue

Tissue biopsies are defined as the 'gold standard' in the identification of wound infection. Biopsies are quantitative yielding the total numbers of organism per gram of tissue, and are more likely to determine the causative pathogen, which will be present within the deep tissue (Heggars, 1998). However, contamination of the sample with superficial microorganisms is possible during sampling, the invasive nature of the procedure, the small area sampled (usually 6mm punch biopsy) and its possible negative effects on wound healing are all disadvantages of this technique (Ratcliff and Rodeheaver, 2002).

Although a superficial swab only provides semi-quantitative data and information on the surface contaminants, there is an argument that the causative organism is derived from the surface, and gains entry through to the deep tissue. Therefore swab information may be equally valid. The use of either technique is dependent on the information required by the Clinician, with superficial swabs providing a general overview of the wounds microflora (Bowler, 2003).

1.9.3.2 Biopsy or swab?

Practicality, cost, issues surrounding the impact of the procedure to the patient and impact on the healing process, has limited the use of tissue biopsies, and questioned their use routinely (Bill et al., 2001). Surface sampling is easier and less time consuming to perform than tissue biopsies. It is also less invasive and the cost is approximately one-sixth that of a tissue biopsy (Bill et al., 2001, Bowler, 2003). Of 345 Clinicians in the United States, 54% routinely swabbed the wound, with 42% routinely collecting a swab and tissue biopsy (Bamberg et al., 2002).

It is suggested that tissue biopsies may not be required to determine the bacterial load in acute wounds, as it is was proposed that a correlation exists between quantitative tissue biopsies and semi-quantitative superficial swab analyses (Bowler, 2003). A correlation value of only 22% was originally reported by Robson (1997). However, studies have since suggested that the techniques are more comparable. Bowler and Davies (1999), showed a close correlation between the two, with Ratcliff and Rodeheaver (2002), demonstrating a correlation co-efficient of $r = 0.89$.

More recently, a comparison of tissue biopsy and swab data by Davies et al. (2007), showed between 1 and 6 bacterial isolates were cultured from both techniques, with means of 2.82 and 2.79 isolates, respectively. Differences in the microflora were observed between the swab and biopsy techniques, notably the detection of anaerobic bacteria in tissue biopsies. The authors suggested that this is a true reflection of bacteria colonisation between the sites, as opposed to the two sampling techniques, due to the controlled design of the study. However, although differences were observed, the information provided by the biopsy results did not add any additional value to the analysis performed.

1.9.3.3 Information provided to Clinician

The semi-quantitative nature of swab results can make them difficult to interpret. The reporting nature of the particular laboratory will affect the information provided to the Clinician. Microbial load can be presented numerically, or a series of pluses (+/++/+++) indicating that there is 'light', 'medium' or 'heavy' growth (Kingsley, 2001, Bamberg et al., 2002, Bowler, 2003). In addition, the microbial analysis of either swab or tissue biopsy, can result in both false positive (outside contaminants) and false negative (un-culturable) outcomes.

The presence of a pathogen within the wound does not necessarily mean that it is the causative microorganism of the infection. A recent study by Gardner et al. (2004), showed that the presence of *S. aureus* was associated with high bacterial bioburden in chronic wounds, although there was a lack of correlation between the presence of *S. aureus* and clinical infection in these chronic wounds.

Much debate still exists how to accurately diagnose chronic wound infection, and whether the diagnosis should rely on clinical signs and symptoms, microbiological evaluation or a combination of both. Recent studies on the microbial flora of chronic wounds, suggests that bacteria impact the wound healing process, and that synergies may exist between microorganisms, both non-pathogenic-pathogenic and aerobic-anaerobic interactions. The relevant importance of bacteria as the causative factor of wound infection is also a matter of debate. As the infecting dose is known to vary with the type of organism involved and the way in which it is presented, Bowler (2003), suggested that the 10^5 guideline in chronic wounds should not be used as the primary indicator of infection, as it implies that all types of bacteria are equally capable of causing infection.

1.9.4 Treatment of chronic wound infection

If a Clinician suspects infection, treatment typically commences immediately, as the time taken to complete the microbial analysis (days), allows sufficient numbers of bacteria to grow and a wound infection to develop/progress. The variation in treatment that could be given to an individual patient was highlighted by the responses to five case studies at a Journal of Wound Care wound infection master class (Harding, 1996). This study demonstrated that there was considerable disagreement in whether or not a wound was infected, and demonstrated that there was little consensus in the most appropriate management strategy.

Although there is no single procedure, common approaches to the treatment of a chronic wound infection are summarised. Clinical diagnosis (redness, tenderness, warmth, increased exudate or systemic features, such as fever) confirms infection. Areas of devitalized tissue are debrided, including any surrounding callus. Tissue biopsy samples may be taken, of the ulcer base after debridement. This procedure minimises the effects of colonization of the ulcer surface with commensal organisms. Alternatively, a wound swab is performed. The wound is probed, and if the probe penetrates to the bone osteomyelitis (infection of the bone) is likely. The wound will be x-rayed for foreign bodies, tissue gas or bony abnormalties. Culturing microorganisms from bone specimens or the use of magnetic resonance imaging can be used to confirm any suspicions of osteomyelitis.

If the infection is mild and superficial, the ulcer can generally be managed with oral antibiotics (given for 1-2 weeks) on an Outpatient basis. If infection is severe, multiple antibiotics that target Gram positive, Gram negative and anaerobic bacteria are administrated. In practice, various regimes are used, such as amoxicillin, ciprofloxacillin, metronidazole and ceftaziidime or ciproflaxlin and clindamycin. If osteomyelitis is suspected, anti-bacterial drugs with good bone absorption (e.g. oral clindamycin) should be used. In severe cases of infection, additional surgery may be required to remove of all infected bone.

It is possible to treat wounds topically with anti-microbial wound dressings. Typically these wound dressings contain either iodine or silver (Sibbald, 2003, White et al., 2006). Both antimicrobials are effective bactericidal agents that have been used in the treatment of chronic wounds for many years. Sensitivities towards iodine, its cautionary use in persons with thyroid dysfunction over extended periods of time, and debate surrounding its impact on wound healing processes (Kramer, 1999, Selvaggi et al., 2003), may be a reason for the emergence of a number of silver containing wound dressing for the treatment of infected chronic wounds. These include Aquacel Ag™ (E.R. Squibb & Sons, Princeton, U.S.A.), Acticoat™ (Smith and Nephew, Hull, U.K.) and Silvercel™ (Johnson and Johnson Medical, Gargrave, U.K.), in recent years. This use of antimicrobial dressings may be attributed to the emergence of antibiotic resistant organisms (Muller et al., 2003, Driver, 2004, Stephen-Haynes and Toner, 2007).

These dressings can maintain a moist wound environment or are used in conjunction with other moist wound healing dressings, in order to lower bacterial bioburden levels in wounds. Moist dressings encourage neutrophils and eliminate necrotic tissue, act as a nutritional source for bacteria, whilst alginate dressings have been shown to sequester bacteria, passively reducing microbial load (Bowler and Davies, 1999). The anti-microbial dressings can be used in isolation or in conjunction with systemic antibiotics (Bowler, 2003). A broad-spectrum approach is employed for the elimination of chronic wound infection. However, the Centres for Disease Control and Prevention warns against the indiscriminate use of antibiotics, especially those with a broad spectrum of activity, because of the increased potential to generate antibiotic resistance (Bamberg et al., 2002).

1.10 Hypothesis for PhD

Chronic wound management strategy is aiming for measurements that predict wound-healing outcomes. Measurements used as tools, to assess the progression of wound healing include, clinical determinations, such as wound area, visual assessments, and physical techniques (Mani, 1999, Goldman and Salcido, 2002).

These measurements have also been investigated as prognostic markers of wound healing responses (Kantor and Margolis, 2000). Currently, many factors have been identified that may contribute to the non-healing of chronic wounds, including host-derived proteases and inflammatory cytokines, associated with low levels of growth factors and ECM components, to name but a few. The identification of these biochemical molecules (biomarkers) between acute and chronic wounds, and chronic non-healing and healing wounds, have led to the development of current wound therapies. However, their use as prognostic biomarkers of wound healing outcomes is yet to be determined (Moseley et al., 2004b).

Biomarkers for the prognosis or diagnosis of chronic wound infection, are rarely described (Debats et al., 2006). Research has revealed that the presence of bacteria may contribute to the delayed healing of chronic wounds. Infection is an issue for both acute wounds and chronic wounds. Acute wound infection is considered easy to diagnose, using clinical signs and symptoms of the inflammatory response to microorganisms. However, the underlying inflammatory nature of a chronic wound presents the Clinician with issues in the diagnosis of chronic wound infection (Bamberg et al., 2002, Gardner et al., 2004). We hypothesise that chronic wounds, such as venous leg ulcers and diabetic foot ulcers, will elicit an inflammatory reaction, in response to bacterial bioburden. This response may be measured biochemically, with this response potentially be consistent, providing an alternative means of diagnosing chronic wound infection.

Subsequently this Study aims to establish whether inflammatory molecules, such as proteases and cytokines, can also serve as useful biochemical markers in the diagnosis or even the prognosis of chronic wound infection.

It has been proposed that wounds in patients with diabetes often demonstrate a decreased inflammatory response (Stadelmann et al., 1998a). Previous, unpublished data for the protease, elastase, in diabetic foot ulcer wound fluid, suggested that this biochemical could be a marker of infection. Within the diabetic population (n=10), one subject had a significantly higher elastase activity (approximately 5-fold increase) and was subsequently hospitalised with severe cellulitis.

To ascertain which host biochemical markers can fulfil the role as diagnostic/prognostic markers of chronic wound infection, investigations were undertaken to address this hypothesis.

An *ex vivo* wound fluid collection study was conducted using clinically diagnosed non-infected and clinically infected venous leg and diabetic foot ulcer wounds. Wound fluids from both venous leg and diabetic foot ulcers were collected and bacterial bioburden quantified using microbiological techniques.

Additionally, an *in vitro* model investigated the expression/secretion of identified/novel markers from neutrophils in response to challenge with bacterial supernatant from species commonly associated with chronic wounds.

Chapter 2

Chapter 2

Materials and methods

All reagents were obtained from Sigma-Aldrich Company Ltd. Dorset, U.K., unless otherwise stated.

2.1 *Ex vivo* wound fluid study

2.1.1 Patient enrolment

Patients (≥ 18 years old) attending the Wound Healing Research Unit at The University Hospital of Wales, Cardiff, U.K., participated in the study, clinical protocol CT-ORA-001-02, following approval from the Local Research Ethics Committee. Subjects were included on the basis they had a venous leg or diabetic foot ulcer for ≥ 30 days, of at least 1cm^2 in area, with exudates flow. Subjects were excluded if the target wound demonstrated signs of infection (only applicable for the non-infected wound categories), the wound was dry and/or predominantly healed, exhibited exposed bone and positive osteomyelitis or if the patient had a concurrent illness or condition that may interfere with wound healing, was a known alcohol or drug abuser, receiving dialysis, or had received corticosteroids, immunosurpressive agents, radiation therapy or chemotherapy within the 30 days, prior to enrolment.

Ten (10) subjects with venous leg ulcers and ten (10) subjects with diabetic foot ulcers were enrolled into the clinically non-infected groups. These subjects exhibited no clinical signs of infection. The collected wound fluid samples generated baseline data for each biochemical marker believed to be important in the host response to infection and also used to determine intra-patient variability.

Each subject contributed a single wound fluid collection. Ten (10) subjects with venous leg ulcers and five (5) subjects with diabetic foot ulcers were enrolled into the clinically infected groups. These patients exhibited clinical symptoms of infected chronic wounds, demonstrating a minimum of four of the following, evidence of cellulitis, localised pain, swelling, erythema, increased local skin temperature, unhealthy granulation tissue, abnormal colour, pus and increased exudates. The wound fluid samples were analysed for the same biochemical markers, as in the non-infected groups, and the data also used to determine intra-patient variability and to ascertain if any of these markers could be used in chronic wound infection diagnosis/prognosis.

2.1.2 Microbiological swabs and wound measurements

The wounds of the enrolled subjects in both groups (clinically non-infected and infected) were swabbed for microbiological analysis, at the Department of Oral Surgery, Medicine & Pathology, School of Dentistry, Cardiff University. Analysis included the quantification and characterisation of aerobic and anaerobic bacteria, using standardised microbial culture techniques. Microorganisms were cultured on fresh blood agar, MacConkey 3 agar, Sabouraud dextrose agar and Fastidious Anaerobic agar (LabM, Lancashire, U.K.) at 32 or 37°C, in the presence or absence of oxygen, allowing for the detection of total microflora, entero-bacteria, yeasts and anaerobes, respectively. After the wound swabs were obtained, the wounds were debrided, if necessary, cleansed with normal saline and dried. The area of each wound was then traced onto a wound measuring acetate and a photographic record of the wounds made.

2.1.3 Wound fluid collection

Each venous leg ulcer and diabetic foot ulcer was dressed with RELEASE® and covered with a BIOCLUSIVE® film dressing. The dressing remained *in situ* up to 4h (in the case of the venous leg ulcers) or as long as overnight (in the case of the diabetic foot ulcers). Upon wound dressing saturation, the BIOCLUSIVE® film was removed, and saturated RELEASE® dressing removed using sterile forceps and placed in a sterile vial on dry ice for transfer to an -80°C freezer.

Any non-saturated regions on the RELEASE® dressings were removed using sterile scissors. In order to elute the wound fluids, wound dressings were measured and immersed in 0.1M Tris-HCl buffer, pH 7.4, containing 0.1% Triton X-100 (wound fluid elution buffer, 500µl buffer/cm^2 dressing) and eluted for 2h, at 4°C, under constant agitation. Eluted wound fluid samples were aliquoted and stored at -80°C, until required.

2.1.4 Protein quantification

The protein concentration of each clinically non-infected and infected venous leg and diabetic foot ulcer fluid was determined on wound fluid aliquots (10µl), using the BioRad Protein Assay Kit (BioRad Laboratories Ltd., Hertfordshire, U.K.) at 595nm, using a DU 800 UV/visible spectrophotometer (Beckman Coulter Ltd., Buckinghamshire, U.K.), versus a bovine serum albumin (BSA) standard curve (0.1-2mg/ml). The protein concentration in each wound fluid was analysed in duplicate.

2.2 Cell culture and microbiological techniques

2.2.1 Isolation of neutrophils

Neutrophils were isolated from whole blood by Ficoll-Hypaque density gradient centrifugation, based upon the method of Moseley *et al* (2003). Whole blood (40ml) was removed from healthy, human volunteers, aged 20-30 years, into vacutainers containing EDTA (Becton Dickinson, Meylan Cedex, France) as an anticoagulant.

Blood aliquots (20ml) were gently layered onto a density gradient, consisting of a lower layer of 10ml Histopaque®-1119 (density 1.119g/ml) and an upper layer of 10ml Histopaque®-1077 (density 1.077g/ml), in 50ml conical tubes (Greiner Bio-One Ltd., Gloucestershire, U.K.).

Conical tubes were centrifuged (Durafuge 200, Thermo Electron, Fife, U.K.), at 700g for 30min at room temperature, resulting in the formation of four definite layers. Upon centrifugation, four definitive layers are formed, the third layer containing the neutrophil cells. The top two layers were discarded, the neutrophil layer harvested, and pooled with an equal volume of phosphate buffered saline (PBS). Cells were washed and re-centrifuged to form a cell pellet (700g/5min), at room temperature.

Erythrocyte contamination was removed by adding cold 0.2% sodium chloride (2.5ml) to the cell pellet, with gentle agitation of the tube for 1min. The osmolarity within the tube was corrected by the addition of 1.6% sodium chloride (2.5ml), followed by gentle agitation. An equal volume of PBS (5ml) was added to the tube, and a cells pellet obtained by centrifugation (700g/5min), at room temperature. This step was repeated as necessary, to eliminate remaining erythrocytes.

Neutrophils were washed in PBS, re-centrifuged (700g/5min) and the neutrophil pellet re-suspended in RPMI-1640 medium, supplemented with L-glutamine (2mM) (Invitrogen Ltd., Paisley, U.K.). Cells were assessed for contamination with other cell types, by visual assessment by light microscopy (Olympus CK2 Inverted Microscope, Olympus U.K. Ltd., Middlesex, U.K.), cell viability and cell counting.

2.2.2 Cell viability determination

2.2.2.1 Neutrophil cells

Isolated neutrophil cell viability was assessed using the trypan blue exclusion method (Tennant, 1964). Cells were diluted 1:10 with RPMI-1640 media, supplemented with L-glutamine (2mM), and equal volumes (20µl) of diluted cell suspension and sterile filtered, 0.04% trypan blue (Invitrogen, Paisley, U.K.), mixed together. An aliquot (10µl) of this cell suspension was loaded onto each side of a Neubauer Improved Haemocytometer (x100 magnification, Fisher Scientific, Leicestershire, U.K.), and non-viable (cells stained blue) and viable cells (non-stained cells) counted.

2.2.2.2 Stimulated cells

On supernatant removal from the neutrophil cell monolayer, cells were trypsinised, 500µl trypsin/EDTA (Invitrogen), for 3min incubated at 37°C, after which trypsin was neutralised with 500µl RPMI-1640 media (Invitrogen) supplemented with 10% heat inactivated, low endotoxin fetal calf serum. Equal volumes (20µl) of trypsinised cells and sterile filtered, 0.04% trypan blue, were mixed together and aliquots (10µl) loaded onto each side of a Neubauer Improved Haemocytometer (x100 magnification), and non-viable (cells stained blue) and viable cells (non-stained cells) counted.

2.2.3 Generation of bacterial supernatants

Briefly, supernatant was kindly gifted from the Department of Oral Surgery, Medicine & Pathology, School of Dentistry, Cardiff University. Wound pathogens, *Pseudomonas aeruginosa* and *Staphylococcus aureus* were cultured from the wound swabs of two subjects in this Study and stored on beads at -70°C. These clinical isolates were grown from storage beads in nutrient broth.

Fresh blood plates were streaked out from the overnight culture in nutrient broth, by normal microbiological techniques and representative colonies selected. Colonies were then inoculated into fresh fastidious anaerobic broth (LabM) and incubated overnight at 37°C in aerobic conditions. A dilution was made from this broth into fresh fastidious anaerobic broth and the bacteria grown to mid-log and stationary phases, as determined spectrophotometrically at 660nm, (DU 800 UV/visible spectrophotometer), plotting absorbance verses time to generate bacterial growth curve.

At time points corresponding to both mid-log and stationary growth phases the supernatant was removed from cells by centrifugation at 14000g (Denley centrifuge, Model BS400, Thermo Electron) for 10 minutes. Supernatant was sterile filtered (0.22μm) twice, aliquoted out and stored at –20°C until analysed. Broth control supernatant was prepared following 5 and 24h incubation at 37°C.

2.2.4 Cellular stimulation with *E. coli* lipopolysaccharide

Isolated neutrophils were seeded in RPMI-1640 media (Invitrogen) supplemented with 10% heat inactivated, low endotoxin fetal calf serum, into 24-well tissue culture plates (Greiner Bio-One Ltd) at a cell density of 2 x 10^6 cells/0.5ml or 5 x 10^5 cells/0.5ml.

Cells were then stimulated with equal volumes of commercially available lipopolysaccharide (LPS) *E.coli* 055:B5, which was added at a final concentration of 0.01, 0.1, 1, and 10 μg/ml. Cultures were then maintained for 1h, 4h, 24h, and 48h at 37°C/5% CO_2. Media alone and cells incubated with media only served as negative controls. At the end of each time point, culture media was removed, aliquoted and immediately frozen at -70°C.

2.2.5 Cellular stimulation with bacterial supernatant

Isolated neutrophils were seeded (0.5ml) in RPMI-1640 media supplemented with 10% heat inactivated, low endotoxin fetal calf serum, into 24-well tissue culture plates, at a cell density of 1 x 10^6 cells/ml (5 x 10^5 cells/well).

Cells were stimulated with equal volumes (0.5ml) of bacterial supernatant at both mid-log and stationary growth phase (Chapter 2, Section 2.2.3), added to the cells, neat or diluted 1:10 with cell media. Cultures were then maintained for 4h, and 24h, at 37°C/5% CO_2.

Cells incubated with cell culture media only served as negative control. Cells incubated with *E.coli* 055:B5 LPS, at a final concentration of 0.1μg/ml, also served as a positive control. Neat fastidious anaerobic broth and broth diluted 1:10 were further included as controls, to determine any potential effects that these alone may have had on cellular viability and/or function. At the end of each time point, culture media was removed, aliquoted and immediately frozen at -70°C.

2.3 Analytical techniques

2.3.1 Fluorogenic substrate enzyme assays

The levels of neutrophil-derived elastase like-activity and collagenase-like activity in *ex-vivo* wound fluids and *in vitro* cell culture supernatants were quantified spectrofluorometrically using substrate activity assays. The substrates comprised of short peptides, synthesised to mimic the appropriate enzyme cleavage site and contained a fluorescent reporter group, which was released upon hydrolysis. Enzyme activity was determined by measuring the rate of production of the fluorogenic compound, 7-amino 4-methyl coumarin, with enzyme activity being expressed either as relative fluorescence units per minute (RFU/min) or as relative fluorescence units per minute, when corrected for total protein (RFU/min/mg protein).

In the analysis of *ex vivo* wound fluid samples, the reaction mixture, in black, flat bottomed, microtitre well plates (Corning Costar UK Ltd, Buckinghamshire, U.K.) comprised 20μl sample, 160μl assay buffer and 20μl substrate (final concentration 50μM), with 6 separate replicates being established per sample. Each substrate was prepared at a 10mM stock concentration, diluted to a working concentration of 50μM in the appropriate assay buffer at room temperature. In the analysis of *in vitro* neutrophil cell culture supernatant samples, in order to conserve sample, the reaction mixture, combined in black, flat bottomed, microtitre well plates comprised 5μl sample, 175μl assay buffer and 20μl substrate (final concentration 50μM), with 6 separate replicates being established per sample.

On reaction mixture completion, the microtitre plates were read immediately using a fluorescent plate reader (Fluorolite 1000, Dynex Technologies) at an excitation of 383nm and emission at 455nm, this provided the reading at 0 minutes. The microtitre plate was then covered and incubated at 37°C. Readings were taken at timed intervals over a 1h period.

Neutrophil-derived elastase-like activity was estimated using the fluorogenic substrate methoxy-alanine-alanine-proline-valine-7-amino-4-methylcoumarin (Bachem U.K. Ltd., Merseyside, U.K.), solubilised in methanol (Cullen et al., 2002). The assay buffer required for optimal activity of this enzyme was 0.1M Hepes buffer, pH 7.5, containing 0.5M sodium chloride and 10% dimethyl sulphoxide (DMSO). Elastase standards were established using neutrophil-derived elastase (Calbiochem, Nottinghamshire, U.K.), re-suspended in 50mM sodium acetate buffer, pH 5.5, containing 200mM sodium chloride (elastase reconstitution buffer, ERB), and diluted appropriately in elastase-like activity assay buffer.

Collagenase-like activity was estimated using the fluorogenic substrate succinyl-glycine-proline-leucine-glycine-proline-7-amino-4-methylcoumarin (Bachem U.K. Ltd.), solubilised in methanol (Cullen et al., 2002). The assay buffer required for optimal activity of this enzyme was 40mM Tris-HCl buffer (Fisher Scientific), pH 7.4, containing 200mM sodium chloride and 10mM calcium chloride. Collagenase standards were established using collagenase from *Clostridium histolyticum*, re-suspended in and diluted appropriately, using collagenase-like activity assay buffer.

Alpha-1-anti-trypsin-like activity was estimated using the fluorogenic substrate tos-glycine-proline-lysine-7-amino-4-methylcoumarin (Bachem U.K. Ltd.), solubilised in methanol (Mullins *et al.*, 1984). The assay buffer required for optimal activity of this enzyme was 50mM Tris-HCl buffer, pH 8.2, containing 150mM sodium chloride, 0.05% Tween 20 and 10% dimethylsulphoxide (DMSO) (Kuzmic *et al.*, 2000). Alpha-1-anti-trypsin standards (Calbiochem) was re-suspended in water, and diluted in the appropriate activity assay buffer.

Plasmin-like activity was estimated using the fluorogenic substrate methoxysuccinyl-alanine-phenyl-lysine-7-amino-4-methylcoumarin (Bachem U.K. Ltd.), solubilised in 1mM hydrochloric acid. The assay buffer required for activity of plasmin was 25mM Tris-HCl, pH 8.1, containing 0.5% Triton X-100 (Cullen et al., 2002). Plasmin standards (Calbiochem) was supplied in 100mM sodium phosphate, pH 7.3, 1mM 6-aminohexanoic acid and 25% glycerol, and diluted in the appropriate activity assay buffer.

2.3.2 Zymography

2.3.2.1 Casein/gelatin zymography

The caseinolytic/gelatinolytic activity of *ex-vivo* wound fluid and *in vitro* neutrophil cell culture supernatant samples were assessed by the semi-quantitative technique, zymography (Leber and Balkwill, 1997, Michaud, 1998, Pagano 1999, Snoek van Beurden and Von der Hoff, 2005). This technique measured the activity of an enzyme, as the enzyme cleaves its substrate within the SDS-polyacrylamide gel, resulting in zones of lysis. Homogenous resolving SDS-polyacrylamide gels containing 12.5% casein (Bio-Rad Laboratories Ltd) were used to quantify caseinolytic enzymes, including human neutrophil elastase. Homogenous resolving SDS-polyacrylamide gels containing 10% gelatin (Bio-Rad Laboratories Ltd) were used to quantify the gelatinases (MMP-2 and MMP-9).

Samples and the appropriate standards, were prepared in non-reducing loading buffer (x5), consisting of 0.625M Tris-HCl buffer, pH 6.8, 12.5% glycerol, 2% (w/v) sodium dodecyl sulphate (SDS) (VWR International Ltd. Leicestershire, U.K.) and 1.25% bromophenol blue.

Samples and appropriate standards were loaded onto the appropriate gel and were run at 60mA for 1h in running buffer (0.25M Tris-HCl buffer, pH 8.3, containing 2.0M glycine and 1% SDS). SDS was removed by washing in 2.5% Triton X-100 (2x30min), which allowed the re-naturation of proteins.

Gelatin gels (Bio-Rad Laboratories Ltd) were incubated in activation buffer, 0.04M Tris-HCl buffer, pH 7.5, containing 0.2M sodium chloride and 0.01M calcium chloride, overnight at 37°C. Casein gels (Bio-Rad Laboratories Ltd) were incubated in activation buffer, 0.1M sodium phosphate, pH range 6.8-7.4, containing 0.1% Triton X-100 and 10mM ethylenediamine-tetraacetic acid (EDTA), overnight at 37°C.

Gels were then stained in Coomassie Brilliant Blue (Bio-Rad Laboratories Ltd.), 0.2% Coomassie R250, 50% methanol (VWR International Ltd.), 10% acetic acid (Fisher Scientific), at room temperature and destained in 20% methanol/10% acetic acid, to visualise banding patterns, represented by clear zones of lysis.

2.3.2.2 Casein zymography standards

Elastase standards were made using neutrophil-derived elastase (Calbiochem), re-suspended in 50mM sodium acetate buffer, pH 5.5, containing 200mM sodium chloride (elastase reconstitution buffer, ERB), and diluted appropriately in ERB.
Proteinase 3 standards were made using neutrophil-derived proteinase 3 (Athens Research and Technology, Athens, U.S.A.), re-constituted and diluted in ERB.

2.3.2.3 Gelatin zymography standards

MMP-2 and MMP-9 (gelainase), was purified from fibroblast conditioned media. Fibroblast cells were grown in serum free media for 7 days, media removed and centrifuged (Durafuge), 1500rpm/5min. The resulting supernatant sample is dialysed, using 2L buffer per 10cm dialysis tubing, overnight at 4°C in MMP buffer; 0.05M Tris/HCl, 5mM $CaCl_2$, pH 7.6, containing 0.05% Brij 35 solution and 0.02% Na Azide. This media was gifted from the Department of Oral Surgery, Medicine & Pathology, School of Dentistry, Cardiff University.

2.3.3 Enzyme linked immunosorbent assay (ELISA) quantification

2.3.3.1 ELISA

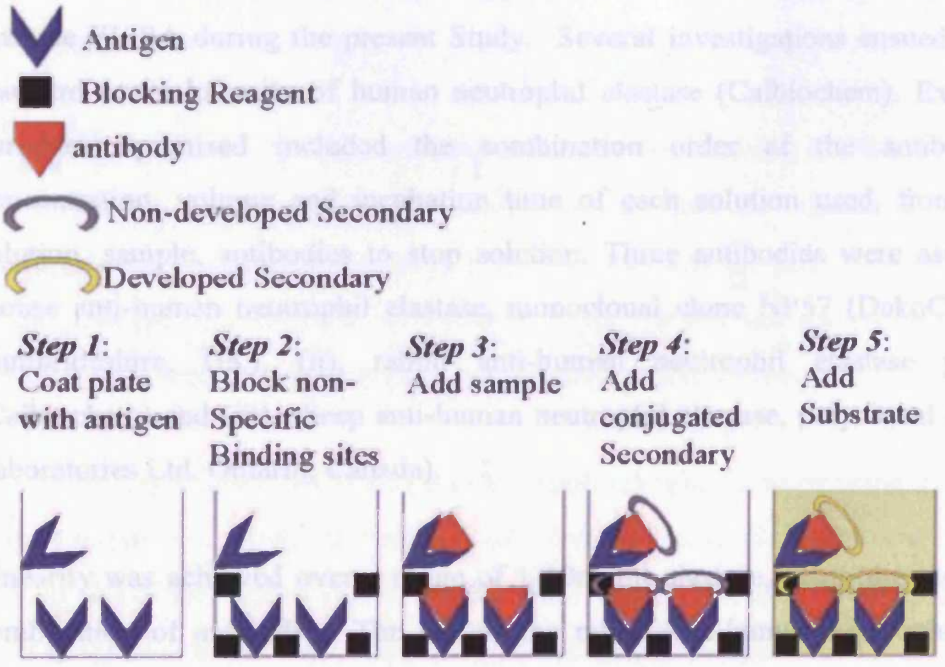
Enzyme-linked immunosorbent assay (ELISA) is a useful and powerful method in estimating ng/ml to pg/ml amounts of a defined substance in a solution. The levels of wound fluid cytokines and cytokines released from simulated neutrophils into cell culture medium, *in vitro*, was determined using commercially available human cytokine immunoassays (Table 2.1).

Table 2.1: Details of ELISAs used in the present Study

Analyte	Source
Alpha-1-antitrypsin	Immunodiagnostik, Oxford Biosystems
IL-6	R&D Systems, Oxfordshire, U.K.
VEGF	R&D Systems, Oxfordshire, U.K.

These assays are based on a quantitative sandwich enzyme immunoassay technique (Figure 2.1). Microtitre plates, coated with antibody, were supplied by the manufacturer. Standards and samples were pipetted into the wells, where the analyte present in standard/sample becomes bound to the antibody. After washing to remove any unbound substances, a labelled secondary antibody, was added to the plate, which binds to the analyte, thus creating the 'sandwich'. Following a wash step to remove any unbound complexes, the substrate was added, reacting with the labelled antibody, resulting in colour development on the plate. The intensity of the colour was directly proportional to the concentration of the analyte. A dose response of the absorbance verses concentration of the standards was generated, resulting in a standard curve. The concentration of the analyte was determined from the curve.

Figure 2.1: Prinicple of ELISA methodology.



2.3.3.2 Elastase ELISA

The lack of a commercially available ELISA that readily detected elastase, as opposed to the elastase:alpha-1-anti-trypsin complex, led to the development of an elastase ELISA during the present Study. Several investigations ensued, assessing standard curve linearity of human neutrophil elastase (Calbiochem). Experimental variables optimised included the combination order of the antibodies, the concentration, volume and incubation time of each solution used, from blocking solution, sample, antibodies to stop solution. Three antibodies were assessed, (i), mouse anti-human neutrophil elastase, monoclonal clone NP57 (DakoCytomation, Cambrideshire, UK), (ii), rabbit anti-human neutrophil elastase polyclonal, (Calbiochem) and (iii), sheep anti-human neutrophil elastase, polyclonal (Cederlane Laboratories Ltd. Ontario, Canada).

Linearity was achieved over a range of 1-10ng/ml elastase, using the mouse:rabbit combination of antibodies. The use of the rabbit anti-human neutrophil elastase, polyclonal antibody as the capture antibody, resulted in a larger range in values, than the mouse anti-human neutrophil elastase, monoclonal clone NP57. However, better sensitivity was achieved with the mouse antibody as capture antibody, and the rabbit antibody as the primary antibody.

Capture antibody, mouse anti-human neutrophil elastase, monoclonal clone NP57, diluted 1:200 in PBS (Invitrogen), were coated (200µl) onto a high binding microplate. Plates were covered and incubated at 4°C overnight, and the capture antibody removed by plate washing with PBS/0.05% Tween 20 (4x400µl). Plates were blocked with 5% w/v skimmed milk powder in PBS (300µl), at room temperature for 1h, and subsequently washed with PBS/0.05% Tween 20 (4x400µl).

Standards or samples were diluted appropriately in sample diluent (100mM Hepes buffer, pH 7.2, containing 100mM sodium chloride, 0.01% BSA and 0.001% Tween 20) and aliquots (100µl) added to each well. Plates were covered and incubated at room temperature for 2h for the elastase present in each standard/sample to bind to the antigen (capture antibody).

Following the removal of any unbound substances by washing in PBS/0.05% Tween 20 (4x400µl), anti-human neutrophil elastase antibody raised in the rabbit (Calbiochem) was diluted 1:1000 in PBS, and added to the plate (200µl), and binds to the elastase. Plates were incubated at room temperature for 2h and washed in PBS/0.05% Tween 20 (4x400µl), to remove unbound antibody.

The rabbit anti-human neutrophil elastase antibody was detected using a horseradish peroxidise-linked, goat, anti-rabbit IgG (whole molecule) secondary antibody, diluted 1:12,000, 200µl per well. Plate were incubated overnight at room temperature and washed in PBS/0.05% Tween 20 (4x400µl).

Substrate solution (Sigma Fast™ OPD, o-phenylenediamine dihydrochloride) was added to each well, 200µl. Colour developed over 1h and the reaction terminated with 3M sulphuric acid (Fisher Scientific) (50µl).

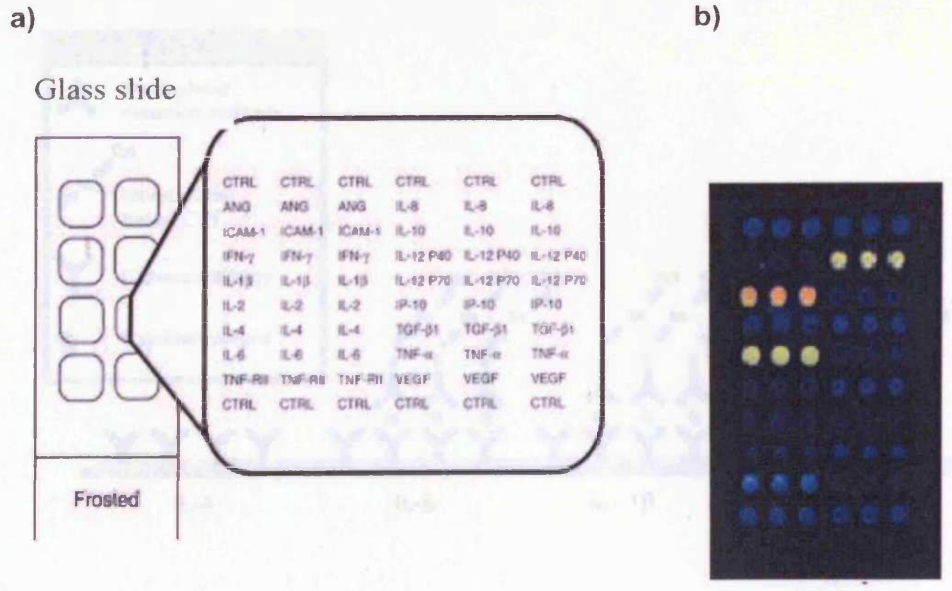
The absorbance of each well was quantified spectrophotometrically at 492nm. The amount of elastase present in each sample calculated using the commercial elastase standard curve.

2.3.4 Cytokine analysis by micro-array

2.3.4.1 Micro-array methodology

Ex vivo wound fluids and *in vitro* neutrophil cell culture supernatant samples, at the 24h time point were measured by micro-array technology. Micro-array analysis was conducted at a testing facility of Whatman® Schleicher & Schuell®, Dassel, Germany, according to standard operating procedures, utilising16-pad FAST® slides, coated with a thin layer of proprietary nitrocellulose, arrayed with all anti-cytokine specificities, in triplicate (Figure 2.2, Table 2.2). Landing light control elements were present on each slide as control elements. Antibodies were attached onto the FAST® slide using a piezo-electric Perkin-Elmer BioChip Arrayer (Perkin-Elmer, Oxfordshire, U.K.). Slides are processed in a FastFrame (64 pads, with 96-well spacing).

Figure 2.2: FAST® slides.
(a) Eight antibody arrays are demonstrated on the glass slide. The configuration of each antibody array on a FAST® slide is shown. Within the array, each antibody is present on the slide in triplicate. There are 12 landing light controls (CTRL) per array. (b) Upon development of an antibody micro-array, fluorescence is detected and the intensity of the fluorescence is measured. In this example, dark blue colour represents no fluorescence and the yellow/orange colour representing a fluorescent signal.

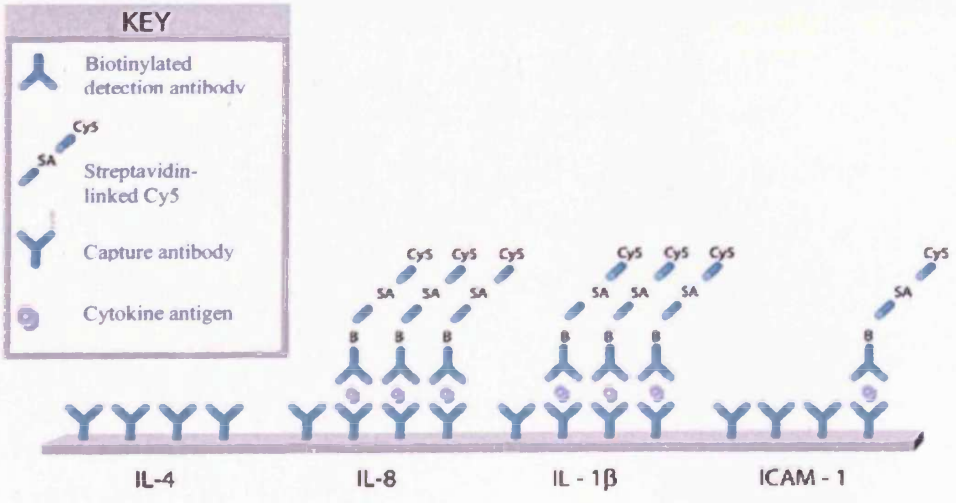


Micro-array methodology (Figure 2.3), was conducted with gentle rocking of slides during incubation periods. Slides were blocked, at room temperature for 15 mins with 70µl Protein Array Blocking Solution (Schleicher & Schuell®). Upon blocker removal, 70µl samples and standards were added to duplicate pads, resulting in 6 replicates per sample/standard.

Slides were incubated at room temperature, for 3-4h, and then washed using Tris-buffered saline-0.05% Tween 20 (TBS-T) (3 x 5min). Slides were treated with 70µl biotinylated detection antibody cocktail, containing one biotinylated antibody corresponding to each of the arrayed capture antibodies (Table 2.2). Slides were then incubated at room temperature, for 1h.

Following the incubation, slides were washed using, TBS-T (3 x 5min). Slides were subsequently incubated, at room temperature, with 70µl of streptavidin-Cy5 conjugate (0.125 µg/ml in TBS-T) for 1h. A last wash step was conducted with TBS-T (3 x 5min), and slides then quickly rinsed in de-ionised water, and dried.

Figure 2.3: The 'sandwich' micro-array method.



Slides were imaged in a Confocal Fluorescent Scanner, AXON GenePix 4000B fluorescent imaging system (Axon Instruments, Sunnydale, U.S.A). Array images were saved as 16-bit TIF files with 10 micron pixel resolution. Images were analysed using Imaging Research ArrayVision software (Whatman® Schleicher&Schuell®, Dassel, Germany). Briefly, spot intensities were determined by subtracting background signal. Spot replicates from each sample condition were averaged and then compared to the appropriate quantitative standard curves. Microsoft Excel was used for additional analysis and data interpretation.

2.3.4.2 Micro-array data analysis

The fluorescent units (signal) obtained for each sample per cytokine, were converted to pg/ml values based on standard curves. The average amount of each cytokine (pg/ml), % Coefficient of Variance and standard deviation (S.D.) were calculated. Samples reading 'over' upon initial analysis were diluted to obtain fluorescent units within the range of the standard curve. Samples with fluorescent units 'under' the range of the standard curve, were given a value equivalent to half of the limit of detection for that particular cytokine (Table 2.3), for the purposes of data analysis.

Table 2.2: Micro-array antibodies were sourced as matched antibody pairs from R&D Systems, Oxfordshire, U.K.

Details of the antibodies raised against the eighteen cytokines measured in the present Study. Each antibody is specific for the human version of the cytokine, with * denoting that the antibody IL-5 can cross-react with some animal species.

Cytokine	Catalogue Number	Clone	Immunogen
Angiogenin	MAB265	14017.7	*E coli* derived rh ANG
IL 10	MAB217	23738	*Sf* 21-derived rh IL 10
ICAM-1	MAB720	14C11	CHO cell-derived rh ICAM-1 extracellular domain
IL12 p40	MAB609	31052	*Sf* 21-derived rh IL 12/IL 23 p40 free monomer
IFNγ	MAB2852	K3.53	*E coli* derived rh IFNγ
IL 12 p70	MAB611	24945	*Sf* 21-derived rh IL 12
IL 1β	MAB601	2805	*E coli* derived rh IL 1β
IL 13	MAB213	32116	*E coli* derived rh IL 13
IL 2	MAB602	5355	*E coli* derived rh IL 2
IP 10	MAB266	33036	*E coli* derived rh IP 10
IL 4	MAB604	3010	*E coli* derived rh IL 4
TGFβ1	MAB240	9016	CHO cell-derived TGFβ1 and latent TGFβ1
IL 5 *	MAB405	TRFK5	Partially purified rm IL 5
TNF-α	MAB610	28401	*E coli* derived rh TNF-α
IL 6	MAB206	HD27	*E coli* derived rh IL 6
TNF r2	MAB726	22210	*E coli* derived rh TNF r2 extracellular domain
IL 8	MAB208	6217	*E coli* derived rh CXCL8
VEGF	MAB293	26503	*Sf* 21-derived rh VEGF

Table 2.3: Limit of detection for each cytokine.
Each cytokine measured has a limit of detection. Upon data analysis, samples were assigned an under value when the amount of cytokine was less than the limit of detection.

Cytokine	Limit of Detection (pg/ml)	Under value (pg/ml)	Cytokine	Limit of Detection (pg/ml)	Under value (pg/ml)
Angiogenin	1	0.5	**IP 10**	10	2.7
IL 10	100	50	**IL 4**	100	2.1
ICAM-1	100	4.8	**TGFβ1**	32	1.2
IL12 p40	1	0.5	**IL 5**	10	1
IFNγ	10	0.2	**TNF-α**	1	0.5
IL 12 p70	10	0.1	**IL 6**	10	5
IL 1β	1	0.5	**TNF r2**	10	2.8
IL 13	100	50	**IL 8**	1	0.5
IL 2	10	2.8	**VEGF**	1	0.5

2.2.5 Western blotting

Ex vivo wound fluids samples were prepared in a reducing loading buffer (5x), consisting of 0.625M Tris-HCl, pH 6.8, containing 5% β-mercaptoethanol, 12.5% glycerol, 2% (w/v) SDS and 1.25% bromophenol blue. Proteins in the samples were reduced, 100°C/2 min and subjected to SDS-PAGE (Mini-Protean-II Gel Electrophoresis System, (BioRad Laboratories Ltd.), on pre-cast, 10% linear Tris-HCl gels (BioRad Laboratories Ltd.), at 60mA for 45 min in running buffer (0.25M Tris-HCl buffer, pH 8.3, containing 2.0M glycine and 1% SDS).

Following separation by SDS-PAGE, gels were electroblotted onto nitrocellulose membranes (BioRad Laboratories Ltd.), using a Trans-Blot Semi-Dry Elelctrophoretic Transfer Cell System (BioRad Laboratories Ltd.), at 90mA, overnight on ice, in Towbins buffer (0.025M Tris, containing 0.192M glycine and 20% methanol) (Towbin et al., 1979).

On transfer, nitrocellulose membranes were blocked with 5% Marvel in PBS, for 1h, at room temperature. The membranes were rinsed in 0.05% Tween 20/PBS, pH 7.2, at room temperature (6x5min), prior to incubation in primary antibody. The nitrocellulose membranes were immuno-probed with primary antibody, anti-human alpha-1-anti-trypsin (Calbiochem), diluted 1:200, in a total volume of 10ml, 0.05% Tween 20/PBS, pH 7.2, at room temperature for 2h.

The membranes were rinsed in 0.05% Tween 20/PBS, pH 7.2, at room temperature (6x5min), prior to incubation in secondary antibody. The nitrocellulose membranes were immuno-probed with secondary antibody, horseradish peroxidise-linked, anti-mouse IgG (whole molecule), diluted 1:11000, in a total volume of 10ml, 0.05% Tween 20/PBS, pH 7.2, at room temperature for 2h. The membranes were then rinsed in 0.05% Tween 20/PBS, pH 7.2, at room temperature (6x5min), prior to incubation in chemiluminscent reagent (Western Blot Detection Kit, BioRad Laboratories Ltd.).

2.4 Statistical analysis of data

Ex vivo wound fluid data was considered as non-parametric and Wilcoxon two-sided probability statistical analysis was used. The exception to this was upon comparison of one ulcer type to another, when the data was considered parametric, and a Students t-test (2 tailed), was applied assuming unequal variance use.

Statistical analysis of *ex vivo* wound fluid data was provided by With Confidence Ltd, U.K. Upon analysis of the biochemical markers in wound fluids, caution must be taken upon interpretation of these results. With so many markers and few patients, a significant result could be due to random variation in the data. Values of $p<0.05$ can only be viewed as potential findings. Larger sample numbers would be required to validate the observations from this Study. *In vitro* sample data was analysed using the Students t-test (2 tailed), assuming unequal variance. Significance was defined at $p<0.05$.

Chapter 3

Chapter 3

Biochemical differentiation between non-infected and infected chronic wounds, based upon clinical diagnosis

3.1 Introduction

The inability of a person's immune response to prevent the proliferation of bacteria and eradicate them results in an infection. Within acute conditions, mechanisms by which bacteria can evade the host immune response (Peschel, 2002) and the elucidation of host defense mechanisms to invasion, has led to the identification of host-derived molecules that aid the diagnosis of infection. An increase in C-reactive protein (CRP) measured in serum (Lind, 2003), indicates an inflammatory response that may be due to an infection. Additionally, these host-derived molecules can also exacerbate the condition, for example, tumour necrosis factor-α (TNF-α) and acute bacterial septicemia have been investigated (Degre, 1996). Chronic conditions, in contrast, can leave a person susceptible to an infection, for example, pneumonia as a consequence of weakened immunity, caused by HIV.

However, little research has been conducted on the host's response to infection within chronic conditions, including wound healing. To date, research has concentrated on biochemical differences between acute and chronic wounds, and chronic non-healing and healing wounds. However, recently a difference in the arginine conversion rate has been shown between chronic infected wounds, with that of both acute and chronic non-infected wounds, as defined by microbiological diagnosis (Debats et al., 2006).

Definition of infection within the chronic wound environment is a subject of debate. Literature would suggest that bacterial bioburden levels $>10^5$ viable organisms per gram tissue would indicate clinical infection (Majewski et al., 1995). This '10^5 guideline' has been emphasized and accepted when diagnosing infection (Bowler, 2003). However, recently, more evidence is emerging that questions the Clinicians' ability to diagnose chronic wound infection based upon clinical signs and symptoms (Gardner et al., 2001a, Lorentzen and Gottrup, 2006 and Davies et al., 2007).

Wilson et al. (1990), claimed that 34% of infected wounds remain undetected, due to differences in the definition of wound infection. Bamberg et al., (2002) also concluded that as many as 20% of clinically diagnosed non-infected wounds can be assigned as infected, upon microbiological content.

With the exception of Debats et al., (2006), no host biochemical molecule has been reported that differentiates between chronic non-infected and infected wounds. In the search for molecules that could act as possible biomarkers of chronic wound infection, it seems likely that potential candidates would encompass either biochemicals associated with acute infection or biochemicals already investigated as markers of delayed wound healing. Any such biochemical would be increased or decreased, due to the presence of bacteria in the wound.

In the present Study, twenty (20) patients with a venous leg ulcer and fifteen (15) patients with diabetic foot ulcer were recruited, from whom samples of wound fluid were collected. Informed consent was obtained, and wound fluids collected, in accordance with clinical protocol CT-ORA-001-02 (Chapter 2, Section 2.1). Briefly, patient information was gathered, quantitative microbiology conducted and the total protein content of each fluid determined, prior to analysis of potential biomarkers.

Wound fluid samples were analysed for the presence and amount of bacteria (both aerobic and anaerobic), fungi and yeast, by standard microbiological techniques (Chapter 2, Section 2.1). This analysis was kindly provided by Dr. Katja Hill and colleagues, School of Dentistry, Cardiff University. The microbiological data was required to investigate possible correlations between the level of a particular marker and bacterial bioburden or the presence of particular bacterial species, which will be the basis of discussion in Chapter 4.

In addition, these chronic wounds were clinically diagnosed as non-infected and infected, as per current standard diagnosis of local infection. The Clinicians opinion as to whether the wound was infected or not was the basis of classification into either group.

The clinical signs and symptoms that led the Clinician to believe the wound was infected were noted. These signs and symptoms included evidence of cellulitis, localized pain, swelling, erythema, increased skin temperature, unhealthy granulation tissue, abnormal odour, pus and increased exudate production (Harding, 1996). A minimum of four clinical signs and symptoms had to be present in the diagnosis of the wound for the Clinician to classify the wound as infected.

Once the wound fluids were assigned an infection classification based on clinical assessment, a comparison was made between the clinically diagnosed non-infected and infected wound fluids for potential biomarkers. Potential biomarkers investigated included, (i) the proteases, elastase and matrix metalloproteinases (MMPs) along with their associated inhibitors, alpha-1-antitrypsin, and tissue inhibitors of metalloproteinases (TIMPs) and (ii) numerous cytokines and growth factors. These particular molecules were selected due to their association with wound chronicity or acute bacterial infections. The rationale for measuring these particular molecules is discussed in turn in the following Sections, with reference to the literature.

3.1.1 Elastase and elastase inhibitors

Elastase is a neutrophilic protease whose major physiological role is to digest bacteria and immune complexes once phagocytosed. Elastase is a serine protease that cleaves many substrates including elastin and fibronectin, components of the ECM (Grinnell and Zhu, 1994, Owen and Campbell., 1995). Neutrophil elastase is contained within the cell, but can be detected extracellularly, following 'frustrated phagocytosis' (Fritz et al., 1986, Doring, 1994), where upon elastase can be secreted from the cell, into the surrounding tissue.

Measurement of elastase in various biofluids and tissues suggests that elevated elastase may be a reliable marker of genital tract infection (Micic et al., 1989), as a predictive marker for the onset of neonatal infections (Matsuda et al., 1995), as an indicator of septicemia after abdominal injury (Duswald et al., 1985), as a general marker of inflammatory diseases (Pradella et al., 1995) and as a marker of inflammatory complications, following surgery (Hofer et al., 1995).

Analysis of the levels and activity of elastase in the clinically diagnosed non-infected and infected wound fluids was performed using a variety of methods, including a fluorogenic assay, zymography and an enzyme-linked immunosorbent assay (ELISA) (Chapter 2, Section 2.3). Quantification of elastase activity was made using a fluorogenic method and by ELISA. Zymography, a substrate-based SDS-polyacrylamide gel electrophoresis (PAGE) technique was also employed as a complementary technique to the fluorogenic data (Chapter 2, Section 2.3). This technique allowed the detection of active elastase complexes, in addition to elastase itself. Interference of other molecules in wound fluids, for example, the naturally occurring elastase inhibitors, such as alpha-1-anti-trypsin (Hlasta and Pagani, 1994, van Gent et al., 2003) means that the measurement of elastase activity may not directly correlate to the actual amount of elastase in each wound fluid.

Alpha-1 anti-trypsin is synthesized and secreted by the liver into serum, where it inhibits plasma proteins circulating in the blood, such as elastase. In addition to its ability to inhibit elastase, alpha-1-anti-tyrpsin is also capable of inhibiting trypsin (Beatty et al., 1980), cathepsin G and proteinase 3 (Dolman et al., 1992, Coakley et al., 2001, Duranton and Bieth, 2003). Uncontrolled activity of any of these proteins results in excessive proteolytic activity that enables ECM degradation and ultimately, may compromise tissue integrity. Genetic deficiencies in alpha-1-anti-tyrpsin have been implicated with a pre-disposition to medical conditions, including pulmonary emphysema and chronic liver disease (Stoller and Aboussouan, 2005).

It is not surprising that alpha-1-anti-tyrpsin has been measured in extracellular fluids associated with inflammatory conditions, such as periodontal disease (Petropoulou et al., 2003), rheumatoid arthritis (Zhang et al., 1990) and acute and chronic wounds (Yager et al., 1997). Alpha-1-anti-tyrpsin levels were found to be highly sensitive and specific for infection, following prosthetic hip surgery, compared to the erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP), indicating a used as a diagnostic tool for infection (Gomez-Navalon et al., 2000). In the present Study, analysis of alpha-1-anti-trypsin was performed by ELISA. Currently, there are no commercially available fluorogenic substrates available that are wholly specific for alpha-1-anti-tyrpsin.

3.1.2 Matrix metalloproteinase and their inhibitors

MMPs are a group of proteolytic enzymes, whose actions include the degradation of ECM components, allowing amongst other functions, cellular migration and angiogenesis (Trengrove et al., 1999) (Chapter 1, Section 1.6).

MMP-2 and MMP-9 are currently being investigated for their role in infectious meningitis (Shapiro et al., 2003), pulmonary infections, including *Mycobacterium tuberculosis* (Quiding-Jarbrink et al., 2001, Hrabec et al., 2002), and *Cryptococccus neoformans* (Majka et al., 2002), as well as the pathogenesis of pseudomonal keratitis (Miyajima et al., 2001). MMPs are associated with ocular infections caused by

Pseudomonas aeruginosa (Kernacki et al., 1997, Dong et al., 2000) and malignant and inflammatory diseases of the stomach, liver, gall bladder and intestine (Bergin et al., 2005, Yanagisawa et al., 2005). Zymographic analysis of MMP-2 and MMP-9 by Makowski and Ramsby, (2003), demonstrated that the amount of MMP activity in synovial fluid is proportional to the presence of polymorphonuclear cells and in response to infection. Tarlton et al., (1997), linked MMP activity and wound healing, concluding that increased MMP activity is associated with an infected acute and a chronic, non-healing wound.

In the present Study, MMP activity in clinically diagnosed non-infected and infected chronic wounds was determined fluorogenically and by substrate gelatin zymography (Chapter 2, Section 2.3). Quantification of active MMP forms were measured fluorogenically for the collagenases, MMP-1, -8, and -13 and gelatinase A and B, MMP-2 and MMP-9, respectively. Semi-quantitative measurement of pro- and active forms of the gelatinases (MMP-2 and MMP-9), and the total amount of the molecule was determined by gelatin zymography. This zymography technique provided a tool for separate analysis of MMP forms, as opposed to the combined activity value of MMP-2 and MMP-9, obtained by fluorogenic analysis. The combined collagenase activity data obtained by fluorogenic analysis was determined as sufficient for this data set. Collagen substrate zymography was not explored in the present Study.

TIMPs are the main endogenous inhibitors of MMPs (Bode and Makos, 2003). TIMPs regulate the action of MMPs, which themselves play a central role in wound healing, tissue repair and remodeling, in response to injury (Nagase and Woessner, 1997). The invasive nature of some tumours has been correlated to an imbalance in the relationship between MMPs and their associated inhibitors (Wojtowicz-Praga et al., 1997). Attempts to address the uncontrolled MMP activity has led to the development of synthetic molecules that mimic the action of TIMPs. This approach has not been adopted for chronic wound repair, with current treatments consisting of alternative substrates for MMPs to act upon (Chapter 1, Section 1.6).

In relation to infection, it has been demonstrated in mice corneal models that TIMP-1 can afford protection (Kernacki et al., 1999) and resistance (Lee et al., 2005), to infection. However, it has also been demonstrated that components of certain bacterial species, are capable of the degradation of TIMP-1 (Grenier, 1996). The levels of TIMPs themselves and the relationship to their respective MMPs has not been investigated between chronic non-infected and infected wounds. The amount of TIMP-1 and TIMP-2, in the present Study, was quantified by enzyme-linked immunosorbent assay (ELISA) (Chapter 2, Section 2.3).

3.1.3 Cytokines

Cytokines are a family of cell signaling molecules that modulate cellular function, associated with inflammatory and wound healing responses (Chapter 1, Section 1.7). The general effects of cytokines are usually contained within the locality of release, which primarily function by autocrine or paracrine actions (Krishnamoorthy et al., 2001, Henry and Garner, 2003).

Specific cytokines, for example tumour necrosis factor-α (TNF-α), TNF-β, interleukins-1 (IL-1) and IL-6 can demonstrate major systemic effects, defined as an endocrine action (Feghali and Wright, 1997). The endocrine actions of cytokines are associated with bacterial sepsis, when the cytokine is produced in large amounts (Waage and Steinshamm, 1993; Matsumoto, 1999) or when produced chronically in lesser amounts, a chronic infection may ensue (Feghali and Wright, 1997). Due to their perceived role in many inflammatory conditions, such as rheumatoid arthritis (Choy et al., 2002) and bacterial infections including *Chlamydia, Listeria, Salmonella, Mycobacterium* and *Klebsiella*, (Degre, 1996), anti-cytokine therapies are currently being evaluated that may eliminate the over-reaction of the immune system to the influence of these cytokines (reviewed by Dinarello, 2003).

Cytokines have been investigated in numerous wound fluid studies, leading to the hypothesis that up-regulation of particular cytokines, is a contributing mechanism behind the chronicity of a wound. Increases in the amount of the pro-inflammatory cytokine, IL-1β, IL-6 and TNF-α in acute verses acute, inflamed (Wagner, 2003), acute verses chronic (Mast and Shultz, 1996) and chronic, non-healing verses chronic healing (Harris et al., 1995, Trengrove et al., 2000), have all been reported. The growth factors, transforming growth factor-β_1 (TGF-β_1) and vascular endothelial growth factor (VEGF) have also been measured in acute wounds (Tarnuzzer and Shultz, 1996, Baker and Leaper, 2000) and chronic wounds (Tarnuzzer and Shultz, 1996). The relationship between TNF-α and its receptor has also been investigated in chronic healing and non-healing wounds (Wallace and Stacey, 1998).

These studies have provided evidence that particular cytokines and growth factors are increased in chronic wounds. This Study aims to determine whether the levels of particular cytokines and growth factors, already reported in the literature to be elevated, can be increased further, due to the host's response to bacteria.

In the present Study, clinically diagnosed non-infected and infected wound fluids were compared, in order to determine whether any cytokine or growth factor levels differentiated between infection status. Typically, the amount of cytokine in a biological sample, such as wound fluid, was quantified by ELISA (Chapter 2, Section 2.3).

Recently advances in micro-array technology have seen the commercialization of cytokine micro-array chips. Advantages of such a technology include the small volume of sample required in order to detect many cytokines in one analysis, and the provision of information on the levels of one cytokine in relation to another in the same analysis. In the present Study, wound fluids were analysed for 18 cytokines and growth factors, which were commercially available on an array chip (Chapter 2, Section 2.3).

A number of the cytokines provided by this micro-array system were of direct relevance to chronic wound healing, as previously cited in the literature. However, the other cytokines provided on the same chip had not been explored in the wound-healing arena and could provide novel candidates for chronic wound infection biomarkers. Due to the recent commercialization of cytokine micro-array chips, no data was available in the literature regarding the levels of cytokines in wound fluids, using this technology. For this reason, a comparison of this relatively new technique was made with the established method of cytokine quantification, ELISA.

The aim of this Chapter, therefore, was to analyse selected proteases, their inhibitors, and cytokines in clinically non-infected and infected venous leg and diabetic foot ulcer wound fluids, that could potentially allow biochemical differentiation between these wounds. Additionally, the clinical diagnosis of infection will be correlated with the number of bacteria within the wound.

3.2 Materials and methods

Experimental conditions detailing the collection method, analysis of wound microflora, and wound fluid protein content, are described (Chapter 2, Section 2.1). Analytical methods used in the quantification of proteases, their inhibitors and a number of cytokines are previously described (Chapter 2, Section 2.3). *Ex vivo* wound fluid data was considered as non-parametric and Wilcoxon two-sided probability statistical analysis was used. Data was considered parametric when comparing venous leg ulcer and diabetic foot ulcer wound fluid data to one another. A Students t-test (2 tailed) was applied assuming unequal variance use. Significance was defined at $p<0.05$.

3.3 Results

3.3.1 Patient information

Within the venous leg ulcer population tested, 7 out of 20 patients were female, with the patient's age ranging from 41-91 years old (Table 3.1). Analysis of quantifiable patient information between clinically non-infected and infected wounds showed no significant difference, ($p>0.05$) as determined by Wilcoxon probability, in patient age (years), median value 75.5 and 75.5 respectively, or wound duration (months), median value 60.0 and 48 respectively. Significant differences in wound area and perimeter were found between the two groups. Wound area (cm^2), tended to be greater in the infected population, median value 51cm^2, compared to 11.5 cm^2, and wound perimeter, median value 39.6cm, compared to 17.3cm.

In the diabetic foot ulcer population, 5 out of 15 patients were female, with the patient's age ranging from 39-85 years old (Table 3.1). Clinically non-infected and infected diabetic foot ulcer wounds showed no significant difference ($p > 0.1$), as determined by Wilcoxon probability, in patient age (years), median value 61.5 and 55.0 respectively, wound duration (months), median value 5.50 and 16.0, respectively. Unfortunately, no data analysis was performed on the size of the wound, either wound area (cm^2) or wound perimeter (cm), as there was an incomplete set of data collected for the diabetic group.

Table 3.1: Patient information
(a) Venous leg ulcer and (b) diabetic foot ulcer wound fluid samples collected by the Wound Healing Research Unit, Cardiff. Patients 1-10 were clinically diagnosed as non-infected. Patients 11-20 were clinically diagnosed as infected. Wound area and perimeter was the only quantifiable wound parameter that distinguishes between clinically non-infected and infected venous leg ulcer wounds based on clinical signs and symptoms (p=0.017). No quantifiable wound parameter distinguishes the two groups within the diabetic foot ulcer wounds.

a)

Venous Leg Ulcer Wounds

	Patient	Gender	Age (years)	Wound Duration (months)	Wound Area (cm2)
Non-Infected Wounds Clinical Diagnosis	1	Female	77	78	11.7
	2	Male	65	360	11.3
	3	Female	78	18	73.1
	4	Female	73	6	23.1
	5	Male	71	96	11.3
	6	Male	77	542	5.4
	7	Male	81	168	14.6
	8	Male	74	9	7.7
	9	Male	71	8	47.8
	10	Male	86	6	5.4
Infected Wounds Clinical Diagnosis	11	Male	87	72	61.2
	12	Male	86	84	40.7
	13	Male	82	204	36.9
	14	Female	91	24	14.1
	15	Female	63	132	7.9
	16	Male	41	48	26.8
	17	Female	90	30	98.7
	18	Female	67	36	205.6
	19	Male	69	131	206.3
	20	Male	48	8	93.0

b)

Diabetic Foot Ulcer Wounds

	Patient	Gender	Age (years)	Wound Duration (months)
Non-Infected Wounds Clinical Diagnosis	1	Male	64	2
	2	Male	81	1
	3	Female	71	6
	4	Male	59	3
	5	Female	58	5
	6	Male	51	36
	7	Male	65	5
	8	Female	57	40
	9	Male	39	53
	10	Male	85	36
Infected Wounds Clinical Diagnosis	11	Female	64	10
	12	Female	40	54
	13	Male	65	3
	14	Male	55	16
	15	Male	50	36

3.3.2 Quantitative microbiology of non-infected and infected venous leg and diabetic foot ulcer wounds

The amount and type of aerobic and anaerobic bacteria, fungi or yeast, in each wound was analysed using standard quantitative microbiology techniques (Chapter 2, Section 2.2). Analysis of wound swabs revealed no significant difference in the number of microorganisms expressed as colony forming units per millilitre (CFU/ml), between the non-infected and infected wounds, in either the venous leg or diabetic foot ulcer populations. The levels of microorganisms in venous leg ulcer wounds ranged from 10^3-10^8 cfu/ml and 10^2-10^8 CFU/ml for diabetic foot ulcer wounds. The number and type of bacteria, fungi or yeast for each wound type are shown in Figure 3.1, for a) venous leg and b) diabetic foot ulcer wounds, respectively. The wound microbiology for each venous leg ulcer or diabetic foot ulcer patient is shown in Table 3.2 and Table 3.3.

The majority of venous leg ulcer wounds contained the aerobic bacteria *Pseudomonas* spp. and *Staphylococcus* spp. In contrast, there was only one diabetic foot ulcer containing *Pseudomonas* spp., a few diabetic foot ulcers containing *Staphylococcus* spp., with the majority of diabetic foot ulcers containing *Corynebacterium* spp.

The vast majority of species detected in both ulcer types, were aerobes, with only 3 venous leg ulcers (patients 4, 9, and 17) and 4 diabetic foot ulcers (patients 9, 11, 14 and 15), containing detectable anaerobic organisms. Of the 32 wounds assessed, a yeast *Candida* spp., was detected in one clinically infected venous leg ulcer (patient 7) and diabetic foot ulcer wound (patient 15). One diabetic foot ulcer wound also contained the fungi, *Actinomyces* spp. (patient 15).

Unfortunately, microbiological data was unavailable for 2 clinically diagnosed infected venous leg ulcers (patients 19 and 20) and 1 clinically diagnosed infected diabetic foot ulcer (patient 12).

Figure 3.1: Bacterial, fungal and yeast characterisation of venous leg and diabetic foot ulcer wound fluid.

Number of bacterial species in clinically non-infected and infected (a) venous leg ulcer and (b) diabetic foot ulcer wound fluid. Total bioburden ranged from 10^3-10^8 CFU/ml and 10^2 and 10^8 CFU/ml in the clinically non-infected and infected venous leg and diabetic foot ulcer wounds, respectively. There was no significant difference in the number of genera, comparing non-infected and infected wounds of either wound type.

The most prominent species detected in venous leg ulcer wound fluid was *Pseudomonas* spp. (12 of 18) and *Staphylococcus* spp. (10 of 18). Three venous leg ulcer wound fluids contained anaerobic bacteria (*) and one wound fluid contained with yeast (-).

The most prominent species detected in diabetic foot ulcer wound fluid was *Corynebacterium* spp. (11 of 14) and *Staphylococcus* (7 of 14). Four diabetic foot ulcer wound fluids contained anaerobic bacteria (*), *Propionibacterium* spp. and *Peptostreptococcus* spp..

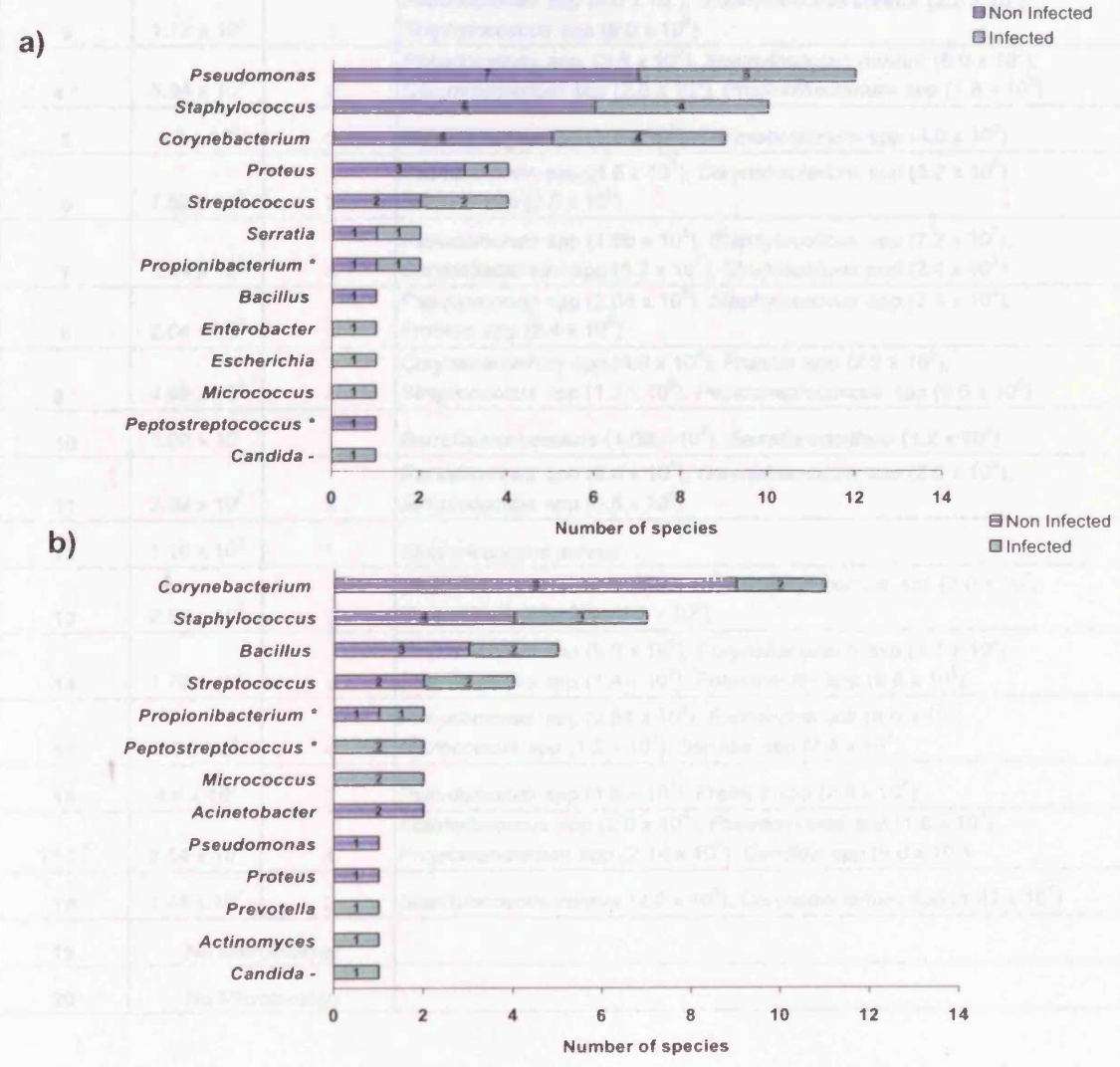


Table 3.2: Wound microflora of clinically non-infected and infected venous leg ulcers.

Total bioburden levels and microbial genera for each individual patients wound are summarized in the table. Some wounds contained anaerobic bacteria (*) and yeast (-). *Proteus* spp. does not produce single colonies on blood agar but rather has a spreading morphology so can be difficult to count.

Patient	Total bioburden (cfu/ml)	Number of genera	Genera
1	3.0×10^6	3	*Pseudomonas* spp (2.0×10^6), *Staphylococcus aureus* (1.0×10^6), *Proteus* spp (Too numerous to count)
2	5.8×10^4	1	*Staphylococcus aureus*
3	1.12×10^5	2	*Pseudomonas* spp (4.0×10^2), *Staphylococcus aureus* (2.2×10^4), *Staphylococcus* spp (9.0×10^4)
4 *	5.94×10^3	4	*Pseudomonas* spp. (3.8×10^3), *Staphylococcus aureus* (6.0×10^1), *Corynebacterium* spp (2.8×10^2), *Propionibacterium* spp (1.8×10^3)
5	4.0×10^6	2	*Pseudomonas* spp (3.6×10^6), *Corynebacterium* spp (4.0×10^5)
6	7.52×10^8	3	*Pseudomonas* spp (3.6×10^7), *Corynebacterium* spp (5.2×10^8), *Bacillus* spp (2.0×10^8)
7	3.14×10^8	4	*Pseudomonas* spp (1.66×10^8), *Staphylococcus* spp (7.2×10^7), *Corynebacterium* spp (5.2×10^7), *Streptococcus* spp (2.4×10^7)
8	2.04×10^8	3	*Pseudomonas* spp (2.04×10^8), *Staphylococcus* spp (2.4×10^4), *Proteus* spp (2.4×10^4)
9 *	4.99×10^8	4	*Corynebacterium* spp (4.9×10^8), *Proteus* spp (2.2×10^6), *Streptococcus* spp (1.2×10^6), *Peptostreptococcus* spp (6.0×10^6)
10	1.09×10^7	1	*Serratia marcescens* (1.08×10^7), *Serratia odorifera* (1.2×10^5)
11	3.98×10^7	3	*Pseudomonas* spp (3.4×10^7), *Corynebacterium* spp (2.0×10^6), *Streptococcus* spp (3.8×10^6)
12	1.16×10^5	1	*Staphylococcus aureus*
13	2.62×10^3	2	*Staphylococcus aureus* (4.0×10^2), *Staphylococcus* spp (2.0×10^3), *Corynebacterium* spp (2.2×10^2)
14	1.79×10^8	4	*Pseudomonas* spp (8.0×10^6), *Corynebacterium* spp (1.7×10^8), *Streptococcus* spp (1.4×10^6), *Enterobacter* spp (6.6×10^3)
15	3.3×10^8	4	*Pseudomonas* spp (2.94×10^8), *Escherichia coli* (8.6×10^3), *Micrococcus* spp (1.2×10^7), *Serratia* spp (2.4×10^7)
16	4.6×10^7	2	*Pseudomonas* spp (1.8×10^7), *Proteus* spp (2.8×10^7)
17 * *	2.14×10^8	4	*Staphylococcus* spp (2.0×10^4), *Pseudomonas* spp (1.8×10^4), *Propionibacterium* spp (2.14×10^8), *Candida* spp (6.0×10^1)
18	1.44×10^7	2	*Staphylococcus aureus* (2.0×10^5), *Corynebacterium* spp (1.42×10^7)
19	No Microbiology		
20	No Microbiology		

Table 3.3: Wound microflora of clinically non-infected and infected diabetic foot ulcers.

Total bioburden levels and microbial genera for each individual patient wound are summarized in the table. Some wounds contained anaerobic bacteria (*).

Patient	Total bioburden (cfu/ml)	Number of genera	Genera
1	1.2×10^6	2	*Corynebacterium* spp (1.2×10^6), *Proteus* spp (4.0×10^3)
2	8×10^2	1	*Bacillus* spp
3	1.2×10^6	1	*Corynebacterium* spp
4	2.8×10^5	3	*Bacillus* spp (2.2×10^5), *Corynebacterium* spp (4.0×10^4), *Acinetobacter* spp (2.0×10^4)
5	4.2×10^8	2	*Corynebacterium* spp (3.6×10^8), *Staphylococcus aureus* (6.2×10^7)
6	4.6×10^8	5	*Corynebacterium* spp (4.6×10^8), *Bacillus* spp (4.0×10^5), *Staphylococcus aureus* (2.0×10^5), *Acinetobacter* spp (2.0×10^5), *Streptococcus* spp (2.0×10^4)
7	8.6×10^8	3	*Corynebacterium* spp (8.6×10^8), *Staphylococcus* spp (2.0×10^6), *Pseudomonas* spp (9.6×10^5)
8	5.0×10^8	2	*Corynebacterium* spp (5.0×10^8), *Staphylococcus* spp (8.0×10^5)
9 *	5.0×10^5	2	*Corynebacterium* spp (6.0×10^4), *Propionibacterium* spp (4.4×10^5)
10	1.4×10^5	2	*Corynebacterium* spp (1.4×10^5), *Streptococcus* spp (8.0×10^2)
11 *	5.2×10^8	2	*Corynebacterium* spp (5.2×10^8), *Propionibacterium* spp (4.0×10^2)
12	No Microbiology		
13	3.9×10^6	4	*Corynebacterium* spp (3.8×10^6), *Staphylococcus* spp (2.0×10^3), *Bacillus* spp (6.0×10^4), *Micrococcus* spp (8.0×10^4)
14 *	1.64×10^7	4	*Staphylococcus aureus* (6.4×10^6), *Micrococcus* spp (6.8×10^6), *Streptococcus* spp (4.0×10^5), *Peptostreptococcus* spp (2.2×10^6)
15 *	1.02×10^8	7	*Staphylococcus* spp (4.6×10^7), *Bacillus* spp (2.0×10^5), *Streptococcus* spp (2.6×10^6), *Actinomyces* spp (3.6×10^7), *Peptostreptococcus* spp (8.0×10^6),

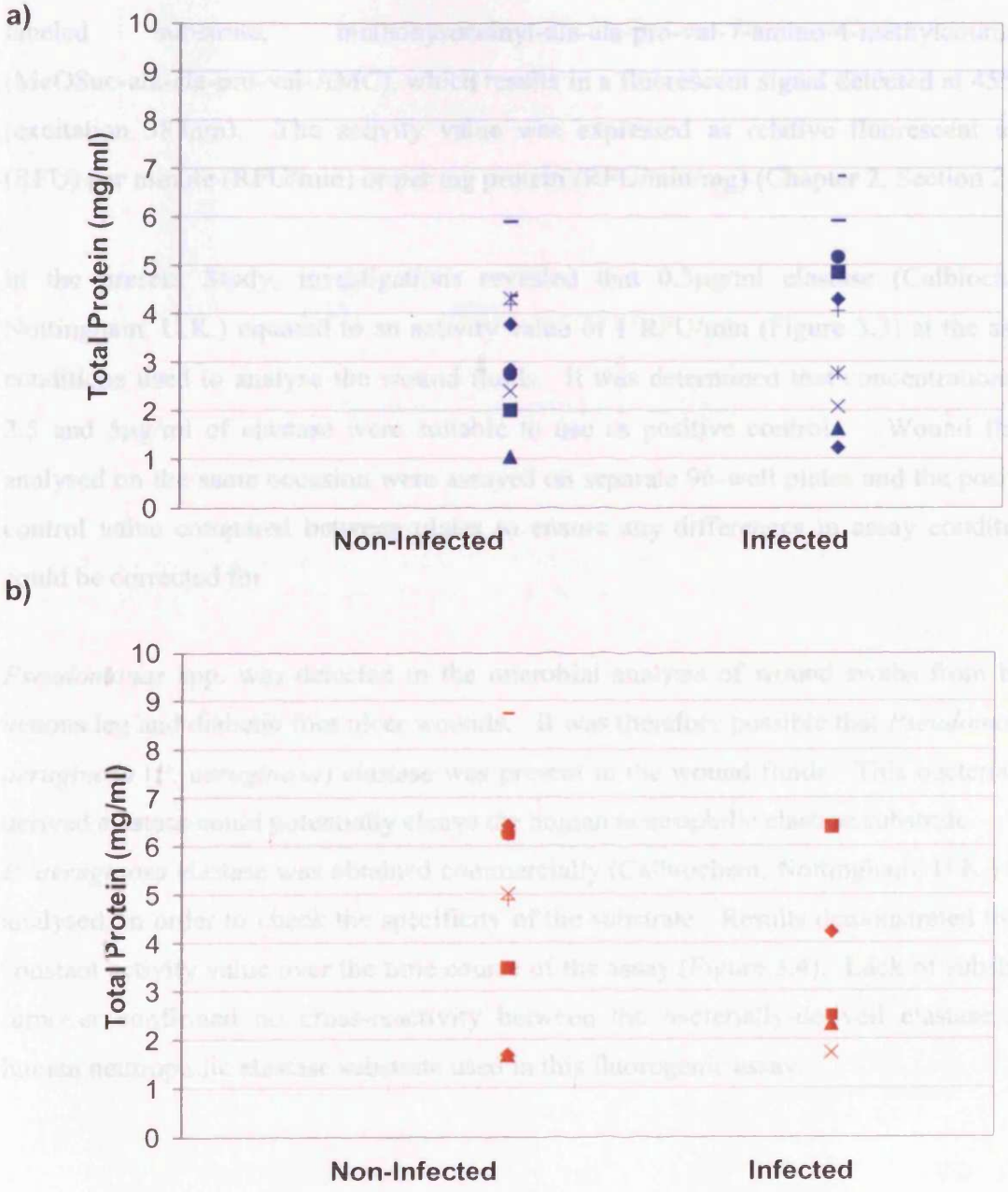
3.3.3 Protein quantification of non-infected and infected venous leg and diabetic foot ulcer wound fluids

Wound fluid protein content was determined using the Bio-Rad Protein Assay Kit (Chapter 2, Section 2.1).

Results demonstrated that the average protein content in venous leg ulcer wound fluid was 3.35 ± 1.06 mg/ml (average±SE, range 1.04-5.89 mg/ml) and 3.87±1.23 mg/ml (average±SE, range 1.22-6.83 mg/ml) in the non-infected and infected wound fluids, respectively (Figure 3.2). The average protein content of non-infected and infected diabetic foot ulcer wound fluid was 4.87±1.51 mg/ml (average±SE, range 1.67-8.74 mg/ml) and 3.43±1.08 mg/ml (average±SE, range 1.72-6.39 mg/ml), respectively (Figure 3.2). No statistical significance difference ($p>0.05$) was found between the non-infected and infected groups, for the venous leg or diabetic foot ulcer wound fluid.

Determination of total protein levels within the samples has allowed for measurements based on sample volume to be corrected for protein. Practically, it has also allowed samples to be standardised for protein content, prior to analysis.

Figure 3.2: Wound fluid protein content.
Average protein concentration for clinically non-infected and infected (a) venous leg ulcer and (b) diabetic foot ulcer wound fluids. Statistical analysis demonstrated no significant difference between the clinically non-infected and infected ulcer wound fluids ($p>0.05$). No statistical difference in protein content was observed between the venous leg and diabetic foot ulcer wound fluids.



3.3.4 Elastase

3.3.4.1 Fluorogenic analysis of elastase activity

3.3.4.1.1 Method conditions

Elastase activity in wound fluids was measured by the cleavage of a fluorogenically labeled substrate, methoxysuccinyl-ala-ala-pro-val-7-amino-4-methylcoumarin (MeOSuc-ala-ala-pro-val-AMC), which results in a fluorescent signal detected at 455nm (excitation 383nm). The activity value was expressed as relative fluorescent units (RFU) per minute (RFU/min) or per mg protein (RFU/min/mg) (Chapter 2, Section 2.3).

In the present Study, investigations revealed that 0.3μg/ml elastase (Calbiochem, Nottingham, U.K.) equated to an activity value of 1 RFU/min (Figure 3.3) at the assay conditions used to analyse the wound fluids. It was determined that concentrations of 2.5 and 5μg/ml of elastase were suitable to use as positive controls. Wound fluids analysed on the same occasion were assayed on separate 96-well plates and the positive control value compared between plates to ensure any differences in assay conditions could be corrected for.

Pseudomonas spp. was detected in the microbial analysis of wound swabs from both venous leg and diabetic foot ulcer wounds. It was therefore possible that *Pseudomonas aeruginosa* (*P. aeruginosa*) elastase was present in the wound fluids. This bacterially-derived elastase could potentially cleave the human neutrophilic elastase substrate.
P. aeruginosa elastase was obtained commercially (Calbiochem, Nottingham, U.K.) and analysed, in order to check the specificity of the substrate. Results demonstrated that a constant activity value over the time course of the assay (Figure 3.4). Lack of substrate turnover confirmed no cross-reactivity between the bacterially-derived elastase and human neutrophilic elastase substrate used in this fluorogenic assay.

Figure 3.3: Activity of commercially available elastase.

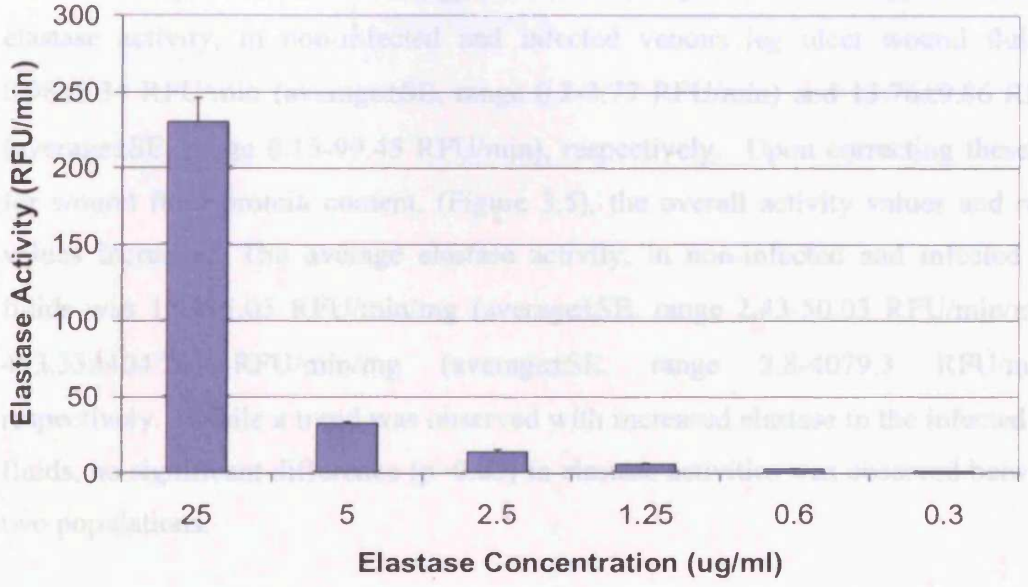
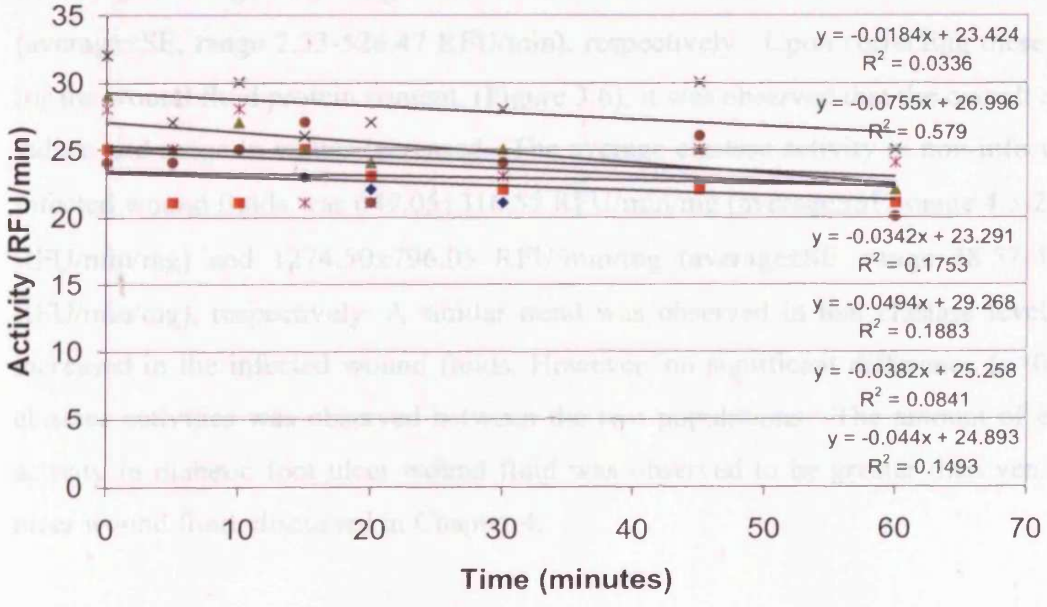


Figure 3.4: *Pseudomonas aeruginosa* elastase activity.
Six samples of *Pseudomonas aeruginosa* elastase were analysed and did not cleave the human neutrophil elastase substrate, MeOSuc-ala-ala-pro-val-AMC.



3.3.4.1.2 Non-infected and infected venous leg ulcer wound fluids

Elastase activities in venous leg ulcer wound fluids based upon volume (RFU/min) and corrected for protein (RFU/min/mg), are shown in Figure 3.5. Results show the average elastase activity, in non-infected and infected venous leg ulcer wound fluids was 0.98±0.34 RFU/min (average±SE, range 0.2-3.77 RFU/min) and 13.76±9.86 RFU/min (average±SE, range 0.15-99.45 RFU/min), respectively. Upon correcting these values for wound fluid protein content, (Figure 3.5), the overall activity values and range in values increased. The average elastase activity, in non-infected and infected wound fluids was 18.4±5.05 RFU/min/mg (average±SE, range 2.43-50.03 RFU/min/mg) and 453.33±404.26 RFU/min/mg (average±SE, range 2.8-4079.3 RFU/min/mg), respectively. While a trend was observed with increased elastase in the infected wound fluids, no significant difference ($p>0.05$) in elastase activities was observed between the two populations.

3.3.4.1.3 Non-infected and infected diabetic foot ulcer wound fluids

Elastase activities in venous leg ulcer wound fluids based upon volume (RFU/min) and corrected for protein (RFU/min/mg), are shown in Figure 3.6. Results show that the average elastase activity, in non-infected and infected wound fluid was 72.82±43.50 RFU/min (average±SE, range 0.53-397.48 RFU/min) and 127.73±101.13 RFU/min (average±SE, range 2.33-526.47 RFU/min), respectively. Upon correcting these values for the wound fluid protein content, (Figure 3.6), it was observed that the overall activity values and range in values increased. The average elastase activity in non-infected and infected wound fluids was 649.05±316.55 RFU/min/mg (average±SE, range 4.3-2274.13 RFU/min/mg) and 1274.50±796.05 RFU/min/mg (average±SE, range 38.57-4118.83 RFU/min/mg), respectively. A similar trend was observed in that elastase levels were increased in the infected wound fluids. However, no significant difference ($p>0.05$) in elastase activities was observed between the two populations. The amount of elastase activity in diabetic foot ulcer wound fluid was observed to be greater than venous leg ulcer wound fluid, discussed in Chapter 4.

Figure 3.5: Elastase activity in venous leg ulcer wound fluids.
Enzymatic cleavage of elastase substrate by venous leg ulcer wound fluids. Results expressed by (a) volume and (b) corrected for total protein in wound fluid.
Each point is the average value of six replicates, where n = 3, with no significant difference ($p>0.05$) between wound fluids from non-infected and infected venous leg ulcer wounds in either analyses.

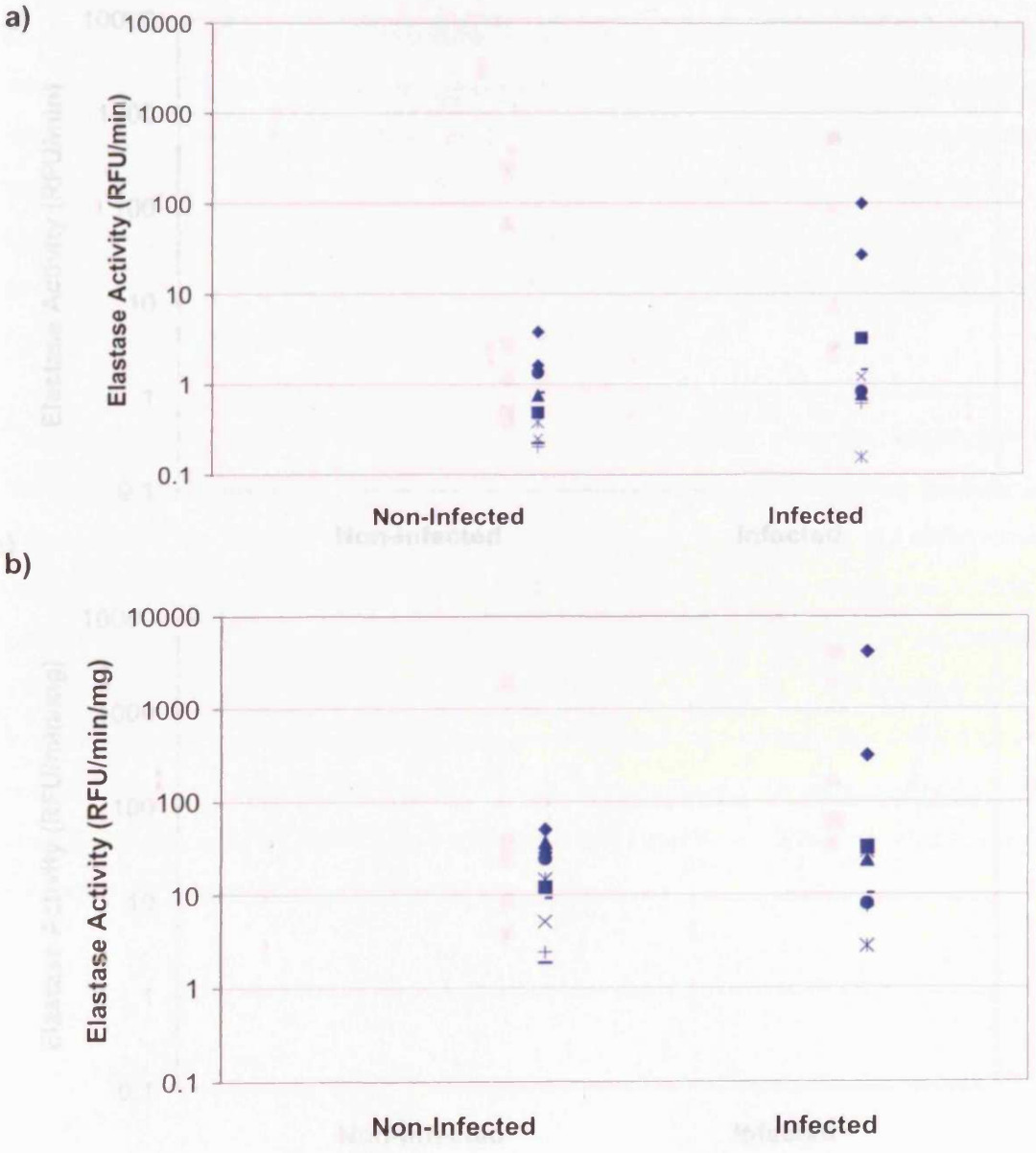
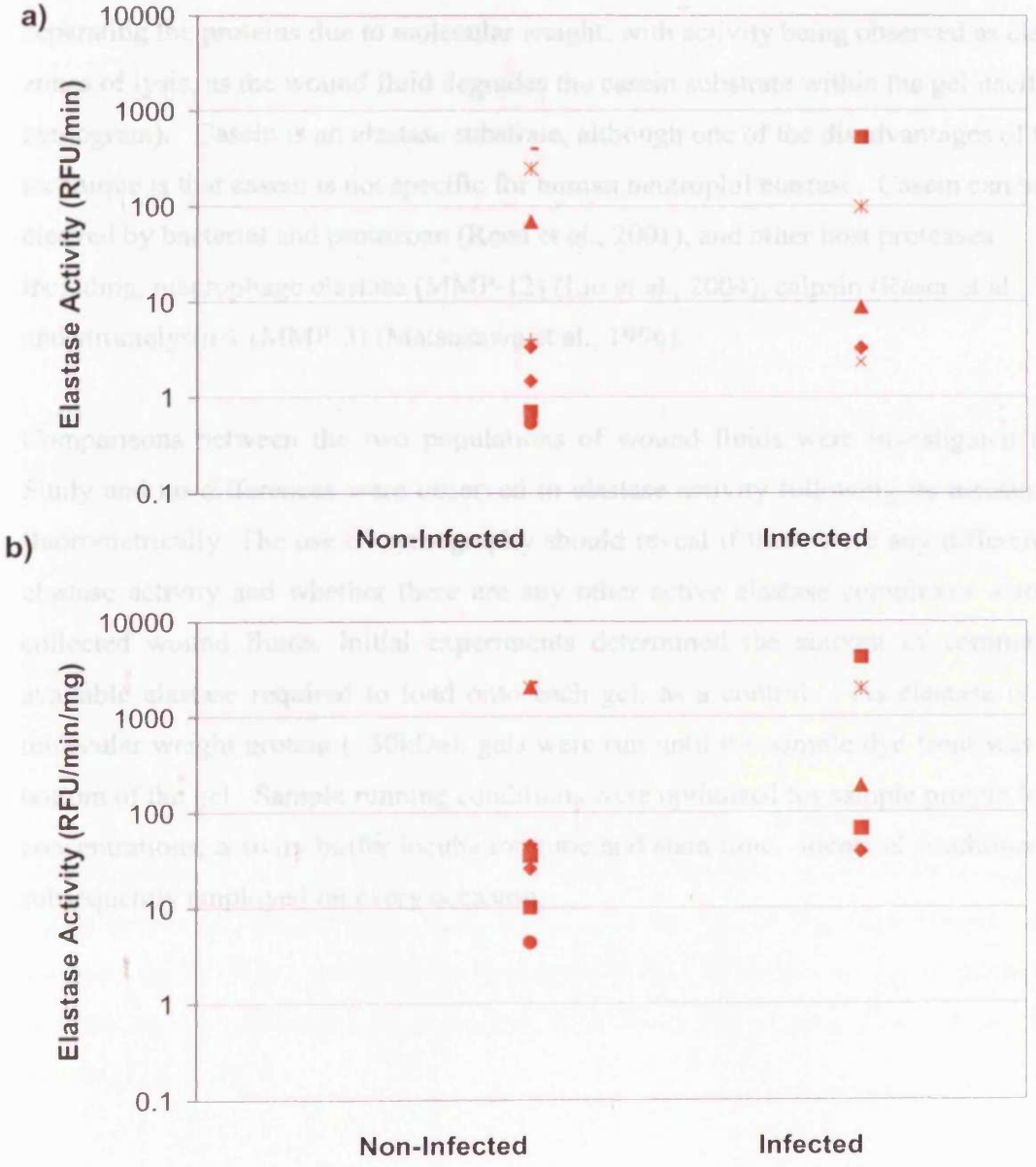


Figure 3.6: Elastase activity in diabetic foot ulcer wound fluid.
Enzymatic cleavage of elastase substrate by diabetic foot ulcer wound fluid. Results expressed by (a) volume and (b) corrected for total protein in wound fluid.
Each point is the average value of six replicates, where n = 3, with no significant difference ($p>0.05$) between wound fluids from non-infected and infected venous leg ulcer wounds in either analyses.



3.3.4.2 Elastase activity determined by zymography

3.2.4.2.1 Method conditions

Wound fluid elastase activity was also be determined by a semi-quantitative technique, via zymography (Chapter 2, Section 2.3). Wound fluid samples were run though linear Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, separating the proteins due to molecular weight, with activity being observed as clear zones of lysis, as the wound fluid degrades the casein substrate within the gel itself (a zymogram). Casein is an elastase substrate, although one of the disadvantages of the technique is that casein is not specific for human neutrophil elastase. Casein can be cleaved by bacterial and protozoan (Reed et al., 2001), and other host proteases including, macrophage elastase (MMP-12) (Liu et al., 2004), calpain (Raser et al., 1995) and stromelysin-1 (MMP-3) (Matsuzawa et al., 1996).

Comparisons between the two populations of wound fluids were investigated in this Study and no differences were observed in elastase activity following its measurement fluorometrically. The use of zymography should reveal if there were any differences in elastase activity and whether there are any other active elastase complexes within the collected wound fluids. Initial experiments determined the amount of commercially available elastase required to load onto each gel, as a control. As elastase is a low molecular weight protein (~30kDa), gels were run until the sample dye front was at the bottom of the gel. Sample running conditions were optimised for sample protein loading concentrations, activity buffer incubation time and stain time. Identical conditions were subsequently employed on every occasion.

Preliminary experiments with wound fluid revealed that the majority of the caseinolytic activity was conserved in the top portion of the gel (Section 3.3.4.2.2). Due to the small amount of caseinolytic activity at the expected molecular weight of elastase, the wound fluid samples were diluted minimally. Consequently, results presented herein for casein zymograms measuring elastase activity are based on wound fluid samples prepared as follows:

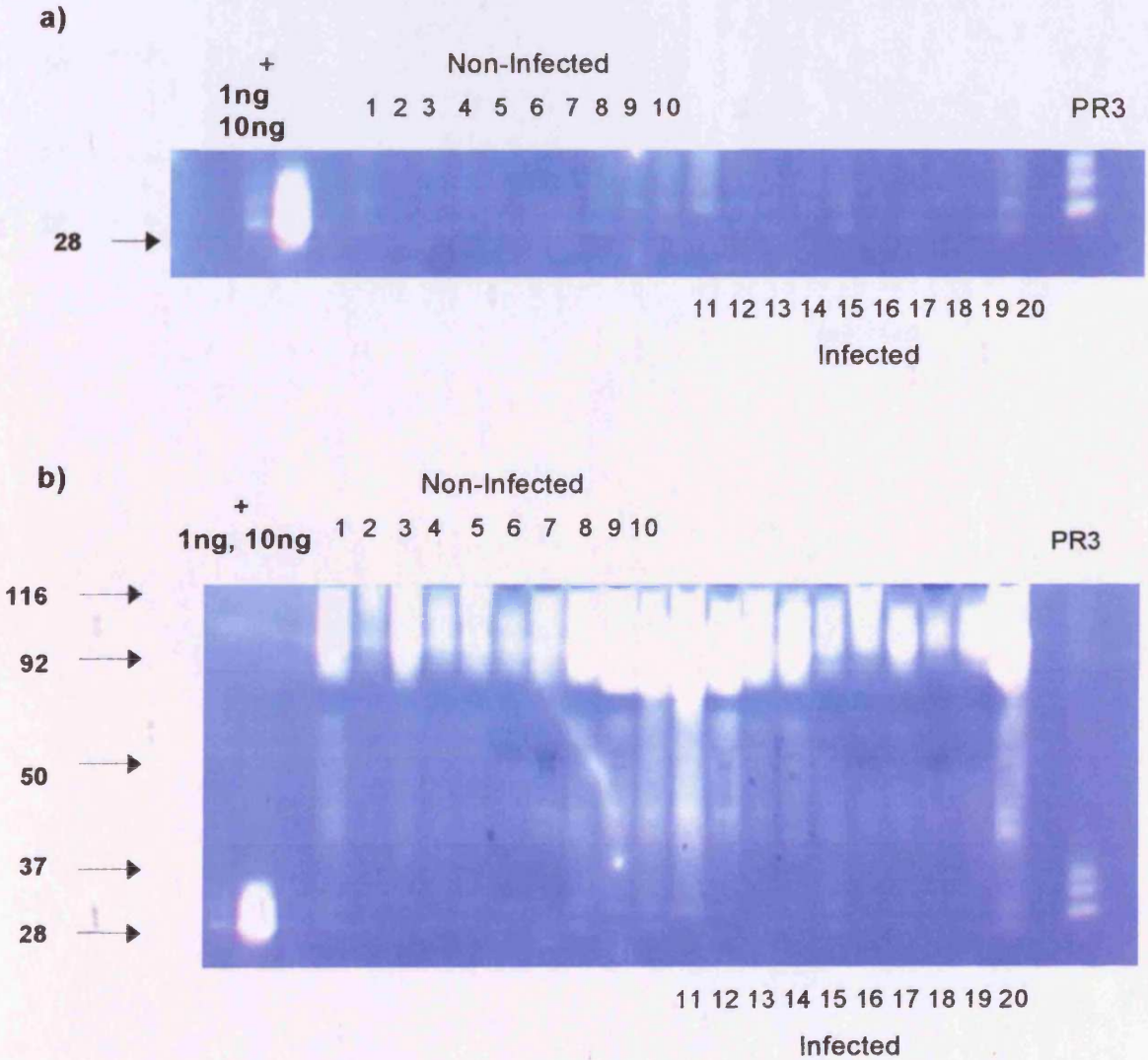
- Zymograms, analysed by protein content, comparing non-infected and infected wound fluid, were loaded at 16μg/10μl, for both venous leg ulcer and diabetic foot ulcers.
- Zymograms, analysed by volume, with each wound fluid was diluted minimally, 20μl wound fluid was added to 5μl treatment buffer, with 10μl loaded.

Duplicate gels were loaded on each occasion and zymograms run on three separate occasions.

3.3.4.2.2 Non-infected and infected venous leg and diabetic foot ulcer wound fluids

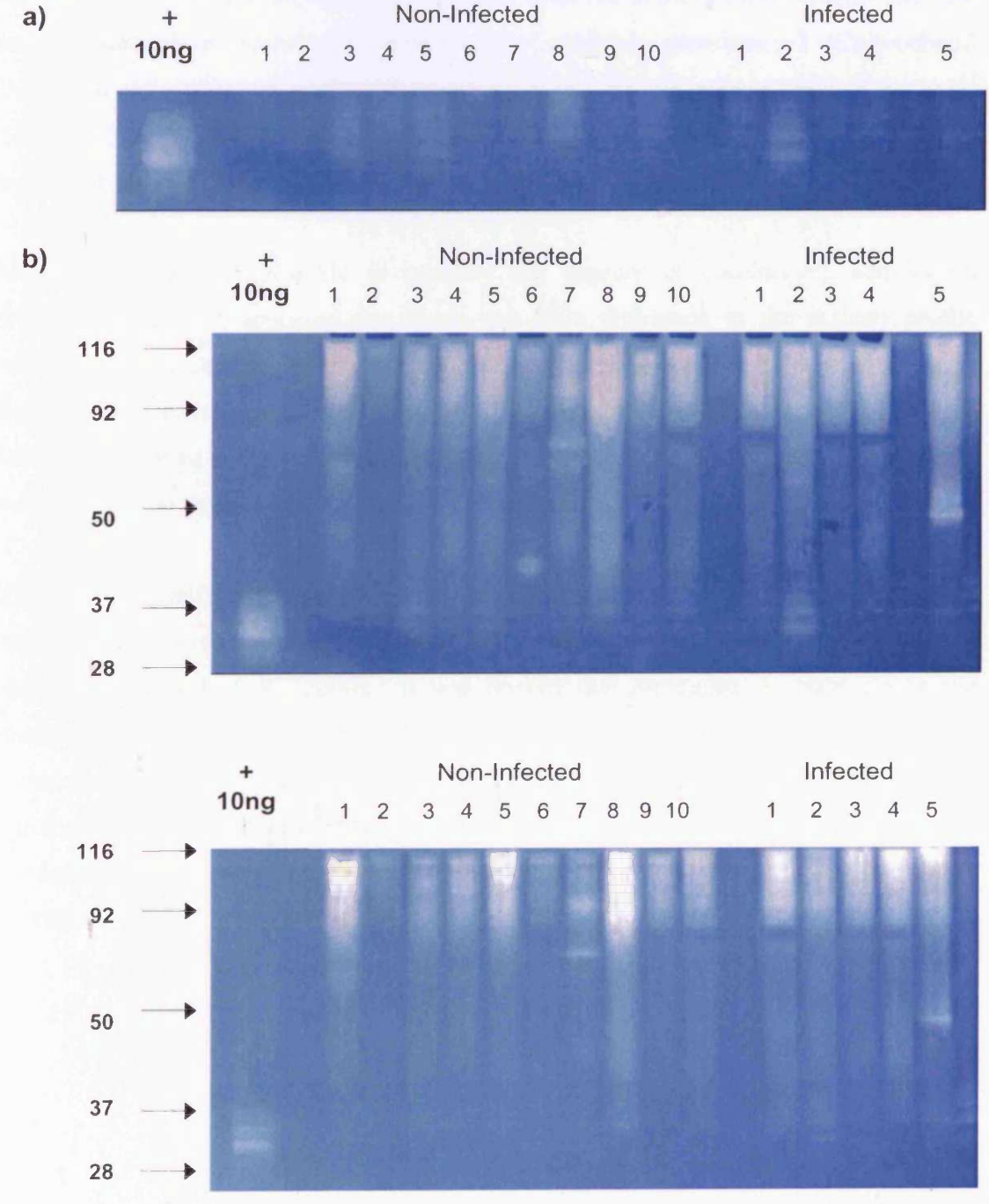
Elastase activity in venous leg ulcer wound fluid based on wound fluid volume, and determined by zymography, are shown in Figure 3.7a. The gel section exhibited zones of lysis in the low molecular weight region on the zymogram (~30kDa), where elastase activity would be expected. Preliminary investigations, however, revealed that the major casinolytic activity in wound fluid from both ulcer types occurred within the higher molecular weight region of the zymogram for venous leg ulcer wound fluid, by volume (Figure 3.7b), and by protein (Figure 3.7c). Diabetic foot ulcer wound fluid elastase activity exhibited similar profiles, when loaded by volume (Figure 3.8a), and by protein (Figure 3.8b).

Figure 3.7: Caseinolytic activity of venous leg ulcer wound fluid.
Representative gels of (a) caseinolytic activity in non-infected (1-10) and infected (11-20) venous leg ulcer wound fluid. Elastase (+, 1ng and 10ng) and proteinase 3 (PR3, 2ng) were used as positive controls. Caseinolytic activity of wound fluid was observed at approximately 30kDa as expected. The most caseinolytic activity in wound fluid appears to occur at high molecular weight (b) and (c), representative gels loaded by (b) volume and (c) by protein n=3. The caseinolytic profiles appeared similar in both non-infected and infected wound fluids.




c)
+
1ng , 10ng
Non-Infected
1 2 3 4 5 6 7 8 9 10
PR3
116
92
50
37
28
11 12 13 14 15 16 17 18 19 20
Infected

Figure 3.8: Caseinolytic activity of diabetic foot ulcer wound fluids.
(a) Representative gels of caseinolytic activity in non-infected (1-10) and infected (11-20) diabetic foot ulcer wound fluids. Elastase (+, 10ng) was used as a positive control. Some caseinolytic activity was observed at approximately 30kDa, as expected. (b) The caseinolytic profiles appear similar in both non-infected and infected wound fluids by (b) volume and (c) by protein.



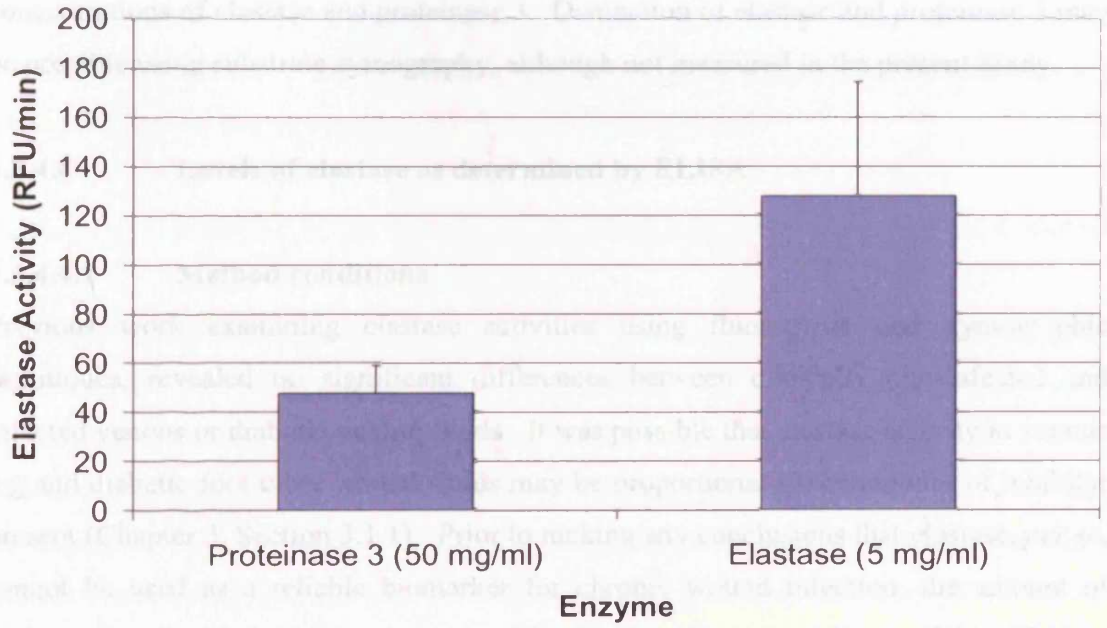
He et al., (1998), proposed that the major caseinolytic activity in burn wound fluid was due to proteinase 3. Their data shows a zone of lysis, between 30-100 kDa, that they attributed to the action of proteinase 3. He et al., (1998) had no explanation as to why proteinase 3 activity was seen at higher molecular weights, however their burn wound caseinolytic profiles appear equivalent to those observed in the chronic wounds analysed here. Caseinolytic activity of commercially available proteinase 3 (Calbiochem) (2ng/10µl) served as an additional positive control on the wound fluid zymograms (Figure 3.7 and 3.8) and migrated to the correct molecular weight, although a small amount of activity could be observed at the higher molecular weights.

Although it was not possible to quantify the amount of caseinolytic activity on zymography gels, it appeared that there was little difference in the activity profile between non-infected and infected wound fluids from either ulcer type (venous leg or diabetic foot ulcers), comparing samples diluted according to volume or protein. To determine whether elastase or proteinase 3 was the major caseinolytic activity in chronic wound fluids, additional investigations were performed.

3.3.4.3 Differentiation of elastase and proteinase 3

Proteinase 3 is a neutrophilic, azurophil granule molecule with a similar specificity to elastase (Campbell et al., 2000). It was proven that proteinase 3 could cleave the elastase fluorogenic substrate, MeOSuc-ala-ala-pro-val-AMC (Figure 3.9), albeit with a lesser preference than elastase. This substrate had been used to determine elastase activity in non-infected and infected wound fluids (Section 3.2.4.1). This activity data previously presented could therefore be a combination of both elastase and proteinase 3 activities.

Figure 3.9: Proteinase 3 activity.
Cleavage of the human neutrophil elastase substrate used in the fluorogenic assay was possible with human proteinase 3. Proteinase 3 is capable of cleaving the same substrate, methoxysuccinyl-ala-ala-pro-val-7-amino-4-methylcoumarin, as elastase.



Distinction between the two molecules fluorogenically has been conducted by Korkmaz et al (2002), using the substrate CrmA-derived substrate, Abz-VADCAQEDDnp. Use of this substrate results in a K_{cat}/Km ratio of proteinase 3 to elastase that allows for selective measurement of proteinase 3 activity in biological samples, containing similar concentrations of elastase and proteinase 3. Distinction of elastase and proteinase 3 may be possible using substrate zymography, although not measured in the present Study.

3.3.4.4 Levels of elastase as determined by ELISA

3.3.4.4.1 Method conditions

Previous work examining elastase activities using fluorogenic and zymographic techniques, revealed no significant differences between clinically non-infected and infected venous or diabetic wound fluids. It was possible that elastase activity in venous leg and diabetic foot ulcer wound fluids may be proportional to the amount of inhibitor present (Chapter 3, Section 3.1.1). Prior to making any conclusions that elastase, *per se*, cannot be used as a reliable biomarker for chronic wound infection, the amount of elastase, in wound fluid was determined by ELISA. Commercially available ELISAs contain a primary antibody that recognizes the elastase:elastase inhibitor complex. In the present Study, an elastase-specific ELISA was developed (Chapter 2, Section 2.3) to measure the amount of free elastase in clinically non-infected and infected wound fluids.

Briefly, the ELISA consisted of an elastase monoclonal capture antibody, raised in the mouse (Dakocytomation, Cambrideshire, U.K.), coated onto high binding 96-well plates, an elastase primary antibody raised in the rabbit (Calbiochem, Nottinghamshire, U.K.) and a secondary anti-rabbit IgG antibody conjugated to horse-radish peroxidase (HRP) (Sigma-Aldrich Company Ltd. Dorset, U.K.). The conjugated antibody allows detection using chromogenic soluble substrate for peroxidase activity, o-Phenylenediamine dihydrochloride (OPD) and urea hydrogen peroxide (Sigma-Aldrich). The ELISA recognized commercially available elastase, over a range of 0-10 ng/ml (Figure 3.10) (Chapter 2, Section 2.3).

The ELISA was verified to ensure no cross-reactivity to *Pseudomonas aeruginosa* elastase, human neutrophil proteinase 3, or albumin levels consistent with those extrapolated from the protein content of wound fluid (data not shown). Elastase was detected in a sample of lysed neutrophils (2 x 10^6 cells, lysed for 15min at 37°C, in 0.5ml RPMI-1640 + 10% serum and 0.5ml lysis buffer (0.1% Triton X-100, 50mM Tris/HCl) (Figure 3.11). This data gave confidence that elastase could be detected in a biological sample that is more representative of wound fluid.

3.3.4.4.2 Elastase levels in non-infected and infected venous leg ulcer wound fluids

Wound fluid dilutions were required, to allow for absorbancies to fall within the range of the standard curve. Interestingly, there did not appear to be a linear relationship upon diluting the wound fluid sample. However, sample linearity was observed in an *in vitro* cell culture sample (Figure 3.11). This lack of linearity upon dilution wound fluids may be explained by its complex nature, as potentially there may be other unidentified proteins, interacting with the elastase antibody, causing a saturation effect of the wound fluid. It was important to ensure that any wound fluid determinations be made at the same dilution factor.

There was no significant difference ($p>0.05$) in the amount of elastase in non-infected and infected venous leg ulcer wound fluid, as quantifed by the ELISA (Figure 3.12). The amount of elastase was 93.7±11.9 ng/ml (average±SE, range 69.3-119.4 ng/ml) and 105.6±19.8 ng/ml (average±SE, range 51.3-145.4 ng/ml), in non-infected and infected wound fluids, respectively. Due to the lack of availability of some wound fluid samples, and the volume of fluid required to conduct these experiments, the numbers of patients in this analysis was limited to those with sufficient levels of fluid available. Additionally, due to the inability to distinguish between non-infected and infected venous leg ulcers using this technique, diabetic foot ulcer wound fluids were not analysed, to determine the amount of elastase by ELISA.

Figure 3.10: Elastase ELISA standard curve.
A number of antibody combinations were investigated to establish a linear standard curve for commercially available human neutrophil elastase (Calbiochem) that could allow for the detection of elastase in chronic wound fluids. The antibody combination selected detected between 0-10ng elastase.

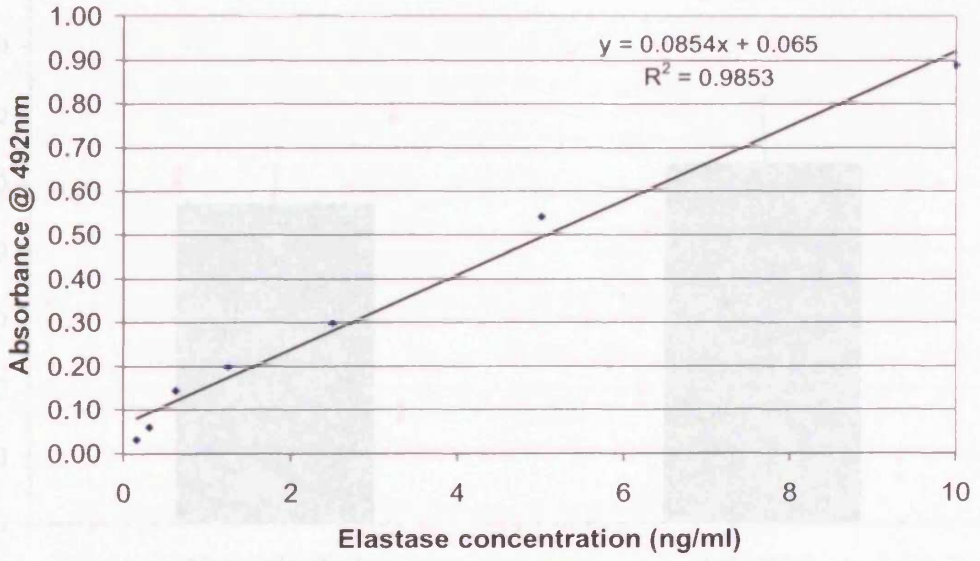


Figure 3.11: Sample linearity of lysed human neutrophils.
Comparison of the actual and predicted amount of elastase in lysed neutrophils, upon dilution. Each point is the average value±SD, n=3. The actual and predicted values for the lysed neutrophils correlate well, demonstrating a linear relationship.

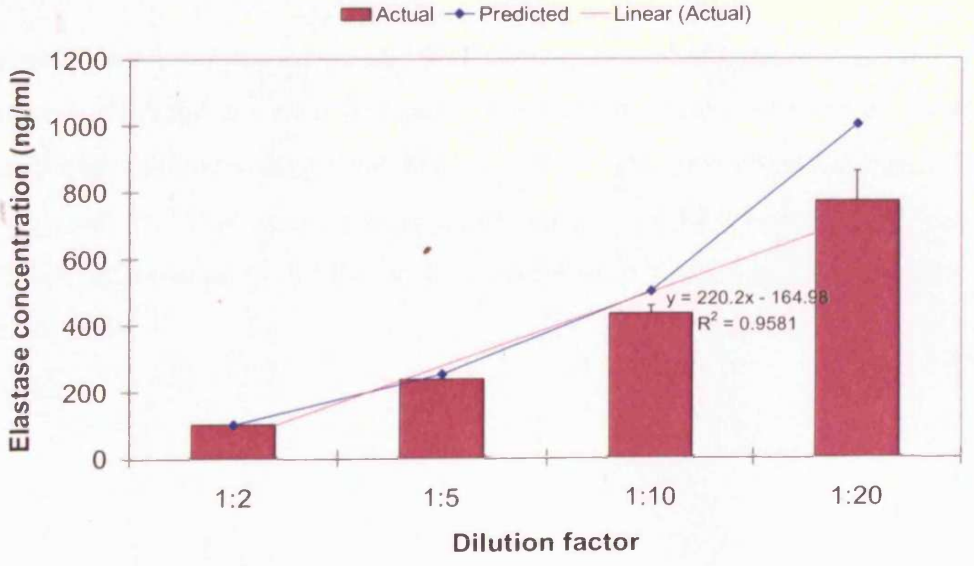
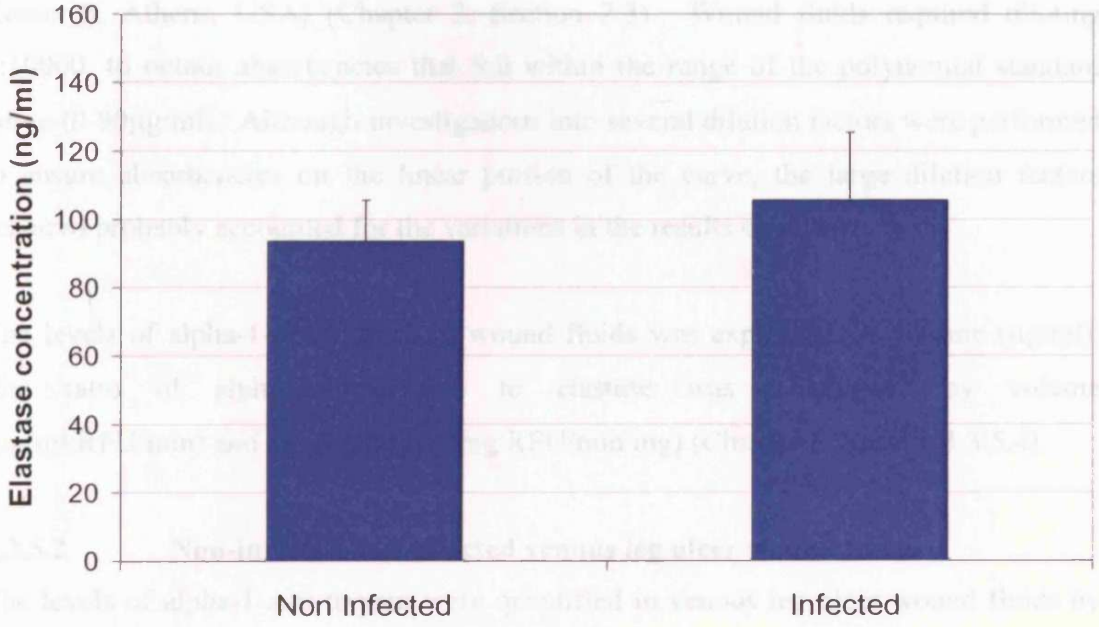


Figure 3.12: Elastase concentration in chronic wound fluids.
The amount of elastase, average±SE, determined by ELISA, in venous leg ulcer wound fluid for non-infected (n=4) and infected (n=4) wound fluids. No significant difference ($p > 0.05$) was observed between the two populations.



3.3.5 Alpha-1-Antitrypsin

3.3.5.1 Method conditions

The levels of alpha-1-anti-trypsin in wound fluid was measured by ELISA (Athens Research, Athens, USA) (Chapter 2, Section 2.3). Wound fluids required diluting 1:10000, to obtain absorbencies that fell within the range of the polynomial standard curve (0-90μg/ml). Although investigations into several dilution factors were performed to ensure absorbancies on the linear portion of the curve, the large dilution factors required probably accounted for the variations in the results obtained.

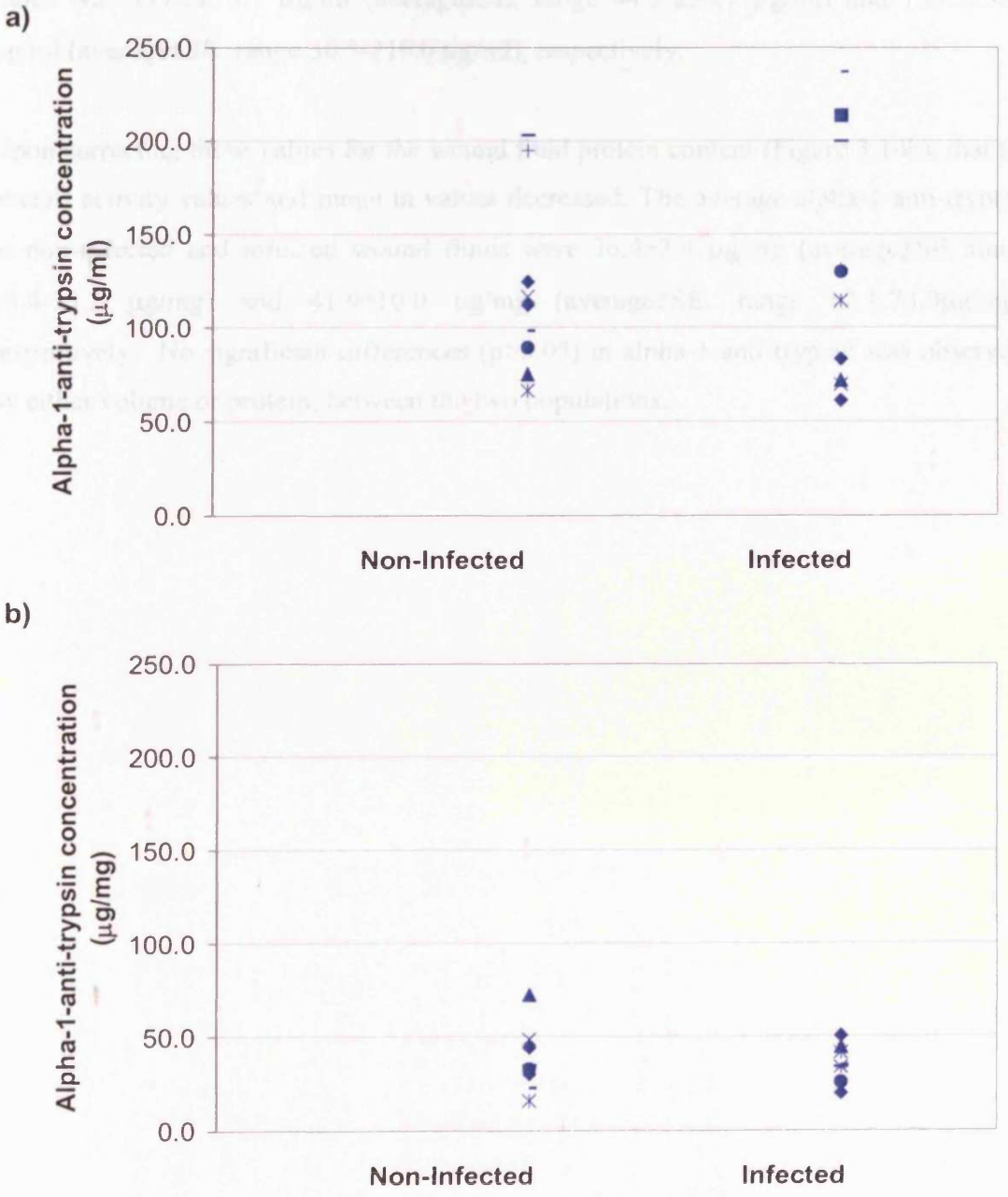
The levels of alpha-1-anti-trypsin in wound fluids was expressed by volume (μg/ml). The ratio of alpha-1-anti-trypsin to elastase was determined by volume (μg/ml:RFU/min) and by protein (μg/mg:RFU/min/mg) (Chapter 3, Section 3.3.5.4).

3.3.5.2 Non-infected and infected venous leg ulcer wound fluids

The levels of alpha-1-anti-trypsin were quantified in venous leg ulcer wound fluids by volume (μg/ml) (Figure 3.13a), and corrected for protein (μg/mg) (Figure 3.13b). Results showed that the average alpha-1-anti-trypsin levels, in non-infected and infected wound fluid was 108±17.9μg/ml (average±SE, range 66.4-203.1 μg/ml) and 131.1±20.4μg/ml (average±SE, range 61.4-236.7 μg/ml), respectively.

Upon correcting these values for wound fluid protein content (Figure 3.13b) the overall activity values and range of values decreased. The average alpha-1-anti-trypsin levels, in non-infected and infected wound fluid were 34.6±6.0 μg/mg (average±SE, range 15.4-72.3 μg/mg) and 35.9±3.0 μg/mg (average±SE, range 19.4-50.4 μg/mg), respectively. No significant differences ($p>0.1$) in alpha-1-anti-trypsin were observed, between the two populations.

Figure 3.13: Alpha-1-anti-trypsin levels in venous leg ulcer wound fluids. Alpha-1-anti-trypsin concentrations as determined by ELISA. Results are expressed by (a) volume and (b) corrected for total protein in wound fluid. Each point is the average value, where n = 3, with no significant difference ($p>0.05$) between wound fluids from non-infected and infected venous leg ulcer wounds, upon analysis by volume or protein.

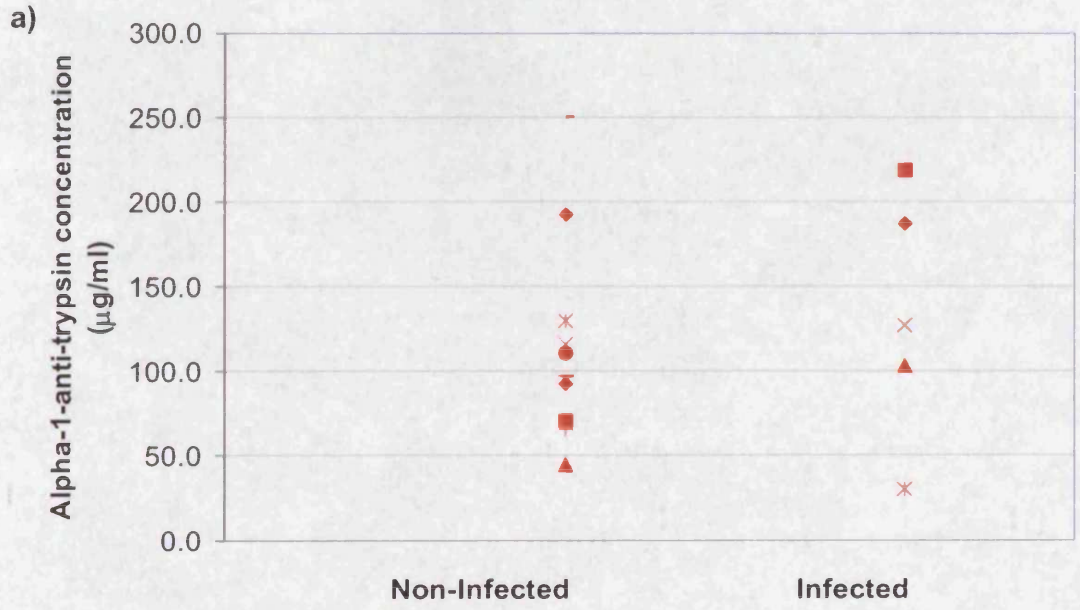


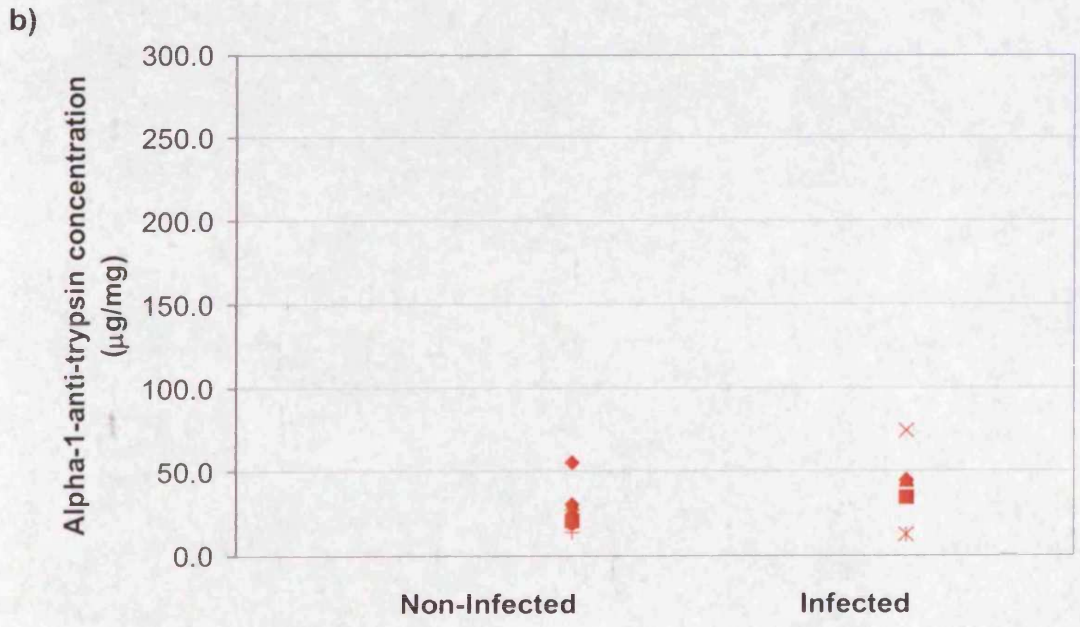
3.3.5.3 Non-infected and infected diabetic foot ulcer wound fluids

Diabetic foot ulcer wound fluid alpha-1-anti-trypsin levels were quantified by volume (μg/ml) (Figure 3.14a), and corrected for protein (μg/mg) (Figure 3.14b). Results showed that the average alpha-1-anti-trypsin levels, in non-infected and infected wound fluids was 117.1±18.7 μg/ml (average±SE, range 44.9-250.7 μg/ml) and 133.5±33.0 μg/ml (average±SE, range 30.3-219.0 μg/ml), respectively.

Upon correcting these values for the wound fluid protein content (Figure 3.14b), that the overall activity values and range in values decreased. The average alpha-1-anti-trypsin, in non-infected and infected wound fluids were 26.4±3.4 μg/mg (average±SE, range 13.4-55.2 μg/mg) and 41.9±10.0 μg/mg (average±SE, range 12.1-74.0μg/mg), respectively. No significant differences ($p>0.05$) in alpha-1-anti-trypsin was observed, by either volume or protein, between the two populations.

Figure 3.14: Alpha-1-anti-trypsin levels in diabetic ulcer wound fluids.
Alpha 1 anti-trypsin concentration, determined by ELISA. Results are expressed by (a) volume and (b) corrected for total protein in wound fluid. Each point is the average value, where n =3, with no significant difference ($p>0.05$) between wound fluids from non-infected and infected venous leg ulcer wounds, upon analysis by volume or protein.





3.3.5.4 Ratio of alpha-1-anti-trypsin to elastase in venous leg and diabetic foot ulcer wound fluids

Secreted elastase from neutrophils, is controlled by the naturally occurring inhibitor, alpha-1-anti-trypsin. Successful control of elastase levels relies on a ratio of at least 1:1 alpha-1-anti-trypsin to elastase (Owen and Campbell, 1995). The data presented herein for venous leg ulcer wound fluids (Figure 3.15a) and diabetic foot ulcer wound fluids (Figure 3.15b) is the ratio of the amount of alpha-1-anti-trypsin (by ELISA) to elastase activity (fluorogenic substrate enzyme assay). The ratio calculated is probably therefore a alpha-1-anti-trypsin: combination of elastase and proteinase 3. Measuring the amount of elastase in wound fluids resulted in an incomplete data set due to the large volume of wound fluid required for the elastase ELISA. Therefore, this ratio was not calculated using the levels of elastase as determined by ELISA.

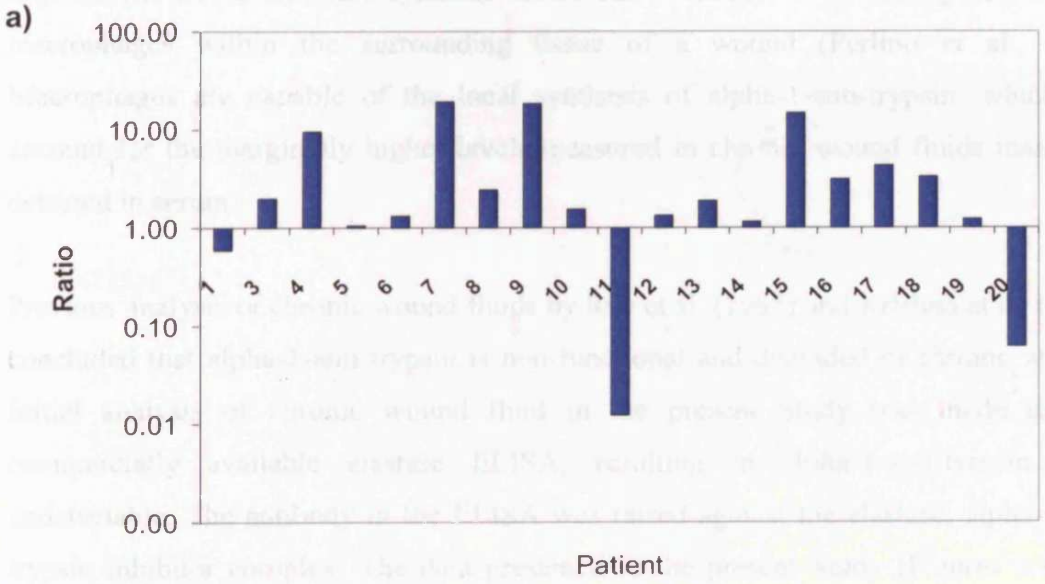
In accordance with previously presented data on elastase and alpha-1-anti-trypsin, there were no significant differences in the ratios of the two molecules ($p > 0,1$) between the clinically diagnosed non-infected and infected venous leg ulcer or diabetic foot ulcer wound fluids. A ratio value of 1 or more indicated a higher amount of alpha-1-anti-trypsin to elastase activity.

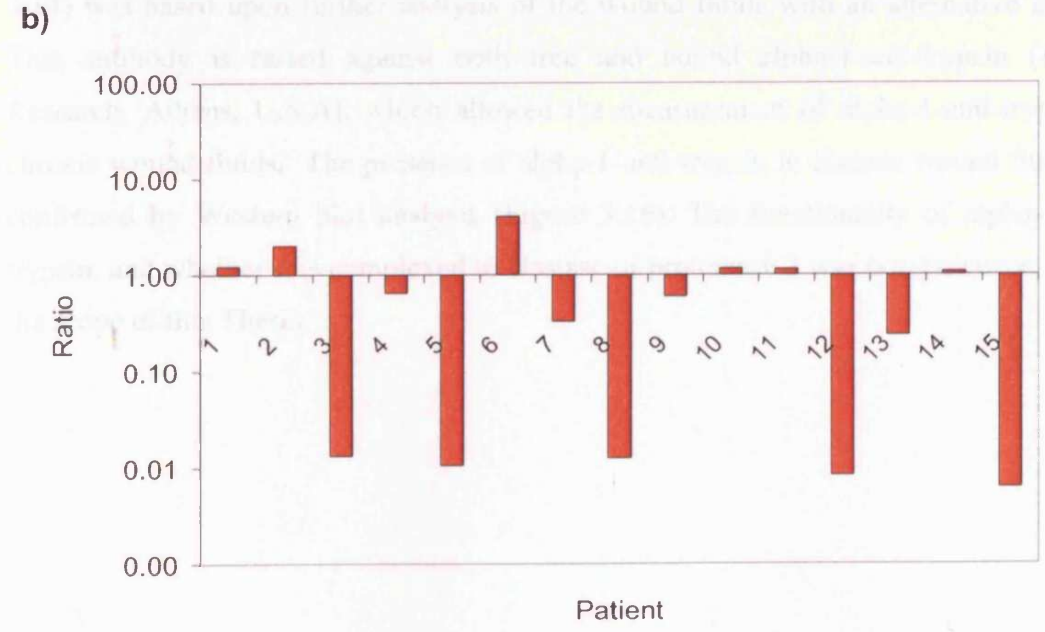
The ratio in venous leg ulcer wound fluids implied that these wound fluid provides a favourable environment for the inhibitor, with the exceptions of patients 1 (clinically non-infected), 11, and 20 (clinically infected). These patients have significantly higher elastase activities that accounted for the low ratio values. The higher elastase activity of diabetic foot ulcer wound fluids, resulted in 9 of 14 wound fluids ratio values favoured elastase. Differences in values between ulcer types, is discussed in Chapter 4.

It appeared that in the present Study, alpha-1-anti-trypsin alone, or a ratio of its amount to elastase activity, is not a reliable diagnostic marker of chronic wound infection, defined by clinical diagnosis.

Figure 3.15: Ratio of alpha-1-anti-trypsin: elastase in venous leg and diabetic foot ulcer wound fluids.

Ratio of levels of alpha-1-anti-trypsin and elastase activity in (a) venous leg ulcer and (b) diabetic foot ulcer wound fluids. No significant difference ($p>0.05$) was observed between non-infected and infected wound fluids. The significantly increased levels of elastase activity in diabetic foot ulcer wound fluids ($p=0.009$) than venous leg ulcer wound fluids, resulted in a ratio value of <1.0 in 9 of 15 wound fluids, indicated a favorable proteolytic environment.



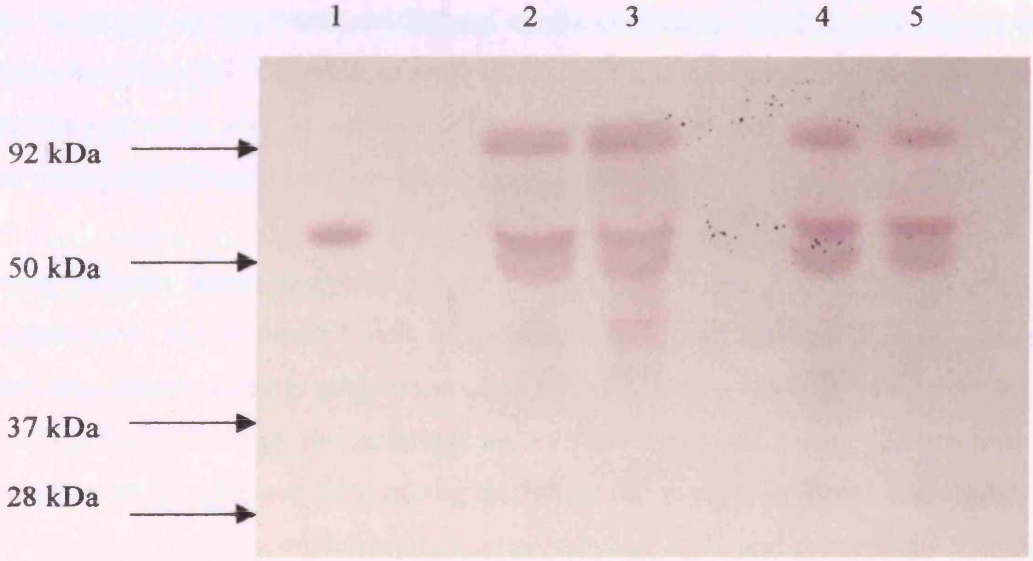


3.3.5.5 Functionality of alpha-1-anti-trypsin in chronic wound fluids

The levels of alpha-1-anti-trypsin determined in wound fluids were equivalent to the systemic levels of alpha-1-anti-trypsin quoted in the literature, between 80-220 μg/ml by nephelometry (Stoller and Aboussouan, 2005). The range of alpha-1-anti-trypsin levels in venous leg ulcer and diabetic foot ulcer wound fluids was between 61-236 μg/ml and 30-250μg/ml, respectively. Alpha-1-anti-trypsin is produced systemically by hepatocytes, however, these systemic levels can be added to by locally concentrated macrophages within the surrounding tissue of a wound (Perlino et al., 1987). Macrophages are capable of the local synthesis of alpha-1-anti-trypsin, which may account for the marginally higher levels measured in chronic wound fluids than those detected in serum.

Previous analysis of chronic wound fluids by Rao et al. (1995) and Krishna et al. (1997), concluded that alpha-1-anti-trypsin is non-functional and degraded in chronic wounds. Initial analysis of chronic wound fluid in the present Study was made using a commercially available elastase ELISA, resulting in alpha-1-anti-tyrpsin being undetectable. The antibody in the ELISA was raised against the elastase: alpha-1-anti-trypsin inhibitor complex. The data presented in the present Study (Figures 3.13 and 3.14) was based upon further analysis of the wound fluids with an alternative ELISA. This antibody is raised against both free and bound alpha-1-anti-trypsin (Athens Research, Athens, U.S.A), which allowed the measurement of alpha-1-anti-trypsin in chronic wound fluids. The presence of alpha-1-anti-trypsin in chronic wound fluid was confirmed by Western blot analysis (Figure 3.16). The functionality of alpha-1-anti-trypsin, and whether it is complexed to elastase or proteinase 3 was not, however, within the scope of this Thesis.

Figure 3.16: Alpha-1-anti-trypsin in chronic wound fluids.
Commercially available Alpha-1-anti-trypsin was detected (lane1). Alpha-1-anti-trypsin was detected in venous leg ulcer (lanes 2&3) and diabetic foot ulcer (lane 4&5) wound fluids by Western blot analysis using the monoclonal, mouse antibody clone MIA1101 (Calbiochem). Two bands at approximately 56kDa and one band at approximately 100kDa, corresponded to alpha-1-anti-trypsin and alpha-1-anti-trypsin complexes, respectively.



3.3.6 Matrix metalloproteinases

3.3.6.1 Zymographic analysis of gelatinolytic activity in wound fluids

3.2.6.1.1 Method conditions

Gelatin zymography was used to semi-quantify (Chapter 2, Section 2.3.2), the gelatinolytic activity of the gelatinases (MMP-2 and MMP-9) in the collected wound fluids. A sample of fibroblast conditioned media containing MMP-2 was run on each occasion as a control. Migration of each MMP form through the gel and their activity at a specific molecular weight accounted for the banding patterns observed on the gels, which allowed distinction between the different forms.

Banding patterns were observed in each wound fluid and control sample, which corresponded to the different forms of gelatinase (pro- and active-) present. Results showed that gelatin zymography detects pro-MMP-2, active-MMP-2, pro-MMP-9 and active- MMP-9. Although the technique measures active MMP forms, the pro-form of the enzyme can become activated during the re-naturation process (Leber and Balkwill., 1997). Proteins were electrophoresed in the presence of SDS, and the proteins renatured using Triton X-100 (Michaud, 1998).

Optimal visualisation of banding patterns was observed after a gel running time of 1h. At this time, the samples remained within the top half of the gel, and each band is sufficiently compact to enable densitometric analysis to be conducted. Analysis of banding patterns yielded a densitometric value for each form of MMP (SigmaGel™, Gel Analysis Software, SPSS Inc, Chicago,USA). A typical densitometric scan consisted of a series of peaks, correlating to the pro- and active- forms of the MMPs of interest.

Wound fluid samples were loaded based upon both volume and protein. To ensure sufficient activity in each wound fluid sample, without causing lack of distinction between forms of MMP that would make densitometric analysis impossible, preliminary experiments were conducted, loading wound fluid samples at various total protein concentrations. Results presented in this Chapter are based on wound fluid samples prepared as follows:

- Zymograms, analysed by protein content, comparing non-infected and infected wound fluids, loaded at 0.5μg/10μl and 2μg/10μl, for venous leg ulcers and diabetic foot ulcers, respectively.
- Zymograms, analysed by volume, with the wound fluid containing the highest total protein diluted to the appropriate protein content for loading (as above) with all other wound fluids in that ulcer type prepared using that same dilution factor.

Duplicate gels were loaded on each occasion and zymograms repeated on three separate occasions.

3.3.6.1.2 Gelatinase activity in non-infected and infected venous leg ulcer wound fluids

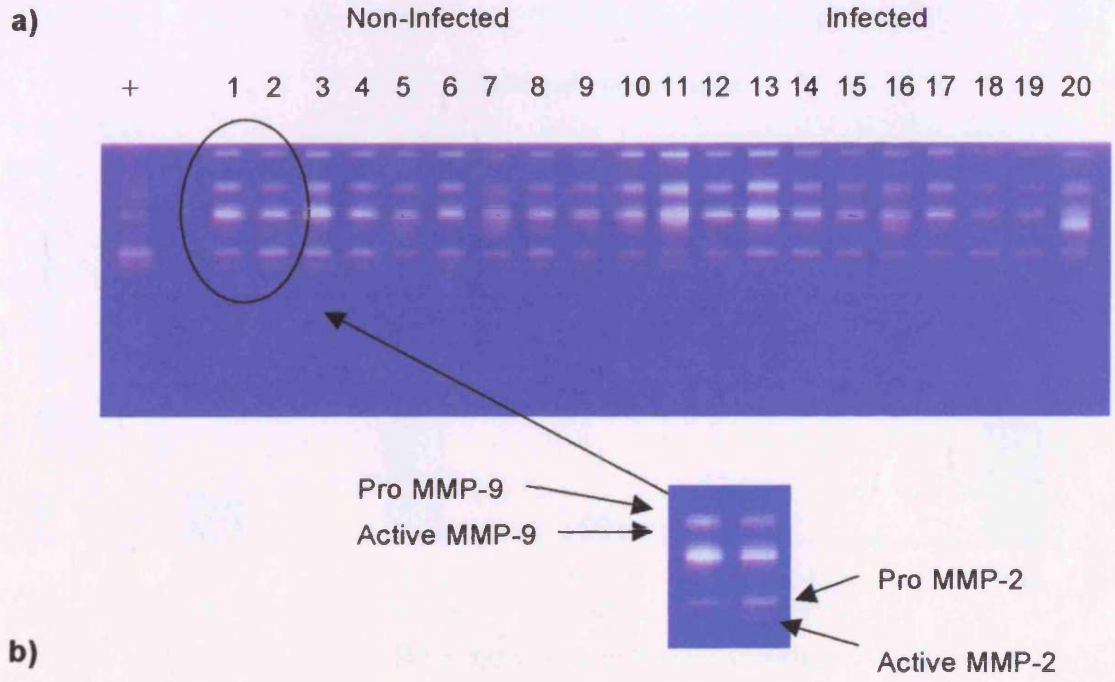
A representative zymogram of venous leg ulcer wound fluid gelatinase activity, loaded by volume is shown (Figure 3.17a). The total gelatinase activity, (Figure 3.17b) and the activity of gelatinases (MMP-2 and MMP-9) (Figure 3.18), for each wound fluid was determined by densitometric analysis..

Comparison of clinically diagnosed non-infected and infected venous leg ulcer wound fluid for gelatinase activity (MMP-2 and MMP-9) did not show a significant difference ($p>0.05$) in samples loaded either by volume or protein content.

Dilution of wound fluid samples by volume results in no active-MMP-2 activity being observed (Figure 3.18). Generally, the densitometric values of MMP-2 and MMP-9 quantified in wound fluids diluted by volume are lower, than for wound fluids measured by protein. The median values for pro- MMP-9 were 168 by volume compared to 289 by protein. The median values for active-MMP-9 were 571, compared to 776 by protein. The median values or pro-MMP-2 is 155, compared to 234 by protein. It appears from these results that the overall reduction in protein content in the wound fluid samples diluted by volume is reflected in the amount of detectable gelatinase activity obtained.

Figure 3.17: Venous leg ulcer wound fluid zymographic analysis for MMP-9 and MMP-2 activities (Average±SE)

(a) Representative zymogram of gelatinase (MMP-2 and MMP-9) activity in non-infected (1-10) and infected (11-20) venous leg ulcer wound fluid. Both pro and active forms of MMP-2 and MMP-9 were detected. (b) Total gelatinase (MMP-2 and MMP-9) activity was plotted and compared to ensure densitometric values reflected visual assessment.



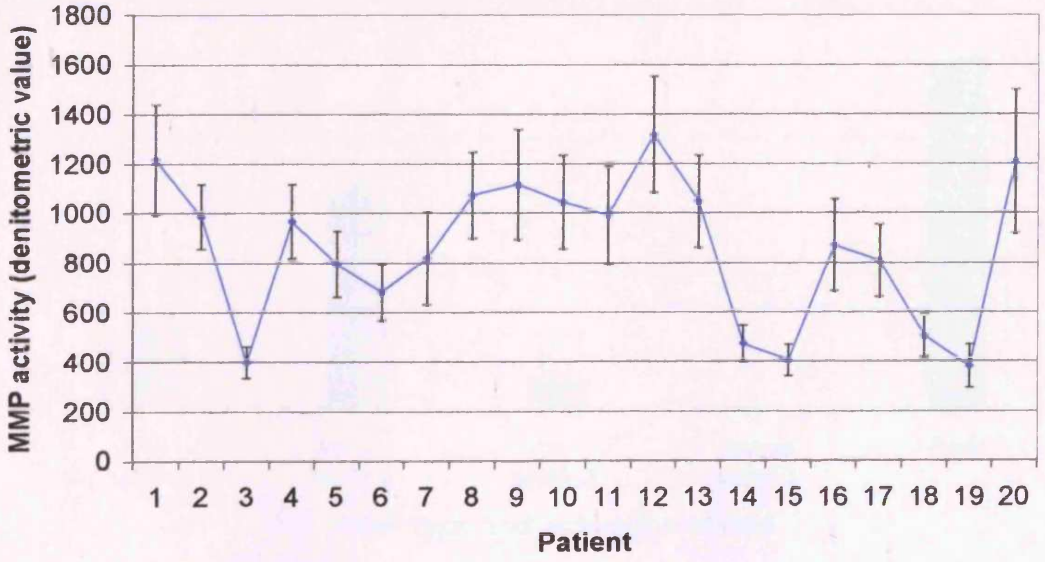
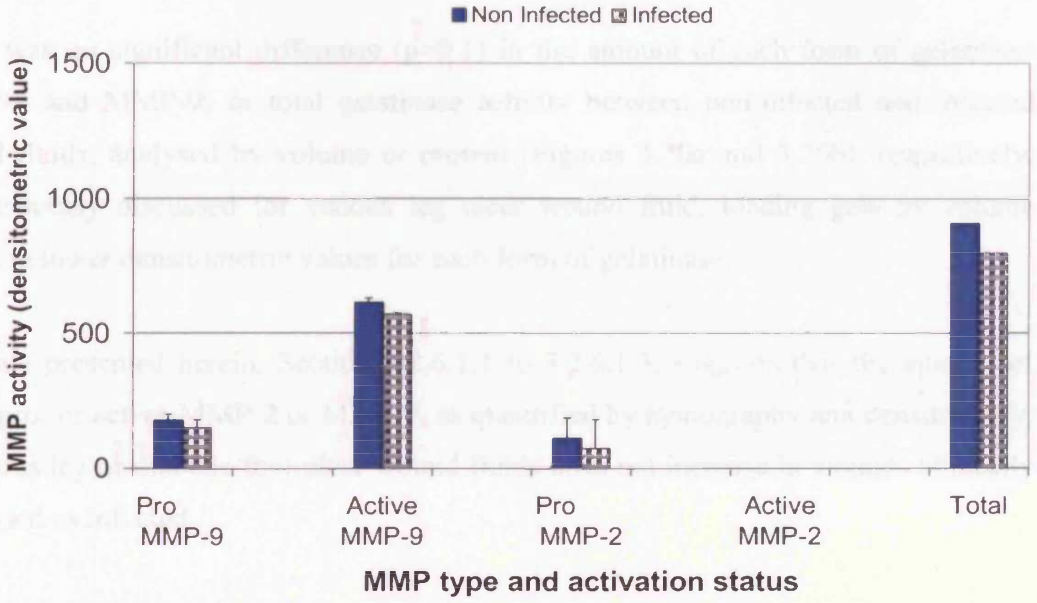


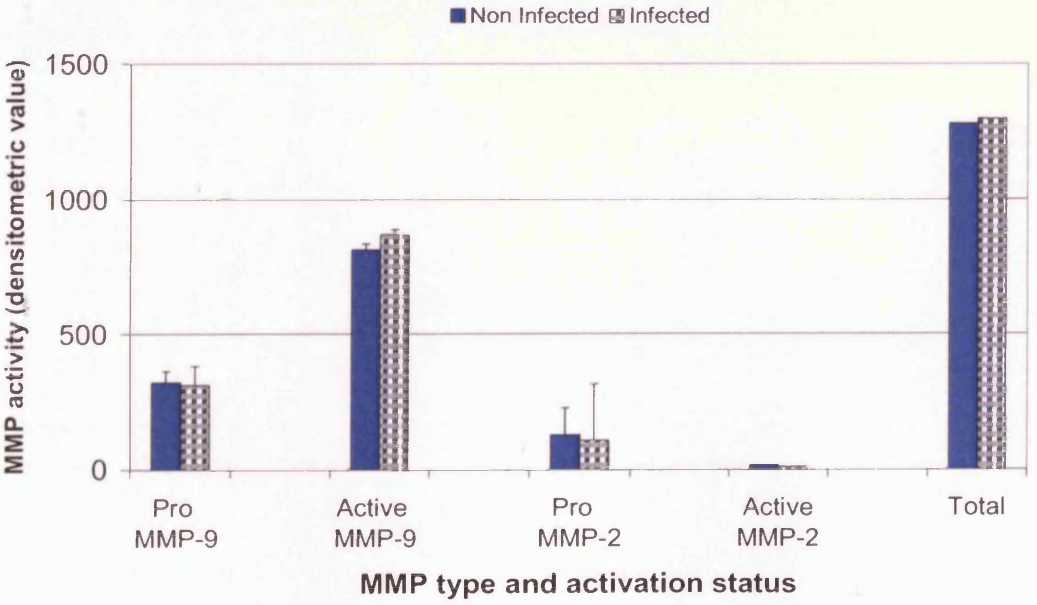
Figure 3.18: Comparison of gelatinase activity in non-infected and infected venous leg ulcer wound fluids.

Comparison of the gelatinase activity, average±SE, for total and each form of MMP in venous leg ulcer wound fluid loaded by (a) equivalent volume or (b) equal protein. There was no significant difference ($p>0.05$) between non-infected or infected wound fluid populations. Loading by protein increased the amount of measurable gelatinase activity obtained.

a)



b)



3.3.6.1.3 Gelatinase activity in non-infected and infected diabetic foot ulcer wound fluids

Comparison of the gelatinase activity, average±SE, for total and each form of MMP in diabetic foot ulcer wound fluid loaded by (a) equivalent volume or (b) equal protein. Gelatinase (MMP-2 and MMP-9) activity was analysed by densitometric analysis of each band, representing the pro- and active- forms of gelatinases (Figure 3.19).

There was no significant difference ($p>0.1$) in the amount of each form of gelatinase (MMP-2 and MMP-9) or total gelatinase activity between non-infected and infected wound fluids, analysed by volume or protein (Figures 3.20a and 3.20b), respectively. As previously discussed for venous leg ulcer wound fluid, loading gels by volume results in lower densitometric values for each form of gelatinase.

The data presented herein, Section 3.2.6.1.1 to 3.2.6.1.3, suggests that the amount of either pro- or active-MMP-2 or MMP-9, as quantified by zymography and densitometry, in venous leg or diabetic foot ulcer wound fluids does not increase in wounds clinically diagnosed as infected.

Figure 3.19: Diabetic foot ulcer wound fluid zymographic analysis of MMP-9 and MMP-2

(a) Representative zymogram of gelatinase (MMP-2 and MMP-9) activity in non-infected (1-10) and infected (11-20) diabetic foot ulcer wound fluids. Both pro and active forms of MMP-2 and MMP-9 were detected. (b) Total gelatinase (MMP-2 and MMP-9) activity (average±SE) was plotted and compared to ensure densitometric values reflected visual assessment.

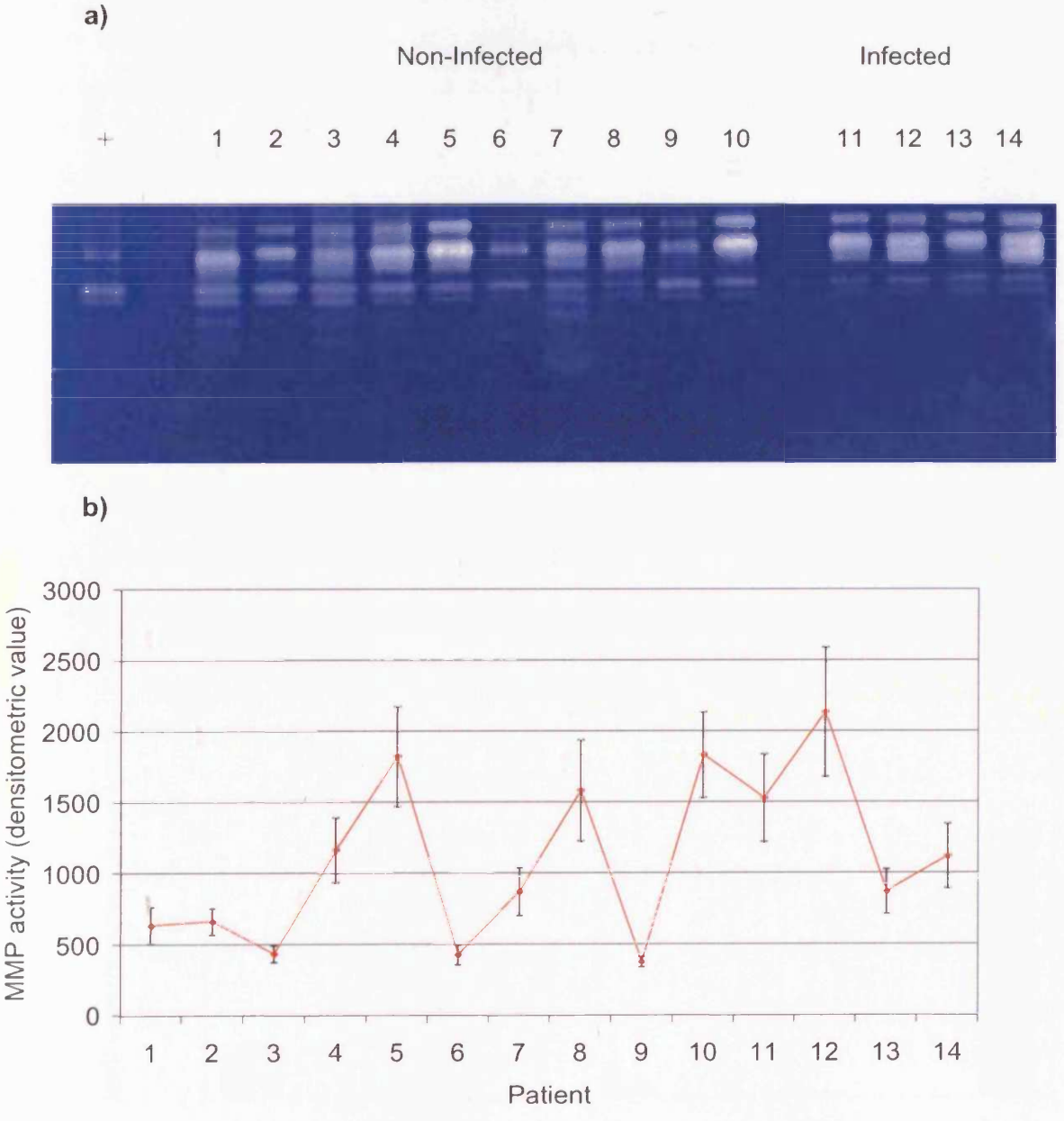
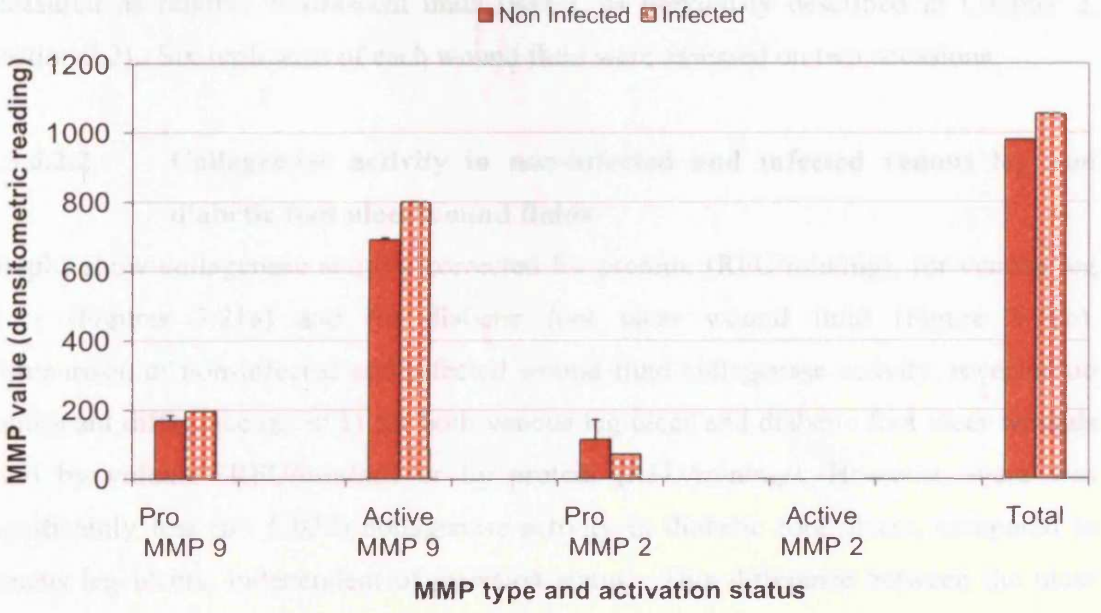
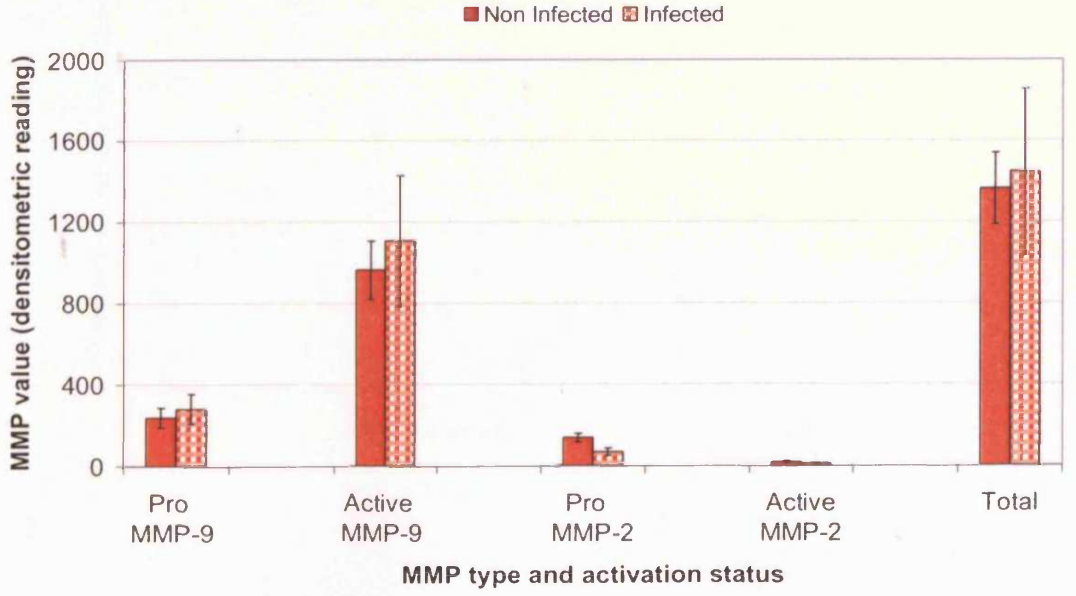


Figure 3.20: Comparison of gelatinase activity in non-infected and infected diabetic foot ulcer wound fluids.
Comparison of the gelatinase activity, average±SE, for total and each form of MMP in diabetic foot ulcer wound fluids, loaded to equivalent volume (a) or equal protein (b). There was no significant difference ($p>0.1$) between non-infected or infected wound fluid populations. Loading by protein increased the amount of measurable gelatinase (MMP-2 and MMP-9) activity obtained.

a)



b)



3.3.6.2 Collagenase activity in wound fluids

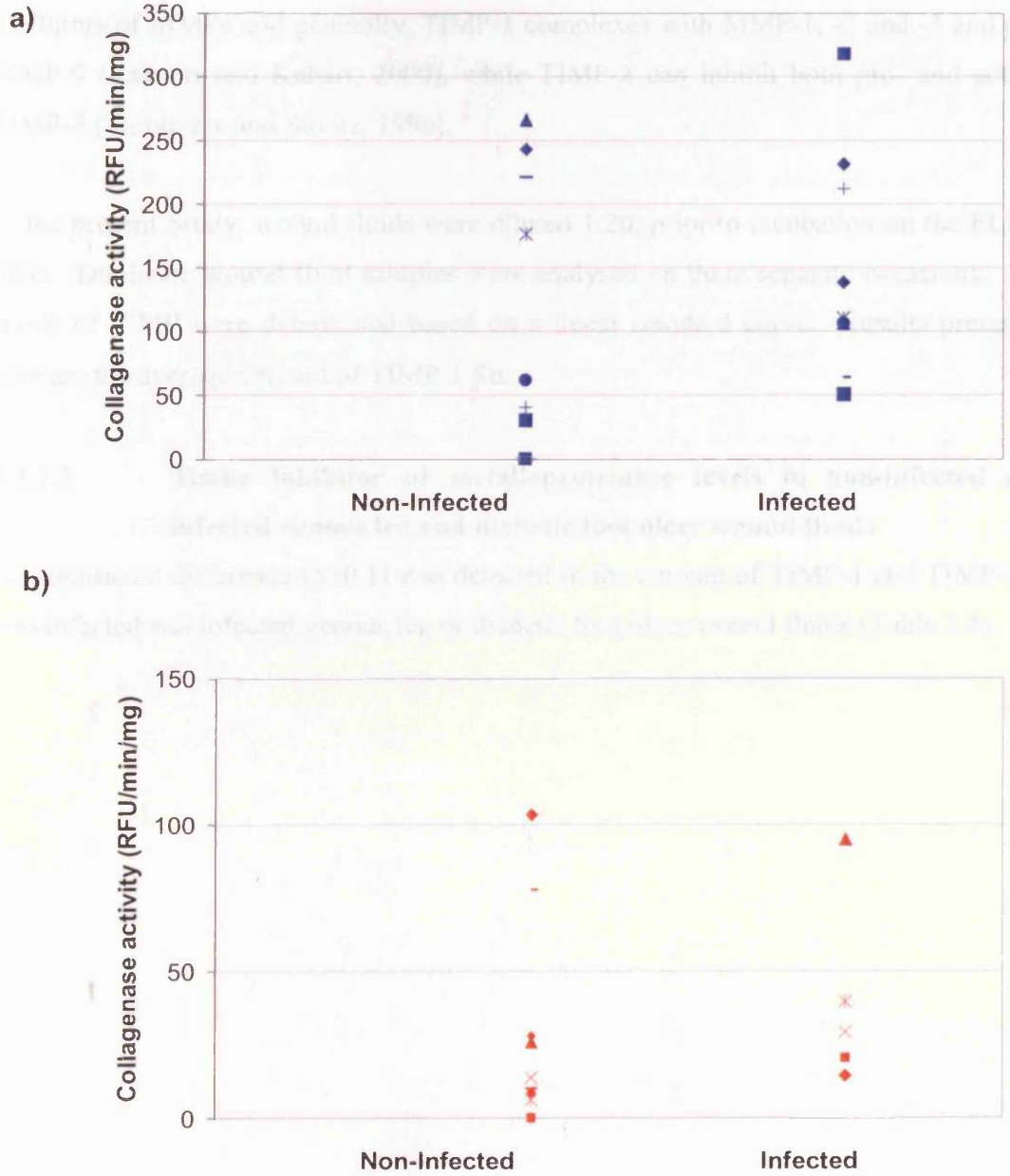
3.3.6.2.1 Method conditions

Analysis of collagenase activity in wound fluids were assessed by wound fluid cleavage of the collagenase substrate, succinyl-gly-pro-leu-gly-pro-7-amino-4-methylcoumarin. Cleavage of this substrate yields a fluorescent signal detected on a fluorometer and measured as relative fluorescent units (RFU), as previously described in Chapter 2, Section 2.2). Six replicates of each wound fluid were assessed on two occasions.

3.3.6.2.2 Collagenase activity in non-infected and infected venous leg and diabetic foot ulcer wound fluids

Graphs show collagenase activity corrected for protein, (RFU/min/mg), for venous leg ulcer (Figures 3.21a) and for diabetic foot ulcer wound fluid (Figure 3.21b). Comparison of non-infected and infected wound fluid collagenase activity, revealed no significant difference ($p > 0.1$) for both venous leg ulcer and diabetic foot ulcer wounds fluid by volume (RFU/min/ml) or by protein (RFU/min/mg). However, there was significantly less ($p = 0.022$) collagenase activity in diabetic foot ulcers, compared to venous leg ulcers, independent of infection status. This difference between the ulcer types for collagenase activity is discussed in Chapter 4.

Figure 3.21: Collagenase activity in venous leg and diabetic foot ulcer wound fluids. Fluorogenic analysis of all (a) venous leg ulcer and (b) diabetic foot ulcer wound fluids. The same trend in results was also seen for analysis by volume. No significant difference ($p>0.1$) between wound fluids from non-infected and infected venous leg ulcer or diabetic foot ulcer wounds in either analyses, where each point is the average values, where n=2.



3.3.7 Tissue inhibitor of metalloproteinase

3.3.7.1 Method conditions

Total TIMP, TIMP-1 and TIMP-2 levels were quantifed in wound fluids by ELISA (R&D Systems, Oxfordshire, UK (Chapter 2, Section 2.3). TIMPs are the endogenous inhibitors of MMPs and generally, TIMP-1 complexes with MMP-1, -2 and -3 and pro-MMP-9 (Ravanti and Kahari, 2000), while TIMP-2 can inhibit both pro- and active-MMP-2 (Tarnuzzer and Shultz, 1996).

In the present Study, wound fluids were diluted 1:20, prior to incubation on the ELISA plate. Duplicate wound fluid samples were analysed on three separate occasions. The levels of TIMP were determined based on a linear standard curve. Results presented here are the average amount of TIMP ± SE.

3.3.7.2 Tissue inhibitor of metalloproteinase levels in non-infected and infected venous leg and diabetic foot ulcer wound fluids

No significant difference ($p>0.1$) was detected in the amount of TIMP-1 and TIMP-2 in non-infected and infected venous leg or diabetic foot ulcer wound fluids (Table 3.4).

Table 3.4: Levels of TIMP-1 and TIMP-2 in venous leg and diabetic foot ulcer wound fluids.

A comparison of non-infected and infected (a) venous leg ulcer and (b) diabetic foot ulcer wound fluid with respect to the levels of TIMP-1 and TIMP-2 (average±S.E., n = 3) were determined not to be significantly different ($p>0.1$), in either ulcer type examined.

a)

	TIMP 1 (ng/ml)		TIMP 2 (ng/ml)	
	Non Infected	**Infected**	**Non Infected**	**Infected**
Ave	24.8	32	28.5	31.2
SE	3.7	5.3	3.3	2.8

b)

	TIMP 1 (ng/ml)		TIMP 2 (ng/ml)	
	Non Infected	**Infected**	**Non Infected**	**Infected**
Ave	38.9	41.3	43.8	32.9
SE	6	8.1	5.7	7.1

3.3.8 Cytokines

3.3.8.1 Cytokine analysis by micro-array

3.3.8.1.1 Method conditions

Micro-array is a recent technology whereby multiple biomolecules, for example, antibodies to cytokines and growth factors, can be immobilized on a suitable surface, such as, a glass slide. In the present Study, eighteen cytokines were 'spotted' onto FAST® slides, coated with a thin layer of proprietary nitrocellulose. Antibodies used in the array were sourced as matched monoclonal antibody pairs from R&D Systems, (Chapter 2, Section 2.3). Each antibody IgG fraction was purified by either protein A or G affinity chromatography. Each antibody was specific for the human version of the cytokine, with the exception of IL-5, which could cross-react with some animal species.

Eighteen cytokines were analysed in venous leg ulcer and diabetic foot ulcer wound fluids by micro-array (Chapter 2, Section 2.3), including; interleukins (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 p40, IL-12p70 and IL-13), angiogenin (angio); interferon gamma (IFN-γ): growth factors, transforming growth factor beta (TGF-β_1) and vascular endothelial growth factor (VEGF); the pro-inflammatory cytokine, tumour necrosis factor alpha (TNF-α) and its soluble receptor p75; an adhesion molecule, intracellular adhesion molecule 1 (ICAM-1), and the chemokine, IFN-γ-inducible-protein-10 (IP-10). Details of the cytokines, summarizing their cellular source and major biological effects are detailed in Chapter 1, Section 1.7.

Briefly, the method comprises antibody arraying on slides, slide blocked to eliminate any non-specific binding, 70µl wound fluid and standards, added to duplicate pads and incubated at room temperature, for 4h. Slides were washed and incubated, at room temperature for 1h, with 70µl biotinylated detection antibody cocktail, containing one biotinylated antibody corresponding to each of the arrayed capture antibodies. Slides were washed and incubated, at room temperature for 1h, with 70µl of streptavidin-Cy5 conjugate. Slides were then washed and dried (Chapter 2, Section 2.3).

Slides were imaged in a confocal fluorescent scanner, AXON GenePix 4000B fluorescent imaging system (Axon Instruments, Sunnydale, USA) and analysed using Imaging Research ArrayVision Software (Whatman® Schleicher&Schuell®, Dassel, Germany). The fluorescent units (signal) obtained for each wound fluid per cytokine, were converted using a standard curve, into pg/ml. The cytokine concentration (pg/ml) per wound fluid was provided by Whatman® Schleicher&Schuell®. Further data analysis was conducted using Microsoft Excel® (Chapter 2, Section 2.3)

Due to limitations on the levels of wound fluid for certain patients, there are variations in the number of times some wound fluids have been assayed. The majority of venous leg ulcer wound fluids were analysed a total of three times, with 6 replicates at each time point assayed, resulting in a total of 18 replicates for each cytokine. Wound fluids that had only been analysed once (6 replicates), or twice (12 replicates), included patients 1, 8, 9, 10, and 12, five wound fluids from a total of twenty.

The number of repeat assays on the diabetic foot ulcer wound fluids was more variable. Wound fluids from patient 2, 3, and 13 were assayed once (6 replicates), patients 1, 4, 11 and 12 wound fluid assayed twice (12 replicates) with 8 wound fluids having sufficient material to be assayed a total of three times (18 replicates).

Initial data analysis revealed considerable variation in the absolute value obtained for wound fluid between runs. This variability was observed in both the diabetic and venous ulcer populations and did not appear to discriminate between non-infected or infected wound fluids. Although differences were observed on individual runs in relation to the absolute amount of cytokine, generally, the increase or decrease of particular cytokines compared to one another remained the same. Due to this variation, it was decided that data could not be merged between runs, but the comparison of non-infected to infected wound fluid samples, was made on a per run basis.

3.3.8.1.2 Cytokine analysis of non-infected and infected venous leg ulcer wound fluids

The minimum and maximum amounts of each cytokine within the clinically non-infected and infected populations are presented (Table 3.5). The data presented herein, is taken from Run 1 venous leg ulcers and diabetic foot ulcers. The data from all three runs could not be combined, due the variation between occasions, as further discussed (Chapter 3, Section 3.4). There was no significant difference ($p>0.05$) (as determined by Wilcoxon probability) between the two populations for any of the cytokines tested. A comparison of the average±S.E for each cytokine (Figure 3.22), between the clinically non-infected and infected wound fluids reveals that both populations followed the same trend in the levels of cytokine, relative to one another.

3.3.8.1.3 Cytokine analysis of clinically non-infected and infected diabetic foot ulcer wound fluids

The minimum and maximum amounts of each cytokine within the clinically non-infected and infected populations are presented (Table 3.6). There was no significant difference ($p>0.05$) between the two populations for any of the cytokines tested. A comparison of the average±S.E for each cytokine (Figure 3.23), between the clinically non-infected and infected wound fluids reveals that both populations followed the same trend in the levels of cytokine, relative to one another.

Table 3.5: Cytokine levels in non-infected and infected venous leg ulcer wound fluids.

Range in the amount of each cytokine (pg/ml) for the non-infected and infected venous leg ulcer wound fluids. Angio showed the greatest range in values in both the non-infected and infected populations. The smallest range in levels was observed with IL-5 in non-infected and IFN-γ in infected wound fluids. The average levels were determined by micro-array, where n=3, with no significant difference ($p>0.05$) between non-infected and infected wound fluids.

Cytokine	Infection Status				Statistical significance
	Non-Infected		Infected		
	Min	Max	Min	Max	Wilcoxon probability
IL-1 beta	6.0	3952.8	387.3	4840.2	0.185
IL 12 p70	0.5	810.3	0.1	58.7	0.157
IL-2	3.6	285.8	2.8	49.5	0.595
IL-13	50.0	13825.0	221.1	2007.8	0.724
IL-4	11.3	715.7	52.4	131.5	0.377
Angio	52.4	23725.0	1869.8	36200.0	0.216
IL-5	1.0	147.5	1.0	41.3	0.288
IFN gamma	1.7	275.0	0.4	17.1	0.185
IL-6	85.2	14375.0	3257.4	30558.1	0.133
ICAM	149.9	4180.2	1032.9	9250.0	0.093
IL-8	21.3	1249.8	380.4	13250.0	0.536
TGF B1	1.2	2127.3	1.2	69.4	0.657
IL-10	216.1	9725.0	16.0	773.3	0.158
TNF alpha	3.8	749.2	7.8	418.6	1.000
IP-10	2.7	371.7	2.7	526.4	0.858
TNF r2	2241.9	254308.7	25280.8	150942.7	0.377
IL 12 p40	93.0	19664.3	45.0	406.5	0.659
VEGF	3075.0	30076.9	5151.0	28128.0	0.791

Figure 3.22: Non-infected and infected venous leg ulcer wound fluids cytokine levels. Average cytokine levels ± S.E, of venous leg ulcer wound fluids as quantified using micro-array, by wound fluid volume. Comparison of non-infected and infected venous leg ulcer wound fluid reveals no significant difference ($p>0.05$) in cytokine levels between the two populations.
Generally the highest levels of cytokine in venous leg ulcer wound fluids included, TNFr2, VEGF, angiogenin and IL-6, with the lowest levels being for IL-5, IFN-γ, IL-12, IL-2, and TGF-β_1.

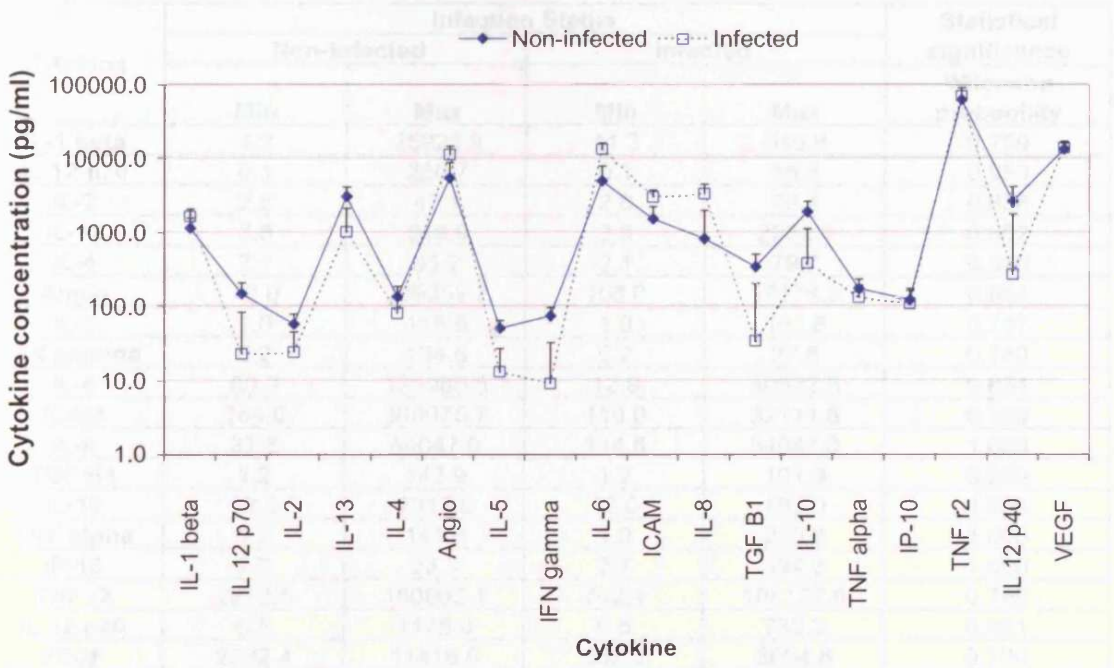
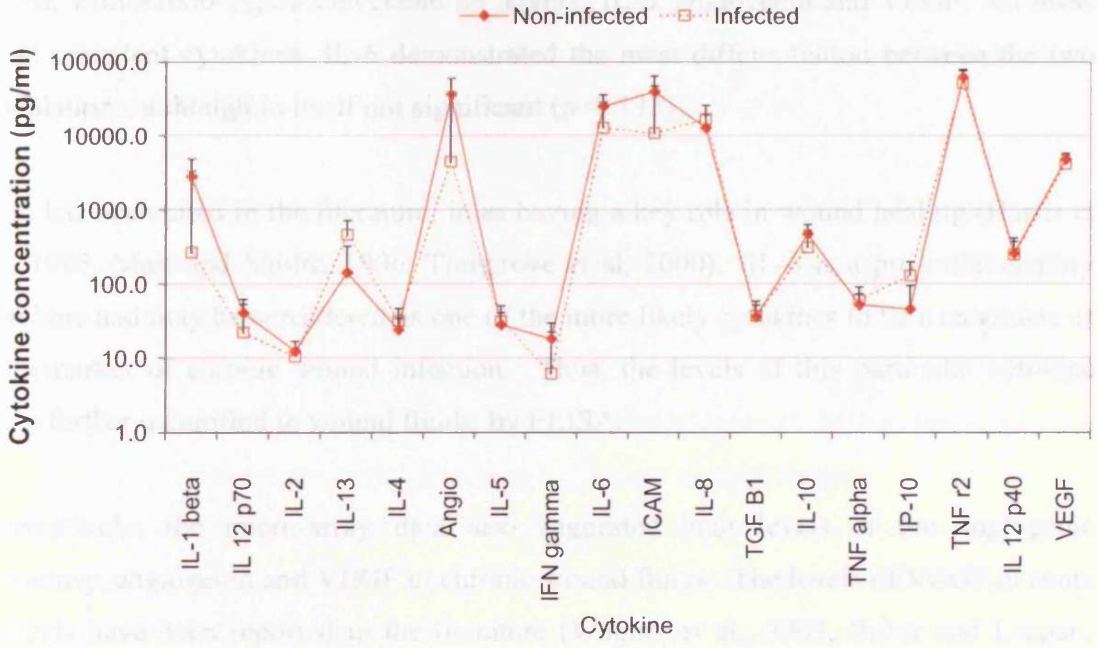


Table 3.6: Cytokine levels in non-infected and infected diabetic foot ulcer wound fluids.

Range in the amount of each cytokine (pg/ml) for the non-infected and infected diabetic foot ulcer wound fluids. ICAM showed the greatest range in values in both the non-infected and infected populations. The smallest range in the levels was observed with IP-10 in non-infected and IL-2 in infected wound fluid. The average levels were determined by micro-array, where n=3, with no significant difference ($p>0.05$) between non-infected and infected wound fluids.

Cytokine	Infection Status				Statistical significance
	Non-Infected		Infected		
	Min	Max	Min	Max	Wilcoxon probability
IL-1 beta	4.2	25926.8	11.3	940.8	0.759
IL 12 p70	0.1	250.7	0.1	85.3	0.950
IL-2	2.8	47.1	2.8	23.4	0.836
IL-13	3.8	549.9	3.8	2252.1	0.662
IL-4	2.1	85.2	2.1	79.7	0.376
Angio	70.0	299259.2	108.0	16174.3	0.854
IL-5	1.0	116.6	1.0	102.8	0.747
IFN gamma	0.2	134.6	0.2	27.8	0.740
IL-6	60.3	120980.5	12.8	40832.5	0.854
ICAM	264.0	309075.7	119.0	32111.8	0.759
IL-8	37.6	54047.0	114.8	54047.0	1.000
TGF B1	1.2	143.9	1.2	101.9	0.950
IL-10	12.0	2017.0	12.0	483.0	1.000
TNF alpha	1.0	141.6	1.0	220.8	1.000
IP-10	2.7	23.7	2.7	594.3	1.000
TNF r2	2272.5	160805.1	742.4	108132.6	0.759
IL 12 p40	0.5	1178.0	0.5	738.3	0.621
VEGF	2232.4	11416.6	562.0	8064.8	0.790

Figure 3.23: Non-infected and infected diabetic foot ulcer wound fluid cytokine levels. Average cytokine levels±S.E, of diabetic foot ulcer wound fluids, as quantifed using micro-array, by volume. Comparison of non-infected and infected diabetic foot ulcer wound fluid reveals no significant difference ($p>0.05$) in cytokine amounts between the two populations.
Generally the highest levels of cytokines in diabetic foot ulcer wound fluid included, angiogenin, IL-6, ICAM, IL-8 and TNFr2, with the lowest levels being for IL-2, IL-4, IL-4, and IFN-γ.



3.3.8.2 Cytokine analysis by ELISA

3.3.8.2.1 Method conditions

Analysis of each of the cytokines between clinically diagnosed non-infected and infected venous leg ulcer and diabetic foot ulcer wound fluids by micro-array, revealed no significant difference in any of the cytokines analysed. However the micro-array analysis did highlight the highest concentration of cytokine in venous leg ulcer wound fluids, with ≥10000pg/ml detectable for TNFr2, IL-6, angiogenin and VEGF. Of these most prevalent cytokines, IL-6 demonstrated the most differentiation between the two populations, although in itself not significant ($p=0.133$).

IL-6 has been cited in the literature, in as having a key role in wound healing (Harris et al., 1995, Mast and Shultz, 1996, Trengrove et al, 2000). IL-6 is a pro-inflammatory cytokine and may be considered as one of the more likely cytokines to be a candidate as a biomarker of chronic wound infection. Thus, the levels of this particular cytokine were further quantified in wound fluids, by ELISA.

Interestingly, the micro-array data also suggested high levels of the angiogenic cytokines, angiogenin and VEGF in chronic wound fluids. The levels of VEGF in acute wounds have been reported in the literature (Wagner et al., 2003, Baker and Leaper., 2000). Recent publications have reported increased serum/plasma VEGF levels in venous leg ulcer patients (Shoab et al., 1998, Murphy et al., 2002, Quatresooz et al., 2003). Increased expression and proteolysis of VEGF has also been cited by Lauer et al (2000), in chronic leg ulcer wounds. These studies support the observation of large amounts of VEGF as determined in the present Study. However, there is currently a lack of data directly correlating VEGF levels and clinical diagnosis of infection and additionally, any comparisons between different ulcer types (Chapter 4). For these reasons VEGF was selected for further analysis.

Total IL-6 and VEGF levels were measured in clinically diagnosed non-infected and infected wound fluid, by ELISA (R&D Systems). All wound fluids were diluted prior to analysis, based upon the range in results determined by micro-array analysis. Consequently, wound fluids used in the analysis of IL-6 and VEGF were diluted 1:200, using calibrator diluent.

However, there appeared to be a discrepancy between the micro-array and ELISA techniques in determining the levels of VEGF in wound fluids. Lower levels of VEGF were detected in wound fluid with the ELISA methodology. Wound fluids were re-analysed at a 1:20, dilution prior to incubation on the VEGF plate.

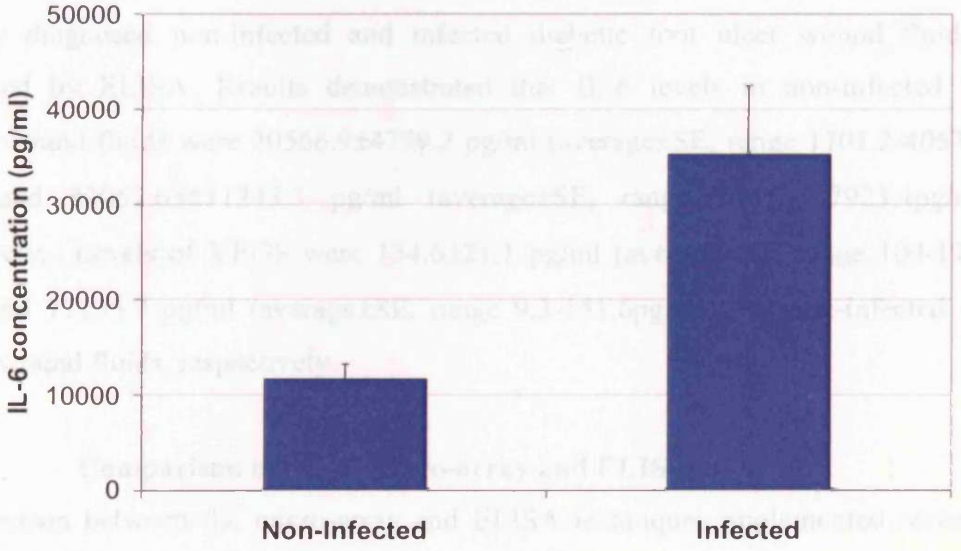
Duplicate wound fluid samples were analysed on three separate occasions. The amount of cytokines was determined based on a linear standard curve. Results presented herein are the average concentrations of IL-6/VEGF ± SE.

3.3.8.2.2 Interleukin-6 and vascular endothelial growth factor levels in non-infected and infected venous leg ulcer wound fluids

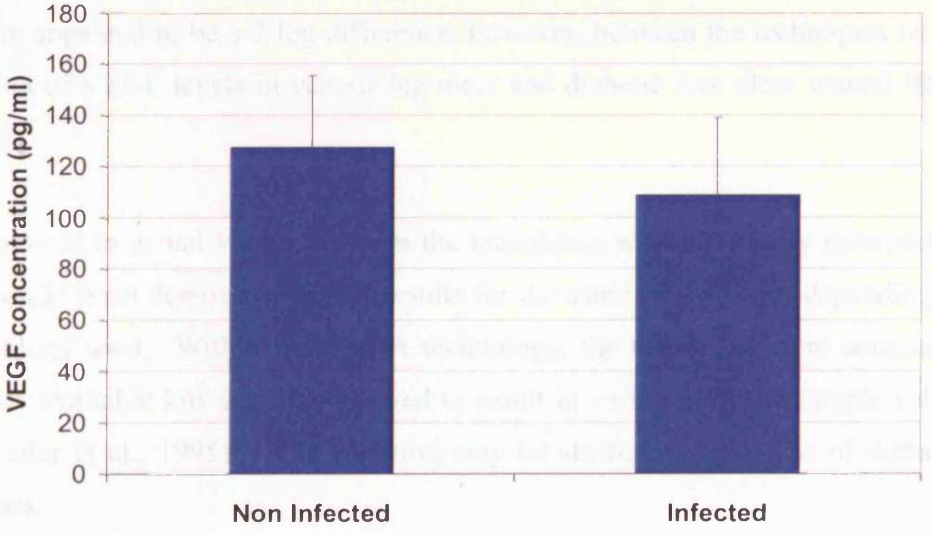
The average levels of IL-6 and VEGF in non-infected and infected venous leg ulcers, determined by ELISA are shown in Figure 3.24. Results showed there was no significant difference ($p>0.05$) in IL-6 (Figure 3.24a) or VEGF (Figure 3.24b) levels between clinically diagnosed non-infected and infected venous leg ulcer wound fluids as determined by ELISA. Results demonstrated that IL-6 levels in non-infected and infected venous leg ulcer wound fluids were 11680.6±1539.9pg/ml (average±SE, range 4881.9-17516.5 pg/ml) and 35295.3±7233.9pg/ml (average±SE, range 13136.5-68753.1 pg/ml), respectively. This data suggested that there was a trend for increased IL-6 in clinically diagnosed infected wound fluids. Levels of VEGF, 127.4±28.1pg/ml (average±SE, range 65.3-201.2pg/ml) and 108.7±30.4pg/ml (average±SE, range 63.6-166.6pg/ml), were comparable, for non-infected and infected wound fluids, respectively.

Figure 3.24: Levels of IL-6 and VEGF in venous leg ulcer wound fluid.
The average levels ±SE of (a) IL-6 and (b) VEGF, as determined by ELISA, for non-infected and infected venous leg ulcer wound fluids. No significant difference ($p>0.1$) was observed between the two populations for either cytokine.

a)



b)



3.3.8.2.3 Interleukin-6 and vascular endothelial growth factor levels in non-infected and infected diabetic foot ulcer wound fluid

The average levels of IL-6 and VEGF in non-infected and infected diabetic foot ulcers, determined by ELISA are shown in Figure 3.24. Results showed there is no significant difference ($p>0.05$) in IL-6 (Figure 3.25a) or VEGF (Figure 3.25b) levels between clinically diagnosed non-infected and infected diabetic foot ulcer wound fluid as determined by ELISA. Results demonstrated that IL-6 levels in non-infected and infected wound fluids were 20566.9±4799.2 pg/ml (average±SE, range 1701.2-40673.1 pg/ml) and 23057.65±11243.3 pg/ml (average±SE, range 1044.2-37923.4pg/ml), respectively. Levels of VEGF were 134.6±21.1 pg/ml (average±SE, range 100-191.4 pg/ml) and 77±35.7 pg/ml (average±SE, range 9.3-151.6pg/ml), for non-infected and infected wound fluids, respectively.

3.3.8.3 Comparison between micro-array and ELISA analyses

A comparison between the micro-array and ELISA techniques implemented revealed that the results obtained by micro-array generally demonstrated a larger range in the levels of detectable cytokines (Table 3.5), than those obtained by ELISA. The average levels of IL-6, as quantified by the two techniques, appeared comparable, for both ulcer types. There appeared to be a 2-log difference, however, between the techniques on the quantification of VEGF levels in venous leg ulcer and diabetic foot ulcer wound fluids (Figure 3.26).

These differences in actual values between the techniques was not wholly unexpected. It has previously been demonstrated that results for the same sample vary depending on the methodology used. Within the ELISA technology, the use of different sources of commercially available kits is acknowledged to result in variation in the sample values obtained (Ledur et al., 1995). This variation may be attributed to the use of different antibody pairs.

Figure 3.25: Levels of IL-6 and VEGF in diabetic foot ulcer wound fluid. The average level±SE of (a) IL-6 and (b) VEGF, as determined by ELISA, for non-infected and infected in diabetic foot ulcer wound fluids. No significant difference (p=>0.1) was observed between the two populations for either cytokine.

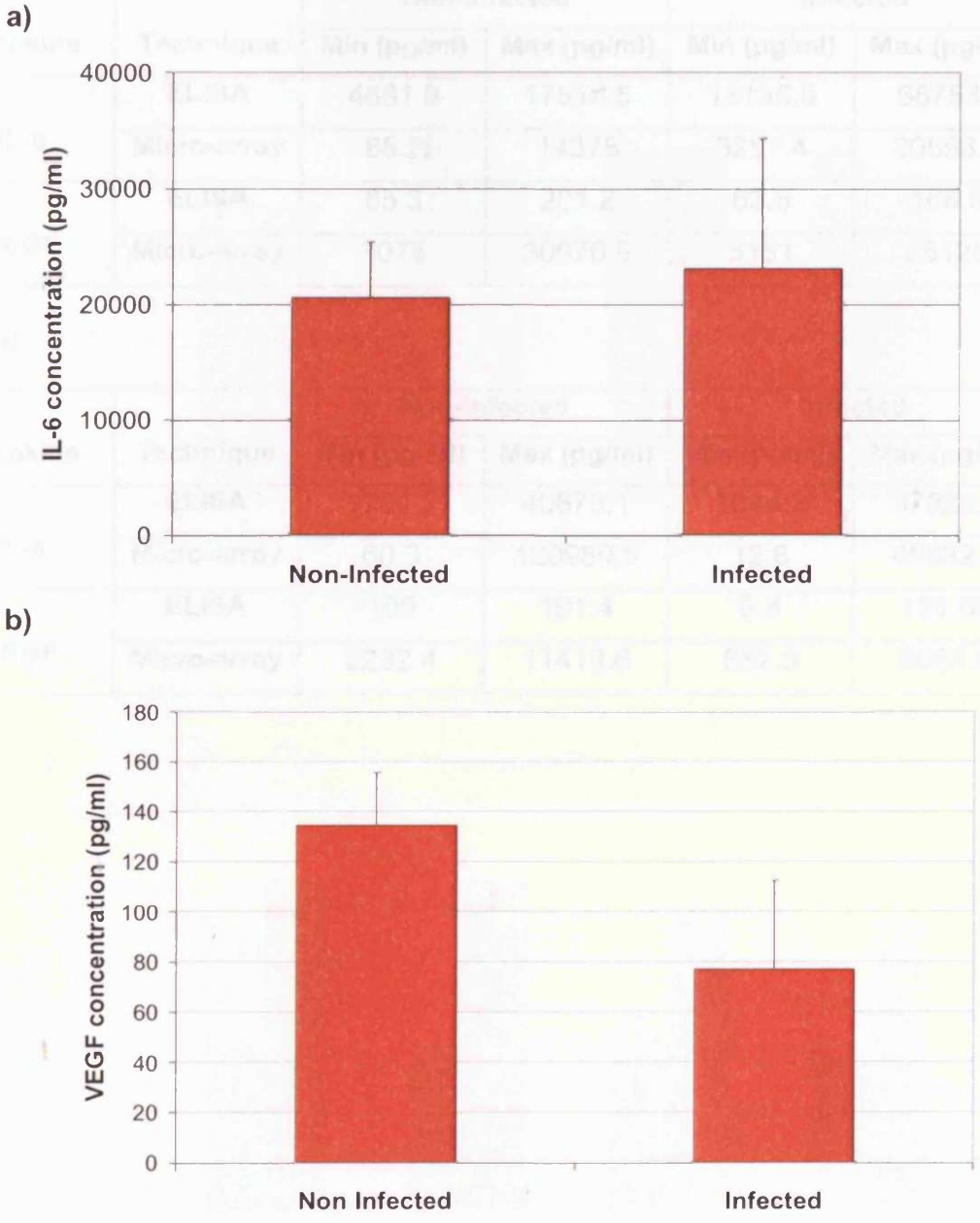


Table 3.5: Concentration of IL-6 and VEGF in (a) venous leg ulcer and (b) diabetic foot ulcer wound fluids, as determined by ELISA and micro-array.

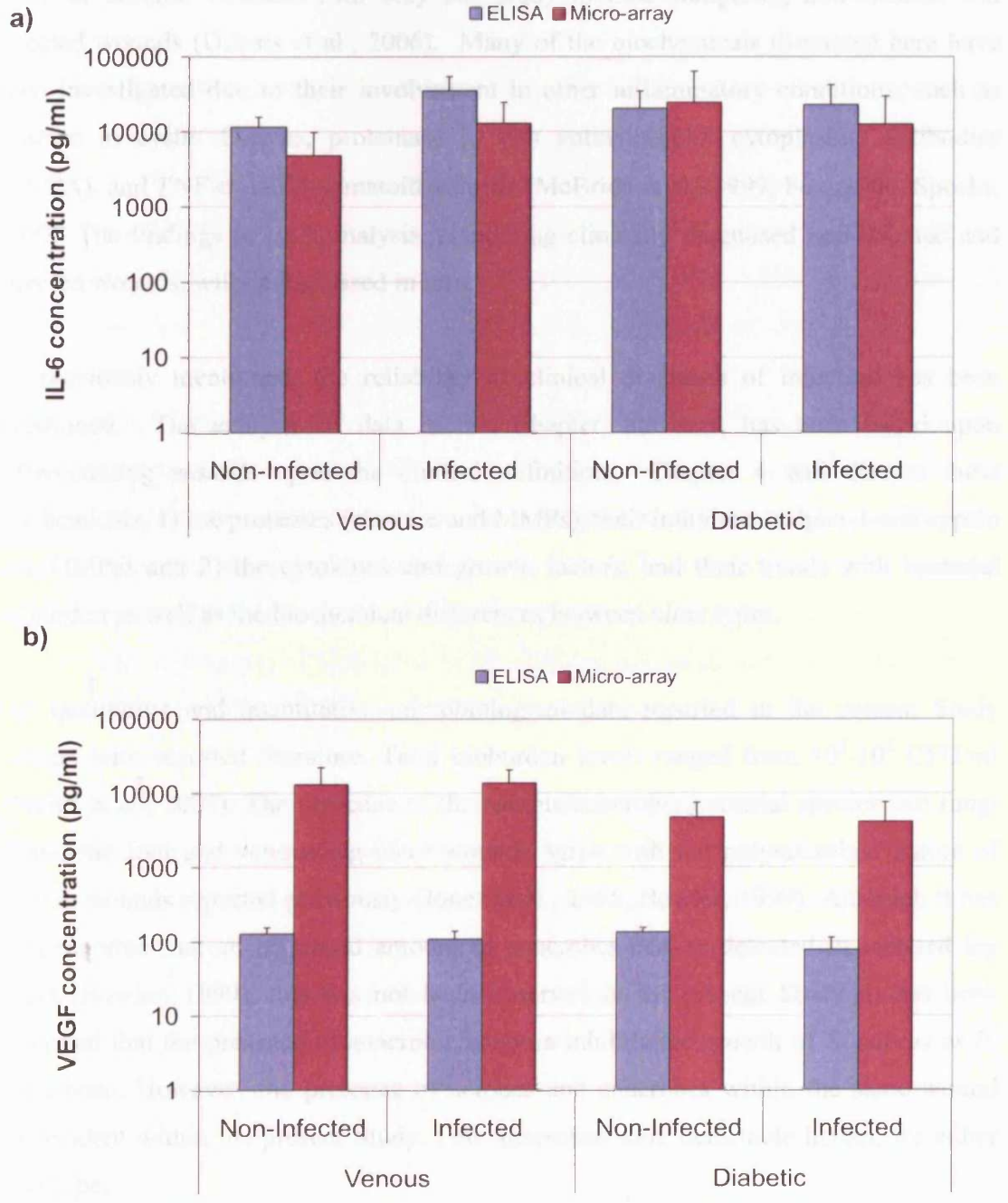
a)

Cytokine	Technique	Non-infected		Infected	
		Min (pg/ml)	Max (pg/ml)	Min (pg/ml)	Max (pg/ml)
IL-6	ELISA	4881.9	17516.5	13136.5	687531
	Micro-array	85.2	14375	3257.4	30558.1
VEGF	ELISA	65.3	201.2	63.6	166.6
	Micro-array	3075	30076.9	5151	28128

b)

Cytokine	Technique	Non-Infected		Infected	
		Min (pg/ml)	Max (pg/ml)	Min (pg/ml)	Max (pg/ml)
IL-6	ELISA	1701.2	40673.1	1044.2	37923.4
	Micro-array	60.3	120980.5	12.8	40832.5
VEGF	ELISA	100	191.4	9.3	151.69
	Micro-array	2232.4	11419.6	562.9	8064.8

Figure 3.26: Comparison of the levels of IL-6 and VEGF (average±SE) by ELISA and micro-array techniques.
Results were comparable between the techniques in the measurement of (a) IL-6. The same trend in results was observed for (b) VEGF, with a 2-log difference in the amount detected. ELISA had lower detection levels than micro-array.



3.4 Discussion

To date, most data in the literature cites biochemical differences between acute and chronic wounds. Few studies have investigated the biochemical differences between the types of chronic wounds, with only one study to date comparing non-infected and infected wounds (Debats et al., 2006). Many of the biochemicals discussed here have been investigated due to their involvement in other inflammatory conditions, such as elastase in cystic fibrosis, proteinase 3 with antineutrophil cytoplasmic antibodies (ANCA), and TNF-α and rheumatoid arthritis (McBride et al., 1999, Fox, 2000, Specks, 2000). The findings of each analysis, comparing clinically diagnosed non-infected and infected wounds, will be discussed in turn.

As previously mentioned, the reliability of clinical diagnosis of infection has been questioned. The analysis of data in this Chapter, however, has been based upon differentiating wounds upon the clinical definition. Chapter 4 will discuss these biochemicals, 1) the proteases (elastase and MMPs), their inhibitors, (alpha-1-antitrypsin and TIMPs) and 2) the cytokines and growth factors, and their trends with bacterial bioburden as well as the biochemical differences between ulcer types.

The qualitative and quantitative microbiological data reported in the present Study concurs with reported literature. Total bioburden levels ranged from 10^2-10^8 CFU/ml (Davies et al., 2007). The presence of the aerobic/anaerobic bacterial species and fungi in diabetic foot and venous leg ulcer wounds agree with the polymicrobial nature of chronic wounds reported previously (Jones et al., 1985, Bowler, 1999). Although it has been reported that an increased amount of anaerobes can be detected in infected leg ulcers (Bowler, 1999), this has not been observed in the present Study. It has been suggested that the presence of anaerobic bacteria inhibits the growth of *S. aureus* or *P. aeruginosa*. However, the presence of aerobes and anaerobes within the same wound was evident within the present Study. Few anaerobes were detectable herein, for either ulcer type.

Similarities in the different types of bacteria was observed between ulcer types, with *Staphylococcus* spp., and *Corynebacterium* spp., the most frequently identified species (Eriksson et al., 1984, Hansson et al., 1995, Bowler, 1998, Schmidt et al., 2000). *Pseudomonas* spp. was the most frequently isolated bacteria in venous leg ulcer wounds (12 of 18 wounds), with only 1 of 14 diabetic wounds containing this bacteria.

This study, however, further demonstrated that wounds clinically differentiated as non-infected and infected, appear to harbour the same number of bacteria. There was a poor relationship between clinical diagnosis of infection and microbiological infection, defined by either bacterial bioburden or the number of bacterial genera contained within the wound. Interestingly, within the venous leg ulcer population, of the 11 subjects with a total bioburden of $>10^7$, only 6 (55%) had exhibited clinical infection.

These findings support the data of Wilson et al., (1990) and Bamberg et al., (2002), who claim that infected wounds ($>10^5$ CFU/ml bacteria) are often mis-diagnosed. The data in the present Study, also suggests that the reverse scenario is equally applicable, that wounds clinically diagnosed as infected, contain $<10^5$ CFU/ml bacteria. Within the venous leg ulcer population, only 2 of the 10 ulcers, clinically diagnosed as non-infected, had low bacterial bioburden ($<10^5$ CFU/ml bacteria), and within the diabetic population, only 1 of the 10 non-infected ulcers had $<10^5$ CFU/ml bacteria.

It is generally accepted, however, that infection is a relationship between bacterial number and virulence against host resistance. Currently, the number and types of bacteria are used to help define infection status, as they are readily measurable, and as such, define treatment options, e.g. the type of antibiotic prescribed (Bowler, 2003). Determination of factors, such as host resistance and overall bacterial virulence, prove more difficult to quantify and have not been measured in this Study.

The data in the present Study suggested that there is no correlation ($p > 0.05$ for venous leg and diabetic foot ulcers) between the clinical and microbiological diagnosis of wound infection. As such, the levels of particular biochemicals have also been related to the total number and types of bacteria (Chapter 4).

Of the clinical parameters correlated to infection, ulcer wound area and perimeter were significantly increased in infected venous leg ulcer wounds. Ulcer size was not found to be attributed to a single microorganism or the level of bioburden within the wound (Hansson et al., 1995). However, increased ulcer size correlated with *Pseudomonas* spp. contaminated wounds, is reported (Madsen et al., 1996).

Protein values were obtained ultimately to enable samples to be standardized for protein content, prior to analysis. The range of protein values in venous leg ulcer wound fluids 1.04-6.83 mg/ml and the diabetic foot ulcer wound fluids, 1.67-8.74 mg/ml, in this Study are consistent with amounts previously determined using the same wound fluid collection technique (Cullen et al., 2002). There are several different techniques reported for collecting wound fluids, with perceived advantages and disadvantages associated with each method (Moseley et al., 2004b). Variations in protein concentrations between studies have been attributed to the wound fluid collection technique. The protein levels detected in the present Study, however, appear to be consistent with protein levels measured using other wound fluid collecting techniques (Harris et al., 1995, Trengrove et al., 2000, James et al., 2000).

Analysis of acute and chronic wound fluid (Harris et al., 1995) and chronic non-healing and chronic healing wounds (Trengrove et al., 2000, James et al., 2000) have revealed that total wound fluid protein levels are significantly higher in acute and healing wound populations. Although this elevation in protein levels is interesting, it has not been widely adopted as a clinical diagnostic technique.

Total protein determination provides no information on the relative contribution of specific proteins, perceived as important in the wound healing process. The findings of the present Study appear to suggest that total protein levels alone are not indicative of wound infection, as defined by clinical diagnosis.

Elastase and its main inhibitor were detected in the present Study in both chronic venous leg and diabetic foot ulcer wound fluids. The levels of neutrophil derived elastase-like activity in this Study can be compared with data presented by Cullen et al., (2002), in diabetic foot ulcer fluid. The range in neutrophil derived elastase-like activity was 0.42-523.8 RFU/min (Cullen et al., 2002), compared to 0.53-526.47 RFU/min, in the present Study. The data revealed that a similar range of neutrophil derived elastase-like activity was measured in both studies.

The present Study has further investigated and confirmed that this activity value may be a combination of elastase and proteinase 3. The presence of proteinase 3 appeared to be predominant in levels, compared to elastase, by zymography. This observation leads to a possible hypothesis that proteinase 3 may be more important than elastase in the degradation of ECM components in the chronic wound environment. However, the relative importance of proteinase 3 in chronic wound fluid was not investigated further herein.

Elastase has previously been measured in chronic wound fluid by casein zymography, and by fluorogenic assays (Mast and Shultz, 1996), which concluded that chronic wounds have elevated elastase levels than acute wounds. Earlier studies by Grinnell et al., (1992) and Grinnell and Zhu (1994), attributed the presence of degraded fibronectin in chronic venous leg ulcer wound fluids and burn wound fluids, respectively, to the activity of elastase.

Further to this work, Herrick et al., (1997), correlated increased ECM degradation with the up-regulation of elastase in both acute aged wounds and venous leg ulcer fluids. These publications provide evidence that increased elastase levels may contribute to the pathophysiology of non-healing chronic wounds. Contradictory to this data, Weckroth et al., (1996), concluded that although gelatinase and collagenase activities were higher in chronic wound fluids, there was no difference in the activity of the serine proteases, elastase and cathepsin G, derived from acute wounds.

Evidence in the literature does suggest that ulcer exudate leukocyte counts show a predominance of polymorphonuclear neutrophil-origin cells (Palolahti et al., 1993). Therefore, an increase in serine protease levels may be expected in chronic ulcer wound fluid, compared to acute wounds, due to the perceived prolonged inflammatory nature of a chronic wound (Agren et al., 2000). As neutrophils are phagocytes whose role is to eliminate bacteria from a wound site, an increase in the levels of neutrophil derived proteases at a site of infection may be apparent.

However, the results presented here seem to indicate that there was no significant difference in neutrophil derived elastase-like activity (determined fluorometrically, by ELISA or zymography) between chronic wound fluids.

Additionally, the levels of alpha-1-anti-trypsin in the clinically non-infected and infected chronic wound fluid or a ratio of alpha-1-anti-trypsin to elastase did not appear to distinquish between clinically non-infected and infected wound fluids.

The endogenous inhibitor of elastase, alpha-1-anti-trypsin levels normally exceed protease levels, thereby controlling proteolysis. Genetic and local factors can vary these concentrations within individuals (Stoller and Aboussouan, 2005). Levels of alpha-1-anti-trypsin in the present Study ranged from 30-250μg/ml.

Several *in vitro* studies have shown that alpha-1-anti-tyrpsin appears to be susceptible to inactivation by both host and microbial proteases (Vissers et al., 1988, Kaminishi et al., 1995, Grenier, 1996, Nie and Pie, 2004). Degradation and inactivity of α-1-antitrypsin is reported in chronic wound fluids (Rao et al., 1995, Krishna., et al, 1997). The resulting fragments upon cleaveage of alpha-1-anti-trypsin have been shown to activate neutrophils to induce pro-inflammatory effects (Janciauskiene et al., 2004). Therefore, in addition to its inability to control protease action, alpha-1-anti-trypsin degradation may induce inflammatory cells to increase their activity.

The amount of alpha-1-antitrypsin and α-2-macroglobulin (elastase inhibitors) in plasma, acute and chronic wound fluid, have also investigated by Yager et al., (1997). Western blot analysis showed alpha-1-antitrypsin from plasma, migrated to a reveal a major band at approximately 52kDa and a minor band of 60kDa. Acute wound fluids showed the same bands, but contained an additional immuno-reactive band at 77kDa.

Increased levels of the 77kDa band can also be observed in chronic wound fluids and it is suggested that this is an elastase:alpha-1-antitrypsin complex. This same banding pattern was observed within the present Study, indicating the presence of alpha-1-anti-trypsin and additionally, in complex form to elastase. Results from the present Study also revealed low molecular weight fragments, indicating increased cleavage of the alpha-1-antitrypsin, as previously observed in chronic wound fluid (Yager et al., 1997). It is therefore possible that the ELISA technique used in the quantification of alpha-1-anti-trypsin may detect fragments of the molecule.

Several techniques were used in the present Study to measure either the activity and /or levels of elastase and alpha-1-anti-trypsin in wound fluids. Direct comparisons between the results obtained in this study and those previously reported is difficult, as most data is observational and semi-quantitative. Often contrasting methods have also been employed to collect and measure these molecules.

A ratio of elastase activity to the levels of alpha-1-anti-trypsin was calculated from the data presented in this Study. Ideally, this ratio would compare either the activity values or the amount of each molecule on a molar basis. A comparison of activity values has not been possible, as no satisfactory way of determining alpha-1-anti-trypsin activity in wound fluids was available. Substrates that detect alpha-1-anti-trypsin are also capable of being cleaved by other molecules, including plasmin and trypsin-like proteases, previously reported as present in wound fluids (Palolahti et al.,1993, Cullen et al., 2002). If an appropriate substrate could be found for alpha-1-anti-trypsin, the alpha-1-anti-trypsin activity value could potentially be compared to the activity of elastase, proteinase 3 or a combination of the two molecules.

A ratio comparing the molar:molar amounts of alpha-1-anti-trypsin to elastase could potentially be made. The levels of elastase could be quantified using the elastase ELISA, developed during the present Study, as it does not cross-react with proteinase 3. An equivalent assay would also need to be developed for proteinase 3, in order for the relative importance of proteinase 3 be investigated.

No significant difference was observed between clinically diagnosed non-infected or infected venous leg or diabetic foot ulcer wound fluids, using the ratio value calculated in this manner. As other biochemical markers remained to be investigated, no further exploratory or developmental work was conducted on elastase, proteinase 3 or alpha-1-anti-trypsin.

The ECM component, collagen is degraded by collagenases 1 (MMP-1), mainly synthesized and released by fibroblasts, but also macrophages, keratinocytes and endothelial cells, and neutrophil-derived collagenase 8 (MMP-8). Subsequent cleavage of the collagen structure leads to spontaneous denaturation to gelatin. Gelatin is further cleaved by a number of enzymes, including the 92kDa and 72kDa gelatinases, MMP-2 and MMP-9 (Weckroth et al., 1996).

Research has revealed that the activity of both collagenases and gelatinases is higher in chronic venous leg ulcer and diabetic foot ulcer wound fluids than acute wound fluids (Weckroth et al., 1996, Barone et al., 1998, Trengrove et al., 1999, Agren et al., 2000, Pirila et al., 2007).

Determination of collagenase activity in wound fluid studies has been conducted using a variety of methods. In the present Study, collagenase-like activity was measured using a fluorogenic peptide, with activity value concurring with a previous study of diabetic foot ulcers (Cullen et al., 2002). Collagenase activity has been measured by other researchers using the Azocoll substrate activity assays (Ladwig et al., 2002), or the use of specific collagen fragments (Yager et al., 1996). The differences in techniques utilized means it is difficult to compare actual activity values between studies.

In the present Study, no significant differences were observed on comparing collagenase-like levels between the two populations of chronic wounds, from clinically non-infected and infected venous leg ulcers and diabetic foot ulcers. Thus, further analysis to determine which collagenase protease may be important in chronic wound infection, was not investigated.

Trengrove et al., (1999), Yager et al., (1996) and Wysocki et al., (1993) have analysed gelatinolytic activity in wound fluids, comparing chronic and acute, or chronic non-healing and healing wounds. Significantly lower gelatinase activities have been identified in acute and healing, chronic wound fluids. Zymographic analysis of wound fluids revealed similar gelatinolytic profiles for both acute and pressure ulcer wounds, compared to wound fluids from venous leg ulcers. Venous leg ulcer wound fluids appear to have multiple gelatinase species present (Wysocki, 1993,1999, Bullen et al., 1995). The present Study showed similar profiles for venous leg ulcer and diabetic foot ulcer wound fluids. The same pro- and active- MMP forms were observed in each ulcer type.

Zymographic analysis of all chronic wound fluids in the present Study exhibited high MMP-9 activity, compared to MMP-2 activity. It has been demonstrated that the zymography technique favours the detection of MMP-9 activity (Ladwig et al., 2002). Very little active MMP-2 was readily detectable in any of the chronic wound fluids studied. This may be explained partially by the technique itself, but may also be a reflection of gelatinase expression.

The expression of MMP-2 and MMP-9 has been linked to different phases of the wound healing process, as determined in acute wound healing studies. The expression of MMP-2 (72kDa gelatinase), mainly fibroblast derived, has been reported as consistent over the course of wound healing (Agren, 1994) and is perceived to be an important mediator in the re-modelling phase of healing (Salo et al., 1994). The lack of MMP-2 expression in the present Study may be indicative of the failure of these wounds to heal. MMP-9, expressed by migrating keratinocytes and macrophages, (Ravanti and Kahari, 2000), in contrast, appears to be associated with the early events of wound repair (Oikarinen et al., 1993, and latterly during the re-epithelialisation process (Agren, 1994).

Although the wounds in the present Study range in duration from 6 to 542 months, their chronic nature implied that each wound may be involved in healing processes consistent with the early stages of wound repair. This suggests that MMP-9 may dominate over the expression of the wound remodeling protease, MMP-2. Findings of several studies seem to show that during re-epithelisation processes, MMP-1 and MMP-9 exhibit high expression. As wound healing progresses, the levels of these particular proteases decrease, as MMP-13 and MMP-2 contribute to the re-modelling phase (Ravanti and Kahari, 2000). Pro-MMP-9 appears to correlate with non-healing, therefore more likely to indicate impaired healing (Moseley et al., 2004a).

In the present Study, there was no significant difference in the levels of MMP activity, either gelatinolyic or collagenolytic, between clinically non-infected and infected venous leg ulcer or diabetic foot ulcer patient populations. A difference in gelatinase activity has previously been observed in wound fluid from normal acute and infected mastectomy wound fluid (Tartlon et al., 1997). Activation of MMPs detected in inflammatory fluids, from a number of disease etiologies, correlate with both the number of neutrophils at the site and the presence of infection (Makowski and Ramsby, 2003). The present Study however has not determined such a relationship in the infection status of chronic wounds.

The level of TIMP-1 and TIMP-2 were analysed in this Study and consistent with previous findings, no significant difference was observed between clinically non-infected and infected chronic venous leg ulcer or diabetic foot ulcer wound fluids. There was no significant differences ($p>0.1$) comparing the levels of TIMP-1 and TIMP-2 within each ulcer type. The levels of TIMP-1 and TIMP-2 ranged from 6.3-80.5ng/ml, as determined by ELISA. This is a low amount of TIMP is again consistent with other studies (Bullen et al., (1995), Nwomeh et al., (1998)).

Cytokine levels were determined in the present Study by both micro-array and ELISA techniques. Analysis of the micro-array results, suggested that regardless of chronic ulcer aetiology, a similar cytokine expression profile was observed. Large amounts of pro-inflammatory cytokines (IL-6, IL-8 and to a lesser extent, IL-1β), angiogenic factors, (angiogenin and VEGF), and cell surface receptors (ICAM-1 and TNFr2), were detected in the chronic wound fluids.

Cytokines with low levels of expression detected in this study are those involved with various roles in mediating the immune response. These roles include recruiting helper T-cells (IL-2), the differentiation of T-cells (IL-4), suppression of pro-inflammatory cytokines (IL-13, TGF-β_1, IL-10), regulation of antibody production (IL-5, IFN-γ), the inhibition of viral replication (IFN-γ), suppression of cell proliferation (TGF-β_1), and the blocking of angiogenesis (IP-10) (Chapter 1, Section 1.6). This may in itself, again, indicate the inflammatory nature of the chronic wound environment.

Of the eighteen cytokines analysed by micro-array, six cytokines had previously been measured in, either acute or chronic wound fluids, by ELISA. These six cytokines, IL-1β, IL-6, TNF-α and its receptor TNFr2, TGFβ_1 and VEGF, have previously been identified and measured by several investigators as potential candidates to explain the patho-physiology of chronic wounds (Tarnuzzer and Shultz, 1996, Wallace and Stacey, 1998, Trengrove et al., 2000, Drinkwater et al., 2003, Werner and Grose, 2003). Studies have compared the levels of these cytokines between acute and chronic, or chronic healing and non-healing wound environments. The key roles these particular cytokines play in healing processes, such as inflammation and angiogenesis, may explain why these cytokines have been measured historically. The levels of each cytokine as quoted in the literature will be discussed in turn.

The levels of IL-1β, on average in chronic wound fluids have been determined at 2500 pg/ml (Mast and Shultz, 1996). Wagner et al. (2003), demonstrated an increase in IL-1β, with > 1000pg/ml in inflamed tissue compared to acute wounds. IL-1β levels have been detectable at 7307-72487pg/ml and 686-51205pg/ml, in healing and non-healing chronic venous leg ulcer wound fluids respectively (Trengrove et al., 2000), or alternatively, bioactive IL-1β at 2.48pg/ml and 5.35pg/ml, respectively (Harris et al., 1995), showing that it is the functionality of these molecules that is important.

IL-6 levels, on average in chronic wounds, have been quoted at 30pg/ml (Mast and Shultz, 1996). Higher levels of IL-6, (24113-210000pg/ml) have been measured by ELISA in chronic venous leg ulcer wound fluids (Trengrove et al., 2000). Large levels of biologically active IL-6 in non-healing, (54404±5216pg/ml) and healing (39233±21344pg/ml) wounds have also been quantified by Harris et al., (1995).

Mast and Shultz, (1996), quoted an average of 500pg/ml TNF-α in chronic wound fluid. Wallace and Stacey, (1998), measured 549.4-3549.2pg/ml in chronic venous leg ulcer wound fluids. TNF-α levels in non-healing and healing chronic venous leg ulcers have been cited at 1030-13257pg/ml and 550-7540pg/ml, respectively (Trengrove et al., 2000). The level of TNF-α to it's receptor TNFr2 found that a ratio of less than 1:300, indicated a non-healing wound (Wallace and Stacey, 1998). TNFr2 was determined at 15874- 31264 pg/ml.

In the present Study, the levels of each of the pro-inflammatory cytokines, IL-1β, IL-6, TNF-α and its receptor TNFr2, appeared to correlate to the levels previously reported in the literature. However, a greater range in values were observed, with lower levels of each cytokine detected. The range in the levels of IL-1β and IL-6 in the present Study were 6-25926pg/ml and 85-120980pg/ml, respectively.

The level of TNF-α in venous leg ulcers, was low, (4-749pg/ml), in comparison to the level of TNFr2 (2.241-2540.308ng/ml). The low level of TNF-α detected suggests that it is bound to its receptor, TNFr2. Analysis of patient-matched TNF-α to TNFr2 levels, suggested that a number of wounds within the present Study, both clinically diagnosed non-infected and infected wounds, are non-healing, as defined by Wallace and Stacey, (1998). Patients were not followed to healing and therefore correlation of healing to TNF levels could not be confirmed herein. However, comparison of TNF-α levels to the levels of other pro-inflammatory cytokines, as assessed by micro-array, begs the question, are the level of TNF-α playing a contributing role in the inflammatory process, associated with the pathophysiology of chronic wounds?

The levels of $TGF\beta_1$ in healing and non-healing chronic venous leg ulcer wounds are lower, at 3.9-22.5pg/ml and 4.4-32.4 pg/ml, respectively, compared to the both IL-1β (686-72487pg/ml) and IL-6 levels (24113-210000pg/ml) (Trengrove et al., 2000). Tarnuzzer and Schultz, (1996), found that on average, 5.26±1.69 nmol/l of $TGF\beta_1$ were present in chronic wounds. Again micro-array data from the present Study concurs with those previously cited by other investigators, with lower levels of TGF-β being measured, indicating that some of the wounds in this Study may be in a non-healing state.

The levels of VEGF by ELISA, in acute, breast and colon wound fluids, were reported at 287-4386pg/ml and 0-7212pg/ml, respectively (Baker and Leaper, 2000). A comparison of acute to inflamed wounds found levels to be <2000pg/ml and >3000 pg/ml, respectively (Wagner et al., 2003). The expression of VEGF has been measured in chronic wounds histologically, at the gene and protein level (Peschen et al., 1998, Lauer et al., 2000, Drinkwater et al., 2003). A correlation between decreasing serum VEGF levels and healing in venous leg ulcer patients under compression therapy was shown (Murphy et al., 2002).

The levels of VEGF in non-healing chronic ulcers and healing chronic venous leg ulcers, by ELISA, were quantified at 67.17±13.87 ng/mg and 32.19±13.87 ng/mg, respectively (Drinkwater et al., 2003). The levels of VEGF in venous leg ulcer ranged from 5151-30077pg/ml and 63-201pg/ml by micro-array and ELISA analysis, respectively, in the present Study. Diabetic foot ulcer wound fluids had significantly lower ($p<0.001$) levels of VEGF, compared to venous leg ulcer wound fluids (Chapter 4, Section 4.3.9).

Overall, the levels of VEGF, in addition to the other 17 cytokines measured, suggested that none of these cytokines are ideal candidates with which to differentiate between clinically non-infected and infected wounds.

The present Study has provided data on the levels of cytokines, previously not measured in wound fluids. Micro-array technology allowed for simultaneous analysis of 18 cytokines that would not have been possible using traditional ELISA methodologies. A cytokine profile has been generated for each patient's wound fluid. This profile could further be investigated for inter and intra-cytokine relationships. Analysis of these profiles provided information on the relative amount of one cytokine to another, with the knowledge that each wound fluid had been subjected to the same experimental conditions.

However, the overall range in the levels of these cytokines in the wound fluids analysed, revealed a disadvantage with this technology. On occasion, certain wound fluids required dilution, due to this high abundance of particular cytokine/s. Yet, the dilution of wound fluids to allow determination of the amount of that particular cytokine, resulted in another cytokine being below the limit of detection. This in itself is an inherent issue in any technique, where multiple analytes are being investigated simultaneously. Some variation in the micro-array data between runs, was observed on occasion. This was mainly attributed to the change in some of the dilution factors used, however, it was too expensive, and the lack of sufficient sample for this issue to be rectified. For the purposes of the present Study, comparisons between cytokine levels were checked between runs (in non-diluted samples). The data presented herein is a representative run, wherein the levels of cytokine between the clinically diagnosed non-infected and infected wound fluids, were compared.

The comparison between micro-array and ELISA results revealed significant difference in the determination of VEGF levels. Additional studies have shown that the amount of cytokine detected in the same sample is dependent on the type of ELISA used (Ledur et al., 1995, Wallace and Stacey, 1998). This study, therefore, further validates comparison of data between two populations, using the same technique. Analysis of the ELISA data generated in the present Study suggests there was no significant difference in the levels of any of the cytokines between clinically diagnosed non-infected and infected wound fluids, as concluded previously using micro-array technology.

3.4.1 Summary of findings

- The clinical diagnosis of infection did not correlate with the number of bacteria in either wound types (venous leg ulcer or diabetic foot ulcer), with a range of 10^2-10^8 CFU/ml, detected in each clinically defined population.

- Protein content in clinically non-infected and infected venous leg ulcer or diabetic foot ulcer wound fluids were not significantly different.

- Chronic wound fluids contained the serine proteases, elastase and proteinase 3. Measuring the activity of both these molecules, revealed no significant difference between clinically diagnosed non-infected and infected venous leg ulcer or diabetic foot ulcer wound fluids. There also appeared to be no significant differences in the levels of their naturally occurring inhibitor, alpha-1-anti-trypsin, or additionally in the ratio of these proteases to their inhibitor, between the two populations.

- Collagenase and gelatinase (MMP-2 and MMP-9) activities were quantified, with the levels of their respective inhibitors, TIMP-1 and TIMP-2. Analysis revealed no significant difference in the activity/levels of these biochemicals, between clinically diagnosed non-infected and infected venous leg ulcer and diabetic foot ulcer wound fluids.

- Levels of cytokines and growth factors in chronic wound fluids, determined by micro-array, generally concurred with levels previously cited in the literature. The data revealed high levels of angiogenic (angiogenin and VEGF) and cell surface receptor cytokines (ICAM and TNFr2) in the chronic wound fluids, in addition to the previously investigated pro-inflammatory cytokines (IL-1β and IL-6).

- No significant differences were observed between the clinically diagnosed non-infected and infected venous leg ulcer and diabetic foot ulcer wound fluids, for any of the 18 cytokines analysed by micro-array. Two cytokines, IL-6 and VEGF, were also quantified by ELISA, and revealed no significant differences between the clinically diagnosed non-infected and infected wound populations.

This general screen of potential biochemicals, in relation to clinically non-infected and infected venous leg ulcer and diabetic foot ulcer wounds, revealed no significant differences in their levels, that would allow them to serve as diagnostic markers for wound infection status, based upon clinical diagnosis. Thus, either one cannot differentiate the infection status of a chronic wound based on the underlying biochemistry or the clinical diagnosis of infection is not accurate. Previous literature suggests that misdiagnosis of infection may not be uncommon, and if this is also the case in the present Study, this, in combination with a limited sample number, may explain the lack of significance differences observed.

Chapter 4

Chapter 4

Biochemical differentiation between non-infected and infected chronic wounds, based upon microbial diagnosis

4.1 Introduction

The findings of Chapter 3 revealed no significant differences in either the levels or activity of each of the biochemical markers analysed, (i) the proteases (elastase and matrix metalloproteinases, MMP), along with their associated inhibitors (alpha-1-antitrypsin, and tissue inhibitor of matrix metalloproteinase, TIMP) and (ii) numerous cytokines and growth factors, interleukins (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, Il-12 p40, IL-12p70 and IL-13), angiogenin (angio); interferon gamma (IFN-γ); growth factors, transforming growth factor beta (TGF-β_1) and vascular endothelial growth factor (VEGF); the pro-inflammatory cytokine, tumour necrosis factor alpha (TNF-α) and its soluble receptor p75; an adhesion molecule, intracellular adhesion molecule 1 (ICAM-1), and the chemokine, IFN-γ-inducible-protein-10 (IP-10), between clinically non-infected and infected wounds. However, these findings may be explained by the lack of correlation between the levels and types of microbiological contamination and the clinical signs and symptoms, used to diagnose the wounds.

The reliability of clinical signs and symptoms in the diagnosis of chronic wound infection has been questioned previously, with the misdiagnosis of microbiologically-defined chronic wound infection possible (Wilson et al., 1990, Gardner et al., 2001a, Bamberg et al, 2002, Davies et al., 2007, Lorentzen and Gottrup, 2006). The data, from the present Study (Chapter 3), was therefore analysed to ascertain whether any of these particular biochemical markers correlated with the number of aerobic and anaerobic bacteria in the wound, the combination of bacteria, or the presence of a particular bacterial species. In the present Study, small numbers of fungi and anaerobic bacteria were detected. Therefore, no analysis was performed on these groups individually, but their numbers were included in calculating total bioburden. Thus, the present Chapter correlated the levels of the various biochemical markers to the microbial content of the chronic wounds.

4.1.1 Bacterial bioburden in the diagnosis of wound infection

Currently, the levels and types of bacterial contamination of a wound is used as secondary information, to aid the Clinician in wound management, if they consider a wound infected. Suspicions of wound infection may be confirmed when a wound swab or biopsy is positive, with microorganisms from the wound cultured over a number of days (Bowler, 2003). Currently diagnosis of wound infection relies upon the combination of both clinical signs, and the detection of microorganisms, using standard microbiological techniques.

A recent study has shown a difference in the arginine conversion rate, with increased levels of citruline, ornithine and a number of amino acids in chronic, infected wounds, compared to those of both acute and chronic non-infected wounds, as defined by microbiological diagnosis at the level of 1×10^3 CFU/ml (Debats et al., 2006). Upon initiation of the present Study, no host biochemical molecules have been reported that differentiate between chronic, non-infected and infected wounds.

Upon defining infection microbiologically, ideally, a statistical assessment for the amount of bacteria at a level equal to or greater than $1x10^5$ CFU/g tissue would be made. However, literature has suggested that differentiation at a level equivalent to 1 x 10^6 CFU/ml is acceptable. Large numbers of bacteria were cultured from wounds in the present Study and as such, differentiating at the level of $1x10^5$ CFU/ml was not statistically valid. Subsequently, microbiological infection was defined as a total bacterial bioburden of $\geq 1x10^6$ and $\geq 1x10^7$ CFU/ml, for both venous leg ulcer and diabetic foot ulcer wounds.

A comparison of each biochemical parameter, to the presence of certain microorganisms, was performed. Possible pathogens, cultured from the wound swab, that may be responsible for infection were included in the analysis, *Pseudomonas* spp., *Staphylococcus* spp. (Chapter 1, Section 1.8.1) and *Streptococcus* spp., whose infecting dose is reported to be lower than the other possible wound pathogens (Heggars, 1998).

Additionally, the commensal, microorganism, *Corynebacterium* spp. was also included, as it was isolated from 9/18 venous leg ulcer and 11/14 diabetic foot ulcer wounds (Chapter 3, Section 3.3.2).

Due to the variation in microbial flora between the two ulcer types and the numbers of microorganisms present, the venous leg ulcer wounds were divided according to the levels of the particular microorganism, *Pseudomonas* spp., *Staphylococcus* spp. and *Corynebacterium* spp., with the divison set at a level of $\geq 1x10^7$ CFU/ml. Conversely, each biochemical in the diabetic foot ulcer population was compared in populations divided upon the basis of the presence or absence of a particular microorganism, namely *Staphylococcus* spp., *Streptococcus* spp. and *Corynebacterium* spp.

As the literature suggests chronic wounds tend to be polymicrobial in nature (Bowler et al., 2001), with the presence of four or more microorganisms in a wound correlated to non-healing chronic wounds (Trengrove et al., 2000, Davies et al., 2004), the level of each biochemical marker was also compared to the number of bacterial genera within the wound.

In summary, microbial infection within the venous leg ulcer wounds was defined as follows:-

1) Total bacterial bioburden of $\geq 1x10^6$ and $\geq 1x10^7$ CFU/ml.
2) *Pseudomonas* spp., *Staphylococcus* spp., *Streptococcus* spp. and *Corynebacterium* spp. at a level of $\geq 1 \times 10^7$ CFU/ml.
3) Three or more bacterial genera.

Microbial infection within the diabetic foot ulcer wound fluids was defined as follows:-

1) Total bacterial bioburden of $\geq 1x10^6$ and $\geq 1x10^7$ CFU/ml.
2) The presence of *Staphylococcus* spp., *Streptococcus* spp. and *Corynebacterium* spp.
3) Three or more bacterial genera.

4.2 Materials and methods

Experimental conditions for the analysis of wound micro-flora have previously been described (Chapter 2, Section 2.1.2). The measurement of proteases, their inhibitors and a number of cytokines and growth factors were performed as previously described (Chapter 2, Section 2.3 and Chapter 3).

4.3 Results

4.3.1 Microbial content of venous leg and diabetic foot ulcer wounds

4.3.1.1 Total bioburden

The total bioburden in venous leg ulcer wounds ranged between 10^3-10^8 CFU/ml and 10^2-10^8 CFU/ml for diabetic foot ulcer wounds (Figure 4.1). Within this analysis, microbiologically defined wound infection is represented by a total bioburden of $\geq 1x10^6$ and $\geq 1x10^7$ CFU/ml, for both venous leg and diabetic foot ulcers. At the level of $\geq 1x10^6$ CFU/ml, 13 of 18, and at a level of $\geq 1x10^7$ CFU/ml, 11 of 18 venous leg ulcer wounds were classified as microbiologically infected. At the level of $\geq 1x10^6$ CFU/ml, 7 of 14, and at the level of $\geq 1x10^7$ CFU/ml, 8 of 14 diabetic foot ulcer wounds were classified as being microbiologically infected.

Chronic wounds had been diagnosed clinically for the presence or absence of infection, with patients being assigned into two groups with either non-infected (patients 1-10) or infected (patients 11-20) wounds, respectively (Chapter 2). The correlation between total bioburden and clinical assignment found that it was possible to have high levels of total bioburden within the wound and clinically assign these patients as non-infected (Figure 4.2). Seven of the 10 clinically non-infected venous leg ulcer wounds would be reassigned as infected upon microbial analysis revealing total bioburden levels $\geq 1x10^6$ CFU/ml.

Additionally, 2 of the 10 infected wounds, would be reassigned as non-infected. Within the diabetic foot ulcer population, all patients assigned as clinically infected had total bioburden levels of $\geq 1x10^6$ CFU/ml, correlating 100%. However, 6 of the 10 clinically non-infected wounds also had high ($\geq 1x10^6$ CFU/ml) total bioburden.

Figure 4.1: Total bioburden in venous leg and diabetic foot ulcer wounds. All wounds contained less than 1.0 x 10^9 CFU/ml, represented as < 1E +09 on the graph. A high proportion of the wounds in both ulcer types in the present study contained a bioburden equal to or greater than 10^5 CFU/ml. Infection was defined microbiologically at a bioburden level equal or greater than 10^6 and 10^7 CFU/ml, for statistical analysis.

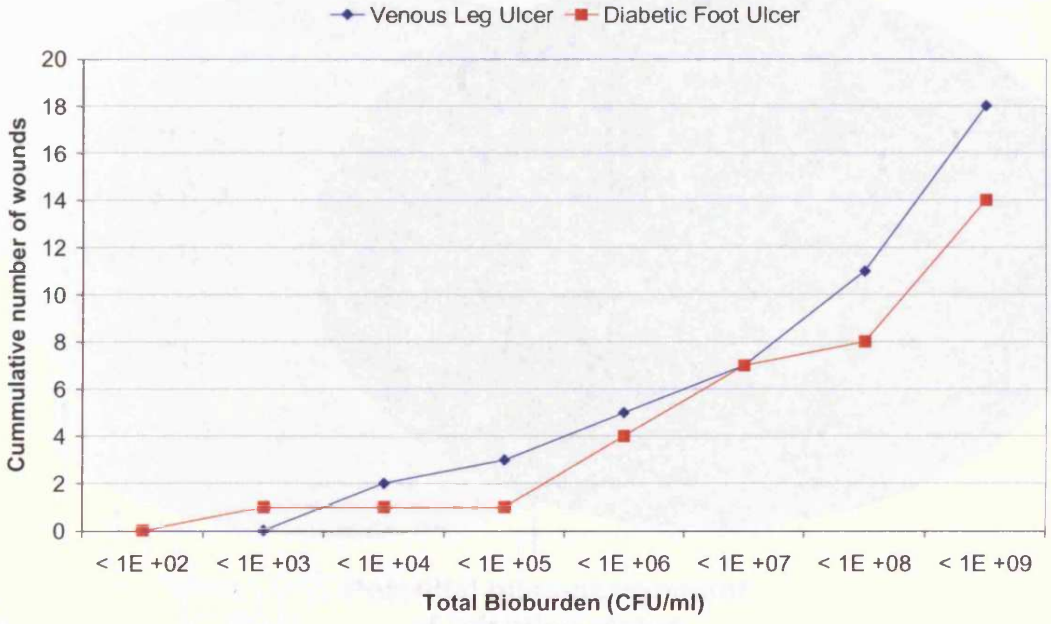
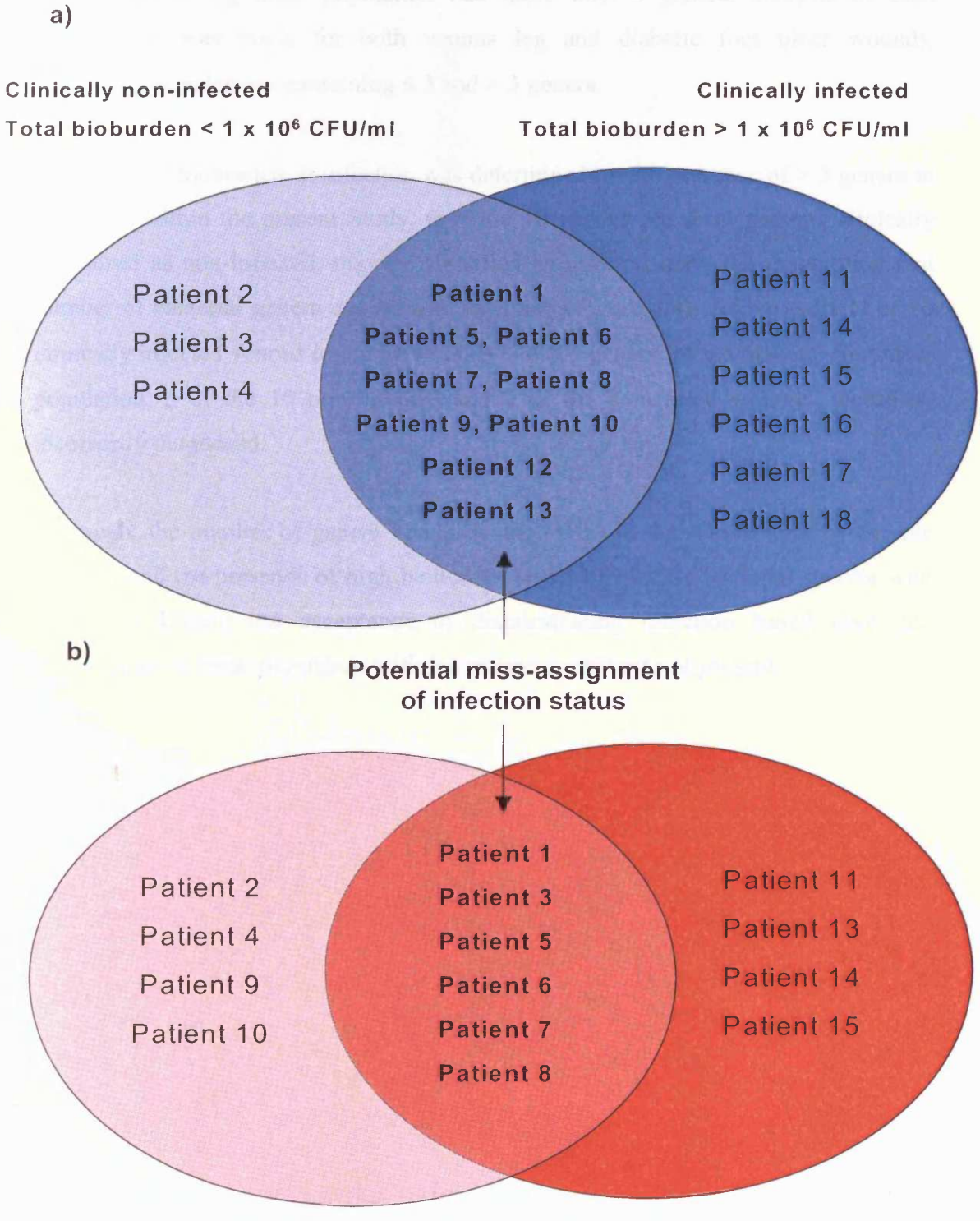


Figure 4.2: Clinical and microbiological comparisons.
Correlation between clinical diagnosis and total bioburden revealed 9/18 wounds may be misdiagnosed for (a) venous leg ulcer wounds. Additionally, 6/14 wounds may be misdiagnosed for (b) diabetic foot ulcer wounds. Patients 1-10 were clinically diagnosed as non-infected, patients 11-20 clinically diagnosed as infected. No microbiological information was available for venous leg ulcer, patients 19 and 20 and for diabetic foot ulcer patient 12.



4.3.1.2 Number of Genera

The number of microbial genera in venous leg ulcer wounds ranged from 1-4, while in diabetic foot ulcers, up to 8 different genera were detected within the same wound. The number of wounds containing the same number of genera, for each ulcer type, venous leg and diabetic foot ulcer wounds is presented in Figure 4.3. As no subjects in the venous leg ulcer population had more than 4 genera, analysis of each biochemical was made for both venous leg and diabetic foot ulcer wounds, comparing populations containing ≤ 3 and > 3 genera.

As with total bioburden, if infection was determined on the presence of > 3 genera in a wound, within the present Study, 6 of the 10 venous leg ulcer patients clinically diagnosed as non-infected, may be classified as infected upon the assumption that number of bacterial genera equate with infection (Figure 4.4). Additionally, 4 of 10 clinically infected venous leg ulcer wounds had ≤ 3 genera. In the diabetic foot ulcer population, 3 of the 10 non-infected and 1 of the 4 infected wounds, would be incorrectly diagnosed.

Although, the number of genera has been implicated in the non-healing of chronic wounds, and the presence of high bioburden levels of specific bacterial species with ensuing infection, the acceptance of discriminating infection based upon the combination of microorganisms within a wound is unknown at present.

Figure 4.3: Microbial genera in venous leg and diabetic foot ulcer wounds. All venous leg ulcer wounds contained ≤ 4 genera, with between 3 and 6 wounds representing each number of genera. Conversely, diabetic foot ulcer wounds were found to contain up to 8 different genera, with between 1 and 6 wounds representing each number of genera.

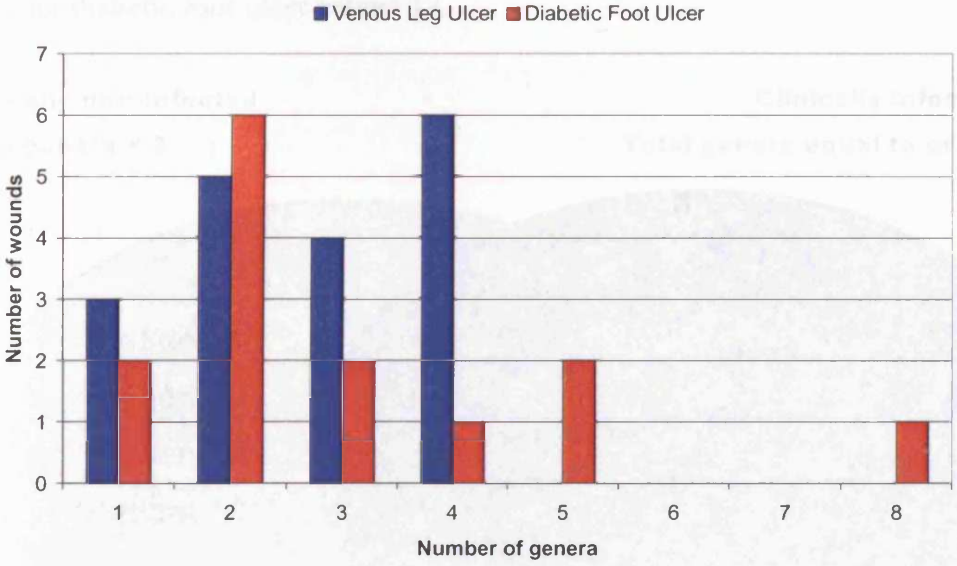
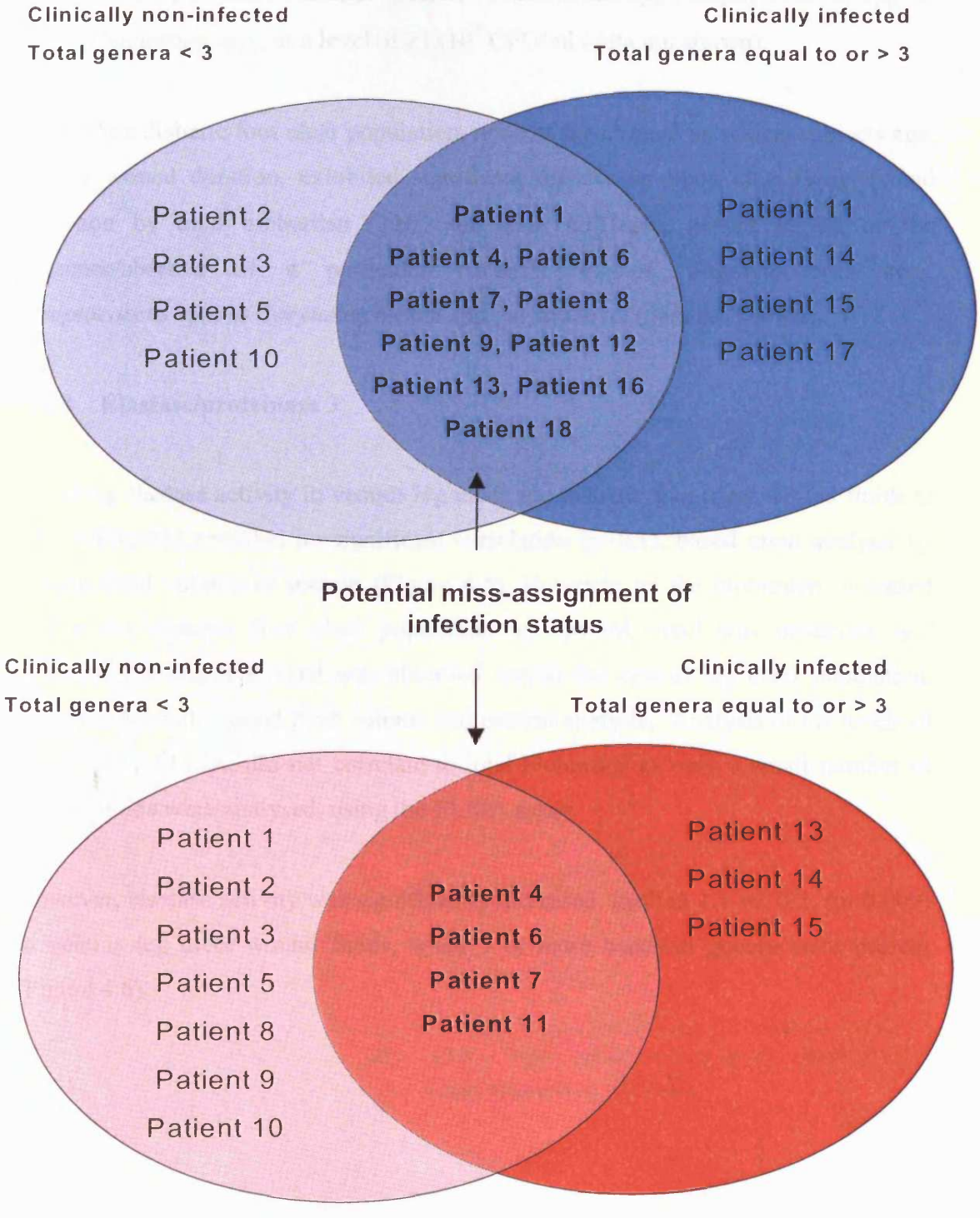


Figure 4.4: Clinical and total genera.
Correlation between clinical diagnosis and total bioburden revealed 9/18 wounds may be misdiagnosed for (a) venous leg ulcer wounds. Additionally, 6/14 wounds may be misdiagnosed for (b) diabetic foot ulcer wounds. Patients 1-10 were clinically diagnosed as non-infected, patients 11-20 clinically diagnosed as infected. No microbiological information was available for venous leg ulcer, patients 19 and 20 and for diabetic foot ulcer patient 12.



4.3.2 Clinical variables to microbiologically defined wound infection

Within the venous leg ulcer population, none of the clinical variables, subjects age, sex, wound duration, area or perimeter, exhibited significant differences upon classifying wound infection by total bioburden, ($\geq 10^6$ and $\geq 10^7$ CFU/ml), genera, (≤ 3), or to a particular bacterial species, *Pseudomonas* spp., *Staphylococcus* spp. or *Corynebacterium* spp., at a level of $\geq 1 \times 10^7$ CFU/ml (data not shown).

Within the diabetic foot ulcer population, none of the clinical variables, subjects age, sex or wound duration, exhibited significant differences upon classifying wound infection by total bioburden ($\geq 10^6$ and $\geq 10^7$ CFU/ml), genera (≤ 3), or the presence/absence of a particular bacterial species, *Staphylococcus* spp., *Streptococcus* spp. or *Corynebacterium* spp., at any level (data not shown).

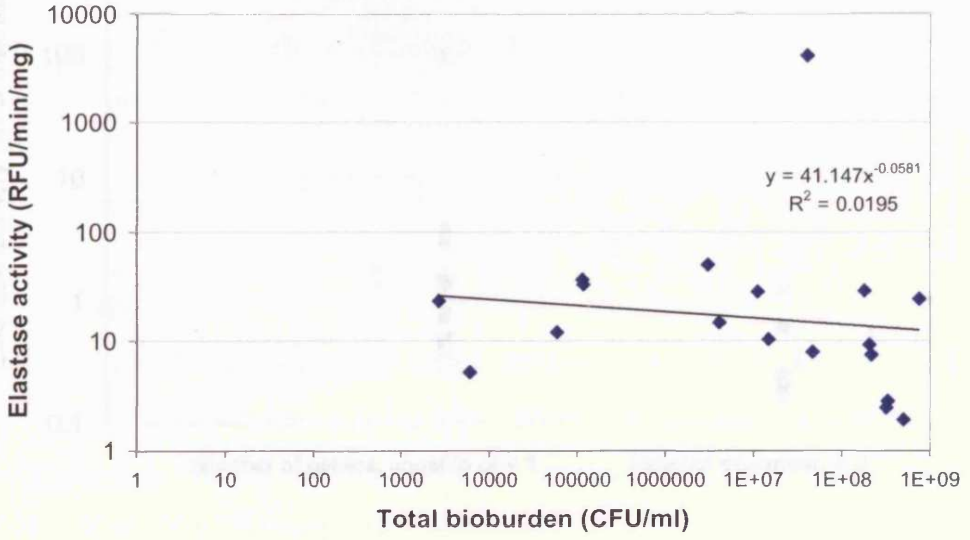
4.3.3 Elastase/proteinase 3

Trending elastase activity in venous leg ulcer and diabetic foot ulcer wound fluids to total bioburden revealed no significant correlation ($p>0.1$), based upon analysis by wound fluid volume or protein (Figure 4.5). However, as the bioburden increased within the diabetic foot ulcer population an upward trend was observed, and conversely a negative trend was observed within the venous leg ulcer population, observed by both wound fluid volume and protein analysis. Analysis of the levels of elastase, by ELISA, did not correlate to total bioburden as only a small number of wound fluids were analysed, using the ELISA assay.

However, elastase activity was significantly increased, median 1.1 vs. 0.2, ($p=0.009$) in venous leg ulcer wound fluids, when 3 or more bacterial genera were present (Figure 4.6).

Figure 4.5: Elastase activity correlated to total bioburden.
No correlation was evident between elastase activity, corrected for wound fluid protein, for each (a) venous leg ulcer and (b) diabetic foot ulcer wound fluid and total bioburden ($p > 0.1$). Clearly, a positive trend was observed in the diabetic foot ulcer population, although the sample number was too small to determine statistical significance.

a)



b)

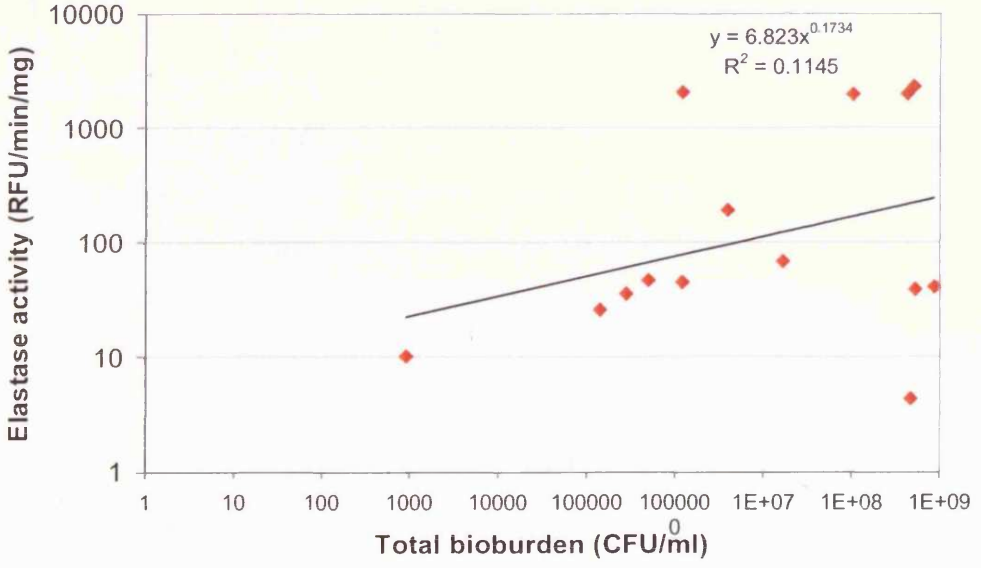
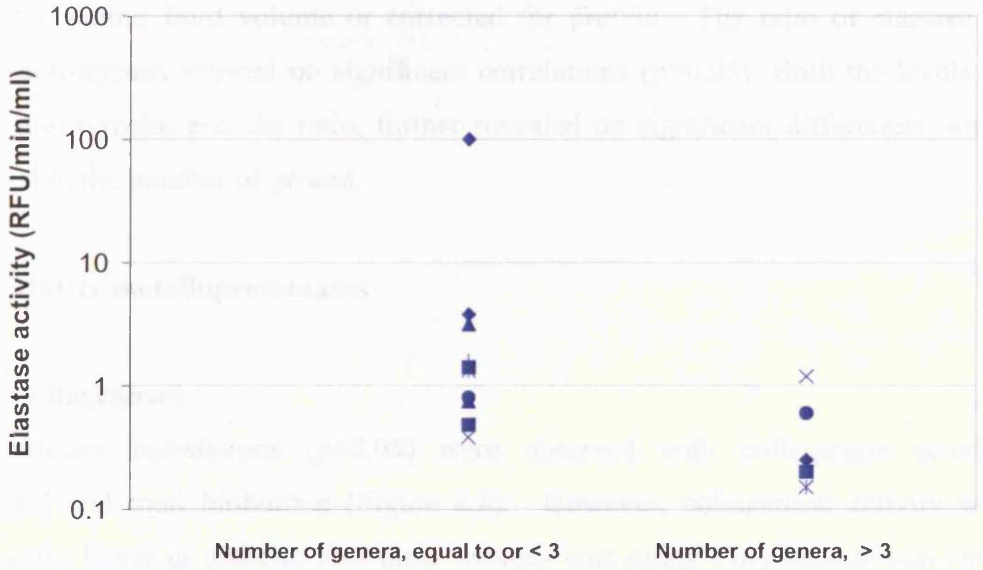


Figure 4.6: Elastase activity and number of genera in venous leg ulcer wounds. Significant differences in elastase activity (p=0.009) were observed in venous leg ulcer wound fluids, defined by the number of microbial genera within the wound. 28.33±8.19 (average ± SE) elastase activity (RFU/min/ml) was detected in wounds with ≤ 3 genera (n = 12) compared with 0.4±0.16 elastase activity (RFU/min/ml) in wounds containing > 3 genera (n = 6).



4.2.4 Alpha-1-anti-trypsin

The levels of alpha-1-anti-trypsin trended to total bioburden, for venous leg and diabetic foot ulcer wounds, are shown in Figure 4.7. No statistical correlations ($p > 0.05$) were observed for either ulcer to total bioburden, for the levels of alpha-1-anti-trypsin by wound fluid volume or corrected for protein. The ratio of elastase to alpha-1-anti-trypsin showed no significant correlations ($p>0.05$). Both the levels of alpha-1-anti-trypsin, and the ratio, further revealed no significant differences when correlated to the number of genera.

4.3.5 Matrix metalloproteinases

4.3.5.1 Collagenases

No significant correlations ($p>0.05$) were observed with collagenase activity (RFU/min) and total bioburden (Figure 4.8). However, collagenase activity was significantly lower in diabetic foot ulcer wounds containing *Corynebacterium* spp., ($n=11$) with 3.46 vs. 14.06 RFU/min ($p=0.029$).

4.3.5.2 Gelatinases

There was no significant correlations in the total gelatinase activity ($p>0.05$) to total bioburden, (Figure 4.9), by either wound fluid volume or protein. Of each form of gelatinase analysed, pro-MMP-2 was significantly lower in the microbiologically defined infected diabetic foot ulcer wounds at a level of $>1x10^6$ CFU/ml, median 75.00 vs. 159.25 pg/ml ($p=0.0017$). However, this relationship was not significant at a level of $>1x10^7$ CFU/ml, median 59.5 vs. 118.50 pg/ml ($p=0.074$). Therefore, it is unlikely to be a biochemical marker of chronic wound infection.

4.3.6 Tissue inhibitor of matrix metalloproteinase

No significant correlations ($p > 0.05$) were observed with either TIMP-1 or -2 levels and total bioburden, in either ulcer type (Figure 4.10). Significantly higher ($p=0.020$), median, 38.43 vs. 23.61 ng/ml, TIMP-2 levels were evident in the 11 of 14 diabetic wounds, in the presence of *Corynebacterium* spp. (Figure 4.11).

Figure 4.7: Alpha-1-anti-trypsin correlated to total bioburden.
The levels of alpha-1-anti-trypsin was measured in each (a) venous leg ulcer and (b) diabetic foot ulcer wound fluid were plotted against the total bioburden present in the wound, as determined by quantitative microbiology.
No correlation was evident between the concentration of alpha-1-anti-trypsin and total bioburden in either the venous leg ulcer or diabetic foot ulcer populations.

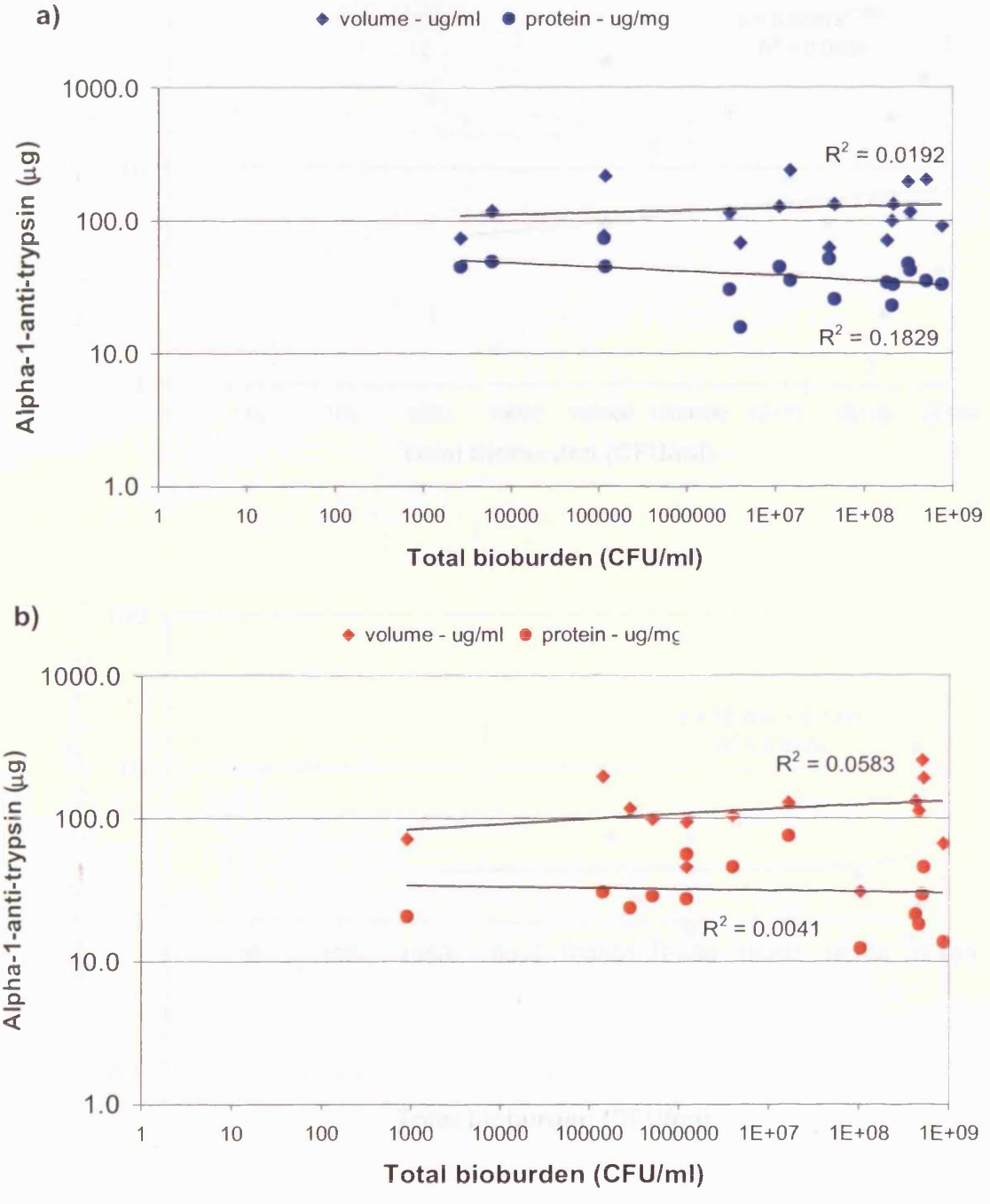
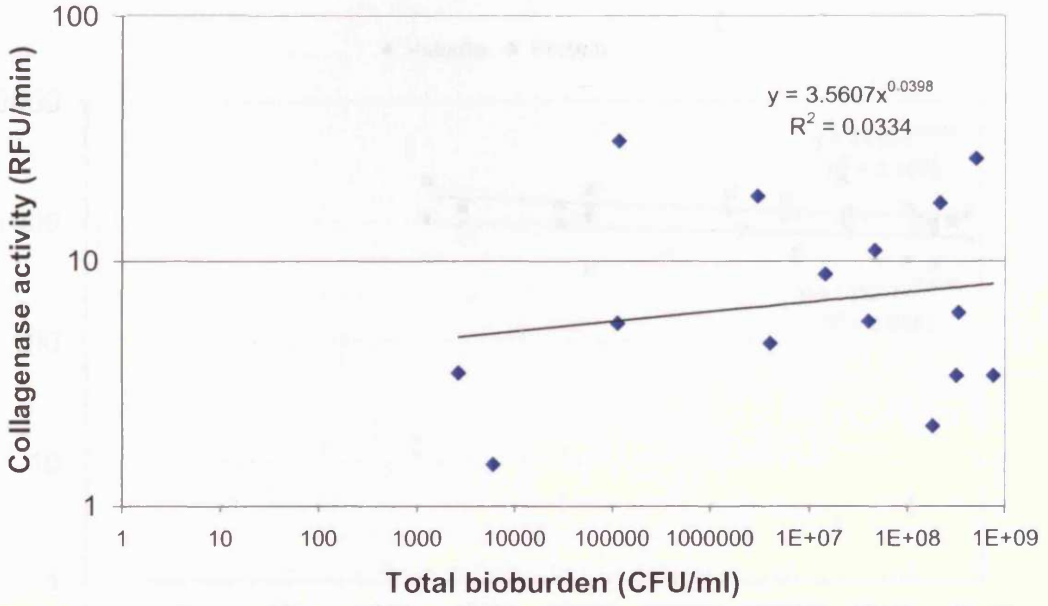


Figure 4.8: Collagenase activity (RFU/min) correlated to total bioburden. No trends were observed between the collagenase activity determined by fluorogenic analysis and the level of bioburden within (a) venous leg ulcer or (b) diabetic foot ulcer wounds.

a)



b)

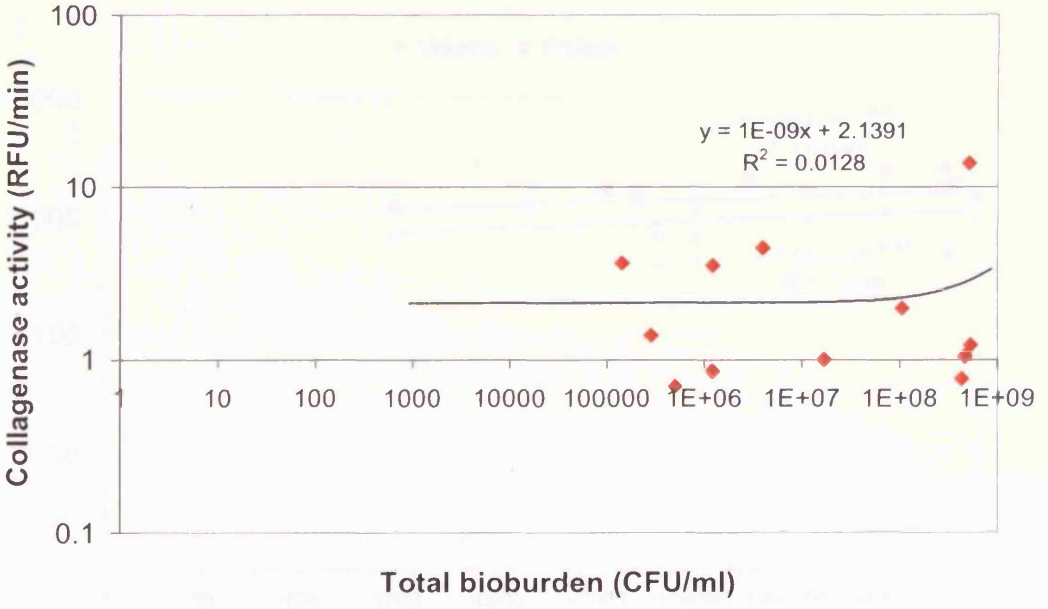
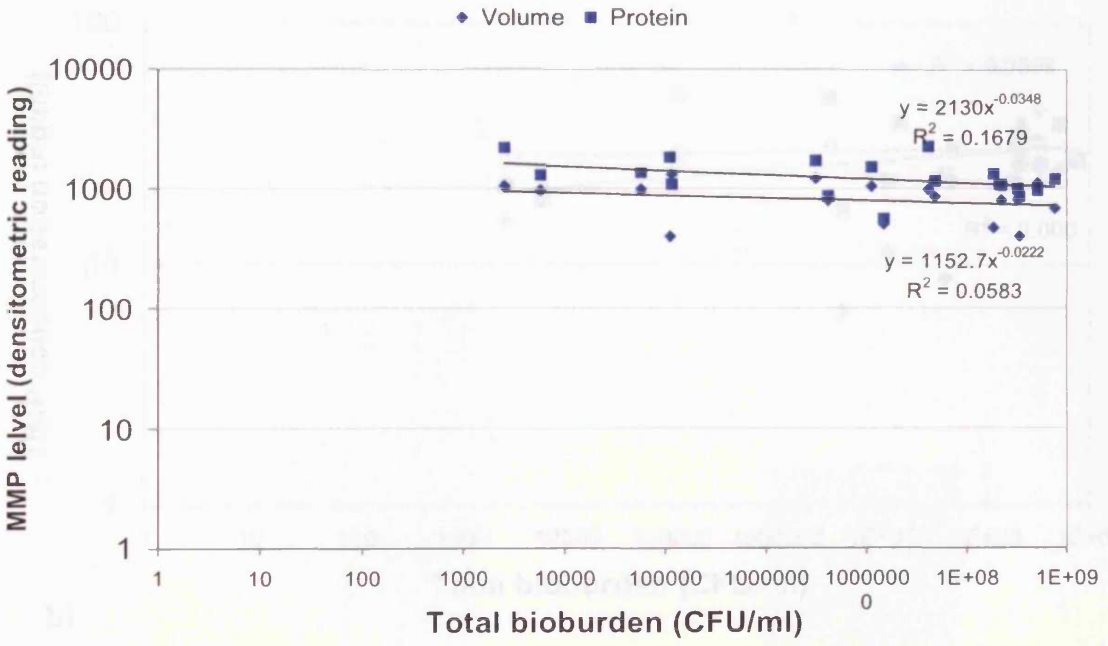


Figure 4.9: Total gelatinase (MMP-2 and MMP-9) activity correlated to total bioburden.
No trends were observed between the gelatinase activity determined by densitometric analysis and the level of bioburden within (a) venous leg ulcer or (b) diabetic foot ulcer wounds.

a)



b)

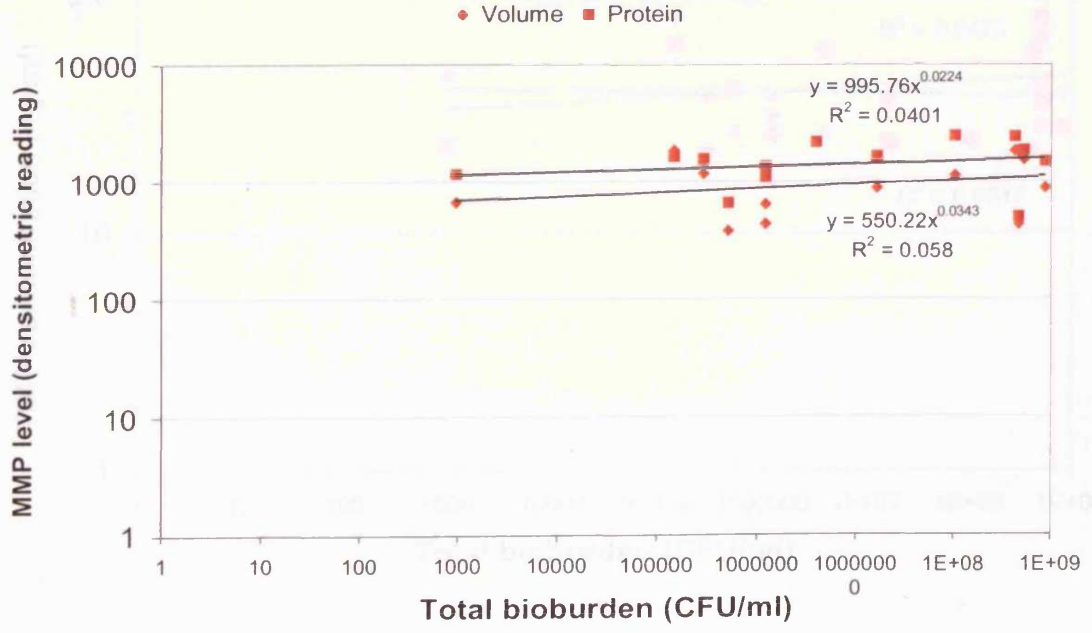
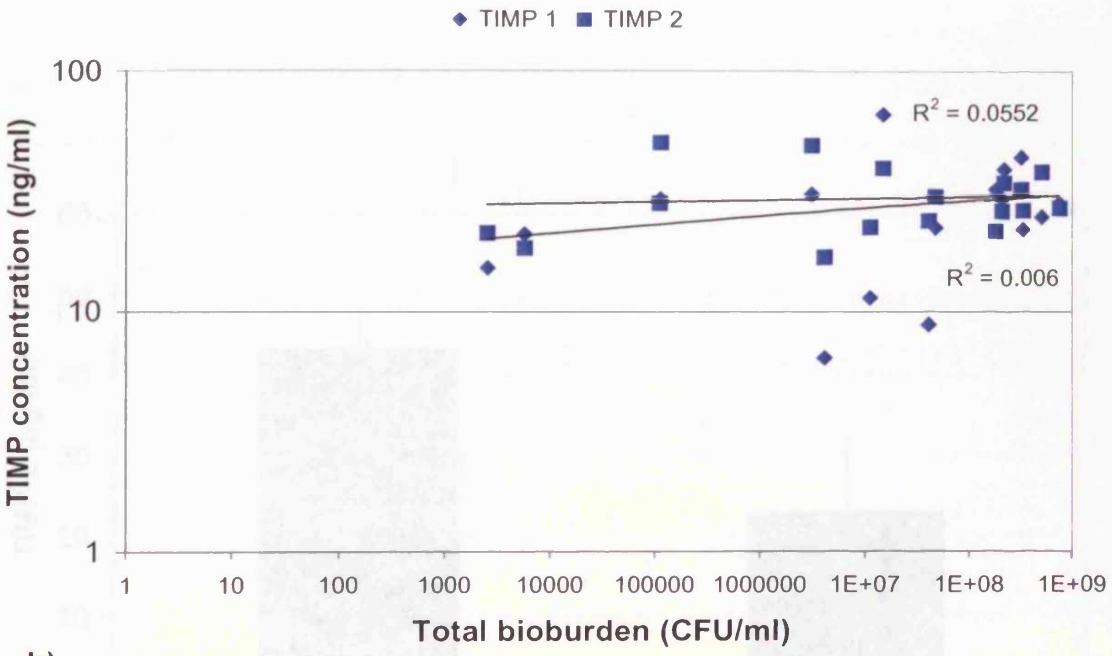


Figure 4.10: TIMP-1 and TIMP-2 levels correlated to total bioburden.
No trends were observed between the levels of TIMP 1 or TIMP 2 to the level of bioburden within (a) venous leg ulcer or (b) diabetic foot ulcer wounds.

a)



b)

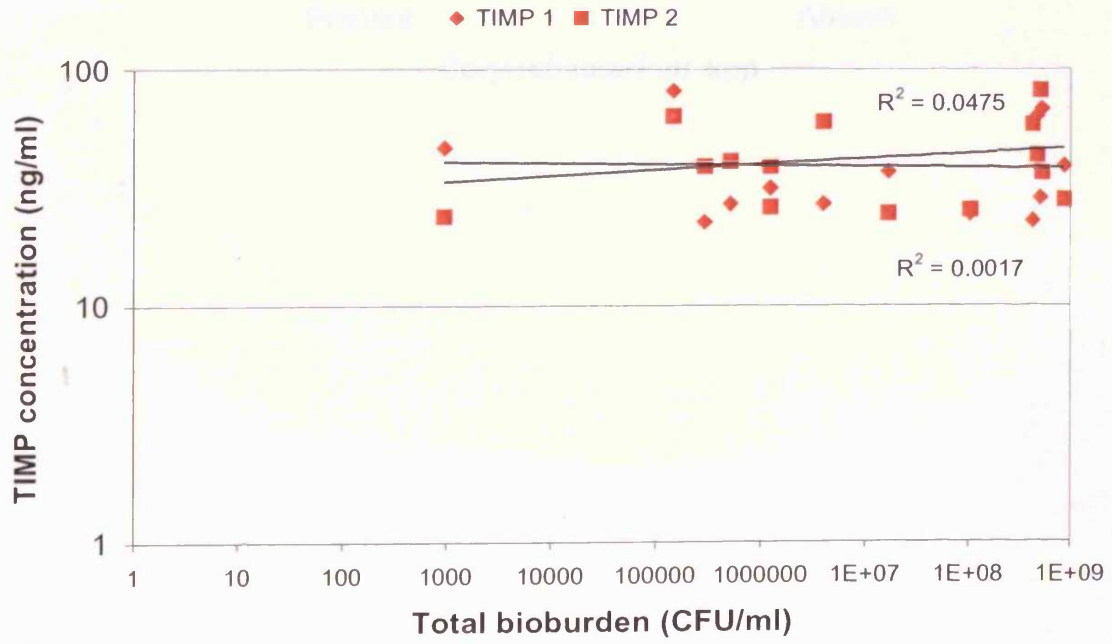
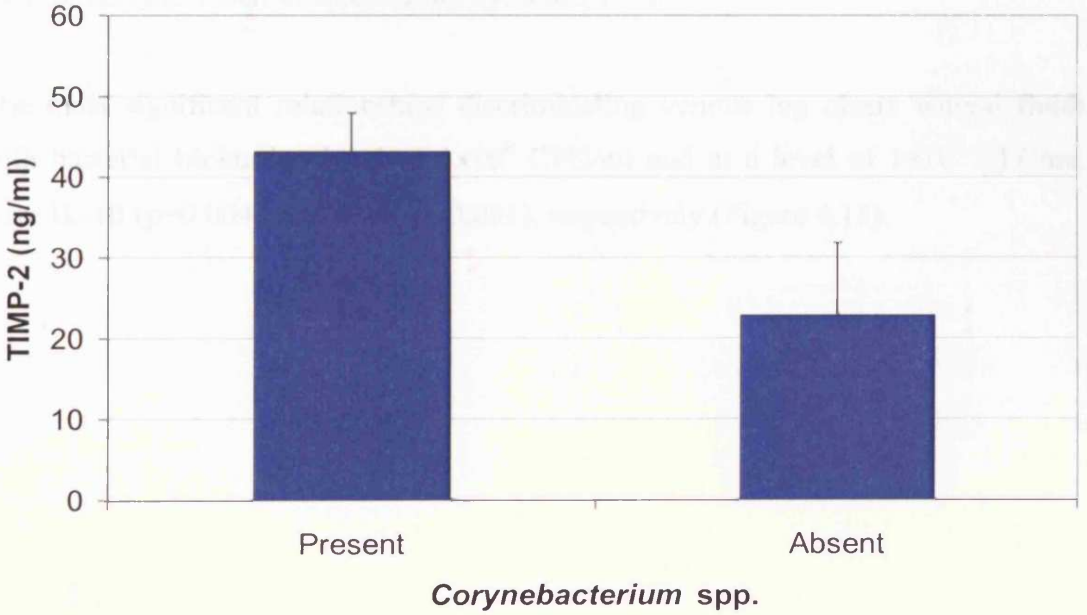


Figure 4.11: TIMP-2 levels in diabetic foot ulcer wounds containing *Corynebacterium* spp.

A significant decrease, 43.2±4.8 (average ± SE) verses 22.8±9.1 (average ± SE) in the levels of TIMP-2 ($p = 0.020$) was observed in diabetic foot ulcer wound fluids, in the presence ($n = 7$) compared with the absence ($n=7$) of *Corynebacterium* spp., respectively.



4.3.7 Cytokines

4.3.7.1 Venous leg ulcer wound fluids

Within the venous leg ulcer population, a significant increase in a number of cytokines was observed as total bioburden increased (Table 4.1). Namely, the pro-inflammatory cytokines, IL-1β, IL-6 (Figure 4.12a), the angiogenic cytokines, angiogenin and VEGF (Figure 4.12b), cell surface receptors, ICAM and TNFr2, and TNF-α, and the T-cell differentiating cytokine, IL-4.

The most significant relationships discriminating venous leg ulcers wound fluids with bacterial bioburden level of $1x10^6$ CFU/ml and at a level of $1x10^7$ CFU/ml, were IL-1β ($p=0.004$), and IL-4 ($p=0.001$), respectively (Figure 4.13).

Table 4.1: The levels of cytokine in microbiologically defined non-infected and infected venous leg ulcer wound fluids.

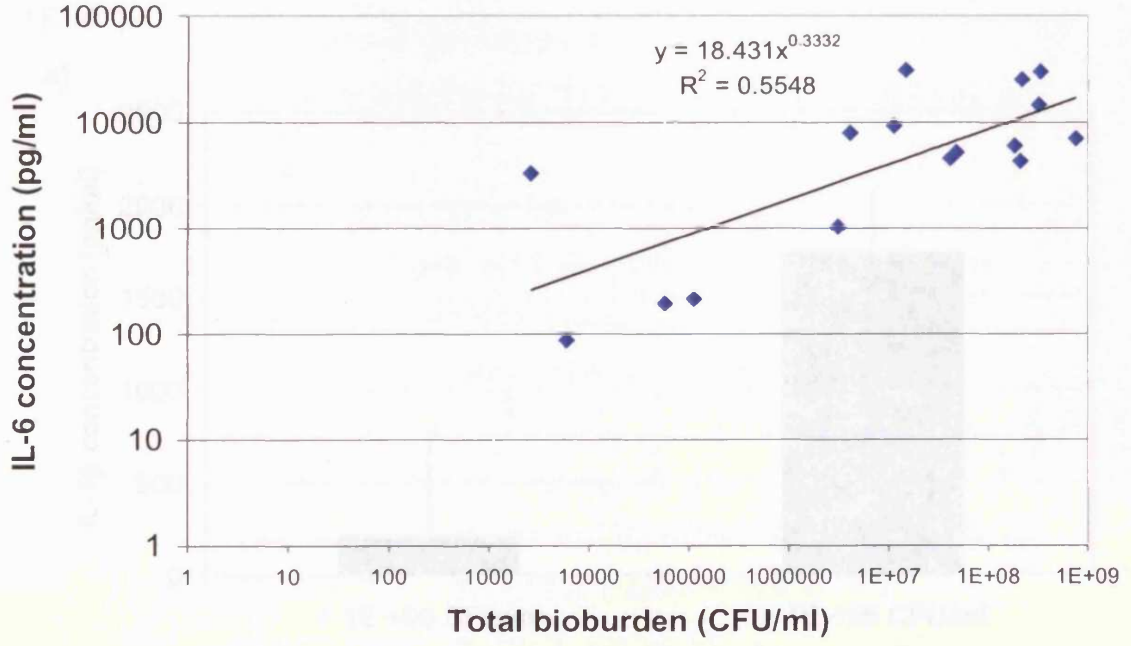
Median values for each cytokine in venous leg ulcer wound fluids that had a significant relationship with the levels of bacterial bioburden.

	Total Bioburden (CFU/ml)		**Significance (p values)**	**Total Bioburden (CFU/ml)**		**Significance (p values)**
Cytokine	$>10^6$	vrs $<10^6$		$>10^7$	vrs $<10^7$	
IL-1β	1250.2	215.4	**0.004**	1309.0	348.1	**0.011**
IL-4	75.4	26.2	**0.006**	83.7	40.6	**0.001**
Angiogenin	5282.0	561.6	**0.025**	6768.0	973.2	**0.011**
IL-6	7328.5	203.6	**0.006**	7997.2	614.4	**0.008**
ICAM-1	1604.2	396.9	**0.009**	1644.3	653.8	**0.003**
TNF-α	127.7	20.9	**0.018**	167.1	40.0	**0.034**
TNFr2	54114.0	7718.0	**0.006**	58427.0	21880.0	**0.015**
VEGF	14385.0	4205.0	**0.013**	14385.0	7070	**0.034**
n =	**13**	**5**		**11**	**7**	

Figure 4.12: The positive correlation between IL-6 and VEGF levels in venous leg ulcer wound fluids and total bioburden.

Increasing levels of (a) IL-6 and (b) VEGF, in venous leg ulcer wound fluid with increases in total bioburden.

a)



b)

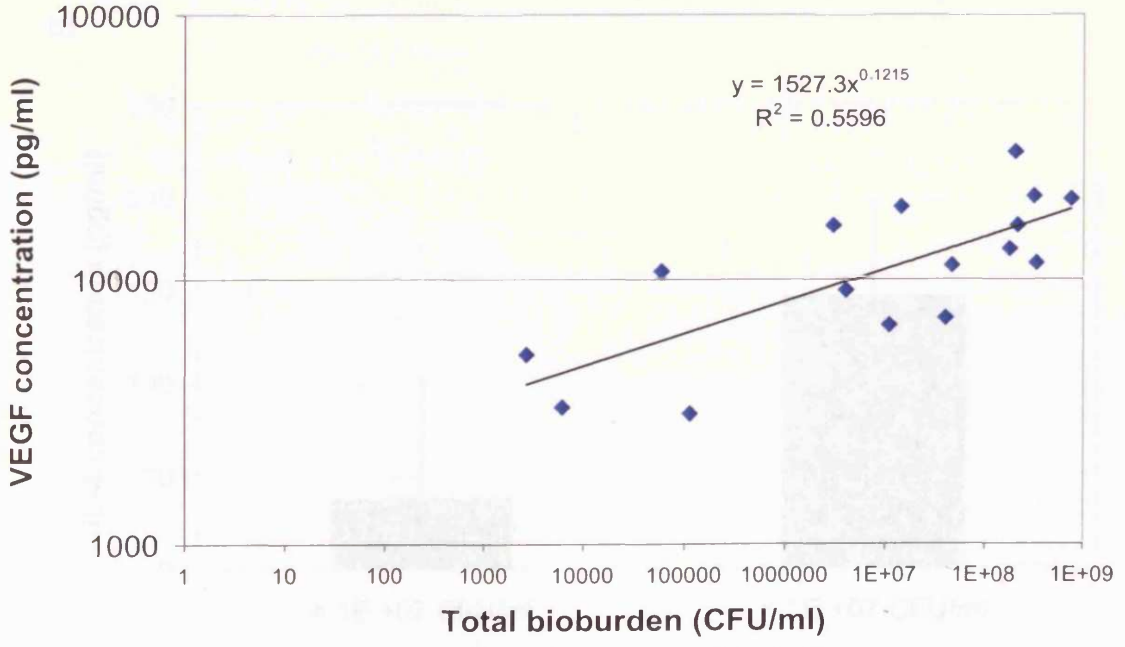
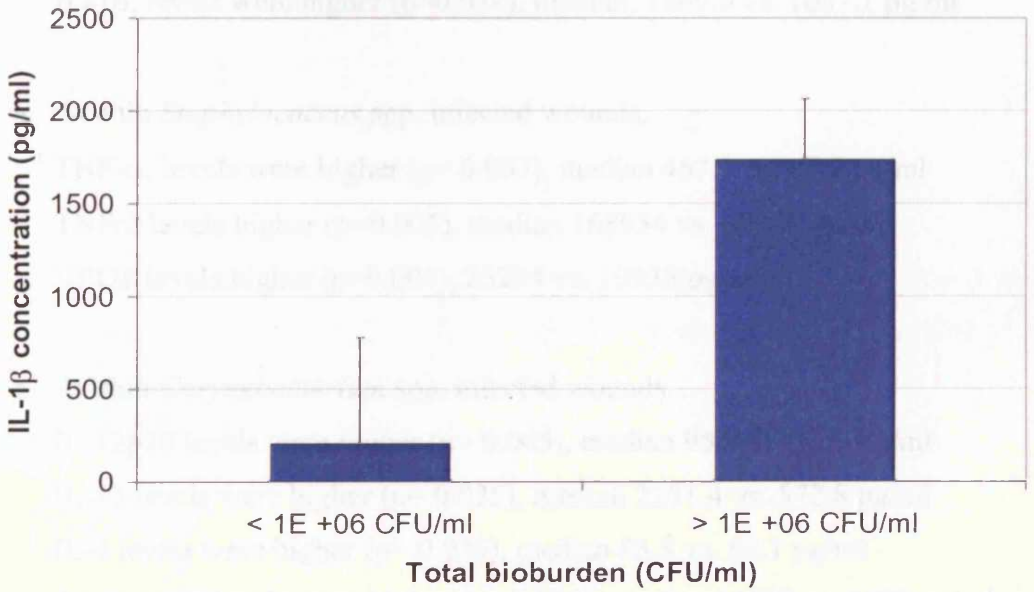


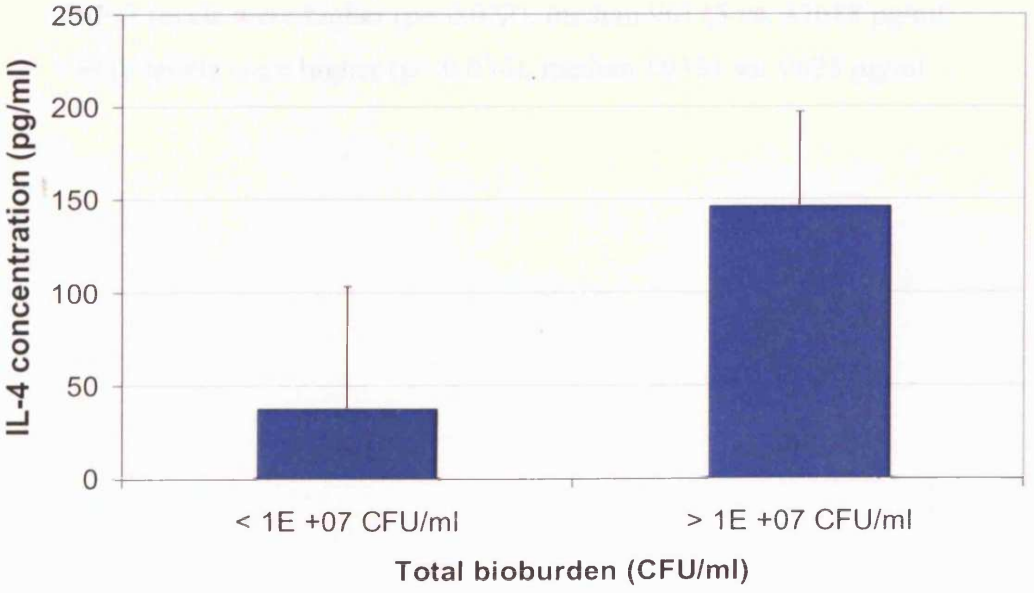
Figure 4.13: Levels of IL-1β and IL- 4 in venous leg ulcer wound fluid (average ± S.D.), verses levels of total bioburden.

Of the 18 cytokines analysed by micro-array, 8 cytokines demonstrated significantly higher levels in the microbiologically defined infected than non-infected wounds (Table 4.1). At the level of $1x10^6$ CFU/ml (represented as 1E +06), (a) IL-1β had the most significant difference (p=0.004), while (b) IL-4, had the most significant difference (p=0.001), between infection classified at $1x10^7$ CFU/ml (represented as 1E +07).

a)



b)



Positive correlations of these 8 cytokines with increasing total bioburden, were observed, IL-6 (Figure 4.12a) and VEGF (Figure 4.12b), as examples of this trend. Additionally, analysis of cytokine levels in the presence of particular bacteria at a level of $\geq 10^7$ CFU/ml revealed that;

- Within *Pseudomonas* spp. infected wounds,
  IL-1β, levels were higher (p=0.034), median, 1489.0 vs. 1087.1 pg/ml

- Within *Staphylococcus* spp. infected wounds,
  TNF-α, levels were higher (p= 0.007), median 467.1 vs. 67.2 pg/ml
  TNFr2 levels higher (p=0.005), median 168954 vs. 39041 pg/ml
  VEGF levels higher (p=0.004), 25294 vs. 10935 pg/ml

- Within *Corynebacterium* spp. infected wounds.
  IL-12p70 levels were higher (p= 0.045), median 95.4 vs. 18.9 pg/ml
  IL-13 levels were higher (p= 0.025), median 2281.4 vs. 572.8 pg/ml
  IL-4 levels were higher (p= 0.025), median 93.8 vs. 60.1 pg/ml
  Angiogenin levels were higher (p= 0.034), median 15137 vs. 3109 pg/ml
  TGFβ1 levels were higher (p= 0.024), median 127.9 vs. 22.5 pg/ml
  IL-10 levels were higher (p= 0.045), median 904.7 vs. 362.9 pg/ml
  TNFr2 levels were higher (p= 0.022), median 96125 vs. 37688 pg/ml
  VEGF levels were higher (p= 0.034), median 19351 vs. 9825 pg/ml

4.3.7.2 Diabetic foot ulcer wound fluids

In contrast, to the venous leg ulcer wounds, the diabetic foot ulcer population had asignificant decreases in the levels of a number of cytokines were observed as total bioburden increased (Table 4.2). These included:-

- IL-2,IL-4, IL-5, IL-10, IL-12p40, IL-12p70, and IL-13.
- TNF-α and its receptor TNFr2.
- IP-10.
- IFN-γ.
- TGFβ.

The most significant relationships discriminating wound fluids at a bioburden level of $1x10^6$ CFU/ml and at a level of $1x10^7$ CFU/ml, were IL-2 ($p=0.002$) and IP-10 ($p=0.010$), respectively (Figure 4.14).

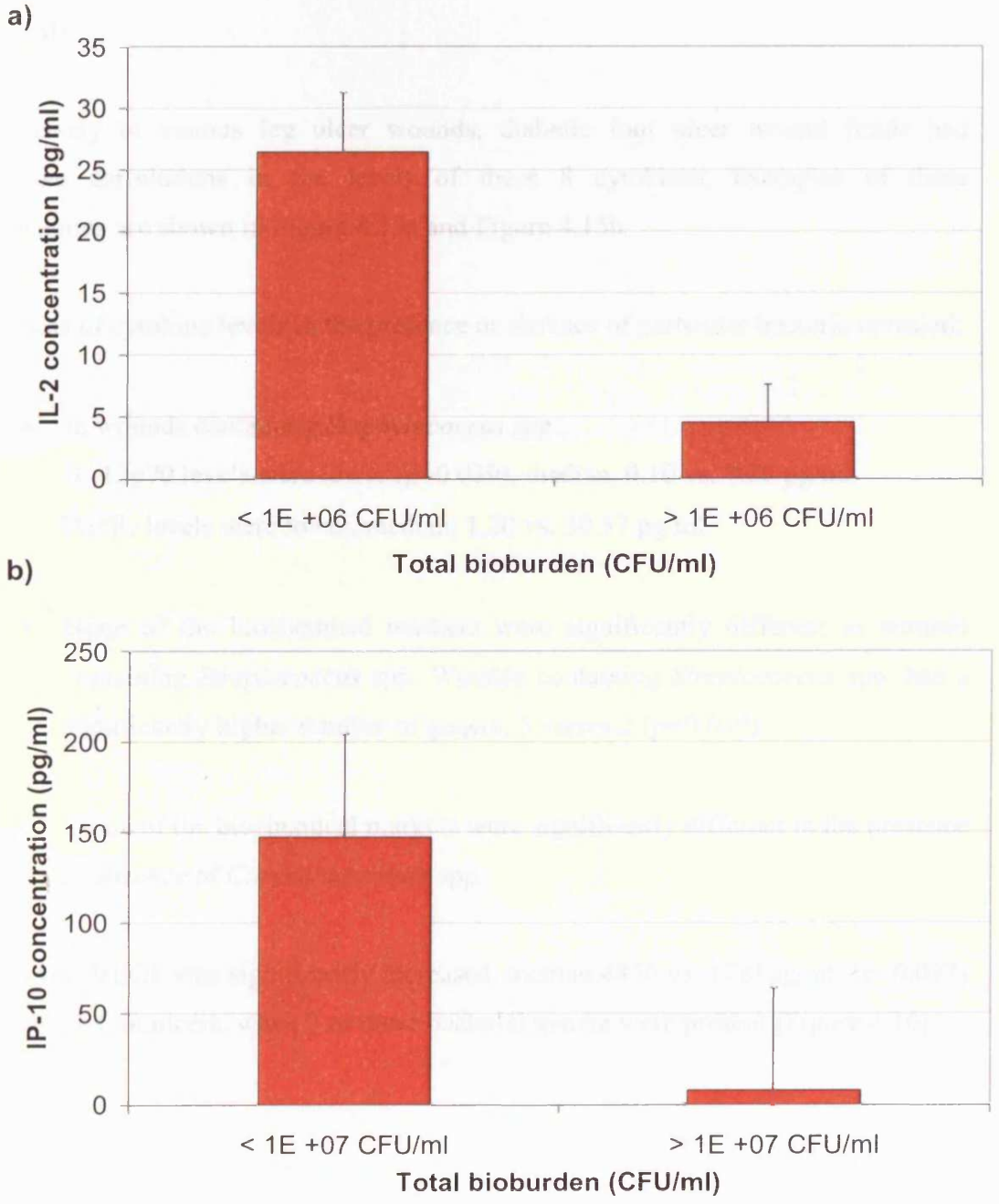
Table 4.2: The levels of cytokine in microbiologically defined non-infected and infected diabetic foot ulcer wound fluids.

Median values for each cytokine in diabetic foot ulcer wound fluids that had a significant relationship with the levels of bacterial bioburden. ns = not significant

	Total Bioburden (CFU/ml)		**Significance (p values)**	**Total Bioburden (CFU/ml)**		**Significance (p values)**
Cytokine	$>10^6$ vs.	$<10^6$		$>10^7$ vs.	$<10^7$	
IL-2	2.80	26.29	**0.002**	2.8	20.34	**0.011**
IL-4	2.10	67.35	**0.033**			
IL-5	1.00	65.05	**0.008**	1.00	26.12	**0.014**
IL-10	255.5	630.5	**0.038**			
IL-12 p40	2.50	600.00	**0.027**	2.00	318.28	**0.033**
IL-12 p70	0.10	70.19	**0.006**	0.10	23.24	**0.020**
IL-13	5.73	178.57	**0.017**			
IP-10	8.52	72.21	**0.100* ns**	2.70	66.00	**0.010**
IFN-γ	0.20	21.54	**0.010**	0.20	4.64	**0.034**
TGFβ1	1.20	84.54	**0.006**	1.20	65.93	**0.020**
TNF-α	14.57	102.13	**0.076* ns**	2.22	83.62	**0.040**
TNFr2	14290.3	109999.6	**0.040**			
n =	**7**	**7**		**8**	**6**	

Figure 4.14: Levels of IL-2 and IP-10 in diabetic foot ulcer wound fluid (average±S.D.), verses levels of total bioburden.

Of the 18 cytokines analysed by micro-array, a total of 12 cytokines demonstrated significantly higher levels in the microbiologically defined infected than non-infected wounds, at a level of $1x10^6$ and/or $1x10^7$ CFU/ml. (a) IL-2 had the most significant difference (p=0.002) and (b) IP-10 had the most significant difference (p=0.010), between infection classified at the level of $1x10^6$ and $1x10^7$ CFU/ml (represented as 1E +06 and +07), respectively.



Within the diabetic foot ulcer population, cytokines IP-10 and TNF-α, were not found to be significantly decreased at a bioburden level of $1x10^6$ CFU/ml but were significantly different at a level of $1x10^7$ CFU/ml. This change in significance may be due to the type of statistical analysis employed, compounded by the limited number of samples in each population analysed. Nevertheless, consistency was observed at both bioburden levels for IL-2, IL-5, IL-12p40, IL-12p70, IFN-γ and TGF-β_1.

Conversely to venous leg ulcer wounds, diabetic foot ulcer wound fluids had negative correlations in the levels of these 8 cytokines, examples of these correlations are shown in Figure 4.15a and Figure 4.15b.

Analysis of cytokine levels in the presence or absence of particular bacteria revealed;

- In wounds containing *Staphylococcus* spp.,
  IL-12p70 levels were lower (p=0.039), median, 0.10 vs. 7.88 pg/ml.
  TGFβ_1 levels were lower, median, 1.20 vs. 30.57 pg/ml.

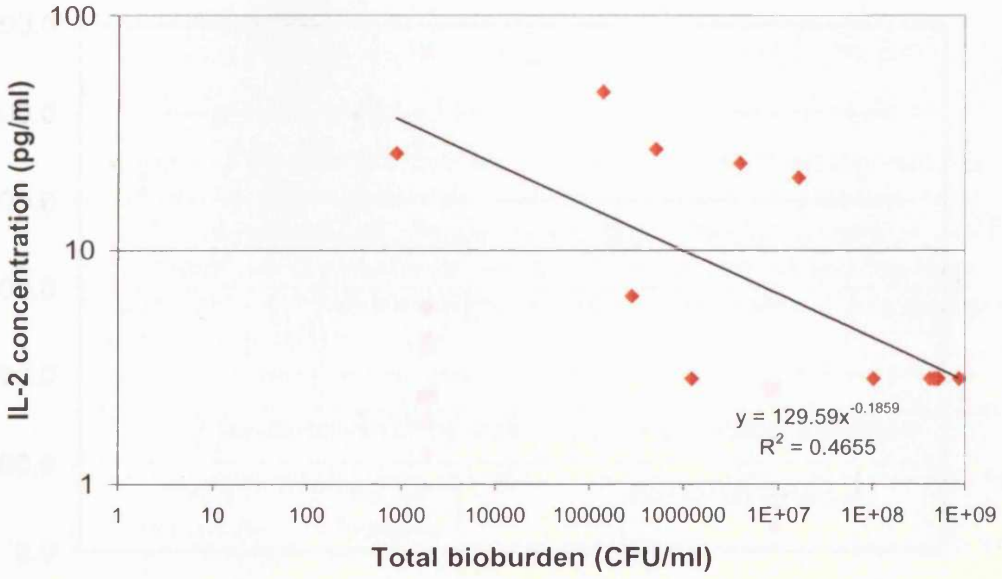
- None of the biochemical markers were significantly different in wounds containing *Streptococcus* spp. Wounds containing *Streptococcus* spp. had a significantly higher number of genera, 5 verses 2 (p=0.039).

- None of the biochemical markers were significantly different in the presence or absence of *Corynebacterium* spp.

However, VEGF was significantly increased, median 4856 vs. 1781pg/ml, (p=0.037) in diabetic foot ulcers, when 3 or more bacterial genera were present (Figure 4.16).

Figure 4.15: The negative correlation of IL-2 and IL-12p70 in diabetic foot ulcer wound fluids.
The decreasing levels of (a) IL-2 and (b) IL-12p70, in diabetic foot ulcer wound fluids, as total bioburden increases.

a)



b)

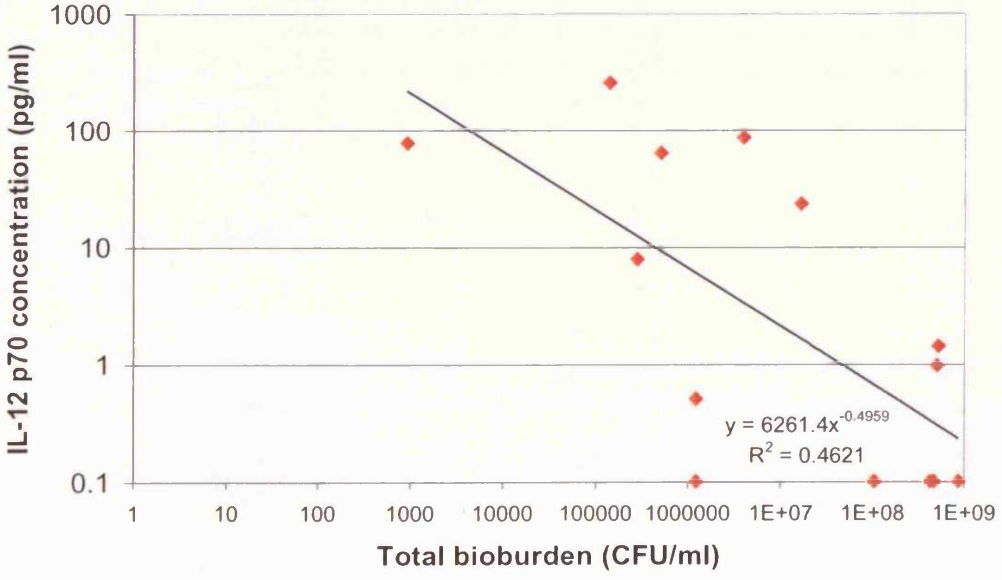
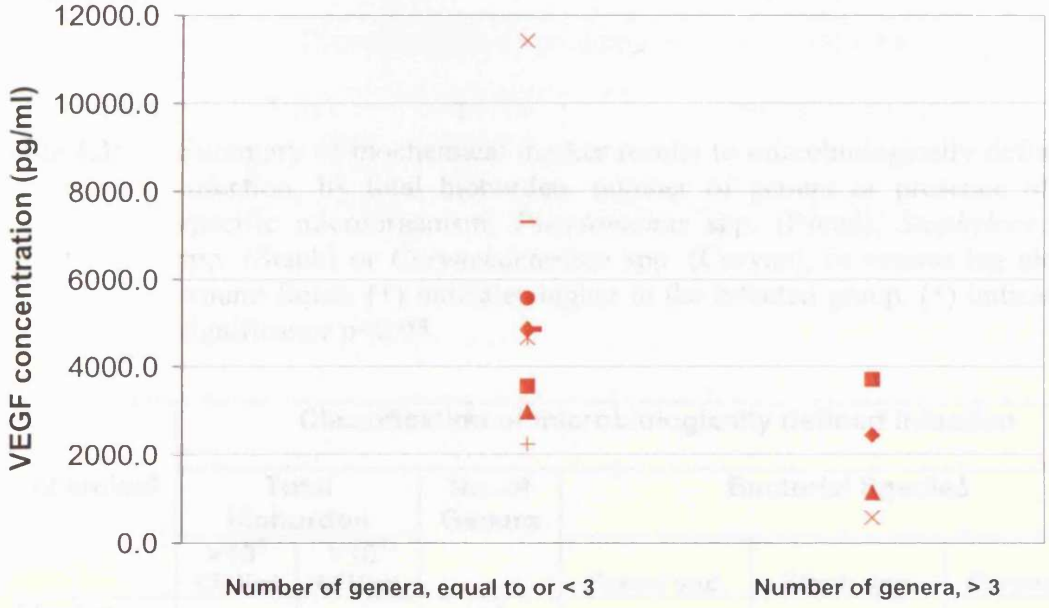


Figure 4.16: Number of genera and VEGF levels in diabetic foot ulcer fluids. A significant increase, 5268.5±816.81 (average±SE) verses 1959.8 ± 1225.2 (average ± SE) in the levels of VEGF ($p = 0.037$) was observed in diabetic foot ulcer wounds, in wounds with more than 3 bacteria genera presence (n=4) compared with wounds containing three bacteria genera or less (n=9).



4.3.8 Summary of biomarkers correlated with wound microflora

A number of biomarkers were significantly different between venous leg ulcer (Table 4.3) and diabetic foot ulcer (Table 4.4) wounds, differentiated by wound microflora (Section 4.4).

Table 4.3: Summary of biochemical marker results to microbiologically defined infection, by total bioburden, number of genera or presence of a specific microorganism, *Pseudomonas* spp. (Pseud), *Staphylcoccus* spp. (Staph) or *Corynebacterium* spp. (Coryne), in venous leg ulcer wound fluids. (+) indicates higher in the infected group. (*) indicates significance $p<0.05$.

Biochemical	Classification of microbiologically defined infection					
	Total bioburden		No. of Genera	Bacterial Species		
	$>10^6$ CFU/ml	$>10^7$ CFU/ml		*Pseud spp.*	*Staph spp.*	*Coryne spp.*
Elastase			+*			
Angiogenin	+*	+*				+*
VEGF	+*	+*			+*	+*
ICAM-1	+*	+*				
TNF-r2	+*	+*			+*	+*
IL-1β	+*	+*		+*		
IL-6	+*	+*				
TNF-α	+*	+*			+*	
IL-4	+*	+*				+*
IL-10						+*
IL-12p70						+*
IL-13						+*
TGFβ1						+*

Table 4.4: Summary of biochemical results to microbiologically defined infection, by total bioburden, number of genera or presence of a specific microorganism, *Streptococcus* spp. (Strep), *Staphylcoccus* spp. (Staph) or *Corynebacterium* spp. (Coryne), in diabetic foot ulcer wound fluid. (+) indicates higher in the infected group, (-) indicates lower in the infected group. (*) indictes Significance $p<0.05$.

Biochemical	Classification of microbiologically defined infection					
	Total bioburden		No. of Genera	Bacterial Species		
	$>10^6$ CFU/ml	$>10^7$ CFU/ml		*Strep spp.*	*Staph spp.*	*Coryne spp.*
Collagenase						-*
TIMP-2						+*
IL-2	-*	-*				
IL-4	-*					
IL-5	-*	-*				
IL-10		-*				
IL-12 p40	-*	-*				
IL-12 p70	-*	-*		-*		
IL-13	-*					
IP-10	-*					
IFN-γ	-*	-*				
TGFβ1	-*	-*		-*		
TNF-α		-*				
TNFr2	-*					
VEGF			+*			

4.3.9 Biochemical differences between venous leg ulcer and diabetic foot ulcer wounds

4.3.9.1 Proteases and inhibitors

During the comparative analysis of each biochemical marker to wounds either clinically or microbiologically diagnosed as infected (Chapter 3), differences in the levels of the biochemical markers measured were apparent. Thus, the levels or activity of each biochemical marker, were compared between venous leg ulcer and diabetic foot ulcer wounds.

Comparisons were made between the clinically diagnosed non-infected populations (10 venous leg ulcer and 10 diabetic foot ulcer) and between all 20 venous leg and all 15 diabetic foot ulcer wound fluids. The data could be combined, irrespective of the bioburden levels, as the same range of total bioburden was observed between ulcer types. Upon comparing the wound fluid data from the two ulcer types, for the levels and/or activities of the proteases and their inhibitors, significant differences between the serine protease, elastase, the collagenase MMPs, (Figure 4.17), and the MMP (gelatinise) inhibitor, TIMP-2, were determined.

Data revealed that elastase activity was significantly higher ($p=0.0009$), in the clinically, non-infected diabetic foot ulcer population, median 3.4 vs. 0.7 RFU/min, compared to the venous leg ulcer population. This level of significance increased further ($p=0.0002$), upon comparing all 15 diabetic foot ulcer wounds with all 20 venous leg ulcer wounds, median 3.6 vs. 0.8 RFU/min.

Collagenase activity was significantly lower ($p=0.022$), in the clinically non-infected diabetic foot ulcer population, median 1.0 vs.4.6 RFU/min. Again, this level of significance increased ($p<0.001$), upon comparing all 15 diabetic foot ulcer wounds, with all 20 venous leg ulcer wounds, median 1.2 vs. 5.9 RFU/min.

In addition, there appeared to be significantly higher levels of TIMP-2 (p=0.045) in the clinically non-infected diabetic foot ulcers than with venous leg ulcer wound fluids, median 39.4 vs. 26.8 ng/ml. However, upon comparing all 15 diabetic foot ulcer wounds with all 20 venous leg ulcer wounds, no significant difference was observed (p=0.096).

Surprisingly, no difference in the levels of protease inhibitors, alpha-1-anti-tyrpsin or TIMP-1 was observed, despite the activities of their substrates, elastase and collagenase respectively, being significantly different between ulcer types. It is possible that the ELISA antibodies recognised inactive forms of these markers. The lack of a wholly specific alpha-1-anti-trypsin substrate, with reverse zymography was not employed in the present Study and is a limitation of the data presented.

Additionally, although TIMP-2 levels were significantly different between ulcer types, the data showed no significant difference in the activity of either gelatinase, MMP-2 and MMP-9. TIMPs are the endogenous inhibitors of MMPs, with TIMP-1 complexing both gelatinases and collagenase, and TIMP-2 to gelatinase B (both pro- and active-MMP-2). The lower activity of collagenase within the diabetic foot ulcer, may, therefore be due to its decreased synthesis, or cleavage by another molecule.

4.3.9.2 Cytokines

Comparison between the two ulcer types (venous leg ulcer and diabetic foot ulcer wounds) for the levels of the 18 various cytokines, revealed lower values in the 10 clinically, non-infected diabetic foot ulcer population for IL-4, IL-13, IFN-γ, and VEGF (Table 4.5) than the 9 clinically, non-infected venous leg ulcer population. Upon comparing all diabetic foot ulcer wounds (n=15) with the venous leg ulcer wounds (n=18), the same 4 cytokines had significantly lower amounts, in addition to IL-1β, IL-2, and TNF-α, which also showed significance between the ulcer types (Figure 4.18).

Figure 4.17: Comparison of the activity of protease between venous leg ulcer and diabetic foot ulcer wound fluids (average±SE).
In the diabetic foot ulcer wound fluid population, significantly higher elastase (p=0.002) and significantly lower collagenase activities (p=0.022), were determined by Wilcoxon probability, compared with venous leg ulcer wound fluids.

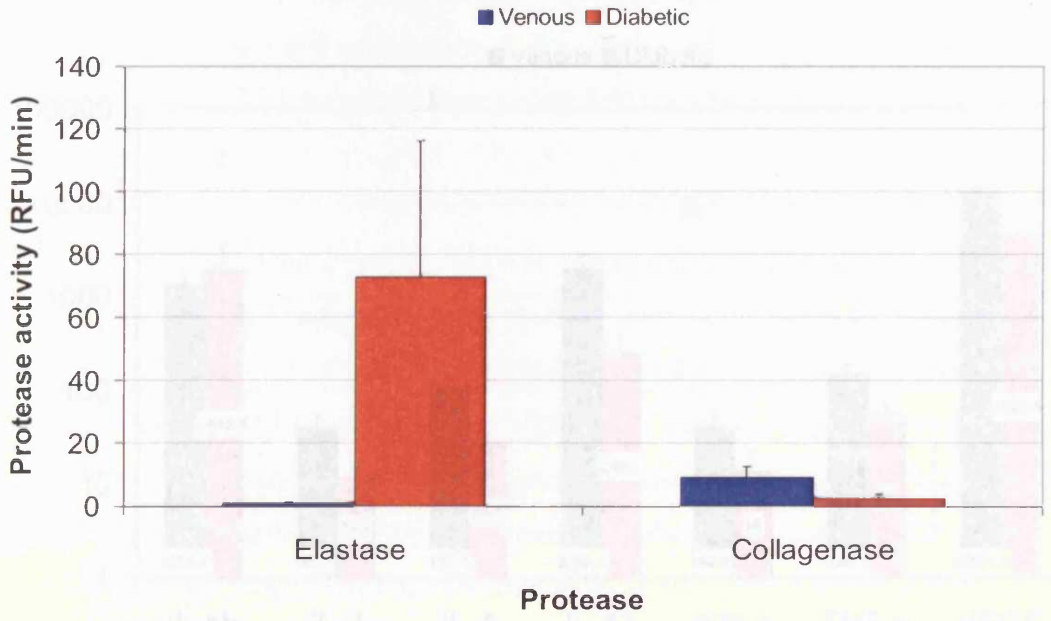
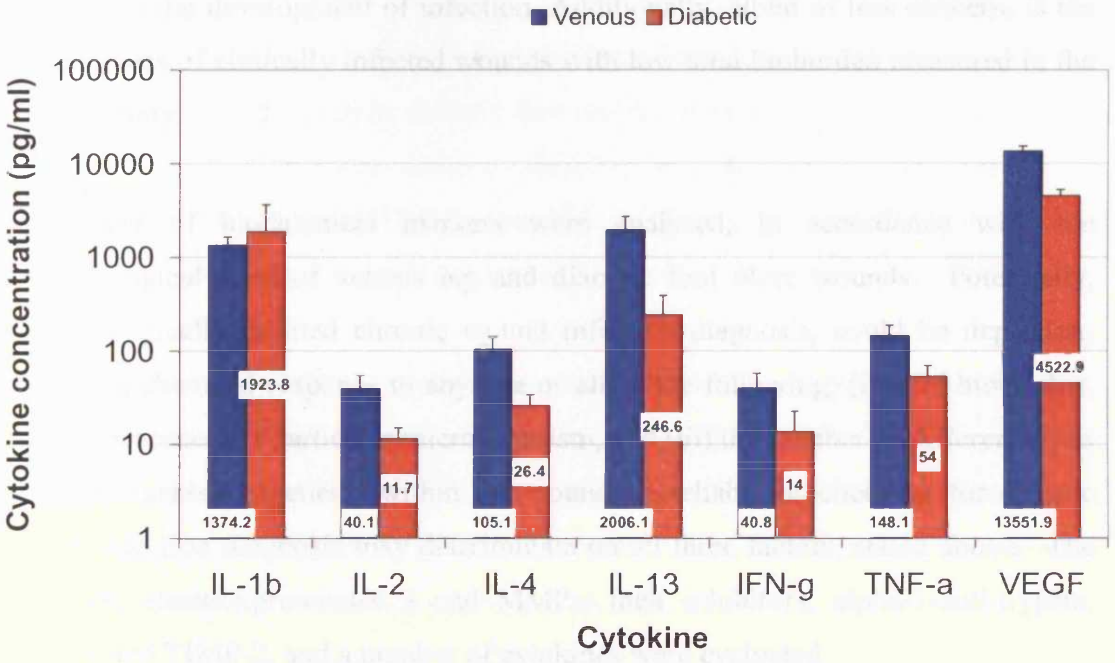


Table 4.5: Comparison of cytokine levels between clinically non-infected venous leg and diabetic foot ulcer wound fluids.
Average levels of cytokine, standard deviation, the minimum, median, maximum and significance between ulcer types, 10 venous leg ulcer (VLU) and 10 diabetic foot ulcer (DFU) by both T-test and Wilcoxon probability are shown. As most parameters have a skew distribution, the Wilcoxon probability is the most reliable.

Cytokine	Ulcer Type	Ave (pg/ml)	SD	Min (pg/ml)	Median	Max (pg/ml)	T-test	Wilcoxon
IL-4	VLU	130.9	222.1	11.3	57.6	715.7	0.150	p=0.019
	DFU	24.0	34.44	2.1	2.8	85.2		
IL-13	VLU	3013	4548.38	50.0	1084.6	13825.0	0.062	p=0.004
	DFU	140.7	192.17	3.8	51.3	549.9		
IFN-γ	VLU	72.7	99.06	1.7	30.7	275.0	0.129	p=0.009
	DFU	17.9	42.7	0.2	0.2	134.6		
VEGF	VLU	13232.7	9081.32	3075.0	10661.5	30076.9	0.016	p=0.027
	DFU	4728.1	2770.35	2232.4	4667.7	11416.6		

Figure 4.18: Comparison of cytokine levels between venous leg ulcer and diabetic foot ulcer wound fluids (average ± SE).

The cytokine, IL-1β was found to be significantly elevated (p=0.002) in diabetic foot ulcer wounds, compared with venous leg ulcer wounds. Significantly lower mean values were observed in the diabetic foot ulcer population for the amount of IL-2 (p=0.046), IL-4 (p=0.002), IL-13 (p=<0.001), IFN-γ (p=0.002), TNF-α (p=0.026) and VEGF (p<0.001), determined by Wilcoxon probability.



4.4 Discussion

The number of clinically diagnosed non-infected wounds with $\geq 1x10^6$ CFU/ml bioburden levels indicates the potential misdiagnosis of chronic wound infection. This assumes the levels of microorganisms in a wound to be the most important pre-requisite in the development of infection. Additionally, albeit of less concern, is the misdiagnosis of clinically infected wounds with low total bioburden measured in the present Study.

A number of biochemical markers were analysed, in accordance with the microbiological flora of venous leg and diabetic foot ulcer wounds. Potentially, microbiologically defined chronic wound infection diagnosis, could be dependent upon a biochemical response to any one or all of the following; (i) total bioburden, (ii) the presence of a particular microorganism, and (iii) the number of different types of microorganisms (genera) within the wound. A reliable biochemical for chronic wound infection diagnosis may discriminate on all three factors, stated above. The proteases, elastase/proteinase 3 and MMPs, their inhibitors, alpha-1-anti-trypsin, TIMP-1 and TIMP-2, and a number of cytokines were evaluated.

Generally, the proteases and their inhibitors were deemed unlikely as candidate markers of microbiologically defined wound infection, as none of these biochemical markers exhibited any significant differences upon total bioburden levels, or the presence of any particular bacterial species in either the venous leg or diabetic foot ulcer wounds (exception of collagenase and TIMP-2 level, with *Corynebacterium* spp.).

Although significantly higher elastase activity was observed in venous leg ulcer wounds containing >3 genera, a lack of correlation with other microbiological data questions its validity as a sole identifier of infection.

The increased number of genera within the wound has been associated with non-healing venous wounds (Trengrove et al., 2000, Davies et al., 2007), and uncontrolled elastase activity is a potential causative factor contributing to the mechanism of chronic wounds (Herrick et al., 1997). However, this data is insufficient to conclude that these two factors are linked.

Differences were also observed in the activity of collagenase (decreased) and the level of TIMP-2 (increased) in diabetic foot ulcers, containing *Corynebacterium* spp. This decrease in collagenase activity could be directly related to this increase in TIMP-2 inhibitor levels. TIMP-2 preferentially cleaves gelatinase-B, however it has been reported to also interact with collagenases (Gomez, 1997, Xue et al., 2006).

Within the venous leg ulcer population, a number of cytokines exhibited significant increases as the wound total bioburden increased (IL-1β, IL-4, IL-6, TNF-α, TNFr2, ICAM-1, angiogenin and VEGF). In the presence of particular bacterial species at a level $\geq 1x10^7$ CFU/ml, particular cytokine levels increased, namely, *Pseudomonas* spp. (IL-1β), *Staphylococcus* spp., (TNF-α, TNFr2 and VEGF) and *Corynebacterium* spp., (IL-4, IL-12p70, IL-10, IL-13, angiogenin, TGFβ1, TNFr2, and VEGF)

The increasing levels of pro-inflammatory cytokines, IL-1β, IL-6 and TNF-α were not wholly unexpected as their increase is well documented within acute infections (Beutler and Grau, 1993, Sautner et al., 1995, Degre, 1996). The expression and secretion of these cytokines, during infection is rapidly induced and repressed, in a timely manner, during host defence responses against microorganisms (Dinarello, 2003). Generally, the increase in these cytokines, and IFN-γ, activates the acute phase response, which in turn increases the plasma concentration of proteins, including complement, c-reactive protein (CRP) and CD14, whose biological functions include the removal of foreign bodies (Lind, 2003, Stavitsky, 2007). Within chronic disease, it appears that the production of cytokines during host defence mechanisms, becomes dysregulated (Dinarello, 2003).

These particular cytokines have also been shown *in vitro*, to enhance bacterial growth (Meduri et al., 1999). The presence of bacteria themselves induces cytokine release from inflammatory cells, *in vitro* and *in vivo* (Birkedal-Hansen, 1993, Cassatella, 1995, Degre, 1996). Therefore, there is the potential of creating a positive feedback loop.

The increased levels of ICAM-1, an inflammatory cell adhesion molecule, measured in wound fluids upon rising total bioburden levels, may be explained as this molecule's biological role includes facilitating cell migration, including that of phagocytes, to sites of inflammation/infection (Levy, 1996).

Additionally, the potential for increased oxygen and nutrients within the wound upon increasing microbial bioburden, to aid in the oxidative burst and other bactericidal actions of immune cells, possibly explains the increased levels of angiogenin and VEGF observed (Van Eden et al., 1999, Dennison and Van Dyke, 2000). Indeed, VEGF levels in plasma are increased in patients with chronic venous insufficiency (Quatresooz et al., 2003).

Increased levels of IL-4, a key cytokine in the differentiation and regulation of T-helper cell populations, were also observed in venous leg ulcer wounds. IL-4 directs Th cells towards a Th2 immune response. Th1 cells are important for driving cell-mediated immunity, with these cells producing a number of cytokines, including IL-2, IL-3, IL-6, IL-12, IFN-γ, TNF-α, GM-CSF and TGF-β. In contrast, Th2 cells produce cytokines, such as, IL-3, IL-4, IL-5, IL-6, IL-10, IL-13, TNF-α, and GM-CSF, helping B-cells to produce antibodies (Playfair and Chain, 2001, Lin et al., 2000). The effect of increasing antibody production by B cells, allows opsonisation of antigens, such as microorganisms, increasing the phagocytic ability of neutrophils and macrophages (Chapter 1, Section 1.5).

Of these particular 8 cytokines (IL-1β, IL-4, IL-6, angiogenin, VEGF, ICAM, TNFr2, and TNF-α), 4 cytokines (IL-1β, TNF-α, TNFr2 and VEGF) were significantly increased in venous leg ulcer wounds containing *Pseudomonas* spp. and *Staphyloccocus* spp., at a level of $1x10^7$ CFU/ml. The most prominent species detected in venous leg ulcer wounds were *Pseudomonas* spp. (12 of 18) and *Staphylococcus* spp. (10 of 18). The ability of these four cytokines to differentiate infection on the basis of both total bioburden and bacterial species, affords them added specificity as wound infection diagnostic markers.

Comparing venous leg ulcer cytokine levels with *Corynebacterium* spp. (9 of 18), demonstrated an increase in additional cytokines, namely IL-1β, IL-4, IL-12 p70, IL-13 and TGF-β_1. The increases reflect a number of pro- and anti-inflammatory cytokines with many un-related to total bioburden (with the exceptions of TNFr2, angiogenin and VEGF), making them unlikely as biomarker for infection. *Corynebacterium* spp. has been associated with bacteramia in immuno-compromised individuals and prostheses infections (Gillepsie and Bamford, 2000). However, its relevance in chronic wound infection is less well understood.

The data presented herein suggested that 8 cytokines can potentially discriminate between microbial bioburden levels within venous leg ulcer chronic wounds. However, due to the small sample size (microbiologically infected n=5 vs. non-infected wounds, n= 13, at the level of 10^6 CFU/ml), a larger study would be required to validate these findings.

Surprisingly, in contrast to the venous leg ulcer cytokine responses, the levels of cytokines significantly decreased as total bioburden within the diabetic foot ulcer wound increased, namely IL-2, IL-4, IL-5, IL-10, IL-12p40, IL-12p70, IL-13, TNF-α, its receptor TNFr2, IP-10, IFN-γ, and TGFβ_1. These appear to be a distinct set of cytokines, from those observed to increase in venous leg ulcer wounds. However, only 6 of these cytokines (IL-2, IL-5, IL-12p40, IL-12p70, IFN-γ and TGFβ_1) showed a significant difference consistently, at both levels of total bioburden ($\geq 1x10^6$ and $\geq 1x10^7$ CFU/ml).

In the presence of *Streptococcus* spp., IL-12p70 and TGFβ_1 levels, were lower. No significant changes in the levels of any cytokine measured were found in wounds containing either *Corynebacterium* spp. or *Staphylococcus* spp., both of which were the most prominent bacterial species in these wounds, being isolated from 11 of 14, and 7 of 14, wounds respectively.

However, within the diabetic foot ulcer population, VEGF was found to be significantly higher in wounds containing >3 genera. As this particular cytokine does not significantly alter upon increases in total bioburden or in the presence of a particular bacterial species, its validity as a biomarker of chronic wound infection is therefore questionable.

At a bioburden level of $1x10^6$ CFU/ml, the decreases in 12 of the 18 cytokines measured, within diabetic foot ulcer wound fluids did not appear to be specific to one type of immune response. Pro- and anti-inflammatory cytokines levels were equally affected, in addition to those that would usually direct a Th1 (IL-12, INF-γ) or Th2 (IL-4) responses.

However, as the level of bioburden increased to $1x10^7$ CFU/ml, significantly lower levels of cytokines, that drive a Th1 response, or are produced by these cells, were observed. Th1 responses are associated with cell-mediated inflammation, tissue damage and the preferential INF-γ-induced classical activation pathway in macrophages. Classically activated macrophages are short-lived cells secreting pro-inflammatory and angiostatic cytokines (Kodelja et al., 1997). However, although a decrease of cytokine associated with Th1 immune response was evident, no increase in the levels of the other cytokines (IL-4, IL-6, TNF-α) measured in the present Study, implied that an inappropriate immune response is present upon challenge with microorganisms.

These changes in cytokine responses may account for the increased susceptibility of diabetic foot ulcers to wound infection (Medina et al., 2005, Peleg et al., 2007). The difference in cytokine levels within the diabetic foot ulcer population, upon increasing total bioburden may be the direct result of the microflora within the wound, compared with venous leg ulcer wounds. However, in the present Study, *Staphylococcus* spp. and *Corynebacterium* spp., were the most prevalent bacteria within both ulcer types.

Pseudomonas spp. were isolated more frequently from venous leg ulcers. *Pseudomonas aeruginosa* proteases have been shown to degrade complement factors (Schmidtchen et al., 2000) and cytokines, IFN-γ (Horvat et al., 1989, Parmely et al., 1990), IL-1, IL-2, and TNF-α (Henderson et al., 1996). Conversely, in the present Study, decreased levels of cytokines were apparent in diabetic foot ulcer wounds, where *Pseudomonas* spp. was only cultured from 1 wound.

It is therefore hypothesised that the underlying pathology of diabetic foot ulcers, may more likely to be a contributing factor, with these ulcers unable to mount an efficient immune response to microbial contamination. Typically, interactions between microorganisms or their products, results in the direct stimulation of host cells, including neutrophils and macrophages, or affecting the local lymphocyte responses (Birkedal-Hansen, 1993).

Compared to acute wounds, both venous leg ulcers and diabetic foot ulcers have been reported to maintain an increased presence of T-cells with abnormal Th (CD4+) and suppressor T-cells (CD8+) subtypes, and increased number of macrophages (Loots et al., 1998). However, diabetic foot ulcers seem to be characterised by an inability to attract sufficient numbers of inflammatory cells into the wound (Medina et al., 2005).

Additionally, inappropriate activation of diabetic macrophages, in an *in vivo* rat model, have been shown to result in a decreased ability to release pro-inflammatory cytokines, IL-1β, IL-6 and TNF-α (Doxey et al., 1998). Impairment of leukocyte function and neutrophils bactericidal function, with reduced superoxide production, in diabetics with poor glycaemic control has also been reported (Peleg et al., 2007). An impaired inflammatory phase of wound repair characterises the diabetic wound healing process (Chen et al., 2005). There are multiple, potential underlying ulcer pathological reasons, affecting cellular function and/or activation status, that may account for the constant or decreased cytokine responses in diabetic foot ulcers, with increasing bioburden.

Differences between ulcer types were highlighted, in the levels of proteases and cytokines, between the two populations. Of the 7 cytokines found to be significantly different between the two ulcer types, 6 cytokines, IL-2, IL-4, IL-13, IFN-γ, TNF-α and VEGF, were detected at lower levels, in diabetic foot ulcer wound fluids.

These decreases in cytokine levels coincided with significantly increased elastase-like activity, in diabetic foot ulcer wounds. It has been demonstrated with IL-6, TGF-β_1 and PDGF-BB, that neutrophil proteases can degrade and inactivate cytokines and growth factors (Yager et al., 1997, Bank et al., 1999). Therefore, the present Study suggests that this increased protease activity may be directly correlated with lower cytokine levels. The increased elastase-like activity measured in diabetic foot ulcers, may also account for the lack of *Pseudomonas* spp. cultured from these wounds. Elastase appears to be preferentially active against Gram-negative bacteria (Belaaouaj and Shapiro, 1999).

There are reports in the literature, of the increased presence of neutrophils (elastase-producing cells), within the ulcer bed of both venous leg and pressure ulcer wounds, yet weak staining of elastase$^+$ granulocytes in diabetic foot ulcers (Galkowska et al., 2005). This increased elastase-like activity measured in diabetic foot ulcer wounds, was surprising, in relation reported literature.

The levels of the cytokines, IL-2, IL-4, IL-13, IFN-γ, TNF-α and VEGF, were higher in venous leg ulcers, in comparison with diabetic foot ulcers. In addition to host-derived proteases, bacterial products have also been shown to degrade host factors including, cytokines. Both ulcer types contained *Staphylococcus* spp., with *Pseudomonas* spp. additionally detected in more venous leg ulcer than diabetic foot ulcer wounds. Proteases from *Pseudomonas* spp, are known to degrade cytokines, and therefore, this suggests that the host-derived proteases, such as the elastase-like activity measured in diabetic foot ulcers, also contribute to lower cytokines levels in chronic wounds.

The presence of large concentrations of IL-1β, and IL-6, associated with a Th1 immune response in both venous and diabetic foot ulcers, may suggest that the immune system is in favour of a Th1 response. *In vivo* data suggests that a Th1 response induces inflammation characterised by tissue damage, whilst Th2 directs wound healing and fibrosis (Sandler et al., 2003).

Irrespective of ulcer type, venous leg or diabetic foot, the data generated from the present Study, suggests that the level of cytokines may potentially serve as biomarkers of microbiologically defined infection. These biomarkers could be measured to provide the basis of a diagnostic test. A variety of tests are used within the field of wound healing to diagnose the wound type and aetiology (Collier, 2001). A diagnostic test is any kind of medical procedure performed to aid in the diagnosis or detection of disease. These tests can range from point-of-care to laboratory-based assays (Moseby, 1997).

Pertinent to the present Study, wound infection status is primarily diagnosed using clinical signs and symptoms, with an assessment of the microbiological content of the wound. Therefore, a measure of host cytokine levels to aid the Clinicians diagnosis of infection and would provide valuable information in a timely manner. Cytokine levels can be measured easily, with a limited requirement for a dedicated laboratory, correct sample preparation/storage media, and usually within hours compared with the days required to culture micro-organisms. These cytokines may also be more specific to infection than the clinical signs and symptoms currently relied upon.

The cytokines themselves appear to discriminate between ulcer types, and the difference in the contributing microflora between venous leg ulcer and diabetic foot ulcer wounds, may account for the varying cytokine levels and responses, observed.

4.4.1 Summary of findings

- No significant differences in the levels or activity of proteases or their inhibitors, upon increasing bioburden, in either venous leg ulcer or diabetic foot ulcer wound fluids.

- Elastase activity was significantly increased in venous leg ulcer fluids, when 3 or more bacterial genera were present.

- Collagenase activity was significantly lower and TIMP-2 levels were higher, in diabetic foot ulcer wound fluids containing *Corynebacterium* spp.

- Increased levels of cytokines; angiogenin, IL-1β, IL-4, IL-6, VEGF, ICAM-1, TNFr2 and TNF-α were detectable in venous leg ulcer wound fluids as total bioburden increases. All other cytokine levels remained the same as bioburden increased.

- Decreased levels of cytokines, IL-2, IL-5, IL-12p40, IL-12p70, IFN-γ and TGF-β_1, were measured in diabetic foot ulcer wound fluids, as total bioburden increased. All other cytokine levels remained the same as bioburden increased.

Due to the differing cytokine responses between venous leg ulcers and diabetic foot ulcers to total bioburden, and the distinct microflora of each ulcer type, it was not possible to combine the venous leg and diabetic foot ulcer data into larger populations, as hoped, in order to draw more valid conclusions. The distinct microflora observed between the two ulcer types could account for the differing biochemical responses. However, these differences could equally be due to the underlying pathology of these particular ulcer types, or potentially, be due to a combination of both these factors.

Chapter 5

Chapter 5

Evaluation of neutrophils as a source of proteases and cytokines during bacterial infection, *in vitro*

5.1 Introduction

The central role of the neutrophil cell within the host immune system is primarily concerned with the eradication of foreign matter, including microorganisms from the body. Neutrophils can produce and respond to a variety of signals, eliciting a response (Cassatella et al., 1999, Segal, 2005). These signals are usually in the form of cytokines, with the response consisting of, although not limited to, the release of proteases, and production of cytokines themselves. These responses provide the host with multiple approaches to defend against microbial pathogens. A general introduction to the neutrophil and its role in inflammation and infection has been provided previously (Chapter 1, Sections 1.1.2 and 1.5.3).

This Chapter presents data derived from an *in vitro* model, designed to investigate the effect of extracellularly secreted molecules from two bacteria commonly associated with chronic wounds, investigated in the present Study. *Pseudomonas aeruginosa (P. aeruginosa)* and *Staphylococcus aureus (S. aureus)* on isolated neutrophils, using the bacterial cell wall component, lipopolysaccharide (LPS), as the positive control. The response of neutrophils to the acellular 'bacterial supernatants' of *P. aeruginosa* and *S. aureus* was determined by measuring their release of cytokines. A general introduction of the cytokines measured in the present Study, has been provided previously (Chapter 1, Section 1.7).

5.1.1 Neutrophils

Within the present Study, neutrophils were isolated from human blood, for use *in vitro*. Neutrophils contain a variety of anti-microbial substances that directly serve to eradicate microorganisms. These include proteases, which can interact, through the process of phagocytosis/secretion, with the bacterial cell (Xu and Hakansson, 2002). Neutrophils can also synthesise and secrete a number of cytokines that regulate the immune system, to facilitate the elimination of microorganisms by increasing neutrophil migration to the area of infection, in addition to influencing neutrophil functions, such as, degranulation, the release of protease and the production of highly reactive oxygen species (ROS) (Witko-Sarat et al., 2000).

5.1.1.1 The protease complement of neutrophils

Neutrophils are currently reported to express six neutral proteinases:- four serine proteinases - elastase, cathepsin G, proteinase-3 (PR3) and urokinase-plasminogen activator (uPA) and two matrix metalloproteinases (MMP-8 and MMP-9) (Barrick et al., 1999). Atypically, the neutrophil completes the synthesis of these proteinases whilst in the bone marrow, storing them within the cell in azurophil, secondary/specific and tertiary/gelatinase granules (Xu and Hakansson, 2002, Mayer-Scholl et al., 2004). Storage of a proteinase allows for its rapid release from the cell.

Elastase and cathepsin G are the most extensively studied neutral proteinases of neutrophils. The levels of elastase per neutrophil had been determined as 3pg (Janoff, 1985), but more recently, it has been reported that in their fully active form, neutrophils contain within their azurophil granules, approximately 1pg of elastase and cathepsin G per neutrophil (Owen and Campbell, 1995). Collagenase (MMP-8), gelatinase B (MMP-9) and uPA are located within the secondary and tertiary granules.

Activation of neutrophils by various biologically relevant cellular activators, such as formyl-methionyl-leucyl-phenylalanine (fMLP) and lipopolysaccharide (LPS), reviewed in Chapter 1 (Section 1.5.3.2), allows the expression of elastase, cathepsin-G, PR3 and uPA, at the cell surface (Barrick et al., 1999), and the release of MMP-8 (Witko-Sarsat et al., 2000) and MMP-9 (Gasser et al., 2003).

The main physiological function of neutrophil-derived proteases include aiding the migration of neutrophils to the site of inflammation/infection (Dallegri and Ottonello, 1997). Decreased neutrophil migration into a site of inflammation has been observed by inhibiting the function of MMP-9 with TIMP-1 (Delclaux et al., 1996). The neutrophil-derived proteases, elastase and cathepsin G, act within the cell inside the phagolysosomes, to degrade and destroy micro-organisms (Fritz et al., 1986).

The importance of proteases in the destruction of microorganisms has been highlighted recently (Reeves et al., 2002), whereby survival of *S. aureus* was observed in protease (elastase and cathepsin G)-deficient, yet normal superoxide producing and iodinating-capacity mice. This study demonstrated that during the process of phagocytosis, ROS generation and myeloperoxidase (MPO) activity were not themselves sufficient to kill microorganisms, such as *S. aureus* and *C. albicans*. Recent data has also demonstrated that elastase, cathepsin G and MPO can be located within extracellular fibres of activated neutrophil cells, termed neutrophil extracellular traps (NETs), which disarm and kill both Gram-negative (*Salmonella typhimurium* and *Shigella flexneri*) and Gram-positive (*S. aureus*) bacteria, extracellularly (Brinkmann et al., 2004).

5.1.1.2 The cytokine complement of neutrophils

Cytokines are the basic regulators of neutrophil function. Neutrophils have receptors for a number of cytokines and evidence from *in vitro* and *in vivo* studies suggest that cytokines modulate many neutrophil functions:-

- Cytokines, specifically colony stimulating factors (CSFs); granulocyte-GSF (G-GSF), granulocyte-monocyte CSF (GM-CSF) and multiple-GSF (M-GSF, or alternatively interleukin-3), appear to change the rate of bone marrow production and release of neutrophils, differentiating neutrophils from progenitor granulocyte, monocyte cell, and aid proliferation and neutrophil survival (Steinbeck and Roth, 1989).
- Enhance the ability of neutrophils to localise at sites of inflammation, via chemokines including interleukin-8 (IL-8) (Normark et al., 2001) and possibly indirectly through the action of IL-1 and tumour necrosis factor (TNF-α). IL-1 and TNF-α have been shown to cause increased neutrophil infiltration, although neither factor is chemotactic. These cytokines must stimulate the production of other endogenous chemotactic factors (Steinbeck and Roth, 1989).
- Increase adherence to endothelial cells under action of TNF-α (Steinbeck and Roth, 1989) and the bacterial cell wall component, LPS (Martin, 1997).
- Maintain neutrophils at the site of inflammation. It is believed that the cytokine, interferon-γ (IFN-γ), has the ability to prevent adherence and decrease migration, thereby keeping neutrophils localised to the site of infection. (Steinbeck and Roth, 1989). Activated neutrophils show signs of increased survival, with avoidance of programmed cell death (Colotta et al., 1992).
- Increase the rate of phagocytosis, in a dose-dependent manner when primed with IL-1, or in the presence of TNF (Steinbeck and Roth, 1989). Additionally, IFN appears to maintain neutrophil phagocytic action.
- Stimulate the production of reactive oxygen species (Remer et al., 2003).
- Stimulate the release of enzymes (e.g. proteinases, as previously described, Steinbeck and Roth, 1989, Cassatella, 1995).

Accumulation of the above factors aid the efficacy of neutrophil bactericidal activity.

In addition to responding to cytokine signals, neutrophils also synthesise and secrete small levels of many cytokines (Table 5.1), including pro-inflammatory cytokines; IL-1, IL-6, IL-8, TNF-α and vascular endothelial growth factor (VEGF), in response to factors such as, LPS, phorbol mysitrate acetate (PMA), fMLP, IL-13 and GM-CSF, and complement C5a (Cassetella, 1995, Cassatella et al., 1993;1999, Takeichi et al., 1994, Dallegri and Ottonello, 1997, Scapini et al., 1999, Cockeran et al., 2002).

Table 5.1: Cytokines synthesised and released by neutrophils.
Cytokines sourced from Cassatella (1995), Scapini et al., (1999). IL-6*, the ability of neutrophils to release IL-6 remains a matter of debate. Authors including Scapini et al., 1999 claim lack of IL-6 indicates the purity of neutrophil isolation procedure.

Pro- and anti-inflammatory cytokines	IL-6 *, TNF-α, IL-1α/ IL-1β, IL-12, IFNα, TGF-β
Angiogenic, fibrogenic or growth factors	VEGF, HB-EGF, BFGF, TGF, C-CSF
Chemokines	IL-8, IL-10, GRO, MIP, CINC

5.1.2 Effects of lipopolysaccharide on neutrophils

Neutrophils are capable of increasing the synthesis and release of particular cytokines when challenged with LPS, a cell wall component of Gram-negative bacteria. LPS is a known stimulator of these cells and is used frequently by investigators for *in vitro* assays (Chapter 1, Section 1.5.3.2). A pre-formed pool of CD14, within the azurophil granule is mobilised upon challenge with LPS or fMLP, and expressed as mCD14, allowing LPS binding to the cell (Liu et al., 2005).

LPS induces mechanisms, such as phagocytosis, fusion of granules to phagosomes and the expression of oxidative burst, with the production of superoxide radicals (Shapira et al., 1994, Remer et al., 2003) that occur instantaneously. Additionally, LPS induces gene expression with the subsequent synthesis of proteins, such as cytokines, for release from the cell, as required (Remer et al., 2003).

Neutrophils synthesise and release pro-inflammatory cytokines, in response to stimulation with various bacterial LPS (Palma et al., 1992). However, the synthesis and release of cytokines is not exclusive to activating agents, such as LPS, as the process of bacterial phagocytosis is sufficient for the neutrophil to synthesise and release large levels of cytokines, such as, IL-8 and macrophage inhibitory protein-1-α (MIP-1α) (Hachicha et al., 1998). The most abundantly produced cytokine by neutrophils, IL-8 (Witko-Sarat, 2000, Gabriel et al., 2002), is an important cytokine that serves as a chemoattractant molecule at sites of infection/inflammation.

Although macrophages produce more cytokines than neutrophils, on a per cell basis, neutrophils are the predominant cell type in inflamed and infected tissues (Cassatella, 1995). *In vivo* studies have confirmed the possible significant contribution of neutrophil-derived cytokines to inflammatory responses, including infection and the wound healing process (Meszaros et al., 2000, Theilgaard-Monch et al., 2004). Secreted inflammatory cytokines from neutrophils (IL-1β, IL-6, TNF-α and the chemokine, IL-8) appear to contribute to the mechanisms of acute inflammation and endotoxic shock *in vivo* (Cassatella, 1995).

However, *in vivo* studies have demonstrated that cytokines can also offer protection from infection, for example, IL-1 appears to increase the infiltration of neutrophils, increasing the survival of mice challenged with *P. aeruginosa* (Steinbeck and Roth, 1989), with IL-2 protecting 80-90% mice from lethal challenge with *E. coli* or *P. aeruginosa*, possibly by affecting macrophage activation, as opposed to altering the rate of infiltration (Steinbeck and Roth, 1989).

5.1.3 Extracellular product of *Pseudomonas* spp. and *Staphylococcus* spp.

P. aeruginosa and *S. aureus* are common bacteria that colonise chronic wounds (Bowler, 1998, Howell-Jones et al., 2005, Davies et al., 2007). *P. aeruginosa* was found to be the major Gram-negative bacteria isolated from leg ulcers (Colsky et al., 1998). Data also suggests, that persistently enlarged ulcers, could in part be attributed to *P. aeruginosa* infection (Danielsen et al., 1998). *S.aureus* infected acute and chronic wounds also exhibit delayed wound healing responses (Madsen et al., 1996, Grimble et al., 2001, Lowy, 2003).

In the present Study, bacterial supernatants of clinical wound isolates from the present Study, *P. aeruginosa* and *S. aureus*, were used during *in vitro* evaluations. These two particular bacteria were the most prevalent bacterial species isolated from venous leg ulcers in the present Study, with 12 of 18 wounds and 10 of 18 wounds, containing *Pseudomonas* spp. and *Staphylococcus* spp. respectively. *Staphylococcus* spp. was the second most frequent bacteria isolated from diabetic foot ulcers, present in 7 of 14 wounds, although only 1 of 14 diabetic wounds contained *Pseudomonas* spp. (Chapter 3, Section 3.3).

Numerous products are secreted extracellularly by both, *P.aeruginosa* and *S.aureus* (Chapter 1, Section 1.8.1). Virulence for both *P. aeruginosa* and *S.aureus* is multifactorial, with certain exoproducts possibly playing a more important role in certain types of infection.

Pseudomonas spp. exotoxin A is the pathogenic element of eye infections, burn wounds and chronic lung infections (Woods et al., 1986, Heggars et al., 1992). Approximately 80-90% of clinical isolates can produce exotoxin A *in vitro*. Exoenzyme S (an ADP ribosyltransferase enzyme) also appears to be responsible for the tissue damage associated with the pathogenicity of lung infection (Nicas et al., 1985). Invasiveness of *P. aeruginosa* in burn infections is due to its alkaline protease, elastase (Wretlind and Pavlocskis, 1983), with the levels of elastase correlating with acute and chronic lung infections (Woods et al., 1986).

Exotoxins and exoenzymes secreted from *S. aureus* include enterotoxins A-H, epidermolytic toxin A and toxic shock syndrome toxin-1 (Chavakis et al., 2002). *S. aureus* also produce four major extracellular proteases: V8 protease, SspA (serine proteases), SspB (cysteine protease) aureolysin (metalloprotease) and Scp (cysteine protease) (Karlsson and Arvidson, 2002). The combination of both proteases and toxins aid the pathogenic capabilities of the bacteria.

Typically bacterial growth curve consists of three phases; i) a lag phase, ii) a logarithmic/exponential phase and iii) a stationary phase (Yates and Smotzer, 2007). *Pseudomonas* spp. express toxins and protease (virulence factors) with transcriptional analysis hinting at increased expression within the stationary growth phase (Van Delden and Iglewski, 1998, Westfall et al., 2004, Waite et al., 2006). Expression of proteases in stationary phase supernatant, has been observed by Schmidtchen et al., (2001). Known staphylococcal toxins are synthesised at the end of the logarithmic growth phase (Veldkamp et al., 2000), regulated by the gene products *agr, sar* and *xpr* (Villavicencio and Wall, 1996). The synthesis of staphylococcal extracellular proteases is activated by the gene product *agr*, under the regulation of a unique operon, *spl,* its expression occurs during the transition to stationary phase, which concurs with protease production during late exponential and post-exponential growth phases (Reed et al., 2001, Karlsson and Arvidson, 2002). Whether strains of *S.aureus* expressing high levels of protease are more virulent in the clinical setting remains to be established.

Due to the differences in the expression of toxins and protease from bacterial cells, supernatants in the present Study were prepared at both mid-logarithmic (mid-log.) and stationary growth phases. A comparison between the two phases, with respect to their ability to produce a cytokine response, were subsequently assessed.

The present Study investigated whether soluble components expressed and released by bacteria isolated from chronic wounds, could induce inflammatory cells to release cytokines. Data from the present clinical Study (Chapter 4), demonstrated that within the venous leg ulcer population, an increase in particular cytokines, angiogenin, VEGF, intracellular adhesion molecule (ICAM), TNF receptor2 (TNFr2), IL-4 and the pro-inflammatory cytokines, IL-1β, IL-6 and TNF-α, correlated to wound infection, as defined by the microbiological content of the wound. Previous studies have suggested a link between pro-inflammatory cytokines and delayed wound healing, with wound infection negatively impacting on wound healing processes (Chapters 1, 3 and 4).

IL-6 was found to be one of the most abundant cytokines in the venous leg ulcer wounds (Chapter 3), positively correlating to the diagnosis of chronic wound infection. The increased levels of IL-6 in clinically diagnosed venous leg ulcer wounds were not found to be significant. However, when correlated to total bioburden, at a level of $\geq 1x10^6$ and $\geq 1x10^7$ CFU/ml, significant increases were observed. Additionally, *in vitro,* IL-1β, IL-6 and TNF-α, have the ability to enhance the growth of *S. aureus* and *P. aeruginosa* (Meduri et al., 1999). The pro-inflammatory cytokine, IL-6, was therefore chosen initially, as a measure of the validity of the *in vitro* model system, implemented herein.

In addition, 18 cytokines were analysed by micro-array, including, interleukins (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, Il-12p40, IL-12p70 and IL-13), angiogenin (angio); IFN-γ; growth factors, transforming growth factor beta (TGF-β_1) and VEGF; TNF-α and it's soluble receptor p75 (TNFr2); the adhesion molecule, ICAM-1, and the chemokine, IFN-γ-inducible-protein-10 (IP-10).

This wide array of cytokines evaluated, allowed for quantification of the levels of a number of cytokines/growth factors, previously unreported in the literature, in relation to stimulation of neutrophils with bacterial supernatants.

Specifically, the expression of neutrophil-derived molecules was investigated in response to bacterial supernatant using this *in vitro* model system. As the contribution of cytokines on a per cell basis for monocytes/macrophages is known to exceed that of neutrophils (Cassatella, 1995, Xing and Remick, 2003), this implies that neutrophils make a minor to non-existent contribution to the production of cytokines. However, neutrophils account for 60-70% of blood leukocytes, and 90% of granulocytes are neutrophils (Schroder and Rink, 2003). In response to infection, neutrophils are the first cell-type to migrate into the tissue and therefore, become the predominant cell-type. The ability of neutrophils to migrate does not appear to be affected by age (Schroder and Rink, 2003). Therefore, the contribution of neutrophil-derived cytokine may be as important as those of derived from the monocytes/macrophages.

The aims of this Chapter, were therefore, to model the responses observed clinically in the wound fluids of venous leg ulcers, where an increase in the expression of particular cytokines were observed. This *in vitro* model investigated the effects of an acellular bacterial 'supernatant', consisting of extracellularly secreted molecules from both *P. aeruginosa* and *S. aureus*, on neutrophils. The bacterial supernatants were collected at the mid-log. and stationary phases of bacterial growth, and the effects compared in order to determine if the host inflammatory response is primarily due to bacterial type and number or if the phase of growth is also an important factor.

5.2 Materials and methods

The generation of bacterial supernatant and culture conditions for the isolation and stimulation of neutrophils with bacterial supernatants and LPS have been described previously (Chapter 2, Section 2.2). Methods used in the analysis of biochemical markers have also described previously (Chapter 2, Section 2.3).

5.3 Results

5.3.1 Method conditions

Neutrophils were stimulated with bacterial supernatant, while LPS served as the positive control in all experiments. The number of cells required, the concentration of LPS needed, and the duration and conditions required for LPS to induce a response were investigated. The pro-inflammatory cytokine, IL-6, released from cells in response to LPS was measured, by ELISA (R&D Systems, Oxfordshire, U.K.). Neutrophils were isolated from two human donors, subject 1 and subject 2, both healthy females, between the ages of 20-30 years old.

5.3.1.1 Bacterial supernatant

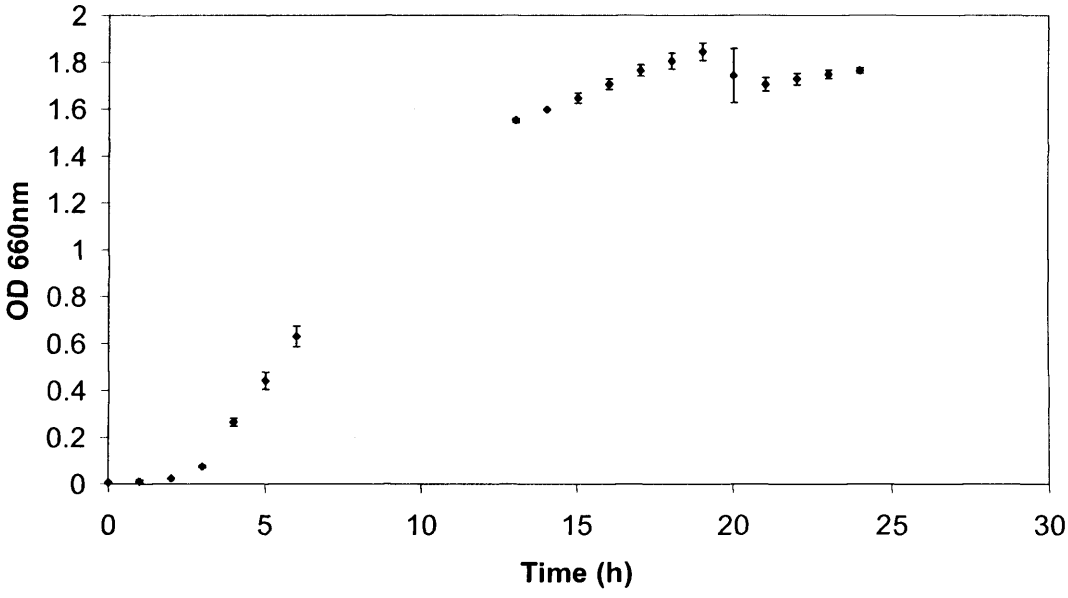
Wound pathogens, *Pseudomonas aeruginosa* and *Staphylococcus aureus* were cultured from the wound swabs of two subjects, venous leg ulcer patient **x and y,** respectively. These bacterial clinical isolates were inoculated into fastidious anaerobic broth and the bacteria grown to mid-log and stationary phases, determined spectrophotometrically, plotting absorbance verses time to generate bacterial growth curve (Figure 5.1). Mid-log growth was observed after 5 hours incubation at 37°C with stationary growth phase achieved by 24 hours, for both *Pseudomonas aeruginosa* and *Staphylococcus aureus*. A broth control supernatant was prepared following 5 and 24h incubation at 37°C.

Table 5.2: The time (h) at which mid.-log and stationary growth phase *Pseudomonas aeruginosa* and *Staphylococcus aureus* supernatant were collected. The amount of bacterial cells (CFU/ml) at each time point corresponded to the growth phase at that time. Similar time and amounts of bacteria were observed for both organisms at mid. log and stationary growth phases. Control supernatant of bacterial broth was prepared following 5 and 24h incubation at 37°C.

	Bacterial Supernatant			
	Pseudomonas aeruginosa		***Staphylococcus aureus***	
Growth Phase	Colony forming units/ml	Time (h)	Colony forming units/ml	Time (h)
Mid. log	6.5×10^8	6	2.6×10^8	5
Stationary	3.8×10^8	24	1.7×10^8	24

Figure 5.1: Bacterial growth curve for clinical isolates of (a) *Pseudomonas aeruginosa* and (b) *Staphylococcus aureus*.
Growth curves (n=3, average ± S.E.) followed the typical three phases; (i) a lag phase, (ii) a logarithmic/exponential phase (mid-log) and (iii) a stationary phase (Yates and Smotzer, 2007). Mid-log and stationary growth phases were identified.

a)



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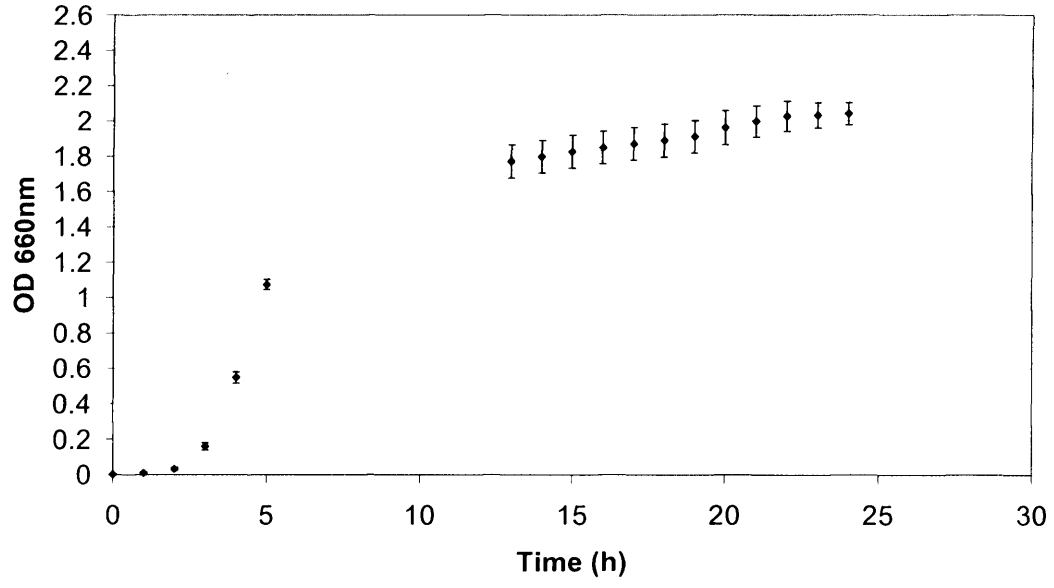
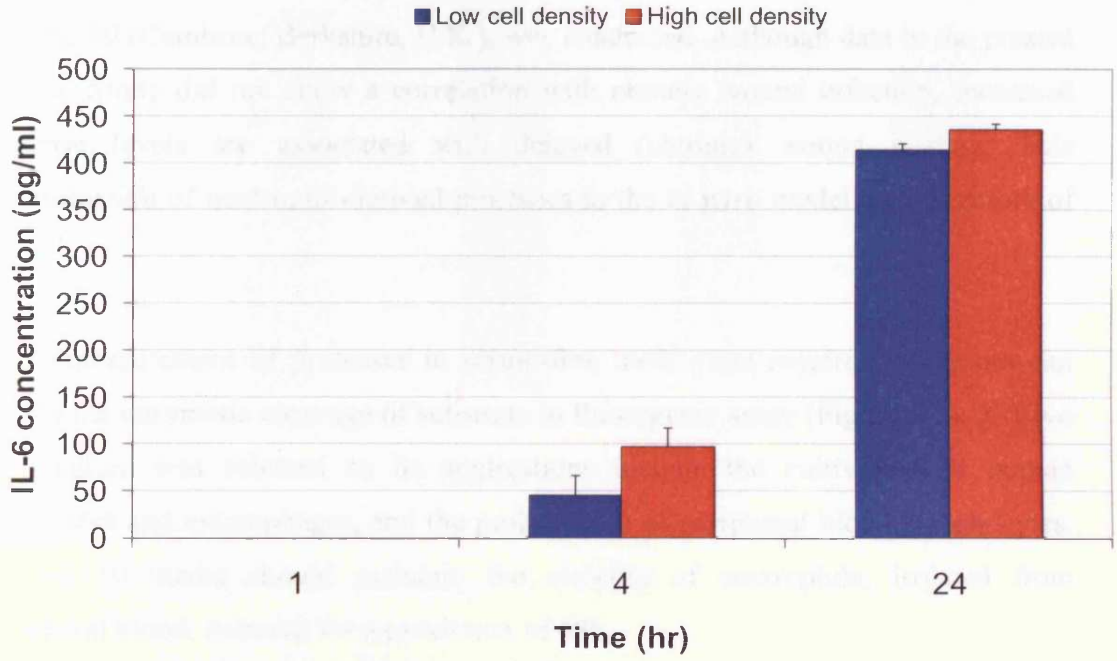


5.3.1.2 Cell density

Stimulation of neutrophils by bacterial supernatants and LPS was performed in conditions that were physiologically relevant. The typical adult blood film contains approximately 3-7x10^6 cells/ml neutrophils (George-Gay and Parker, 2003). A concentration of neutrophils based on the lower amount present in the blood (2x10^6 cells/ml) was selected initially. However, in order to best utilize each primary neutrophil isolate, the cell density was decreased. Decreasing the concentration of cells four-fold, to 0.5x10^6 cells/ml, appeared to have little effect on the overall levels of cytokine secreted by neutrophils (Figure 5.2). However, there was a difference between the two cell densities, noted at the 4h time point, yet by 24h, this difference was minimal.

Figure 5.2: Cell seeding density and IL-6 production by neutrophils.
Detection of IL-6 (average±SE) in cell culture neutrophil supernatant, stimulated in the presence of 0.1μg/ml LPS at two cell densities; $2x10^6$ cells/ml and $0.5x10^6$ cells/ml at 37°C/5% CO_2 for 1, 4 and 24h. There appeared to be little difference in the levels of cytokine released between the two cell densities. Increasing amounts of IL-6 were released as time progressed.



5.3.1.3 Effects of serum

In addition to measuring cytokines, the use of the serum-free media was assessed, as this would allow the measurement of the expression of proteases, by enzyme methods from the same *in vitro* samples. An investigation to change the type of media from RPMI-1640 +10% fetal calf serum (FCS) to a serum-free based media, X-Vivo 10 (Cambrex, Berkshire, U.K.), was conducted. Although data in the present clinical Study did not show a correlation with chronic wound infection, increased protease levels are associated with delayed (chronic) wound healing. This determination of neutrophil-derived proteases in the *in vitro* model was therefore of interest.

Thus, measurement of proteases in serum-free media was required, as serum can inhibit the enzymatic cleavage of substrate in fluorogenic assay (Figure 5.3). X-Vivo 10 medium was selected as its applications include the cultivation of human monocytes and macrophages, and the proliferation of peripheral blood lymphocytes. X-Vivo 10 media should maintain the viability of neutrophils, isolated from peripheral blood, cultured for a maximum of 48h.

Assessment of X-Vivo 10 medium, revealed that it appeared to enhance elastase activity when measured using the fluorogenic activity assay (Chapter 2, Section 2.3), in comparison with values obtained for either elastase activity buffer itself or RPMI-1640 media (Figure 5.4). Levels of elastase activity for RPMI-1640 and X-Vivo 10 media were found to be 23.0±5.4 and 228.4±16.6 RFU/min at 5μg/ml elastase, 2.4±1.3 and 38.1 ± 4.9 at 1μg/ml elastase, 1.2 ± 0.3 and 19.7 ± 2.2 RFU/min at 0.5μg/ml elastase, 0 and 0.4±0.5 RFU/min at 0.05μg/ml elastase, respectively.
This enhanced activity could not be explained by X-Vivo 10 medium cleaving the substrate itself, or be accounted for by the pH values of X-Vivo 10, RPMI-1640 and elastase activity buffer, found to be pH 7.26, pH 7.5 and pH 7.5, respectively. It may be that some component of the media stabilised the enzyme preventing autolytic degradation. As the constituents of X-Vivo 10 medium are commercially sensitive, the precise reason for the observations obtained are therefore unknown.

Figure 5.3: Serum interference in fluorogenic determination of elastase activity. Elastase activity (average ± SD, n=3) was inhibited when incubated in cell culture media (RPMI-1640) containing fetal calf serum (FCS) at concentrations from 1-10%.

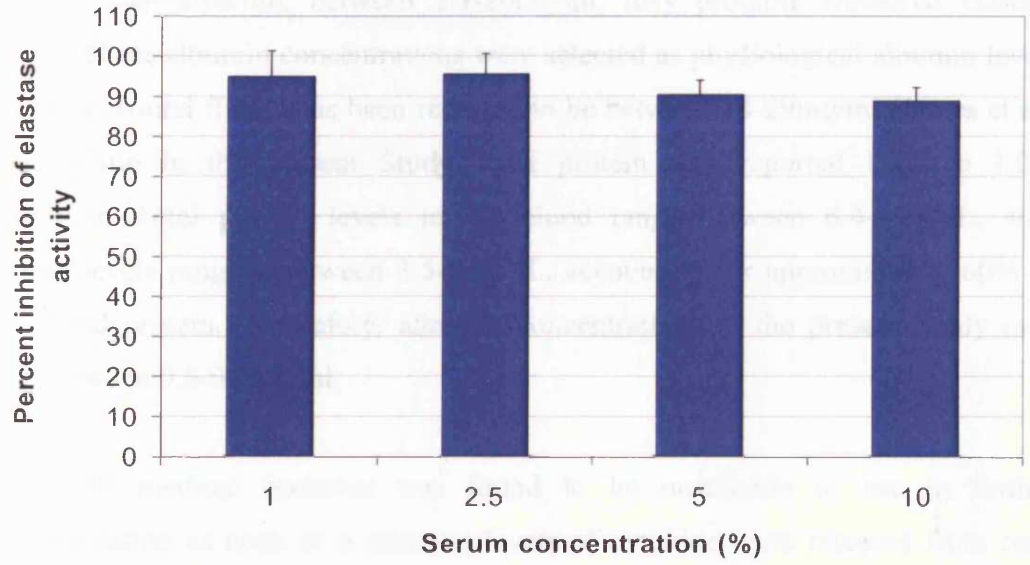
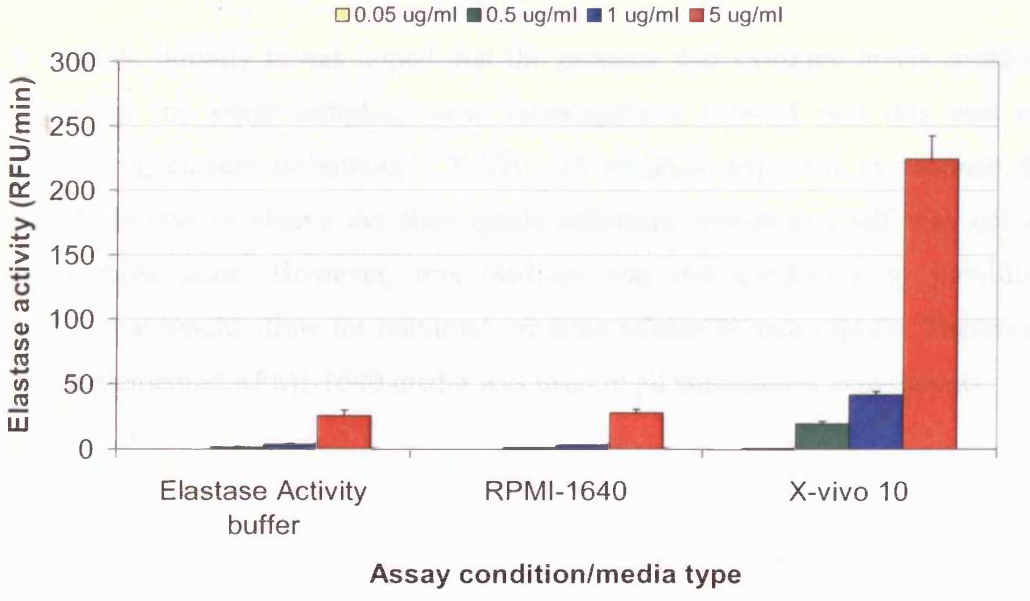


Figure 5.4: Enhanced turnover of substrate by elastase in cell culture media, X-Vivo 10 (average ± SD, n = 2).

Elastase activity (RFU/min) upon cleaving substrate MeOSuc-ala-ala-pro-val-AMC, by X-Vivo 10 media, compared to RPMI-1640 media and elastase activity buffer (0.1M Hepes buffer, pH 7.5, containing 0.5M Sodium Chloride and 10% DMSO). Significant (approximately 4-fold), increases in elastase activity were observed when assayed using X-Vivo 10 media, at the concentrations of elastase tested, 0.5µg/ml ($p<0.0001$), 1µg/ml ($p<0.0001$) and 5µg/ml ($p<0.0001$), compared to RPMI-1640.



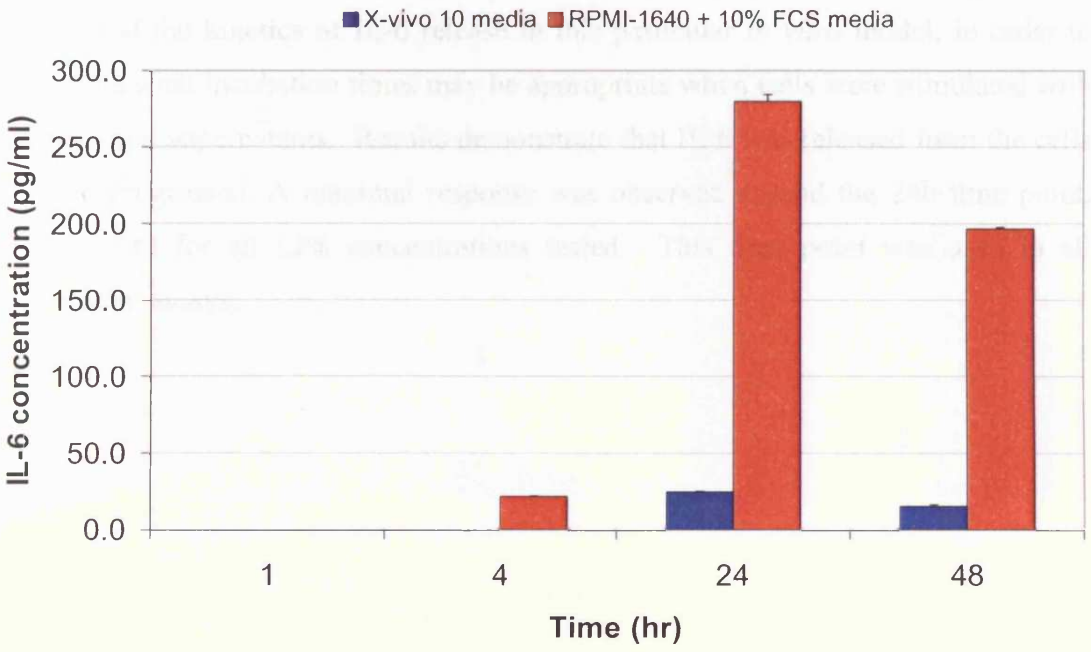
An investigation of X-vivo 10 medium by SDS-PAGE protein gel electrophoresis indicated a predominant band corresponding to the molecular weight of albumin. As such, additional fluorogenic activity assays which incubated albumin with elastase suggested that albumin, between 0.3-20mg/ml, may promote enhanced elastase activity. These albumin concentrations were selected as physiological albumin levels in chronic wound fluids, has been reported to be between 14-29mg/ml (James et al., 2000), while in the present Study, total protein was reported between 1.04-8.74mg/ml. Total protein levels in the blood range between 6.4-8.3g/dL, with albumin levels ranging between 3.5-5.0g/dL, accounting for approximately 60% of total blood protein. Therefore, albumin concentrations in the present Study may range between 0.6-0.2mg/ml.

X-Vivo 10 medium however was found to be unsuitable to use in further experimentation as none or a minimal levels of cytokine were released from cells stimulated with LPS in X-Vivo 10 medium, as opposed to serum-supplemented media (Figure 5.5). Cells, such as monocytes, do not absolutely require serum proteins to be stimulated by LPS, as CD14-independent LPS signalling pathways exist (Lynn et al., 1993). Therefore, it appears that in the present Study, serum is required for the release of cytokines by neutrophils. The serum protein LPS binding protein (LBP), complexes with LPS (Chapter 5, Section 5.1), binding to the cells and activates signalling pathways, to allow the expression of cytokines.

Ideally, while initially it was hoped that the protease and cytokine levels could be quantified in the same samples, these investigations showed that this was not possible using current techniques. X-Vivo 10 medium appeared to enhance the ability of elastase to cleave the fluorogenic substrate, which in itself was not an insurmountable issue. However, this medium was not conducive to providing conditions that would allow for maximal cytokine release by neutrophils. Therefore, serum supplemented RPMI-1640 media was used in all stimulation experiments.

Figure 5.5 IL-6 release from neutrophil cells in serum-supplemented (RPMI-1640 + 10% FCS and serum-free (X-Vivo 10) media, collected between 1h and 48h.

Results demonstrated no or minimal release of IL-6 from cells in serum free, X-Vivo 10 media, compared to serum supplemented media, RPMI-1640 +10% FCS, over 1, 4, 24 and 48h. Neutrophils were stimulated with 0.1µg/ml LPS.

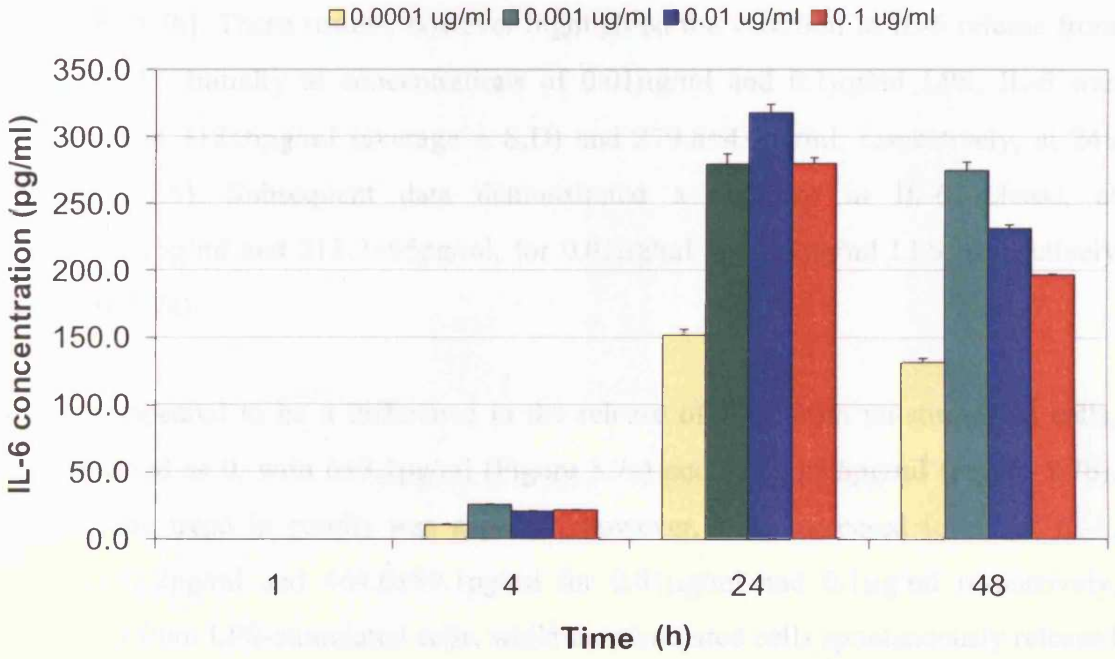


5.3.2 Interleukin-6 release from neutrophils, stimulated with lipopolysaccharide

5.3.2.1 Kinetics of interleukin-6 release from neutrophils

The production of IL-6 was quantified over time. In the present Study, neutrophils were incubated in the presence of LPS for a period of up to 48h. It was important to understand the kinetics of IL-6 release in this particular *in vitro* model, in order to anticipate what incubation times may be appropriate when cells were stimulated with the bacterial supernatants. Results demonstrate that IL-6 was released from the cells as time progressed. A maximal response was observed around the 24h time point, (Figure 5.6) for all LPS concentrations tested. This time point was used in all subsequent assays.

Figure 5.5: Time course for IL-6 production by neutrophils.
Levels of IL-6 (average ± SD) detected in cell culture supernatant over time for neutrophils stimulated in the presence of various concentrations of LPS at a cell density of $0.5x10^6$ cells/ml at 37°C/5% CO_2 for 1, 4, 24 and 48h. An increasing amount of IL-6 was released up to 24h, with data suggesting a maximal IL-6 response from cells, at 0.01μg/ml LPS.



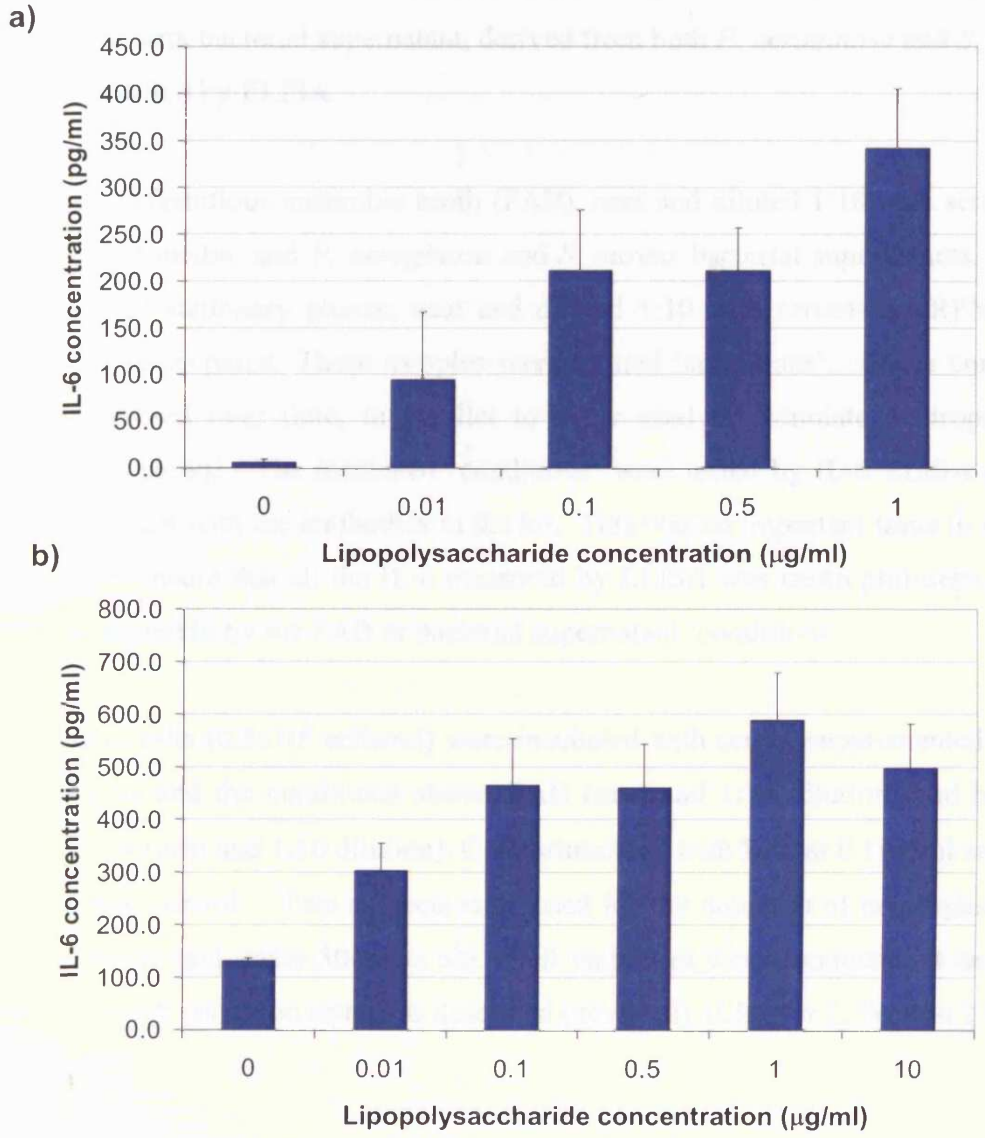
5.3.2.2 Dose-dependent interleukin-6 release from neutrophils when stimulated by LPS

Results demonstrated that a dose-dependent release of IL-6 by neutrophils from 0.01μg/ml-0.1μg/ml LPS (Figure 5.7a and 5.7b). There was no significant difference ($p>0.1$) in the release of IL-6 at LPS concentrations, ranging from 0.1μg/ml-10μg/ml (Figure 5.7b). These results, however highlighted the variation in IL-6 release from subject 2. Initially at concentrations of 0.01μg/ml and 0.1μg/ml LPS, IL-6 was released at 318±6pg/ml (average ± S.D) and 279.8±4.6pg/ml, respectively, at 24h (Figure 5.6). Subsequent data demonstrated a decrease in IL-6 release, at 94.2±73.1pg/ml and 211.2±65pg/ml, for 0.01μg/ml and 0.1μg/ml LPS, respectively (Figure 5.7a).

There appeared to be a difference in the release of IL-6 from un-stimulated cells, represented as 0, with 6±3.1pg/ml (Figure 5.7a) and 131.1±5.5pg/ml (Figure 5.7b). The same trend in results was apparent, however, with increased levels of IL-6, 302.1±52.2pg/ml and 464.6±89.1pg/ml for 0.01μg/ml and 0.1μg/ml respectively, released from LPS-stimulated cells, while un-stimulated cells spontaneously released IL-6. The release of IL-6, however, remained dose-dependent, following activation with LPS (Figure 5.7).

On all occasions, the response appeared to be maximal between 0.01μg/ml-0.1μg/ml. The increased response (consistently at least a two-fold difference) between the unstimulated and cells stimulated with 0.1μg/ml LPS, was deemed sufficient for the purposes of the present Study. LPS, at a concentration of 0.1μg/ml, was subsequently used as a positive control for bacterial supernatant responses.

Figure 5.7: Neutrophil dose-dependent release of IL-6.
Release of IL-6 (average ± SD, n = 3) from neutrophils, on two occasions (a) and (b) respectively, stimulated with LPS at concentrations 0.01-10μg/ml, at a cell density of $0.5x10^6$ cells/ml at 37°C/5% CO_2 for 24h.



5.3.3 Levels of interleukin-6 released from neutrophils determined by ELISA.

5.3.3.1 Method conditions

The levels of cytokine in cell culture media samples, harvested from neutrophils stimulated with bacterial supernatant, derived from both *P. aeruginosa* and *S. aureus*, were quantified by ELISA.

Samples of fastidious anaerobic broth (FAB), neat and diluted 1:10 with serum-free RPMI-1640 media, and *P. aeruginosa* and *S. aureus* bacterial supernatants, both at mid-log and stationary phases, neat and diluted 1:10 with serum-free RPMI-1640 media, were prepared. These samples were termed 'conditions'. These conditions were incubated over time, in parallel to those used to stimulate neutrophils (as described below). The incubated 'conditions' were tested by IL-6 ELISA and did not cross-react with the antibodies in the kit. This was an important issue to confirm in order to ensure that all the IL-6 measured by ELISA was neutrophil-derived, and not contributed to by the FAB or bacterial supernatant 'conditions'.

Neutrophil cells ($0.5x10^6$ cells/ml) were incubated with serum-supplemented RPMI-1640 media and the conditions above, FAB (neat and 1:10 dilution) and bacterial supernatant (neat and 1:10 dilution). Cells stimulated with LPS at 0.1μg/ml served as the positive control. Two subjects were used for the donation of neutrophils; both were females, and under 30 years old. Cell viabilities were conducted at each time point, for each condition tested, as described previously (Chapter 2, Section 2.5).

5.3.3.1.1 Duration

Neutrophils were stimulated for 1, 4, 24 and 48h, and the IL-6 quantified, by ELISA. Results showed that no cytokine was released at 1h, although IL-6 was detectable at 4h, with maximal cytokine release between 4-48h (Figure 5.8). This trend for IL-6 release from neutrophils exposed to bacterial supernatant correlated with the IL-6 release observed previously in cells incubated with LPS (Figure 5.6). Therefore, in the present Study, conditioned neutrophil media supernatant was collected at two time points only, 4 and 24h.

Additional work investigating the precise time of maximal release between 4-48h could have been assessed. However, due to the time required to isolate neutrophils from peripheral blood, prepare 24-well plates with cells and conditions, and the subsequent collection of conditioned samples and cell viability determination, post-incubation, only 4 and 24h sample collection time points were carried out.

5.3.3.1.2 Effect of neat bacterial supernatants

Upon incubating neutrophils from both donors with bacterial supernatants, data showed that neutrophil exposure to neat bacterial supernatant at the stationary growth phase for both bacteria (*P. aeruginosa* and *S. aureus*), produced very little, if any IL-6 (Figure 5.9). This lack of IL-6, with stationary phase supernatants, correlated with cell viability data, with only small numbers of viable neutrophil cells remaining at these particular conditions.

Figure 5.8: Effect of incubation time on the release of IL-6 from neutrophils, following stimulation with bacterial supernatant

Release of IL-6 (average ± SD, n = 3) from neutrophils, stimulated with FAB, neat bacterial supernatant, *P. aeruginosa* and *S. aureus* at mid-log. growth phase, and LPS at 0.1µg/ml, at 37°C/5% CO_2, for 1, 4, 24 and 48h at a cell density of $0.5x10^6$ cells/ml.

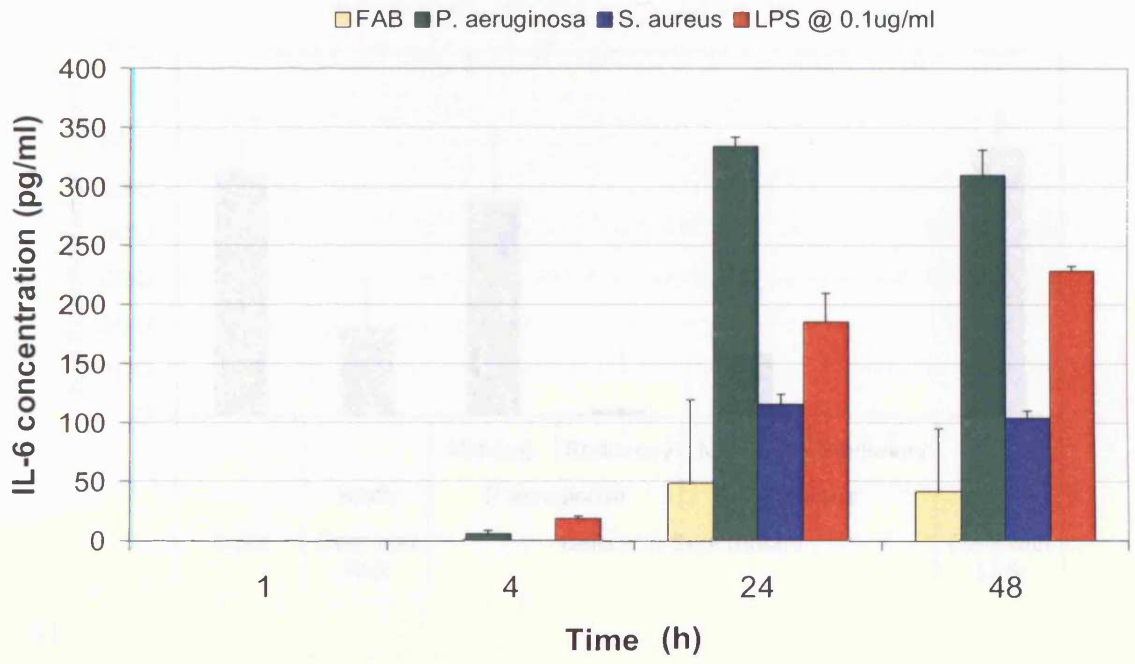
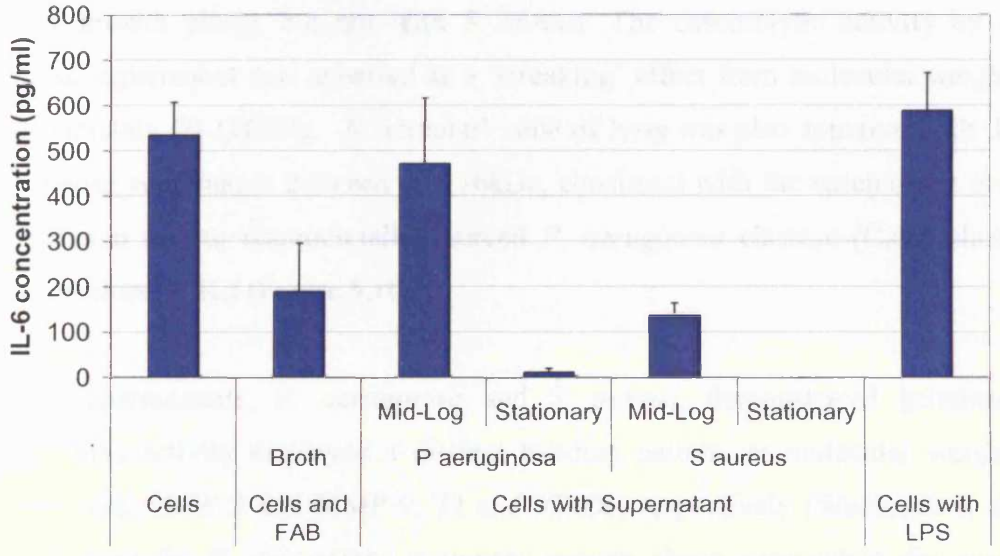


Figure 5.9: Effect of bacterial supernatant on release of IL-6 from neutrophils. Release of IL-6 (average ± SE, n = 3) from neutrophils, isolated from (a) subject 1 and (b) subject 2, stimulated with neat bacterial supernatant at 37°C/5% CO_2, for 24h at a cell density of $0.5x10^6$ cells/ml. Small amounts or no IL-6 were detected in cell media from neutrophils and neat bacterial supernatant.

a)



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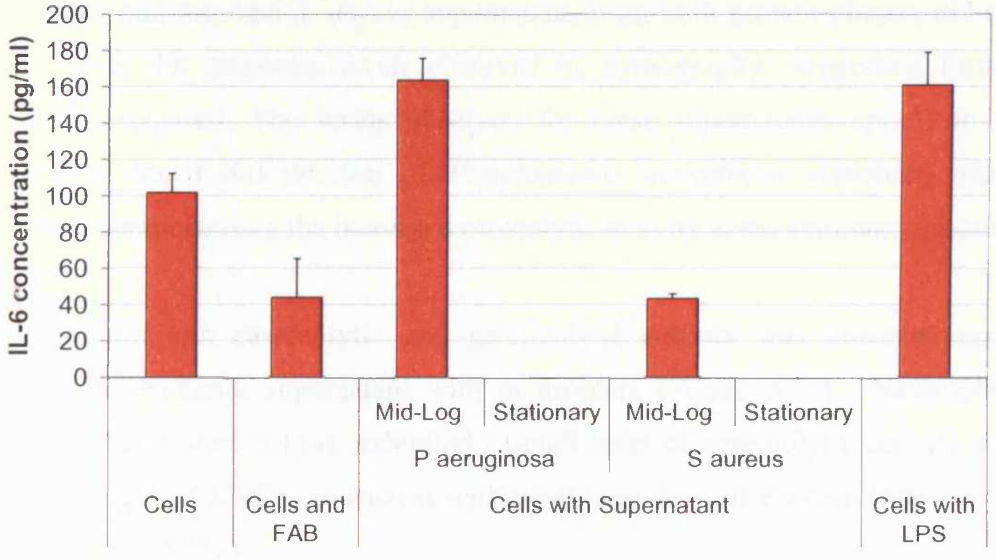


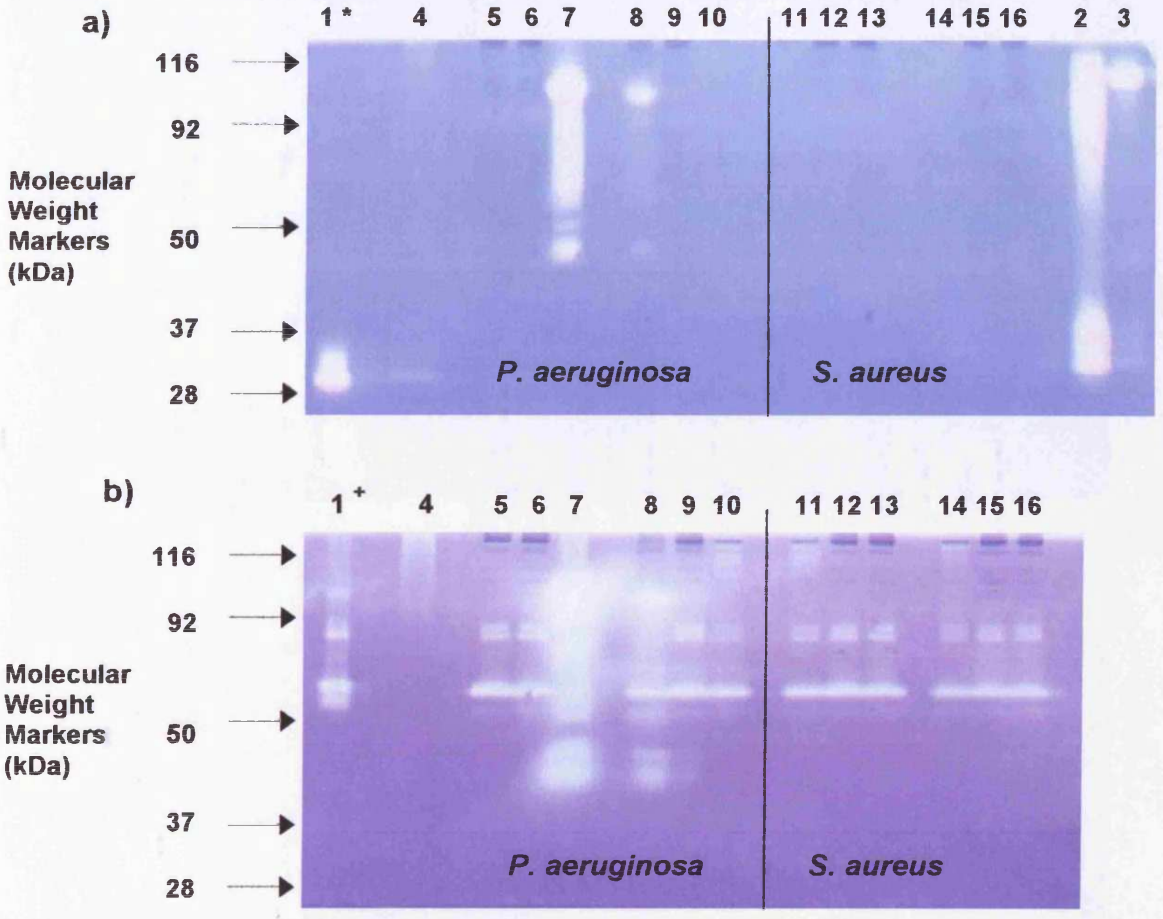
This lack of cell viability may be a result of extracellular toxins or enzymes, such as, proteases present in the bacterial supernatants used, thus subsequent analysis was performed investigating the proteolytic content of *P. aeruginosa* and *S. aureus*, using gelatin and casein zymography (Figure 5.10).

Caseinolytic activity was observed for the supernatant of *P. aeruginosa* at the stationary growth phase, but not with *S. aureus*. The caseinolytic activity by *P. aeruginosa* supernatant was apparent as a 'streaking' effect from molecular weights of approximately 50-116kDa. A 'circular' zone of lysis was also apparent with the *P. aeruginosa* supernatant between 92-116kDa, consistent with the caseinolytic area of degradation due to commercially sourced *P. aeruginosa* elastase (Calbiochem, Nottinghamshire, U.K.) (Figure 5.10a).

Bacterial supernatnants, *P. aeruginosa* and *S. aureus*, demonstrated gelatinase activity. This activity displayed a distinct banding pattern, at molecular weights consistent with MMP-2 and MMP-9, 72 and 92kDa, respectively (Weckroth et al., 1996). Diluting the *P. aeruginosa* stationary growth phase supernatant decreased both the caseinolytic and gelatinolytic activity. However, the amounts expressed by *P.aeruginosa* mid-log. and *S. aureus* supernatant, from both growth phases, did not appear to alter the gelatinise levels observed by zymography, suggesting further dilution was required. The levels observed for these supernatants appear to be equivalent to the 1:100 dilution of *Pseudomonas aeruginosa* stationary phase supernatant, demonstrating the increased proteolytic activity at the stationary phase.

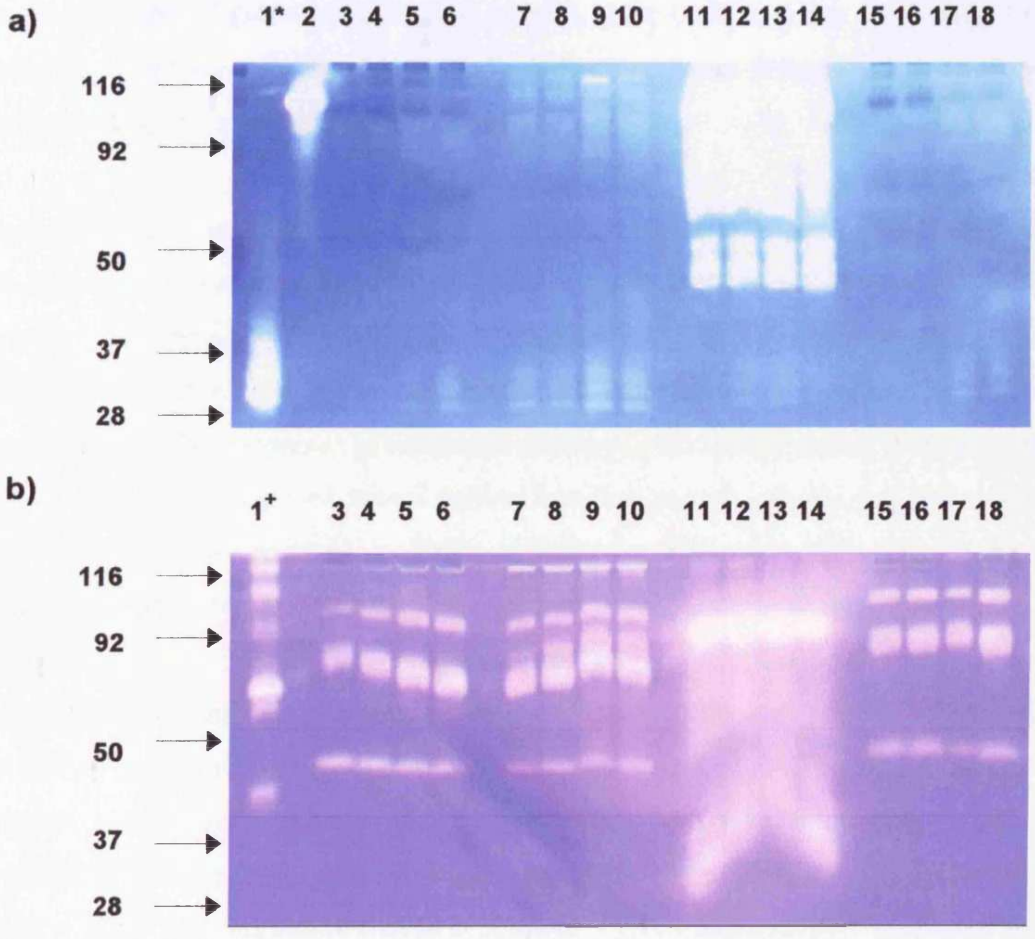
An increase in both caseinolytic and gelatinolytic activity was observed upon incubating the bacterial supernatant with neutrophils (Figure 5.11). Neutrophils themselves (3-6, Figure 5.11a), exhibited a small level of caseinolytic activity at a molecular weight of 30kDa, consistent with neutrophil-derived elastase (depicted in column 1, Figure 5.11a).

Figure 5.10: Caseinolytic and gelatinolytic activity of *P.aeruginosa* and *S. aureus* supernatants.
(a) Caseinolytic activity was observed in *P. aeruginosa* supernatant at stationary growth phase, loaded onto the gel neat and diluted 1:10. (b) All bacterial supernatants, both neat and diluted samples, demonstrated gelatinase activity, especially *P. aeruginosa* supernatant, at stationary growth phase.



1*	Human neutrophil elastase (control)	1+	Human MMP-2 & MMP-9
2	Human neutrophil proteinase 3 (control)		
3	Pseudomonas elastase (control)		
4	FAB (control)		
5	*P. aeruginosa* supernatant	Mid-log	Neat
6			1 in 10 dilution
10			1 in 100 dilution
7	*P. aeruginosa* supernatant	Stationary	Neat
8			1 in 10 dilution
9			1 in 100 dilution
11	*S. aureus* supernatant	Mid-log	Neat
12			1 in 10 dilution
13			1 in 100 dilution
14	*S. aureus* supernatant	Stationary	Neat
15			1 in 10 dilution
16			1 in 100 dilution

Figure 5.11: Protease expression in neutrophil media stimulated with bacterial supernatants.
(a) Caseinolytic and (b) gelatinolytic activity was measured from the conditioned media of neutrophils that had been incubated with *P. aeruginosa* supernatant and LPS for 1, 4, 24 and 48h at 37°C/5% CO_2. An increase in proteolytic activity was apparent when cells were conditioned with mid-log. growth phase supernatant, and further increased activity in the presence of stationary growth phase supernatant. There appeared to be a time-dependent increase in proteolytic activity.



1*	Human neutrophil elastase (control)	1+	Human MMP-2 & MMP-9 (control)
2	*Pseudomonas* elastase (control)		

3-6	Neutrophil cells		1, 4, 24 and 48h
7-10	Neutrophil cells and *P. aeruginosa*	Mid-log	1, 4, 24 and 48h
11-14	Neutrophil cells and *P. aeruginosa*	Stationary	1, 4, 24 and 48h
15-18	Neutrophil cells and LPS (0.1μg/ml)		1, 4, 24 and 48h

Gelatinase activity from neutrophils, stimulated with bacterial supernatant appeared to correlate with pro-MMP-9 and active MMP-9. Rapid release of MMP-9 by neutrophils in response to LPS has previously been shown by gelatin zymography (Pugin et al., 1999). A band, at the lower molecular weight of approximately 50kDa was also observed on the gelatin zymogram. No banding pattern was observed at 62kDa, corresponding to the molecular weight of MMP-2. As neutrophils are not known to express MMP-2 (Nglia et al., 2007), this result was not surprising. The 62kDa band detected in the supernatant of *P. aeruginosa* (Figure 5.11b), was not present in samples incubated with neutrophils.

It could be clearly seen that mid-log. growth phase supernatants, incubated over time with neutrophils appeared to increase the expression of proteases (Figure 5.11). An increase in elastase-like activity at low molecular weights (Figure 5.11a, lane 7-10), and gelatinase activity, at higher molecular weights (Figure 5.11b, lane 7-10), was also observed. The increased gelatinolytic activity at stationary phase, (Figure 5.10b, lanes 11-14), was associated with a diffuse banding pattern, which may indicate the presence of different protease isoforms or indeed a difference in the glycosylation pattern of these proteins.

It is hypothesised that the observed increase in caseinolytic and gelatinolytic activity was due to the contribution of neutrophil-derived proteases. The larger levels of proteases in stationary growth phase supernatants, incubated with neutrophils, in comparison with mid-log. growth phase supernatants could explain the lack of cell viability. This data suggested that neat stationary phase supernatants incubated with cells were detrimental to cytokine release. Degradation of cytokines by both host and bacterial proteases has been documented previously (Yager et al., 1997, Bank et al., 1999, Barbula et al., 1999, Parmeley et al., 1990).

In the present Study, neutrophil viability was maintained upon incubating the cells with diluted *P. aeruginosa* and *S. aureus* stationary growth phase bacterial supernatants. Supernatants diluted 1:10 and 1:100. However, the 1:100 dilution induced minimal cytokine expression. Therefore, in order to measure cytokine release from cells that maintain their viability over 24h, bacterial supernatant was diluted 1:10 with cell culture media, RPMI-1640. This allowed valid comparisons between the supernatant growth phases and type of bacteria to be made.

5.3.3.2 Levels of interleukin-6 released from neutrophils, determined by ELISA

The data presented herein is the average ± S.D., calculated for each subject from the three separate stimulations at the 24h time-point. Results show a minimal amount of cytokine was released at 4h, as previously determined (Figure 5.8), and therefore 4h data was not presented herein. The levels of IL-6 per $1x10^6$ cells were calculated to allow comparisons to be made, and to control for any differences in the actual viable cell number, when incubated with bacterial supernatants, between subjects on each occasion.

In addition, only 1:10 dilution data was presented here, due to loss of cell viability and therefore lack of IL-6 release from cells, incubated with neat stationary phase supernatants (Chapter 5, Section 5.3.3.1.2).

Although a significantly ($p=0.018$) lower levels of IL-6 was released on run 2, subject 1 (Figure 5.14), this data was still included in the analysis presented herein.

Upon stimulating neutrophils with bacterial supernatant, diluted 1:10, the release of IL-6 was significantly increased ($p=0.01$) from neutrophils, in response to LPS (1336±492pg), that served as the positive control, compared to cells incubated with broth (762±310pg) for subject 1 (Figure 5.12a).

However, upon averaging all three separate experimental runs, for subject 2, there appeared to be a significant increase ($p=0.004$) in release of IL-6, from LPS stimulated cells (297±30pg), compared to unstimulated cells (204±37pg), yet a significant decrease ($p=0.007$), compared to cells incubated with broth (608±232pg) (Figure 5.12b).

Previously, the release of IL-6 in response to LPS had been investigated. No determination had been made with cells incubated with FAB. Therefore, the contribution of increased IL-6 release from cells and FAB for subject 2 was unexpected, as it was not anticipated that a broth used to support bacterial growth would stimulate cells to increase their cytokine production. Additionally, the increase in IL-6 (from 204pg/ml to 297pg/ml), for neutrophils stimulated with LPS was less than the two fold increase, as previously observed (Figure 5.8), which was also unexpected.

The response of neutrophils to bacterial supernatant was analysed for both subjects 1 and 2, compared with cells incubated with broth and significant differences highlighted. However, significant differences, compared with unstimulated cells were also highlighted.

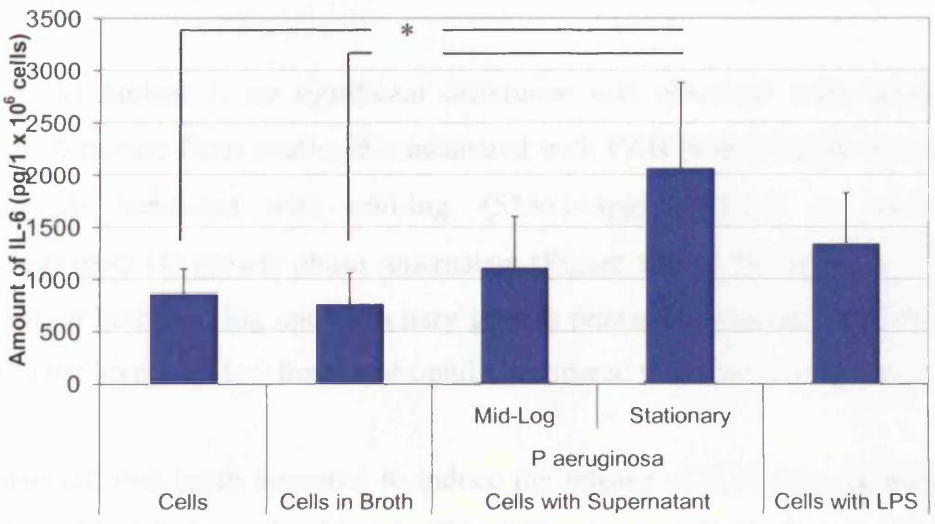
5.3.3.2.1 *Pseudomonas aeruginosa*

Significant increases in the levels of IL-6 released from neutrophils isolated from subject 1, were apparent (Figure 5.12a), upon incubation with *P. aeruginosa* supernatant, at stationary growth phase (2059±824pg), compared to both un-stimulated cell (864±238pg) (p=0.0046) and cells incubated with FAB (762±310) (p=0.003).

The ability of *P. aeruginosa* supernatant, at stationary growth phase, to increase the levels of IL-6 from neutrophils was also observed upon the use of neutrophils from the other donor, subject 2 (Figure 5.12b). The increased levels of IL-6 (1258±511pg) was significant when compared to un-stimulated cells (204±37pg) (p=0.0012) and cells incubated with FAB (608±232pg) (p=0.0080). Additionally, mid-log. supernatants were shown to significantly increase the levels of IL-6 from neutrophils (553±56pg) (p>0.0001) compared to un-stimulated neutrophils (204±37pg) for subject 2.

Figure 5.12: IL-6 release from neutrophil cells stimulated with *P. aeruginosa.* Release of IL-6 (average ± SD, n = 3) from neutrophils, measured by ELISA, (a) subject 1 and (b) subject 2, upon incubation with *P.aeruginosa,* at mid-log. and stationary growth phases. Significant increases (*) in IL-6 release from neutrophils incubated with stationary phase supernatant were observed for subject 1 (p=0.003) and subject 2 (p=0.0080), compared to cells incubated with FAB.

a)



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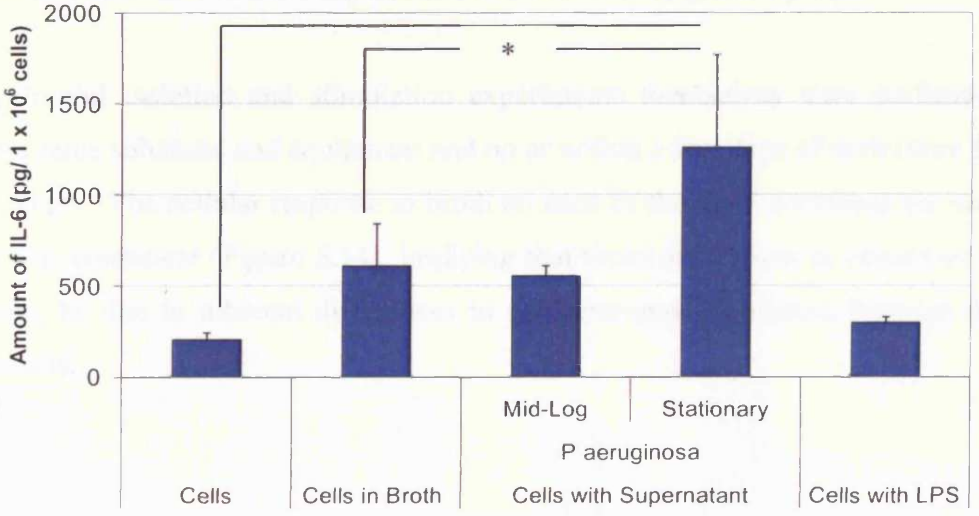


5.3.3.2.2 *Staphylococcus aureus*

Significant increases in the levels of IL-6 released from neutrophils, isolated from subject 1, were observed (Figure 5.13a), upon incubation with *S. aureus* supernatant, at stationary growth phase (1580±190) compared to both unstimulated cell (864±238) ($p=0.00006$) and cells incubated with FAB (762±310) ($p=0.0001$).

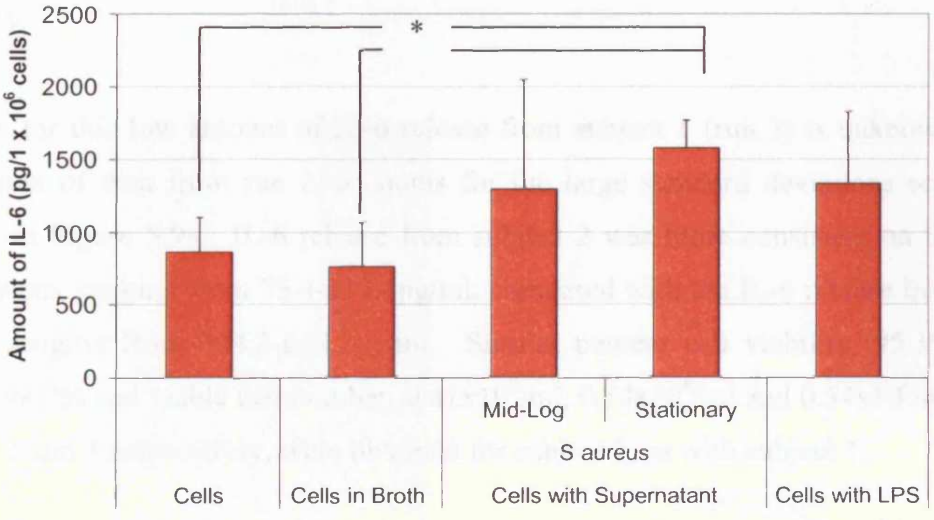
In contrast to subject 1, no significant difference was observed with subject 2, between IL-6 release from neutrophils incubated with FAB (608±232pg), compared to neutrophils incubated with mid-log. (533±144pg) ($p=0.24$) or stationary (725±155pg) ($p=0.14$) growth phase supernatant (Figure 5.13b). However, *S. aureus* supernatant, at both mid-log and stationary growth phase, did significantly increase ($p>0.0001$) the levels of IL-6 from neutrophils, compared to subject 2 cells alone.

It was observed that broth appeared to induce the release of IL-6 from neutrophils donated by subject 2, but not subject 1. This difference in cellular responses in the presence of FAB accounted for the lack of significant increase in IL-6 release in response to *S. aureus* bacterial supernatant at the stationary growth phase.

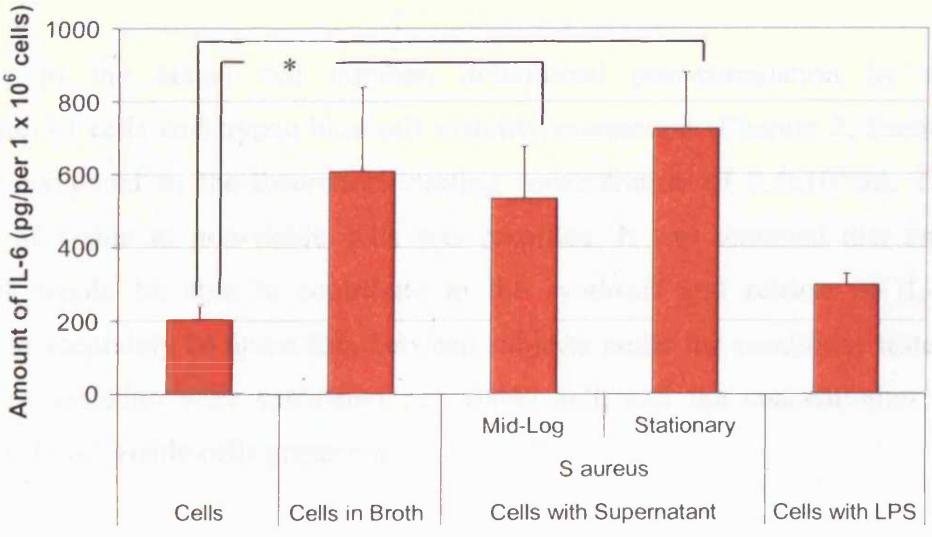
The neutrophil isolation and stimulation experiments themselves were performed using the same solutions and equipment and on or within a few days of each other for both subjects. The cellular response to broth on each of the three occasions for each subject was consistent (Figure 5.14), implying that these differences in responses to broth may be due to inherent differences in the neutrophil responses, between the two subjects.

Figure 5.13: IL-6 release from neutrophil cells stimulated with *S. aureus*. Release of IL-6 from neutrophils, measured by ELISA, of (a) subject 1 and (b) subject 2 upon stimulation with *S. aureus* at mid-log. and stationary growth phases. Significant increases (*) in IL-6 release from neutrophils incubated with stationary phase supernatant were observed for subject 1 ($p=0.0001$), compared to cells with broth. No significant difference was observed in the levels of IL-6 for subject 1, mid-log. or subject 2, mid-log. and stationary growth phases, compared to the levels of IL-6 from cells incubated with FAB.

a)



b)



5.3.3.3 Variability in cell responses per neutrophil isolation

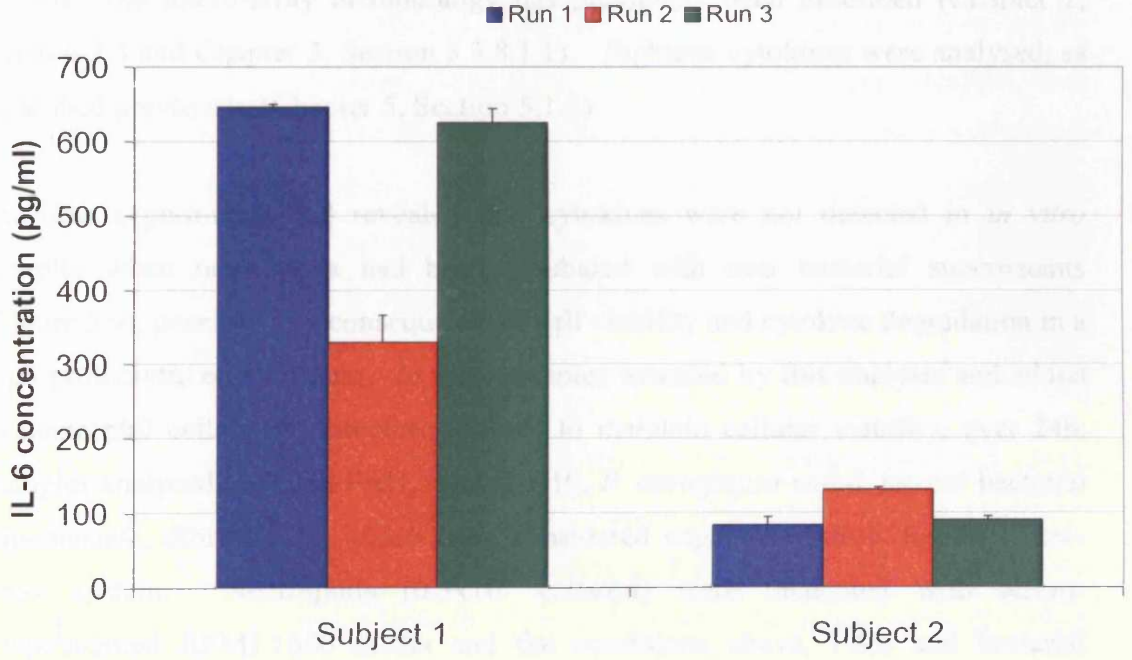
Analysis of data revealed some variation between neutrophil isolations (Figure 5.14). Intra-subject variation was observed with a significant decrease in IL-6 release from unstimulated neutrophils for subject 1 (run 2), compared to the other two occasion, (runs 1 and 3). This decrease was not associated with changes in cell viability, at 91.4%, 90.3% and 95.5%, or in the actual viable cell number observed on each occasion, $0.64x10^6$/ml, $0.56x10^6$/ml and $0.63x10^6$/ml, for runs 1, 2 and 3, respectively.

The reason for this low amount of IL-6 release from subject 1 (run 2) is unknown. The inclusion of data from run 2, accounts for the large standard deviations seen previously in Figure 5.9a. IL-6 release from subject 2 was more consistent on the three occasions, ranging from 75.4-133.1pg/ml, compared with the IL-6 release from subject 1, ranging from 304.2-649.2pg/ml. Similar percent cell viability, 95.3%, 100% and 98.2% and viable cell number, $0.41x10^6$/ml, $0.54x10^6$/ml and $0.54x10^6$/ml, for runs 1, 2 and 3 respectively, were obtained for subject 2, as with subject 1.

Inter-subject analysis revealed significantly ($p=0.0005$) lower amounts of IL-6 released, 101.57 ± 23.8pg/ml and 534 ± 159.4pg/ml, from un-stimulated neutrophils from subject 2, compared to subject 1. This variation indicates differences in the basal levels of cellular synthesis and secretion of IL-6 between the two subjects.

Differences in the actual cell number, determined post-stimulation by the trypsinisation of cells and trypan blue cell viability assessment (Chapter 2, Section 2.5), were compared to the theoretical starting concentration of $0.5x10^6$/ml. The proportion of viable to non-viable cells was recorded. It was assumed that only viable cells would be able to contribute to the synthesis and release of IL-6. Therefore, to accurately compare data between subjects under the conditions tested, the levels of cytokine were calculated per viable cell, and the concentration of cytokine per $1x10^6$ viable cells presented.

Figure 5.14: Cellular expression of IL-6 from both subjects neutrophils. Neutrophil cells were isolated from subject 1 and subject 2, incubated for 24h at 37°C/5% CO_2, at a cell density of $0.5x10^6$ cells/ml. Intra- and inter-subject variation in IL-6 ((average ± SD, n = 3) release from neutrophils was observed. Subject 1 had significantly lower levels ($p=0.018$) of IL-6 on run 2, compared with runs 1 and 3. No significant differences ($p>0.1$) were observed between run 1 and 3, for subject 1 or runs 1, 2 and 3, for subject 2. Lower levels of IL-6 released from subject 2 neutrophils, compared to subject 1 possibly indicative of inherent differences in neutrophil responses between individuals.



5.3.4 Levels of cytokine released from neutrophils, determined by micro-array

5.3.4.1 Method conditions

The levels of cytokines in cell culture samples, harvested from neutrophils, stimulated with bacterial supernatants from both *P. aeruginosa* and *S. aureus,* were also quantified using the cytokine micro-array. This technique allows for multiple cytokines to be analysed in one sample, opposed to the single analysis of IL-6 by ELISA. The micro-array methodology has previously been described (Chapter 2, Section 2.3 and Chapter 3, Section 3.3.8.1.1). Eighteen cytokines were analysed, as described previously (Chapter 5, Section 5.1.3).

Previous experiments had revealed that cytokines were not detected in *in vitro* samples when neutrophils had been incubated with neat bacterial supernatants (Figure 5.9), possibly as a consequence of cell viability and cytokine degradation in a high proteolytic environment. *In vitro* samples assessed by this analysis and added to neutrophil cells were therefore, diluted to maintain cellular viability, over 24h. Samples analysed included FAB, diluted 1:10, *P. aeruginosa* and *S. aureus* bacterial supernatants, diluted 1:10, which were considered negative controls for the micro-array system. Neutrophils ($0.5x10^6$ cells/ml) were incubated with serum-supplemented RPMI-1640 media and the conditions above, FAB and bacterial supernatant, at 1:10s. Additionally, cytokines were also measured from cells, stimulated with LPS, at 0.1μg/ml.

Due to limitations of cost in conducting this type of analysis, samples were rationalised. Samples were analysed from both neutrophil donors, subject 1 (run 2) and subject 2 (run 1), only at the 24h time-point. Duplicate samples were analysed in triplicate, resulting in 6 data points for each cytokine, on one ocassion. The data presented herein, is the average and S.D calculated for each sample. Within this analysis, all samples fell onto the standard curve for each cytokine, with the exception of one sample result for angiogenin for subject 2, at mid-log. *S. aureus* bacterial supernatant, which required a 1:4 dilution.

5.3.4.2 Cellular level of cytokine expression

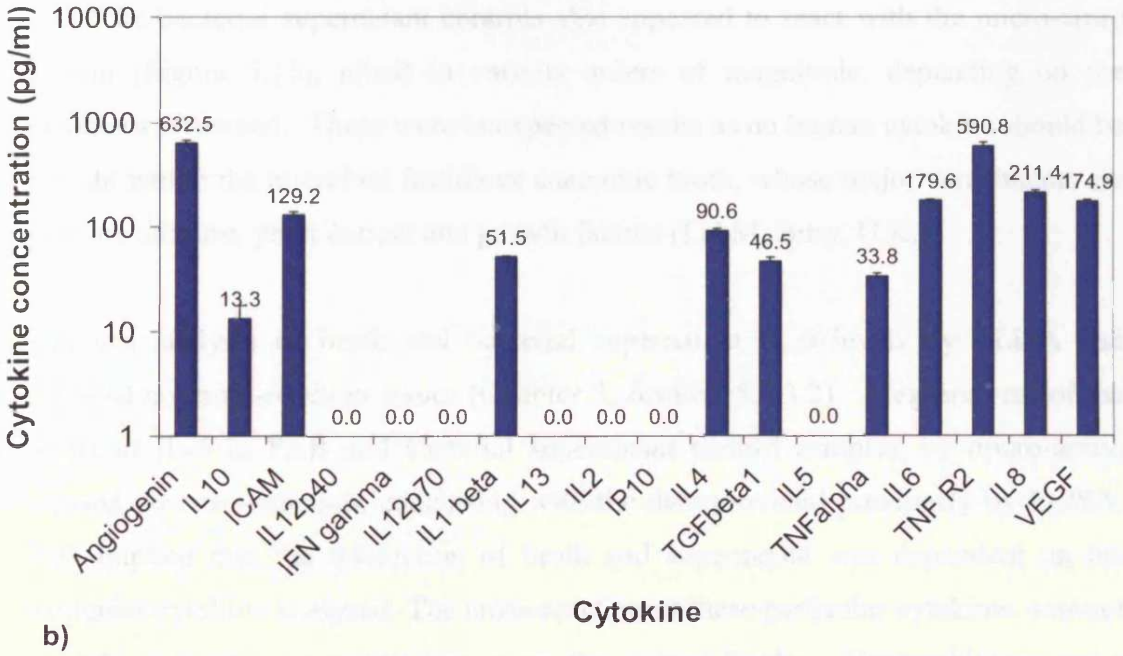
The levels of each cytokine (pg/ml) detected in un-stimulated neutrophils was plotted for subject 1 (Figure 5.15a), and for subject 2 (Figure 5.15b). Analysis revealed that of all the cytokines measured, angiogenin was present in the largest quantities of 632.5±39.3 pg/ml and 2495.4±215.9 pg/ml,for subjects 1 and 2, respectively.

Consistency was observed between subjects in the lack of expression of the cytokines IL-2, IL-5, IL-12p40, IL-12p70 and IFN-γ. The lack of expression for these five particular cytokines, from unstimulated neutrophil cell samples, was concurrent with findings in the literature that the major sources of these cytokines are cells other than neutrophils, including T-lymphocytes, natural killer cells, monocytes/macrophages, basophils, astrocytes, eosinophils, and mast cells (Chapter 1, Section 1.6). Biologically active IFN-γ appears to be released from neutrophils, but only upon stimulation with IL-12 (Ethuin et al., 2004).

Subject 2 also expressed IL-13 and IP-10, whereas no expression of either of these cytokines was observed for subject 1. The release of IL-13 in subject 2 and the release of IL-10 by both subjects was surprising as it is reported that neutrophils do not express these cytokines *in vitro* (Reglier et al., 1998, Cassatella, 1999). The other cytokines measured, were detected in varying amounts, in both subjects.

Figure 5.15: Neutrophil cytokine release profile.
Micro-array analysis of the levels of cytokine, (average ± SD, n = 2), from un-stimulated neutrophil cells isolated from (a) subject 1 and (b) subject 2. Data showed a lack of expression of IL-2, IL-5, IL-12p40, IL-12p70, and IFN-γ. The levels of the other cytokines varied, both in the levels of one cytokine to another, but additionally with respect to the levels of a particular cytokine expressed between subjects 1 and 2.

a)



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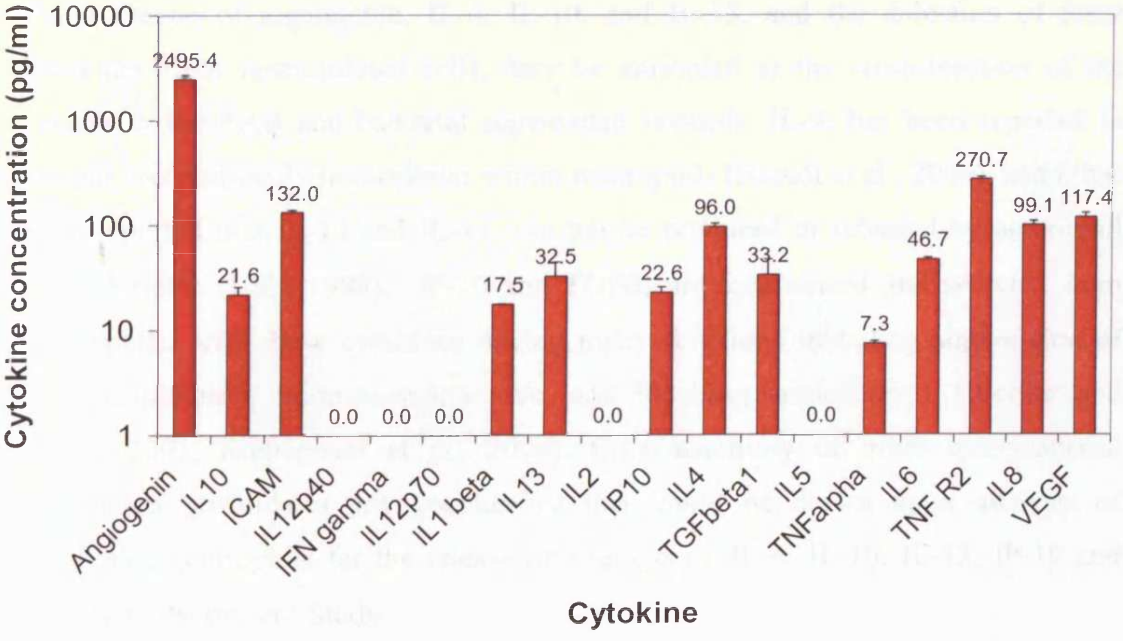


5.3.4.3 Cross-reactivity of fastidious anaerobic broth (FAB) and bacterial supernatant

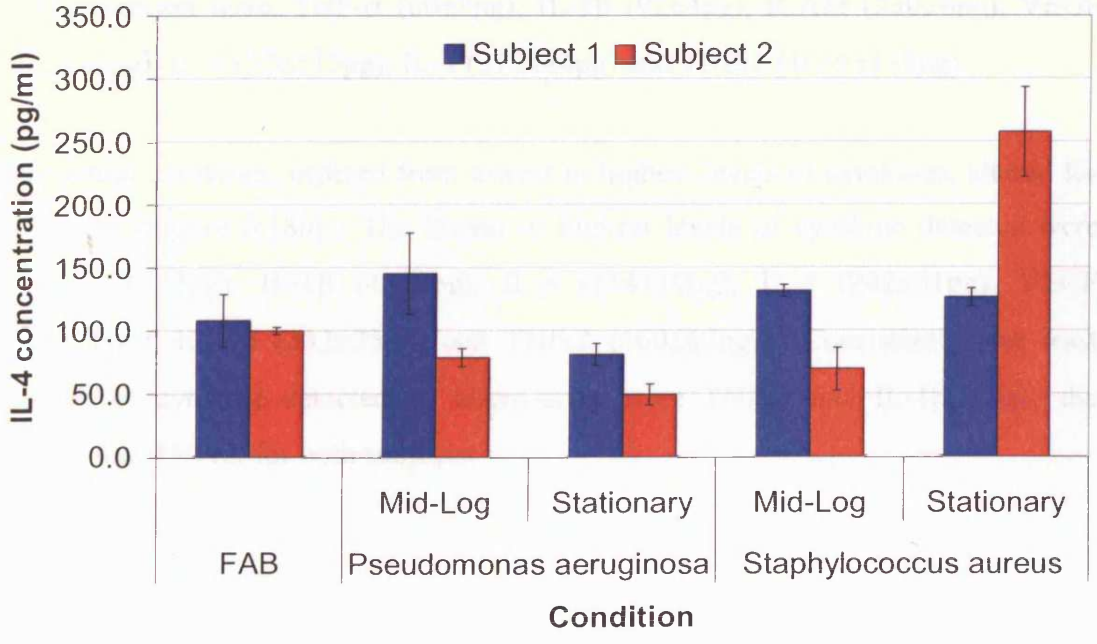
Data suggested that for the number of cytokines measured, FAB, the media used to support bacterial growth whilst generating bacterial supernatant, cross-reacted with the system, yielding positive results equivalent to or better than the neutrophil samples themselves, for angiogenin, IL-4, IL-10, IL-13, IP-10 and TGFβ_1 (Table 5.3). The bacterial supernatant controls also appeared to react with the micro-array system (Figure 5.16), albeit in various orders of magnitude, depending on the cytokines measured. These were unexpected results as no human cytokine should be present within the microbial fastidious anaerobic broth, whose major constituents are peptone mixture, yeast extract and growth factors (LabM, Bury, U.K.).

Previous analysis of broth and bacterial supernatant IL-6 levels by ELISA had revealed no cross-reaction issues (Chapter 5, Section 5.3.3.2). Measurement of the levels of IL-6 in FAB and bacterial supernatant control samples, by micro-array, showed no cross-reaction, concurring with the data provided previously by ELISA. This implied that the interaction of broth and supernatant was dependent on the particular cytokine analysed. The cross-reaction of these particular cytokines was not deemed as having a negative impact on the present Study. Neutrophils are not a major source of angiogenin, IL-4, IL-10, and IL-13, and the detection of these cytokines from unstimulated cells, may be attributed to the cross-reaction of the system to the FAB and bacterial supernatant controls. IL-4, has been reported to remain predominantly intracellular within neutrophils (Brandt et al., 2000), and it has been reported that IL-10 and IL-13, can not be produced or released by neutrophil cells (Reglier et al., 1998). IP-10 and TGFβ_1 are synthesised and secreted from neutrophils, with these cytokines having many functions including suppression of cell proliferation, immuno-suppressive, and blocking angiogenesis (Werner and Grose, 2003, Romagnani et al., 2004). Cross-reactivity of broth and bacterial supernatant invalidates any conclusions that could be drawn upon analysis of stimulated neutrophils for the release of angiogenin, IL-4, IL-10, IL-13, IP-10 and TGFβ_1, in the present Study.

Table 5.3: Cross-reactivity of FAB with human cytokine micro-array.
FAB samples revealed positive results for certain cytokines analysed, namely angiogenin, IL-4, IL-10, IL-13, IP-10, and TGF-β_1, although no human cytokine would be present in this synthetic microbial broth media.

	Amount of cytokine (pg/ml)			
	Neutrophil		FAB control	
Cytokine	Average	Standard deviation	Average	Standard deviation
Angiogenin	632.5	39.3	696.5	63.9
IL-10	13.3	4.4	50.4	5.1
IP-10	0	0	22.2	5.8
IL-13	0	0	650.5	278.5
IL-4	90.6	4.8	108.0	20.7
TGF-β_1	46.5	4.5	60.2	4.5

Figure 5.16: Amount of cytokine (average ± SD, n = 2) detected in FAB and bacterial supernatant controls.
Bacterial supernatant derived from *P. aeruginosa* and *S. aureus* reacted positively with the micro-array system, as demonstrated here with IL-4.



5.3.4.4 Neutrophil cell viability incubated with bacterial supernatant diluted 1:10

Cellular viability for these samples was recorded, with difference between the two subjects being evident (Figure 5.17). The levels of viable cells that could contribute to the cytokine levels measured were therefore different between the two subjects. As a consequence of these observations, the levels of cytokine per viable cell were calculated, which yielded very low concentrations per cell. Therefore, the data presented from this point forward, was expressed as the amount of cytokine produced per $1x10^6$ cells, to enable data to be compared.

5.3.4.5 Comparison of cellular expression in the absence and presence of fastidious anaerobic broth

Of the eighteen cytokines measured by micro-array, seven cytokines did not show any cross-reactivity issues with either the FAB itself or bacterial supernatants. These cytokines, IL-1β, IL-6, IL-8, ICAM, VEGF, TNF-α, and TNFr2, were analysed in further detail (Figure 5.18). These cytokines had no or minimal background values attributed to the broth or bacterial supernatant controls. The levels of these seven cytokines secreted and detected by micro-array per $1x10^6$ neutrophil cells varied. The levels of cytokine from subject 1 neutrophils (Figure 5.18a), lowest to highest levels detected were, TNF-α (60±8pg), IL-1β (92±4pg), ICAM (230±6pg), VEGF (312±26pg), IL-6 (320±15pg), IL-8 (378±44pg) and TNFr2 (1055±188pg).

The actual cytokines, ordered from lowest to highest levels of cytokines, altered for subject 2 (Figure 5.18b). The lowest to highest levels of cytokine detected were TNF-α (17±2pg), IL-1β (43±2pg), IL-6 (114±10pg), IL-8 (242±51pg), VEGF (286±66pg), ICAM (332±35pg) and TNFr2 (660±67pg). Consistently, the least amount of cytokine detected by micro-array were TNF-α and IL-1β, while the largest was TNFr2, for both subjects.

It is possible that neutrophils do not synthesise and release TNF-α or alternatively, the high levels of TNFr2 detected in the system would account for the low levels of TNF-α reported.

However, with these seven cytokines, the response of cells incubated with FAB varied from inhibitory to stimulatory depending on the neutrophil donor, subject 1 or 2. This had been highlighted previously (Chapter 5, Section 5.3.3.2.1, Figure 5.12 and Section 5.3.3.2.2, Figure 5.13). Neutrophils incubated with broth appeared to produce equivalent or significantly less cytokine per $1x10^6$ cells, than un-stimulated neutrophils in subject 1 (Figure 5.18a), with the converse trend for subject 2 (Figure 5.18b), being evident despite equivalent viable cell numbers with both subjects.

In order to determine the stimulatory effect of bacterial supernatants on neutrophil cells, the data presented compares the bacterial supernatant responses with those observed with the neutrophils incubated with FAB. Bacterial supernatants were generated in FAB, therefore, any effects observed from bacterial supernatant-stimulated cells, should be significantly different from this condition. Data suggested that FAB contribute to the level of cytokine liberated from the neutrophil.

Figure 5.17: Neutrophil viability, when incubated with various conditions, FAB, bacterial supernatants and LPS.

There appeared to be some variation in cell viability for subject 1, ranging from 62.6-100% viability, depending on the cells incubation conditions. Cell viability ranged between 93-100% for all conditions in subject 2.

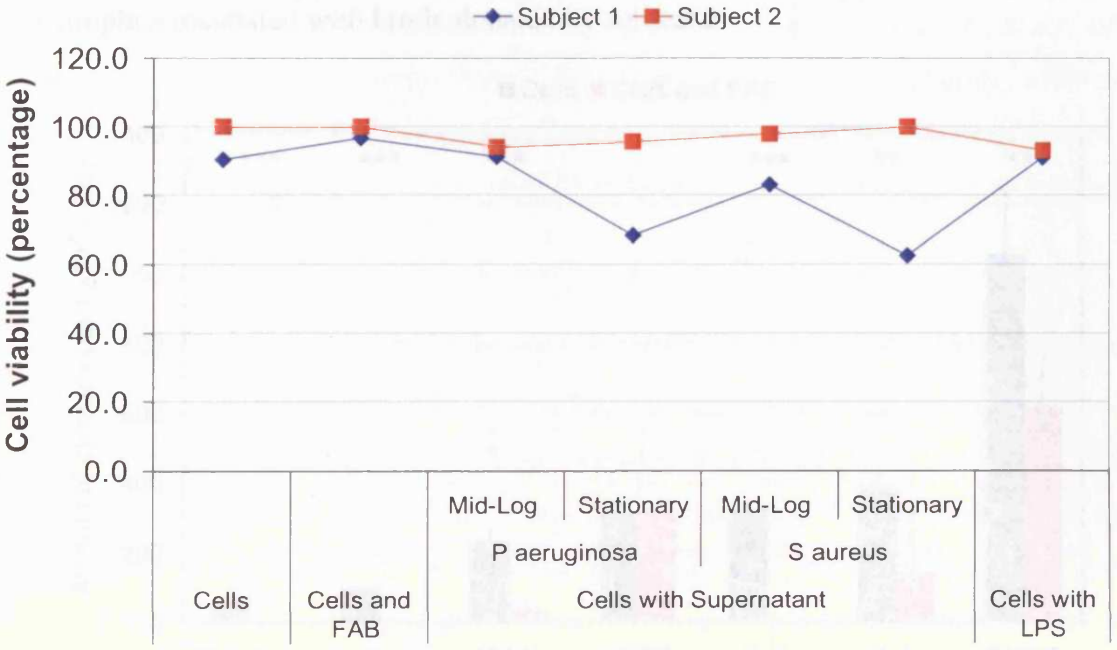
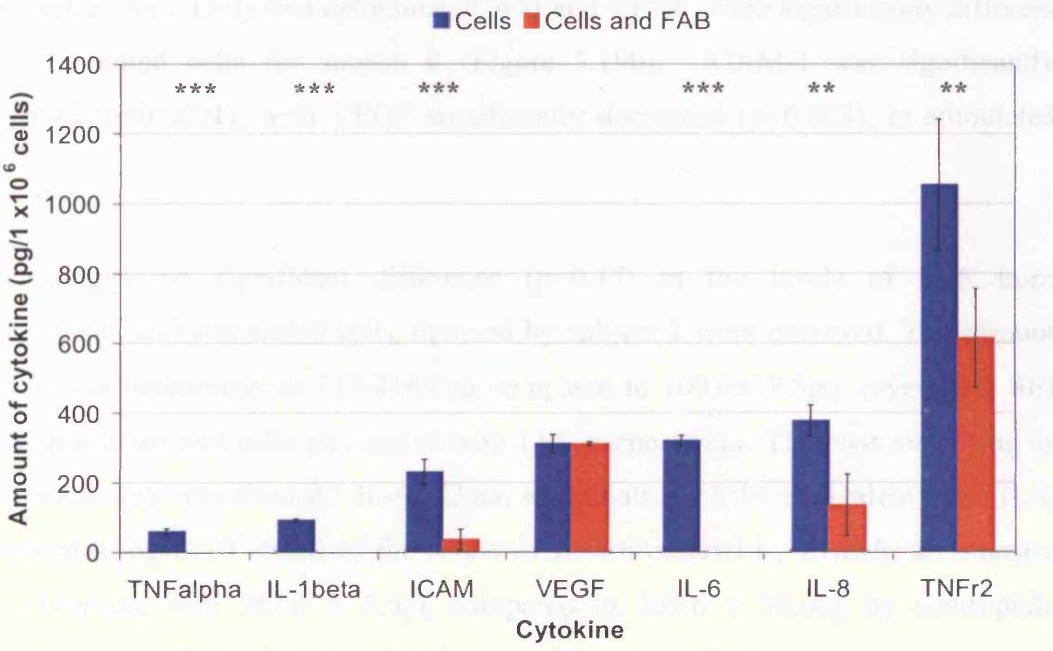
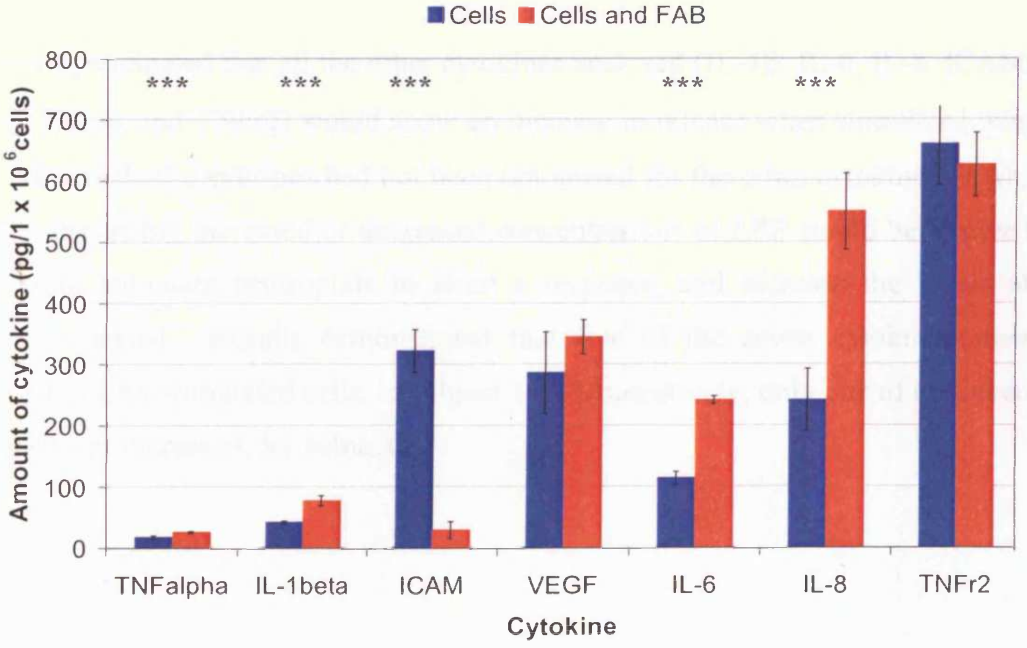


Figure 5.18: Effect of FAB on cytokine release by neutrophils.
The levels of cytokine, per $1x10^6$ cells, by micro-array in neutrophil cell samples from (a) subject 1 and (b) subject 2. The levels of each cytokine was either equivalent or significantly decreased as indicated by ** ($p<0.01$) and *** ($p<0.001$), in neutrophils incubated with broth for subject 1. In contrast, significant increases were observed in the pro-inflammatory cytokines (TNF-α, IL-1β, IL-6 and IL-8) by neutrophils incubated with broth, donated by subject 2.

a)





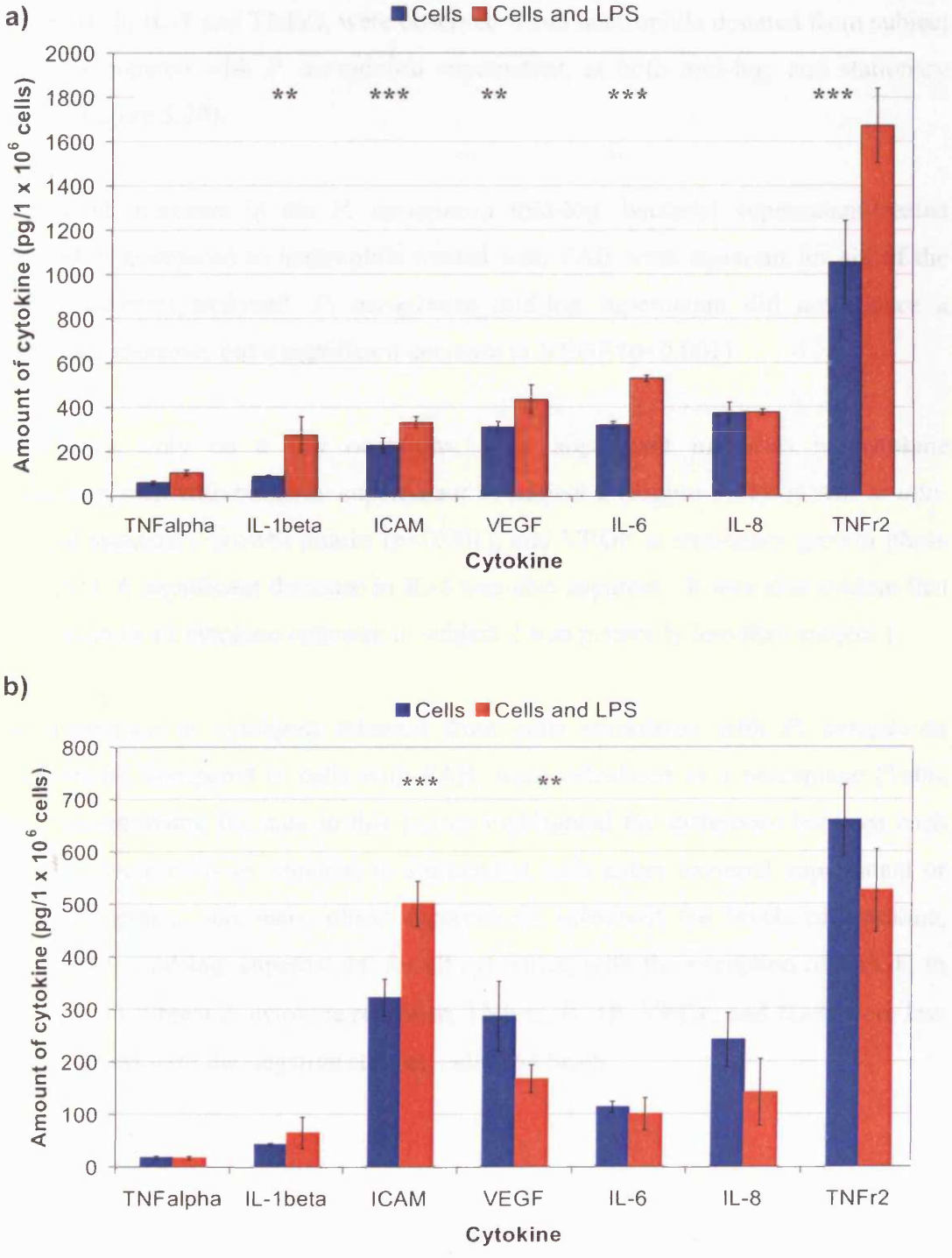
5.3.4.6 Comparison of cellular expression in the absence and presence of lipopolysaccharide

LPS served as the positive control in cell stimulations with bacterial supernatants. Results showed significant increases in the release of IL-1β ($p=0.001$), IL-6 ($p<0.0001$), ICAM ($p=0.0001$) and VEGF ($p=0.0017$), from neutrophils from subject 1 (Figure 5.19a). Only two cytokines, ICAM and VEGF, were significantly different to unstimulated cells for subject 2 (Figure 5.19b). ICAM-1 was significantly increased ($p<0.0001$), with VEGF significantly decreased ($p=0.003$), in stimulated cells.

Interestingly, no significant difference ($p=0.17$) in the levels of IL-6 from unstimulated and stimulated cells, donated by subject 2 were observed. The amount of IL-6 was determined as 113.9±6.0pg compared to 100.6±17.5pg (average ± SE) in un-stimulated and cells stimulated with LPS, respectively. This was surprising as the system was optimised for IL-6. Upon examination of the equivalent data (IL-6 measured in pg/1x10^6 cells) of the levels of IL-6 measured by ELISA, an increase was observed, with 281.6 ± 5.5pg compared to 203.6 ± 28.0pg by neutrophils stimulated with LPS and unstimulated neutrophils, respectively.

It was not anticipated that all the other cytokines analysed (IL-1β, IL-6, IL-8, ICAM, VEGF, TNF-α and TNFr2) would show an increase in release when stimulated with LPS. The method conditions had not been optomised for the other cytokines. It was possible that either increased or decreased concentrations of LPS would be required in order to stimulate neutrophils to elicit a response, and increase the levels of cytokine released. Results demonstrated that five of the seven cytokines were increased in LPS-stimulated cells, in subject 1. Comparatively, only one of the seven cytokines was increased, for subject 2.

Figure 5.19: Effects of LPS on cytokine release, by neutrophils.
The levels of cytokine, per $1x10^6$ cells, by micro-array in neutrophils from (a) subject 1 and (b) subject 2. The levels of each cytokine was either equivalent (TNF-α and IL-8) or significantly increased as indicated by * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$) in cells incubated with LPS for subject 1. In contrast, only ICAM was significantly increased ($p<0.001$) in neutrophils, donated by subject 2.



5.3.4.7 Cytokine release, following stimulation of neutrophils with bacterial supernatant

5.3.4.7.1 *Pseudomonas aeruginosa*

Significantly increased amounts ($p<0.001$) of all 7 cytokines, TNF-α, IL-1β, ICAM, VEGF, IL-6, IL-8 and TNFr2, were observed when neutrophils donated from subject 1 were incubated with *P. aeruginosa* supernatant, at both mid-log. and stationary phases (Figure 5.20).

Significant increases in the *P. aeruginosa* mid-log. bacterial supernatant-treated neutrophils compared to neutrophils treated with FAB were apparent for six of the seven cytokines analysed. *P. aeruginosa* mid-log supernatant did not induce a significant increase, but a significant decrease in VEGF ($p<0.001$).

Conversely, only on a few occasions, were significant increases in cytokine production seen with bacterial supernatant in subject 2 (Figure 5.21). ICAM at mid-log. and stationary growth phases ($p<0.001$), and VEGF at stationary growth phase ($p<0.001$), A significant decrease in IL-8 was also apparent. It was also evident that the magnitude of cytokine response in subject 2 was generally less than subject 1.

The difference in cytokines released from cells stimulated with *P. aeruginosa* supernatants, compared to cells with FAB, were calculated as a percentage (Table 5.4). Summarising the data in this format highlighted the difference between each subjects' neutrophils in response to stimulation with either bacterial supernatant or LPS. Generally, stationary phase supernatants increased the levels of cytokine, compared to mid-log. supernatant, for all cytokines, with the exception of VEGF, in subject 1. In subject 2, cytokine response, TNF-α, IL-1β, VEGF, and IL-8, were less than observed with the negative control, cells and broth.

When neutrophils from subject 1 were stimulated with LPS, an increase in cytokine release was observed ranging from 40-530.5%, In contrast, with subject 2, only 1 cytokine (ICAM) was found to increase (1577%), in response to LPS.

Table 5.4: Average percentage differences in cytokine responses of neutrophils stimulated with *P. aeruginosa* supernatants compared to neutrophils in FAB, for both subjects.

Negative responses are highlighted by a negative sign (-) and are orange in colour, as opposed to green. The majority of cytokines responses are positive, for subject 1, indicating an increase in amount when treated with bacterial supernatant.

		Cytokine						
Subject	Supernatant Growth phase	TNF-α	IL-1β	ICAM	VEGF	IL-6	IL-8	TNFr2
1*	Mid-log	35.1	42.3	325.8	-15.2	309.6	492.4	79.8
	Stationary	28.7	118.0	1485.5	95.0	722.1	1123.2	278.2
	LPS	105.6	278.8	819.1	40.4	530.5	178.9	171.7
2	Mid-log	-19.1	7.0	1043.3	-26.8	7.1	-10.8	-8.4
	Stationary	-100	-43.7	1937.7	92.8	22.0	-36.9	67.5
	LPS	-35.4	-16.7	1577.3	-51.5	-58.4	-74.2	-16.0

* Actual amount of cytokine is stated as the percentage for TNF-α, IL-1β and IL-6. No cytokine was measurable from cells incubated with broth from neutrophils donated by subject 1.

Figure 5.20: Effect of bacterial supernatants, *P. aeruginosa,* on subject 1 neutrophils.
(a) Mid-log. and (b) stationary phase supernatant on cytokine production from $1x10^6$ neutrophils, at 24h, donated by subject 1 (average ± SD). *P. aeruginosa* mid-log and stationary phase significantly (***, $p<0.001$, ** $p<0.01$) increased cytokine production, compared to IL-6 release from cells and FAB. The only exception was *P aeruginosa* mid-log. phase for VEGF.

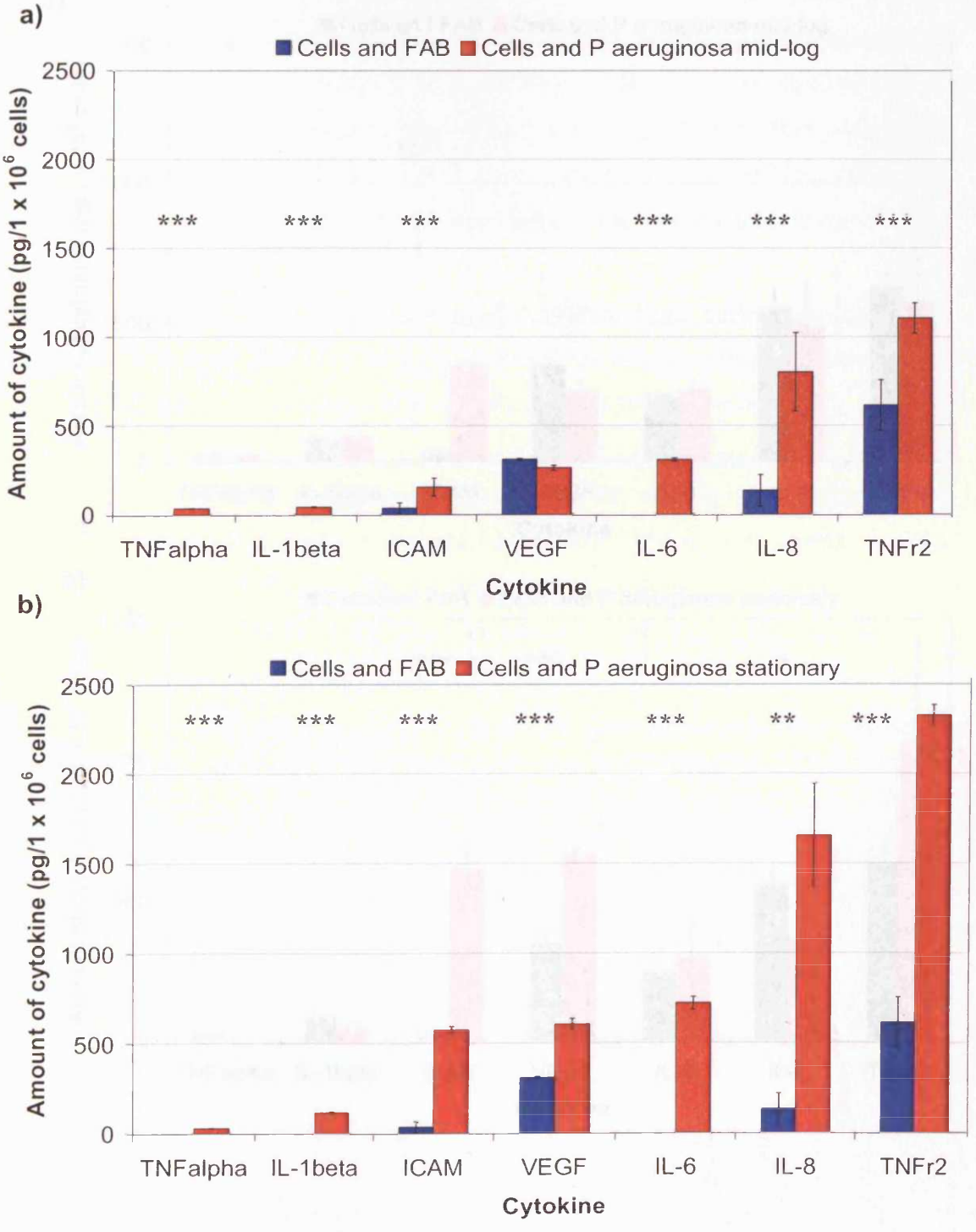
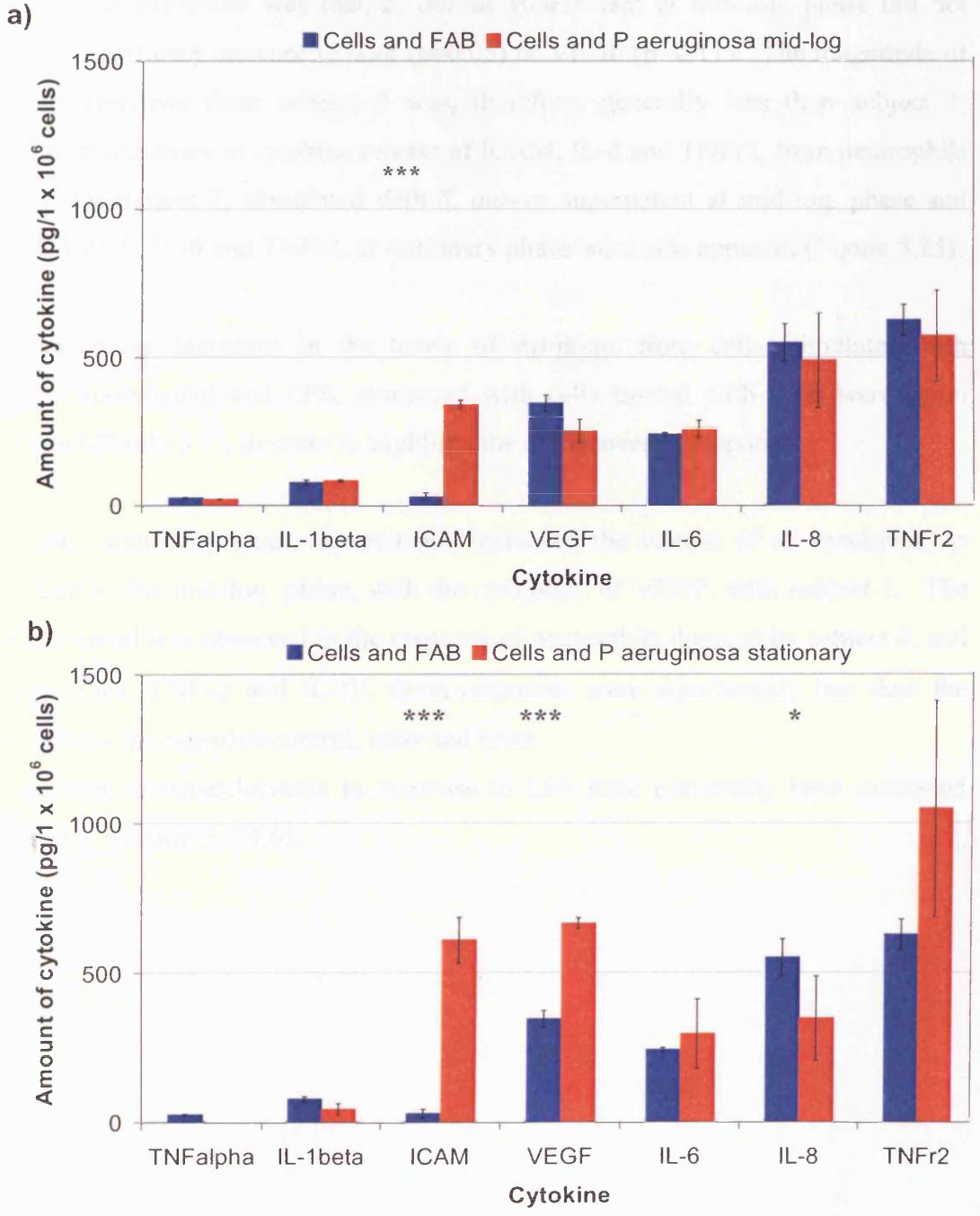


Figure 5.21: Effect of bacterial supernatants, *P. aeruginosa*, subject 2 neutrophils. (a) Mid-log. and (b) stationary phase supernatant on cytokine production from $1x10^6$ neutrophils, at 24h, donated by subject 2 (average ± SD). On occasion, *P. aeruginosa* at mid-log and stationary phase significantly (* $p < 0.05$, ** $p<0.01$ and *** $p<0.001$) increased cytokine production. In the majority of the analysis, the level of cytokine detected in cells with FAB appeared to negate the effect of the supernatant, for subject 2.



5.3.4.7.2 *Staphylococcus aureus*

The same trends were also evident for *S. aureus*, as previously observed with *P. aeruginosa*, with significantly increased amounts of all 7 cytokines, TNF-α, IL-1β, ICAM, VEGF, IL-6, IL-8 and TNFr2, from neutrophils donated from subject 1, incubated with *S. aureus* supernatant at both mid-log. and stationary phases (Figure 5.22). The exception was that, *S. aureus* supernatant at mid-log. phase did not induce a significant increase in IL-8 ($p=0.05$) or VEGF ($p=0.11$). The magnitude of cytokine response from subject 2 was, therefore, generally less than subject 1. Significant increases in cytokine release of ICAM, IL-8 and TNFr2, from neutrophils donated by subject 2, stimulated with *S. aureus* supernatant at mid-log. phase and ICAM, VEGF, IL-6 and TNFr2, at stationary phase were also apparent (Figure 5.23).

The percentage increases in the levels of cytokine, from cells stimulated with bacterial supernatant and LPS, compared with cells treated with FAB were again calculated (Table 5.5), in order to highlight the differences in response.

Generally, stationary phase supernatants increased the release of all cytokines, as compared to the mid-log. phase, with the exception of VEGF, with subject 1. The converse trend was observed in the response of neutrophils donated by subject 2, and for cytokines, TNF-α and IL-1β, these responses were significantly less than the release from the negative control, cells and broth.
The percent increase/decrease in response to LPS have previously been discussed (Chapter 5, Section 5.3.4.6).

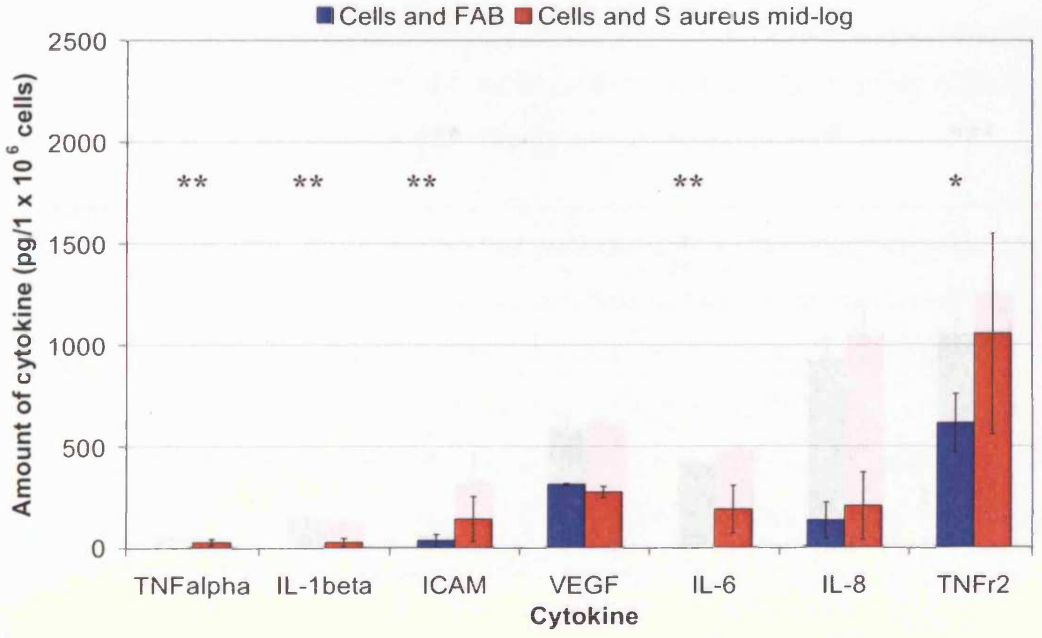
Table 5.5: Average percentage difference in cytokine response of neutrophils stimulated with *S. aureus* supernatants compared to neutrophils in FAB, for both subjects.

Negative responses are highlighted by a negative sign (-) and are orange in colour, as opposed to green. The majority of cytokines responses were negative, when cells were treated with bacterial supernatant compared to cells in broth.

		Cytokine						
Subject	Supernatant Growth phase	TNF-α	IL-1β	ICAM	VEGF	IL-6	IL-8	TNFr2
1	Mid-log	27.0	27.5	288.5	-11.9	189.1	51.6	71.8
	Stationary	68.4	109.9	834.2	67.6	850.2	240.4	212.6
	LPS	105.6	278.8	819.1	40.4	530.5	178.9	171.7
2	Mid-log	-16.8	-10.4	525.1	5.4	17.6	14.1	18.6
	Stationary	-8.2	-27.4	1289.5	19.2	48.1	6.7	40.9
	LPS	-35.4	-16.7	1577.3	-51.5	-58.4	-74.2	-16.0

Figure 5.22: Effect of bacterial supernatant, *S. aureus*, on subject 1 neutrophils. (a) Mid-log. and (b) stationary phase supernatant on cytokine production, per $1x10^6$ neutrophils, at 24h, donated by subject 1 (average ± SD).
S. aureus at mid-log and stationary phase significantly (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$) increased cytokine production, with the exception of *S. aureus* mid-log. for IL-8 and VEGF.

a)



b)

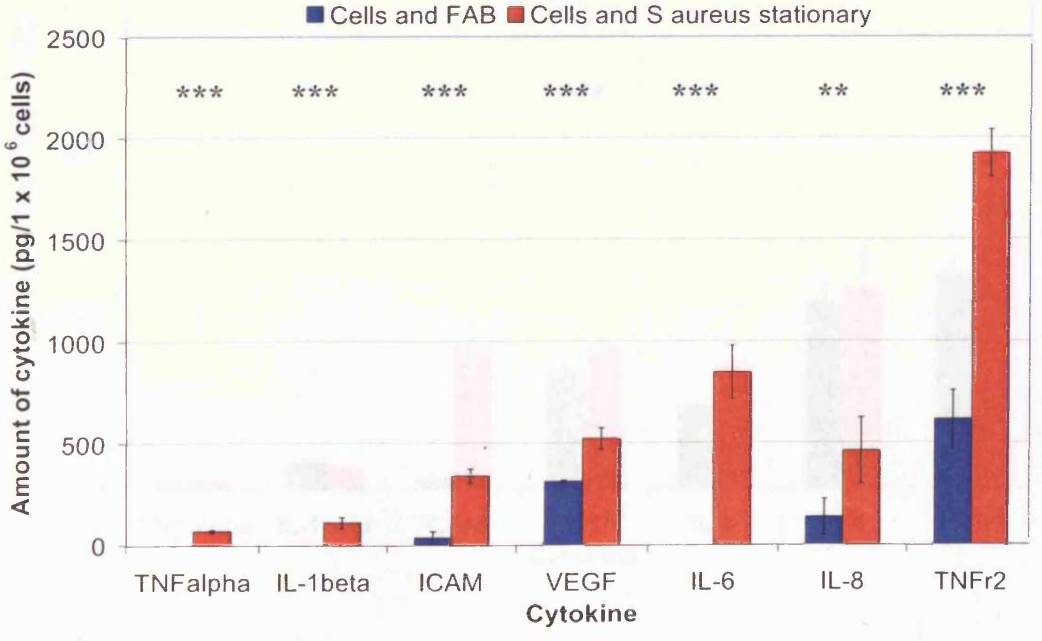
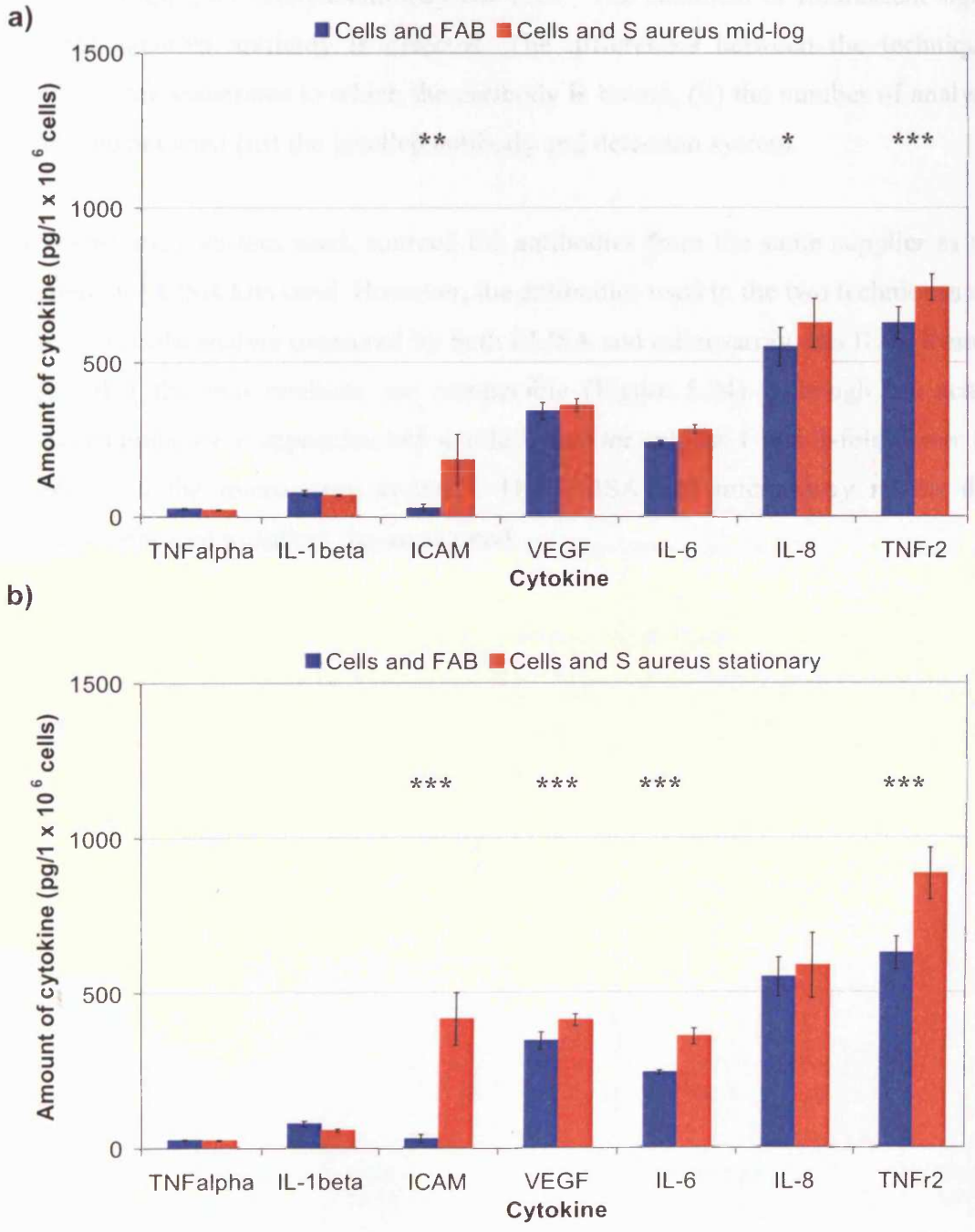


Figure 5.23: Effect of bacterial supernatant, *S. aureus*, on subject 2 neutrophils. (a) Mid-log and (b) stationary growth phase on cytokine production, per $1x10^6$ neutrophils, at 24h, donated by subject 2 (average ± SD). On occasion, *S. aureus* mid-log and stationary phase significantly increased cytokine production as indicated by * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$). The high levels of cytokine detected in cells with FAB appeared to negate the effect of the supernatant.



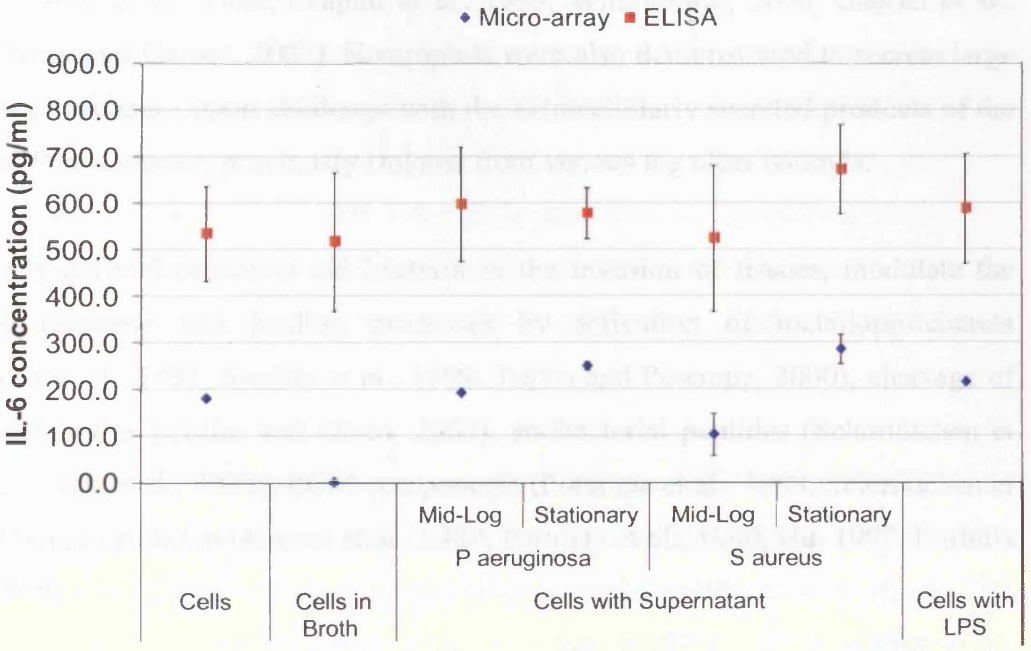
5.3.5 Comparison between ELISA and micro-array techniques

The levels of cytokines, in the present Study, were quantified by both ELISA and micro-array techniques. The two methods rely on the same basic principle, with the analyte of interest binding to an antibody, and a chemical or fluorescent-labelled antibody binding the analyte/antibody complex. The chemical or fluorescent signal from the labelled antibody is detected. The differences between the techniques include (i) the substrates to which the antibody is bound, (ii) the number of analytes that can be detected (iii) the labelled antibody and detection system.

The micro-array system used, sourced the antibodies from the same supplier as the commercial ELISA kits used. However, the antibodies used in the two techniques did differ. The only analyte measured by both ELISA and micro-array was IL-6. Results suggest that the two methods are comparable (Figure 5.24), although the actual values obtained were approximately 4 fold lower for subject 1 and 2-fold lower for subject 2, in the micro-array system. The ELISA and micro-array results did, however, appeared to follow the same trend.

Figure 5.24: Comparison of ELISA verses micro-array.
Levels of IL-6, (average ± SE, 6 replicates), per $1x10^6$ cells, measured by ELISA (n=3) and micro-array (n=1) for (a) subject 1 and (b) subject 2. Results demonstrated that for each subject, the trends in results were similar. However, the difference in response is approximately 4-fold for subject 1, and approximately 2-fold for subject 2.

a)



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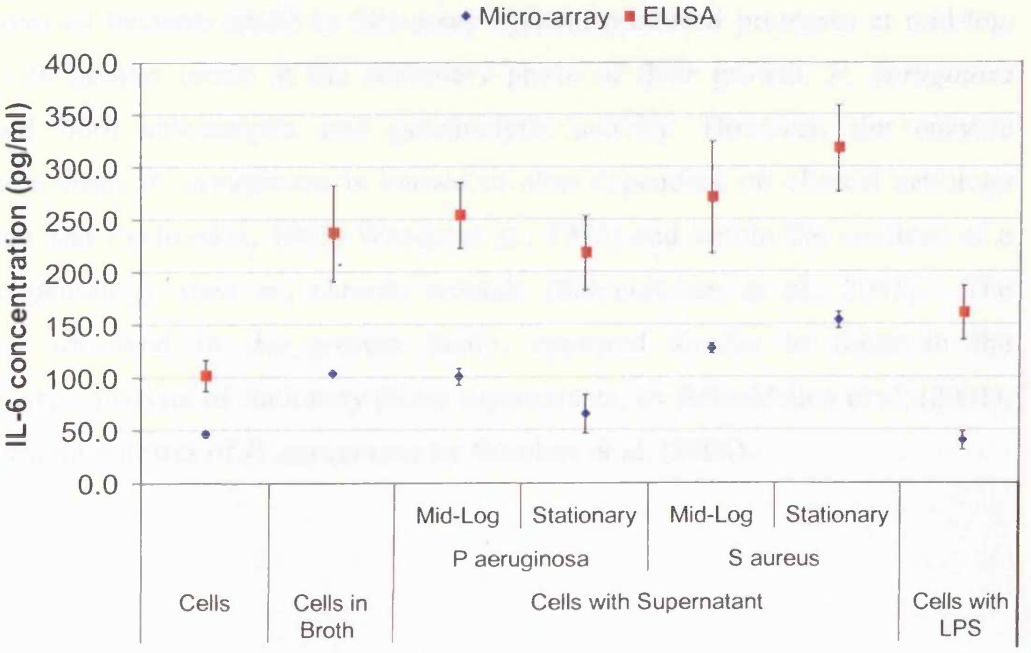

5.4 Discussion

The data presented in this Chapter, supports previous findings, that neutrophils are capable of the synthesis and secretion of numerous cytokines, *in vitro*, including pro-inflammatory cytokines, IL-1β, IL-6, IL-8 and TNF-α and the angiogenic cytokine VEGF (Palma et al., 1992, Scapini et al., 1999, Witko-Sarat, 2000, Gabriel et al., 2002, Henry and Garner, 2003). Neutrophils were also demonstrated to secrete large amounts of protease, upon challenge with the extracellularly secreted products of the bacteria, *P.aeruginosa*, previously isolated from venous leg ulcer wounds.

Bacterially-derived proteases aid bacteria in the invasion of tissues, modulate the immune response and healing processes by activation of metalloproteinases (Okamoto et al., 1997, Santala et al., 1999, Travis and Potempa, 2000), cleavage of immunoglobulins (Collin and Olsen, 2003), antibacterial peptides (Schmidtchen et al, 2002, Frick et al., 2003), ECM components (Potempa et al., 1988, Schmidchen et al., 2003) and cytokines (Horvat et al., 1989, Parmely et al., 1990, Hu, 1997, Barbula et al., 1999).

Both types of bacteria tested in this assay system, produced proteases at mid-log. phase with greater levels at the stationary phase of their growth. *P. aeruginosa* expressed both caseinolytic and gelatinolytic activity. However, the enzyme expression from *P. aeruginosa* is known to alter dependent on clinical aetiology (Wretlind and Pavlovskis, 1983, Woods et al., 1986) and within the confines of a specific pathology such as, chronic wounds (Schmidtchen et al., 2001). The proteases observed in the present Study, appeared similar to those in the zymographic analysis of stationary phase supernatants, by Schmidchen et al. (2001), and overnight cultures of *P. aeruginosa* by Werthen et al. (2004).

No caseinolytic activity was detected in *S. aureus* supernatant, although gelatinolytic activity was observed. Upon infection, *S.aureus* produces cell attachment proteins and protein A, but as the infection persists, the expression of invasive proteins, including proteases has been observed (Wesson et al., 1998).

S. aureus are established at producing cysteine, serine, and metalloproteinase (Section 5.1). In the present Study, the proteases expressed by the *S. aureus* isolate used, appeared to cleave gelatin. The levels of gelatinase activity did not appear to increase in the latter growth phase supernatant, contrary to literature, with the protease complement of this isolate, not able to cleave casein (Veldkemp et al., 1997, 2001, Dubin, 2002). Different levels of proteases have been observed in clinically derived *S. aureus*, as previously described for *P. aeruginosa* (Karlsson and Avidson, 2002).

Cytotoxicity of secreted bacterial products to host cells has also been reported (Marty et al., 2002) and within the present Study, a decrease in neutrophil cell viability, upon their co-incubation with both *P. aeruginosa* and *S. aureus*, was observed. This may be related to the observed high protease activity. As a consequence, supernatants were diluted prior to further cell experimentation.

Incubation of *P. aeruginosa* bacterial supernatants at stationary phase growth, with neutrophil cells, increased the levels of caseinolytic and gelatinolytic activity, of the neutrophil. Neutrophil granule contents, including the proteases, elastase, collagenase and gelatinase, can be released *in vitro*, upon adherence to other cells (Xu et al., 2002), stimulation by bacterial products, such as LPS (Faurshou and Borregaard, 2003) and fMLP (Gasser et al., 2003), or host cells and molecules, such as IL-1, IL-8 and TNF-α (Proost et al., 1991, Topham et al., 1998).

The release of proteases from neutrophils can also occur spontaneously upon cell plating. Caseinolytic activity may be reflective of elastase activity, along with cathepsin G activity, which are freely released as neutrophil azurophil granules translocate to the cell surface. This occurs in response to signals, such as phagocytosis or binding opsonised substrates (Owen and Campbell, 1995, Barrick et al., 1999).

Neutophil gelatinase was detected by zymography. Formyl-methionyl-leucyl-phenylalanine (fMLP) triggered neutrophil release of MMP-9 *in vitro*, has been shown by Western blot analysis and gelatin zymography. Western blot banding patterns at 92, 130 and 220kDa corresponded to the three storage forms; pro-MMP monomer, the MMP-9/lipocalin heterdimer and the MMP-9 homodimer, respectively (Gasser et al., 2003).

In addition to their cleavage and degradative properties of host molecules, bacteria and their secreted products, are also potent activators of numerous host cell types, including neutrophils inducing the expression, synthesis and secretion of cytokines.

The effect of whole bacteria (UV-killed, heat killed) on host cells has been studied previously with respect to cytokine expression (Schlindler et al., 1990, Hachicha et al., 1998, Jiang et al., 1999, Akahoshi et al., 2003). The production of pro-inflammatory cytokines, IL-1β, IL-6 and TNF-α from host cells, including monocyte cells sourced from the blood or established cell lines, can be induced by bacterial surface associate material (Reddi et al., 1996), cell wall components from both Gram-negative and Gram-positive bacteria, such as, LPS (Ferrante et al., 1997, Kreutz et al., 1997, Yoshimura et al., 1997, Gabriel et al., 2002) and peptidoglycan and teichonic acid (Heumann et al., 1994, Kusunoki et al., 1995, Medvedez et al., 1998). Bacterial toxins can also activate cytokines, e.g. *S. pneumoniae* pneumolysin activates IL-8 synthesis and release (Cockeran et al., 2002). Other toxins modulate the immune response by affecting the cytokine secretion of a host cell, e.g. cholera toxin blocks the IL-12 receptor, inducing the specific release of Th2 cytokines .

In the present Study, the effect of the acellular supernatant from two commonly isolated chronic wound bacteria, *P. aeruginosa* and *S. aureus*, on the cytokine release from neutrophils was investigated. Many cytokines are produced by neutrophils (Section 5.1). Micro-array analysis in the present Study, confirmed the production of angiogenin, IL-1β, IL-4, IL-6, IL-8, IL-10, ICAM, TGF-β_1, VEGF, TNF-α and its receptor TNFr2, from un-stimulated cells donated by both subjects. Additionally, IL-13 and IP-10, was detected in cell culture media, from subject 2's neutrophils.

It was difficult to directly compare the results of the present Study with those of others, due to variations in cellular type, source and cell number, in addition to the type and preparation of stimulant (LPS/bacteria/bacterial supernatant) and technique employed in the measurement of the analyte. However, as cytokines act at pico-nanomolar concentrations, results from the present Study, were within the expected range to have a clinically relevant effect.

An increase of cytokine, IL-6, by ELISA, was observed from LPS stimulated cells compared to unstimulated neutrophils for both subjects. ICAM and VEGF levels, by micro-array, significantly increased in neutrophils sourced from both subjects, with increased release of IL-1β, IL-6 and TNFr2, from subject 2 neutrophils. The levels of LPS used in this Study were optimised for IL-6 release, however, upon micro-array analysis, this was not significantly increased for subject 2. The optimisation work in the present Study, was conducted using ELISA methodology, with the same samples analysed by micro-array. No further development was conducted upon the use of this new measuring technique. Cytokines that did not increase their production upon LPS stimulation, included, TNF-α and IL-8. These cytokines were synthesised by neutrophils, but did not increase their secretion, in response to the concentration of LPS, used in the present Study.

The induction of IL-6 release from neutrophils has been demonstrated at both an mRNA level (Cicco et al., 1990), and the protein detected in from cell culture superantant by ELISA upon stimulation or priming with cytokines and LPS (Jablonska et al., 2001, Jablonska and Jablonska, 2002, Liu et al., 2005).

Upon stimulation of neutrophils with bacterial supernatant, the level of IL-6 (by ELISA), were found to be significantly increased, consistently, in both donors in response to *P. aeruginosa* at stationary growth phase. Upon stimulation with *S. auerus* supernatant, significant increases were observed using the stationary growth phase, in subject 1. However the effect of FAB medium incubated with neutrophils, negated the effect of *S. aureus* stationary phase supernatant in subject 2. This inconsistency in response between subjects was observed throughout the subsequent analysis of cytokine responses by micro-array.

Consistency in the significant increase in ICAM levels was observed with both subjects, for both *P. aeruginosa* and *S. aureus*, at both mid-log. and stationary growth phases. This indicated that while other cytokine responses may be dependent on the type of bacteria and the ability of their growth phase dependent expression of cytokine modulating factors, neutrophils do not discriminate between bacterial species in the up-regulation of the adhesion molecule, ICAM, which is essential in cellular migration to an inflamed or infected site (Dallegri and Ottenello, 1997).

Responses to bacterial supernant appeared to be subject dependent. The levels of IL-1β, IL-6, IL-8, TNF-α, TNFr2, ICAM and VEGF, for cells incubated with FAB and cells stimulated with bacterial supernatants, were significantly higher in subject 1. In contrast, the level of cytokine detected in medium, from subject 2 neutrophils incubated with FAB, appeared to negate the ability of the bacterial supernatant to elicit a response. However, it appeared that the greatest responses were growth phase dependent, with stationary phase supernatants inducing a cytokine response superior to the mid.-log growth phase supernatant on almost every occasion.

Additionally, of the 7 cytokines analysed by micro-array, the largest responses were for IL-6, IL-8 and ICAM.

The effect of cell free *S. aureus* supernatant on neturophil cell activation in the expression of chemokines and cytokines, including IL-1β and TNF-α has been demonstrated (Veldkamp et al., 1997, 2000). In addition, the induction of IL-1β, IL-8 and TNF-α in response to the Gram-positive cell wall component, lipoteichoic acid (LTA) from neutrophils has been reported (Hattar et al., 2006).

Activation of neutrophils with LPS via transcriptional profiling, suggests an up-regulation post- IL-8 and LPS treatment. This response could be further increased upon IL-8 treatment of cells, with subsequent exposure to LPS, thereby demonstrating possible synergies between host and bacterial molecules in regulating neutrophil responses (Martinez et al., 2004).

The effect of *P. aeruginosa* products, porins, exomucopolysaccharide, and lipopolysaccharide, on whole blood increased TNF-α and IL-6 levels. Monocytes are believed to be the population of cells, responsible for this cytokine production (Cusumano et al., 1997). Increased release of IL-1β and TNF has also been reported, using bacterial suspensions and LPS on monocytes and macrophages, from bacteria specific to periodontal disease (Socransky and Haffajee, 1991).

It has been reported, that particular bacterial species appear to have the ability to induce differing cytokine responses, correlated with whether they are Gram-negative or Gram-positive organisms (Cui et al., 2000, Hessle et al., 2000, Iwadou et al., 2002). These responses appear to be regulated by different signalling pathways, as demonstrated using monocyte cells (Rabehi et al., 2001). The release of cytokines from mononuclear cells stimulated with LPS and Staphyloccocal enterotoxin B, were IL-1β, IL-6, IL-18, INF-γ and TNF-α, compared with IL-2 and INF-γ, respectively.

A study by Hessle et al., (2000), suggests that the Th1 or Th2 cytokine responses can be modulated with Gram-negative bacteria, inducing the Th1 phenotype with IL-12, and Gram-negative bacteria stimulating the release of IL-10, the inhibitor of the Th1 response (Mocellin et al., 2004). Cytokines induced from a Th1 response are inflammatory and mediate tissue damage (Sandler et al., 2003). The ability to co-ordinate cytokine responses may correlate with the level of virulence of a particular bacteria (Jiang et al., 1999).

Unfortunately the interference of FAB with the micro-array did not allow analysis of any potential differences in the expression of IL-10 and IL-12, from neutrophils stimulated with Gram-negative (*P. aeruginosa*) and Gram-positive bacteria (*S. aureus*), in the present Study.

In the present Study the responses of neutrophils to bacterial supernatants appeared to be more reliant on the subject than the type of bacteria. The type of stimulus affects the amplitude and characteristic of response, but the ability of a neutrophil cell to release cytokines, may depend on the cells functional state (Cassatella, 1995). It is assumed that the expression of these cytokines from neutrophils is representative of basal and activated cellular expression. Variability in the amount of cytokine produced by peripheral blood mononuclear cells, from the same individual on different days, and between subjects, in the production of IL-1β, IL-6 and TNF-α, upon LPS stimulation has been reported previously (Schindler et al., 1990).

However, it is also possible that cells may have been activated during the isolation procedure itself (Venaille et al., 1994, Schroder et al., 2006) or their may be contamination of the cellular fraction analysed with other cytokine producing cells, such as monocytes. The lack of pro-inflammatory cytokines, IL-1β, IL-6 and TNF-α, in neutrophil culture medium is an indicator of purity, by Scapani et al. (1999), with IL-8 accounting for the largest amount of cytokine produced from these cells (Schroder and Rink, 2003, Schroder et al., 2006).

Within the present Study, IL-8 was also detected from neutrophil cells and its expression was greater than that of pro-inflammatory cytokines. However, IL-8 levels were found to be less than angiogenin and ICAM in both subjects, and than TNFr2 in subject 1.

The micro-array analysis has provided a cytokine profile of response for unstimulated cells, relative to one another both subjects were similar, except for IL-13 and IP-10. This technique was further validated, when cytokines, whose major source are cells other than neutophils, were not detected by micro-array. However, it appears that the purity of the isolation method is critical in whether these cells actually produce pro-inflammatory cytokines. The recent study by Schroder et al., (2006), has cast doubt on previous investigations reported in the literature, and indeed the finding of the present Study. The study conducted by Schroder et al., (2006), concludes that neutrophils can not produce IL-6, with any detected in cell culture media, the result of contamination of the neutrophil preparation by other cell types.

However, regardless of which particular cytokines the neutrophils express, evidence does suggest that the neutrophil is not terminally differentiated. Whilst doubt may exist over the actual production of particular cytokines from neutrophils, the micro-array methodology does allow for simulataneous analysis, using small amounts in comparison to ELISA techniques.

Proteomic techniques, used in the measurement of cytokines are being increasingly reported in the literature. A recent study has adopted the use of micro-array in the comparison in the expression of cytokines between healthy and diseased states in gingival crevicular fluid (Sakai et al., 2006). The use of protein micro-array in the analysis of disease states and host:pathogen interactions is less common than the use of DNA micro-arrays (Rappuoli, 2000, Jenner and Young, 2005).

However, changes to RNA levels in neutrophils exposed to bacteria have also been explored by this technique (Yerramilli et al., 2001), in addition to neutrophil transcriptional changes upon migration into sites of inflammation *in vivo* (Theilgaard-Monch et al., 2004). The present Study, has therefore, provided information at, perhaps the more relevant, protein level of expression, and provided data on a wide array of cytokines, comparing donor responses, and their response to two bacterial species supernatants, with consistent sampling and methodology.

In vitro samples analysed by micro-array, exhibited similar trends to results obtained by ELISA. However, the micro-array results were between approximately 2-fold and 4-fold lower, depending on subject. Upon analysis of wound fluids (Chapter 3, Section 3.3.8), IL-6 levels between the techniques were comparable. However, a two-log difference was observed between the techniques for measurement of VEGF.

Data presented herein, demonstrates that cytokines can be induced in response to bacterial supernatants, commonly isolated from chronic wounds, and that this release is significantly increased with stationary phase bacterial supernatants.

While small levels of cytokine appeared to aid bacterial clearance, large amounts encourage bacterial adherence to cells and bacterial growth in a dose-dependent manner (Meduri et al., 1999, Meduri, 2002, Ellmerich et al., 2002). The types of bacteria and their potential ability to influence Th1/Th2 cytokine responses, may dictate the severity of infection and/or tissue damage (Sandler et al., 2003).

Bacterial virulence factors, such as enzymes and toxins, appear to increase in expression, being released from the cell, shortly prior to, or during stationary growth phase of bacterial growth. Bacterial virulence is conferred though mechanisms of cell attachment, the ability to invade tissue, evade host defences and modulate immune responses. The data from the present Study suggests that the growth phase of the bacteria, in addition to bacterial cell number, may be as important in the bacteria's ability to overcome host defence and induce infection.

5.4.1 Summary of findings

- The presence of proteases within bacterial supernatant from *P.aeruginosa* and *S. aureus* correlated with the degradation and/or or inhibition of IL-6 release from neutrophils.

- Effects of both protease and cytokine release is dependent on bacterial growth phase, with stationary phase supernatant inducing larger responses than mid.-log phase supernatant.

- Neutrophils release cytokines, angiogenin, IL-1β, IL-4, IL-6, IL-8, IL-10, ICAM, TGF-β_1, VEGF, TNF-α and its receptor TNFr2, from un-stimulated cells donated by both subjects. Additionally, IL-13, and IP-10 was detected in cell culture media, from subject 2 neutrophils.

- The levels of IL-6, ICAM and VEGF are increased in LPS stimulated cells. In addition, increased release of IL-1β, IL-6 and TNFr2 from subject 2 neutrophils, were observed.

- Variation in neutrophil cytokine expression was observed between donors, when neutrophils were stimulated with either LPS or bacterial supernatant.

- Neutrophils consistently respond with significantly increased expression of a number of cytokines or their receptors, with ICAM > IL-8 > IL-6 > TNFr2 > VEGF, upon stimulation with stationary growth phase bacterial supernatant.

- Increases in cytokine production were also observed with mid.-log phase supernatants, although these responses appear to be both bacterial species and donor-dependent. It is possible that the growth phase of the bacteria may contribute more than bacterial cell number, in the development of infection.

Chapter 6

Chapter 6

General Discussion

Chronic wound healing is characterized by persistent inflammation with abnormal cellular function and activation responses (Moore, 1999, Agren et al., 2000). The inflammatory nature of these wounds combined with the continual presence of microorganisms, can make the diagnosis of chronic wound infection a challenge for the Clinician.

A number of diagnostic methods are currently used to assess wounds and monitor wound healing outcomes (Collier, 2001). Accumulation of such information guides the Clinician's decision-making processes in order for the most appropriate course of treatment is administered. Any measurement that can be relied upon to indicate effective treatment options, improve patient outcomes or provide information more rapidly than existing methodologies/technologies would be of value. Studies of acute and chronic wound environments, and additionally chronic non-healing and healing wounds reveal a number of host biochemical molecules that could potentially serve as diagnostic/prognostic biomarkers of wound healing status (Moseley et al., 2004b). However, currently the information provided to the clinician to diagnose infection in the chronic wound is limited. The present Study, sought to identify host-derived biochemical markers that could be correlated with/to chronic wound infection, and to link these findings to studies which examined the release of these biomarkers by bacterial supernatant stimulation of neutrophils *in vitro*.

Results of the present Study indicated no relationship between clinical diagnosis of infection and the number of bacteria in either venous leg ulcer or diabetic foot ulcer wounds. Additionally, no host biochemical was able to distinguish between clinically diagnosed non-infected and infected wounds (Chapter 3). It was possible however, to measure biochemical differences in host-derived molecules with changes in the microbial number, species or genera within the wound environment (Chapter 4).

The levels of host-derived cytokines, angiogenin, interleukin-1β (IL-1β), IL-4, IL-6, vascular endothelial growth factor (VEGF), intracellular adhesion molecule (ICAM-1), tumor necrosis factor-α (TNF-α) and its receptor, TNFr2, in venous leg ulcer wound fluids and levels of IL-2, IL-5, IL-12p40, IL-12p70, interferon-γ (IFN-γ) and transforming growth factor-β_1 (TGF-β_1), in diabetic foot ulcer wound fluids could be indicative of chronic wound infection, relating to total bioburden levels and/or particular bacterial species within the wound (Chapter 4). Upon culturing *P. aeruginosa* and *S. aureus* bacterial supernatants, at mid-log and stationary growth phase with the neutrophil, increases in the levels/activities of proteases and cytokines were detected (Chapter 5).

Currently, the clinical diagnosis of acute and chronic wound infection relies upon rudimentary, non-specific clinical signs and symptoms (Harding, 1996, Gardner et al., 2001b). Whilst these signs and symptoms are easily identified within the acute setting, their diagnostic ability to distinguish infection in the chronic wound is questioned (Bamberg et al., 2002, Gardner, 2001a).

Whilst, acute wounds tend to be devoid of microorganisms and a positive wound culture, by swab or tissue biopsy, chronic wounds are always colonized with microorganisms (Bowler and Davies, 1999, Bill et al, 2001). The continual presence of microorganisms within the wound appears to delay the healing process. Indeed, within venous leg ulcers, the presence of four or more microorganisms in a wound has been correlated with non-healing responses (Trengrove et al., 2000, Davies et al., 2004).

Infection is a complication of healing, and its resolution, through treatment with systemic antibiotics and topical antimicrobials, is required before chronic wounds can progress successfully towards healing. The aims of the present Study included the identification of host-derived molecules that may serve as useful biomarkers in the diagnosis of chronic wound infection. The identification of such biomarkers may provide an alternative, rapid, specific measurement compared with the current, primary diagnosis of infection using clinical signs and symptoms.

Analysis of the microflora from chronic wounds is not solely relied upon by the Clinician in the diagnosis of chronic wound infection, but is used as a confirmatory tool. Limitations of current microbiological analysis include the time required to culture microorganisms, results tend to be semi-quantitative and usually, a number of known microbial pathogens are present, making it difficult to ascertain which particular bacteria is the causative wound pathogen. Surface sampling of the wound, by swabbing, and within the tissue, by wound biopsy, are the routine microbiological techniques used, in the identification of bacterial pathogens, in wound infection (Hansson et al., 1995, Gardner et al., 2006).

Microbial analysis of swabs, collected in the present Study, demonstrated varied microflora in both venous leg ulcers and diabetic foot ulcers, consistent with the polymicrobial nature of chronic wounds (Bowler et al., 2001). The most frequently isolated microorganisms in venous leg ulcers were *Pseudomonas* spp., *Staphylococcus* spp., and *Corynebacterium* spp. Within diabetic foot ulcers, *Corynebacterium* spp., *Staphylococcus* spp. and *Bacillus* spp., were the predominant species. These species of bacteria are commonly detected in chronic wounds, particularly *Staphylococcus* spp. (Eriksson et al., 1984, Bowler, 1998, Schmidt et al., 2000, Davies et al., 2007).

Swab analysis was preformed, *in lieu* of tissue biopsies, as although biopsies are perceived to enable the identification of the causative pathogen, the presence of many types of bacteria may contribute to the development of wound infection, and it is likely that the pathogen responsible can be found on the surface of the wound.

Although slight variation in the culture of bacteria between swabs and biopsies has been reported, the overall bioburden levels and number of bacterial genera does not appear to alter (Davies et al., 2007). Furthermore, the use of tissue biopsies could not be justified ethically. Whether the use of tissue biopsies would have contributed any additional value, to the microbiological findings in the present Study, is difficult to conclude.

Results from the present Study showed that clinical diagnosis of infection did not correlate with total bacterial bioburden within the wounds. Additionally, no host-derived biochemical, including proteases (elastase, collagenases and gelatinases), their inhibitors (alpha-1-anti-trypsin, tissue inhibitor of matrixmetalloproteinases (TIMP), or cytokines and growth factors, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, Il-12 p40, IL-12p70, IL-13, angiogenin (angio); IFN-γ, transforming growth factor beta (TGF-β_1), VEGF, TNF-α, TNFr2, ICAM-1 and IFN-γ-inducible-protein-10 (IP-10), could distinguish between clinically diagnosed non-infected and infected venous leg ulcers and diabetic foot ulcers.

The present Study design aimed to keep as many variables as possible constant, with a dedicated Clinician involved in the diagnosis of all chronic wounds, the swabbing of wounds, and additional wound measurements taken. However, the present findings indicate that no host-derived marker could define chronic wound infection on clinical signs.

Published literature had highlighted the difficulty for Clinicians in diagnosing chronic wound infection, using clinical signs and symptoms. Additionally, microbiological analysis of chronic wounds show that clinically diagnosed non-infected chronic wounds, have significant bacterial bioburden, ranging from 10^2-10^8 CFU/ml (Ennis et al., 2005, Davies et al., 2007). Assuming bioburden levels of $\geq 1x10^5$ CFU/ml bacteria, are indicative of wound infection, it is possible that chronic wound infection may not be detected (Bamberg et al., 2002, Lorentzen and Gottrup, 2006). These studies, and the present Study, made the assumption that a microbiologically defined parameter, is primarily responsible for wound infection.

Therefore, assuming infection is defined upon the microbiological characteristics of the wound, results of the present Study confirm that chronic wound infection may be wrongly assigned. Of wounds clinically diagnosed as non-infected, 70% of venous leg ulcers and 60% of diabetic foot ulcers, respectively, contained $\geq 1 \times 10^6$ CFU/ml bacteria.

The correlation of bacterial number and infection, at least within the acute wound healing setting has been established, but whether this translates to chronic wound infection is currently debated. Opposing views centre around, (i) the importance of bacterial number (Robson et al., 1990, Robson, 1997) and (ii) the type, and/or synergies that may exist between microorganisms (Bowler, 2003). With that in mind, further analysis was conducted in the present Study, on host-derived bio-molecules, with total bioburden, particular bacterial species and the number of genera, within the wound in an attempt to identify host-derived biomarkers.

No significant differences were observed in the activities or amounts of protease (elastase, collagenases and gelatinases), or their inhibitors (alpha-1-anti-trypsin, tissue inhibitor of matrixmetalloproteinases (TIMP), with total bioburden levels. Elastase-like activity was significantly increased in diabetic foot ulcer wounds containing four or more genera, and differences in collagenase and TIMP-2 levels in *Corynebacterium* spp. wounds. However, as these markers only discriminated on one microbial factor, the significances of which are unknown, the reliability of these particular host-derived factors as biomarkers of infection, would be questionable.

The levels of the pro-inflammatory cytokines; IL-1β, IL-6, and TNF-α; cell surface receptors, TNFr2 and ICAM-1; angiogenic factors, angiogenin and VEGF and IL-4, involved in mediating adaptive immune responses, in chronic venous leg ulcer wound fluid, were significantly increased in wounds containing $> 1 \times 10^6$ CFU/ml total bioburden (Chapter 4). A determination at a level of $>1 \times 10^5$ CFU/ml could not be made, due to low sample number containing less that 1×10^5 CFU/ml.

The increased levels of these particular cytokines in venous leg ulcer wound fluid, implied that an acute, innate, inflammatory response had been initiated to remove bacteria from the wound.

It has been suggested that pro-inflammatory cytokines promote the rate of bacterial growth, enabling bacteria to reach their stationary (exponential) phase earlier, thereby secreting a barrage of virulence factors, including enzymes (Meduri, 2002). Therefore, data from the present Study, showed increasing total bioburden and pro-inflammatory cytokines, are directly linked.

The levels of IL-1β and TNF-α, its receptor TNFr2 and VEGF, were significantly increased when high levels of *Pseudomonas* spp. and *Staphylococcus* spp., were present in the wound, implying greater specificity of these particular cytokines in diagnosing chronic wound infection. The levels of a number of cytokines increased with *Corynebacterium* spp., although these did not translate to increasing total bioburden, with the exceptions of TNFr2, angiogenin and VEGF. Although comparisons have been made to specific bacterial species, it may be inappropriate to diagnose chronic wound infection on this sole definition (Gardner et al., 2004).

Surprisingly, in contrast to the venous leg ulcer cytokine responses, the levels of cytokines significantly decreased as total bioburden within the diabetic foot ulcer wound increased, namely IL-2, IL-4, IL-5, IL-10, IL-12p40, IL-12p70, IL-13, TNF-α, its receptor TNFr2, IP-10, IFN-γ, and $TGF\beta_1$. These decreases were unexpected, and it is difficult to account for them. It is possible that the higher proteolytic environment of the diabetic foot, represented by increased elastase-like activity may explain the lack of both Gram-negative bacteria cultured (Belaaouja and Sharpiro, 1999) and the general decrease in cytokine levels of IL-2, IL-4, IL-13, IFN-γ, TNF-α and VEGF, compared to venous leg ulcer wounds (Yager et al., 1997, Bank et al., 1999). However, the levels of host-derived proteases quantified in the present Study, did not increase with bioburden. This does not preclude the involvement of other host or bacterially-derived proteases or molecules, not measured in the present Study.

Although slight differences in the detectable bacterial species, between venous leg ulcers and diabetic foot ulcers, were evident, both ulcers contained two of the three most prevalent bacteria, *Staphylococcus* spp., and *Corynebacterium* spp. However, diabetic foot ulcer wounds were virtually devoid of *Pseudomonas* spp., highlighting the contribution to the wound environment by Gram-positive bacteria, in diabetic foot ulcer wounds compared to venous leg ulcer wounds.

This decrease in cytokine levels, as total bioburden within the diabetic foot ulcer wound increases, may alternatively imply a dysfunctional immune response. Increased cytokine levels aid the immune system in the removal of bacteria from the body, through the co-ordination of innate, inflammatory responses that can activate host cells, such as neutrophils, to elicit a response. It was observed within the diabetic foot ulcer population, that upon increasing bioburden, cytokine levels remained constant, or decreased. This may be indicative of an inability of the diabetic population to mount an efficient immune response to bacteria, accounting for the reported increased risk of infection, (Medina et al., 2005, Peleg et al., 2007).

Upon comparing venous leg ulcers and diabetic foot ulcers, irrespective of total bioburden, a general lack of a number of cytokines were quantified in diabetic wounds. Of these cytokines, specifically, low levels of IL-2, IL-4 and IFN-γ were observed. IL-2 and IL-4 have roles in the antibody-mediated immune response, to infection, aiding the removal of bacteria from the wound (Steinbeck and Roth, 1989). IFN-γ is capable of activating neutrophils, maintaining their phagocytic action and retaining these cells at sites of infection (Lin et al., 2000). These cytokine responses, therefore, may account for dysfunctional neutrophil function and increased likelihood of bacterial infection, in diabetic foot ulcers (Medina et al., 2005, Peleg et al., 2007).

Analysis of both venous leg and diabetic foot ulcers, comparing cytokines to the number of bacterial genera, did not find a significant result, although elastase-like activity was positively correlated. Since previous studies have linked the number of bacterial genera to non-healing ulcers, this data presented herein suggests that elastase-like activity may be more indicative of healing status than infection (Trengrove et al., 2000, Davies et al., 2004), although wounds were not followed to healing in the present Study.

Analysis of patient-matched blood samples may have identified any cellular differences between venous leg and diabetic foot ulcers, and may have allowed correlations, if any, between the cytokine levels in the local wound environment, with those systemically.

The *in vitro* model system involved the co-culture of bacterial supernatants, from two commonly isolated wound bacteria, *P. aeruginosa* and *S. aureus*, with neutrophils (Chapter 5). Protease production from both these bacteria was increased at the stationary growth phases compared with mid-log growth phase. The incubation of supernatants with neutrophils, induced an increase in host-derived proteases (elastase, gelatinases), a key element in the arsenal of host defense mechanisms that contribute to the elimination of bacteria (Reeves et al., 2002).

The increased protease expression *in vitro*, did not reflect the lack of protease production in clinically or microbiologically defined infected chronic venous leg ulcers or diabetic foot ulcer wounds. The overall increase in both caseinolytic and gelatinolytic activity within the *in vitro* model system, however, may have indicated the contribution of both bacterially-derived and bacterially-induced host-derived serine and MMPs to the chronic wound inflammatory response. Both these protease families are reported as increased within chronic ulcers, independent of infection status (Mast and Shultz, 1996, Yager et al., 1996, Wysocki et al., 1999).

The increased cytokine expression within the microbiologically-diagnosed infected venous leg ulcer population, however, was mimicked using the *in vitro* model system. Levels of a number of cytokines were increased, with levels as follows: ICAM > IL-8 > IL-6 > TNFr2 > IL-1β > VEGF > TNF-α. The role of adhesion molecules and pro-inflammatory cytokines have been demonstrated in studies involving acute disease/infection models, demonstrating that the binding of bacteria, or the effect of their soluble products, on host cells, initiates host-intracellular pathways, that results in their increased expression (Henderson et al., 1996, Fujihara et al., 2003).

Unfortunately, due to interference of the bacterial growth medium, fastidious anaerobic broth (FAB) and the micro-array system, the levels of cytokines specifically down-regulated in the diabetic foot ulcer wounds, could not be determined *in vitro.*

In future studies, it would be interesting to source neutrophils from people with venous leg and diabetic foot ulcers, and compare the data obtained with these from normal healthy volunteers. A recent study by Hatanaka et al., (2006), has shown that neutrophils from diabetic patients release more cytokines than healthy controls. Whether this is still less than neutrophils from venous leg ulcer subjects is unknown at present, although it and would be an interesting comparison to make.

The *in vitro* system highlighted the importance of bacterial growth phase in inducing a cytokine response. Generally, stationary phase supernatants invoked larger protease and cytokine responses than mid-log phase supernatants. This would concur with the increased expression of virulence factors, including enzymes, shown to be dependent on growth phase for their expression. The difference in cytokine response appeared to be bacterial supernatant growth phase dependent, with similar results observed between the ability of both bacterial species to induce cytokine release. Within the cellular responses of the *in vitro* model, the largest amount of variability was attributed to the difference between donors.

The increased expression of ICAM, however, was consistently increased in both subjects, for both mid-log and stationary growth phase supernatant. The stationary growth phase responses were larger than those of the mid-log supernatant. This could be indicative of the primitive response and basic essential function necessary to attract cells to the sites of inflammation. Cell receptors are required for leukocyte trafficking and while other cytokine responses appear to be dependent on the type of bacteria and the growth phase (therefore virulence), neutrophils do not appear to discriminate upon their expression of ICAM. This molecule performs the basic function enabling cells to attach and migrate to the sites where they are required to perform their functions.
Interestingly, after many years of studying at cytokines, to potentially obtain an aid in the diagnosis of the acute clinical disease, sepsis, attention now seems to now be switching to neutrophil activation/adhesion molecules (Davis, 2005). ICAM expression in venous leg ulcer wound fluid, increased with bacterial bioburden and in response specific bacteria. However, although the level of ICAM expression did not increase, its levels were maintained within the diabetic foot ulcer wound fluids.

Within the chronic wound environment, there are many cell types contributing to the cytokines levels measured in these wound fluids. Whilst cytokine production from neutrophils is believed to be insignificant, compared to the contribution of cytokines from other cells, including macrophages, this is the predominant, initial, cell type, involved in wound infection.

The *in vitro* model demonstrated the production of specific cytokines, by bacterial supernatants, from two commonly isolated bacteria. These responses were usually greater that those of lipopolysaccharide (LPS). Measurement of IL-6 by ELISA and micro-array techniques appeared to follow the same trend, but a limitation of the micro-array analysis was discovered, with the cross-reaction with the microbial growth media, and therefore, all cytokines could not be compared directly.

Additionally, although not the primary objective of the present Study, differences in the activities or levels of host-factors were apparent. A significant decrease in the activity of elastase in venous leg ulcer wound fluid, with significant increases in the activity of collagenase and the levels of angiogenin, IL-2, IL-4, IL-13, IFN-γ TNF-α and VEGF, were observed, compared to, diabetic foot ulcer wounds. It is possible that the increased activity of elastase within the diabetic foot ulcers, directly relates to the decreased levels of cytokine, compared with venous leg ulcer wounds. It has been demonstrated that the proteolytic environment of chronic wounds enables the degradation of ECM components and growth factors (Grinnell et al., 1992, Yager et al., 1997).

In Summary, Chapter 3 of this Thesis concluded that no host-derived biochemical markers correlated to clinically diagnosed, wound infection. However, diagnosing chronic wound infection can be problematical for the Clinician with no decent, reliable parameters to aid this decision making process.

Analysis of host-derived biochemicals to microbiologically-defined wound infection (Chapter 4) revealed that eight cytokines, angiogenin, IL-1β, IL-4, IL-6, VEGF, ICAM-1, TNF-α and TNFr2, within the venous leg ulcer population, increased their levels in response to total bioburden. Additionally, four of these cytokines, IL-1β, VEGF, TNF-α and TNFr2, appeared to correlate both to bioburden and with particular bacterial species. Therefore, from a future work viewpoint, it may be prudent to further explore these cytokines. In contrast, within the diabetic foot ulcer wound population, numerous cytokines, IL-2, IL-5, IL-12p40, IL-12p70, IFN-γ and TGF-β_1, decreased their levels with increasing bioburden. However, although differences in response was detected between ulcer types, the results of the present Study, suggest that cytokines are biomarkers of chronic wound infection.

The increases of particular cytokines, was mimicked *in vitro*, from neutrophils, during their co-culture with a 'acellular' supernatants, derived from *P. aeruginosa* and *S. aureus* (Chapter 5). However, all 18 cytokines measured by micro-array could not be analysed directly, due to the interference of microbial growth media. Therefore, a neutrophil response consistent with the diabetic foot ulcer could not, unfortunately be replicated in this *in vitro* model system.

The quantification of these biomarkers, in chronic venous leg ulcer and diabetic foot ulcer wound fluids, may provide additional, timely, reliable tools that could potentially aid the Clinician in the diagnosis of chronic wound infection. These measurements would rely on a suitable, consistent method for the collection of wound fluid and either a laboratory or *in situ* test, e.g. a point-of-care diagnostic, which would compliment the other clinically relevant signs of infection, currently in use.

Bibliography

Bibliography

Agren MS (1994). Gelatinase activity during wound healing. *Br J Dermatol* 131:634-640.

Agren MS, Eaglestein WH, Ferguson M, Harding KG, Moore K, Saarialho-Kere UK *et al.* (2000). Causes and effects of the chronic inflammation in venous leg ulcers. *Acta Derm Venereol Suppl (Stockh)* 210:3-17.

Akahoshi T, Sasahara T, Namai R, Matsui T, Watabe H, Kitasato H *et al.* (2003). Production of macrophage inflammatory protein 3α (MIP-3α) (CCL20) and MIP-3β (CCL19) by human peripheral blood neutrophils in response to microbial pathogens. *Infect Immun* 71:524-526.

Asensi V, Valle E, Meana A, Fierer J, Celada A, Alvarez V *et al.* (2004). *In vivo* interleukin-6 protects neutrophils from apoptosis in osteomyelitis. *Infect Immun* 72:3823-3828.

Ashcroft GS, Lei K, Jin W, Longenecker G, Kulkarni AB, Greenwell-Wild T *et al.* (2000). Secretory leukocyte protease inhibitor mediates non-redundant functions necessary for normal wound healing. *Nat Med* 6:1147-1153.

Baker EA, Leaper DJ (2000). Proteinases, their inhibitors, and cytokine profiles in acute wound fluid. *Wound Repair Regen* 8:392-398.

Bamberg R, Sullivan PK, Conner-Kerr T (2002). Diagnosis of wound infections: current culturing practices of US wound care professionals. *Wounds* 14:314-327.

Bank U, Reinhold D, Kunz D, Schulz HU, Schneemilch C, Brandt W *et al.* (1995). Effects of interleukin-6 (IL-6) and the transforming growth factor beta (TGF-β) on neutrophil elastase release. *Inflammation* 19:83-99.

Barbula A, Bugno M, Kuster A, Heinrich PC, Travis J, Potempa J (1999). Rapid and efficient inactivation of IL-6 gingipains, lysine- and arginine-specific proteinases from *Polymorphomonas gingivalis*. *Biochem Biophys Res Commun* 261:598-602.

Barone EJ, Yager DR, Pozez AL, Olutoye OO, Crossland MC, Diegelmann RF *et al.* (1998). IL-1α and collagenase activity are elevated in chronic wounds. *Plast Reconstr Surg* 102:1023-1027.

Barrett AJ, Rawlings ND, Woessner FFJr (1998). Handbook of proteolytic enzymes. San Diego, London, Tokyo: Academic Press.

Barrick B, Campbell EJ, Owen CA (1999). Leukocyte proteinases in wound healing: roles in physiologic and pathogenic processes. *Wound Repair Regen* 7:410-422.

Bartunkova J, Tesar V, Sediva A (2003). Diagnositc and pathogenetic role of antineutrophil cytokplasmic autoantibodies. *Clin Immunol* 106:73-82.

Basset C, Holton J, O'Mahony R, Roitt I (2003). Innate immunity andn pathogen-host interaction. *Vaccine* 21:S12-S23.

Beatty K, Bieth JG, Travis J (1980). Kinetics of association of serine proteinases with native and oxidised α−1 proteinase inhibitor and α-1-antichymotrypsin. *J Biological Chemistry* 255:3931-3934.

Belaaouaj AA, Sharpiro S (1999). Mechanisms of Gram-negative bacteria by neutrophil elastase. *Inter conf of Antimicrob Agents Chemother* 39:26-29.

Bello YM, Falabella AF, De Carbalho H, Nayyar G, Kirsner RS (2001). Infection and wound healing. *Wounds* 13:127-131.

Bergin PJ, Sicheng W, Qiang PH, Marianne QJ (2005). Secretion of matrix metalloproteinase-9 by macrophages, *in vitro*, in response to *Helicobacter pylori*. *FEMS Immunol Med Microbiol* 45:159-169.

Beutler B, Grau GE (1993). Tumor necrosis factor in the pathogenesis of infectious diseases. *Crit Care Med* 21:S423-S435.

Bieth JG (1996). Leukocyte elastase. *J Biological Chemistry*. 270:13204-13209

Bill TJ, Ratliff CR, Donovan AM, Knox LK, Morgan RF, Rodeheaver GT (2001). Quantitative swab culture verses tissue biopsy: A comparison in chronic wounds. *Ostomy Wound Manage* 47:34-37.

Birkedal-Hansen H (1993). Role of cytokines and inflammatory mediators in tissue destruction. *J Periodontal Res* 28:500-510.

Blitstein-Willinger E (1991). The role of growth factors in wound healing. *Skin Pharmacol* 4:175-182.

Bode W, Maskos K (2003). Structural basis of the matrix metalloproteinases and their physiological inhibitors, the tissue inhibitors of metalloproteinases. *Biol Chem* 384:863-872.

Boniface K, Lecron J-C, Bernard F-X, Dagregorio G, Guillet G, Nau F *et al.* (2005). Keratinocytes as targets for interleukin-10-related cytokines: a putative role in the pathogenesis of psoriasis. *Eur Cytokine Netw* 16:309-319.

Bonjardim CA (2005). Interferons (IFNs) are key cytokines in both innate and adaptive activiral immune responses - and viruses counteract IFN action. *Microbes and Infection* 7:569-578.

Bowler PG (1998). The anaerobic and aerobic microbiology of wounds: A review. *Wounds* 10:170-178.

Bowler PG (2003). The 10^5 bacterial growth guideline: Reassessing its clinical relevance in wound healing. *Ostomy Wound Manage* 49:44-53.

Bowler PG, Davies BJ (1999). The microbiology of acute and chronic wounds. *Wounds* 11:72-78.

Bowler PG, Duerden BI, Armstrong DG (2001). Wound microbiology and associated approaches to wound management. *Clin Microbiol Rev* 14:244-269.

Brandt E, Woerly G, Younes AB, Loiseau S, Capron M (2000). IL-4 production by human polymorphonuclear neutrophils. *J Leukoc Biol* 68:125-130.

Brantly M, Nukiwa T, Crystal RG (1988). Molecular basis of alpha-1-antitrypsin deficiency. *Am J Med* 84:13-31.

Brem H, Balledux J, Sukkarieh T, Carson P, Falanga V (2001). Healing of venous ulcers of long duration with a bilayered living skin substitute: results from a general surgery and dermatology department. *Dermatol Surg* 27:915-919.

Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS *et al.* (2004). Neutrophil extracellular traps kill bacteria. *Science* 303:1532-1535.

Bullen EC, Longaker MT, Updike DL, Benton R, Ladin D, Hou Z *et al.* (1995). Tissue inhibitor of metalloproteinases-1 is decreased and activated gelatinases are increased in chronic wounds. *J Invest Dermatol* 104:236-240.

Campbell EJ, Campbell MA, Owen CA (2000). Bioactive proteinase 3 on the cell surface of human neutrophils: Quantification, catalytic activity, and susceptibility of inhibition. *J Immunol* 165:3366-3374.

Cassatella MA (1995). The production of cytokines by polymorphonuclear neutrophils. *Immunol Today* 16:21-26.

Cassatella MA, Gasperini S, Bovolenta C, Calzetti F, Vollebregt M, Scapini P *et al.* (1999). Interleukin-10 (IL-10) selectively enhances CIS3/SOCS3 mRNA expression in human neutrophils: Evidence for an IL-10-induced pathway that is independent of STAT protein activation. *Blood* 94:2880-2889.

Cassatella MA, Meda L, Bonora S, Ceska M, Constantin G (1993). Interleukin-10 (IL-10) inhibits the release of proinflammatory cytokines from human polymorphonuclear leukocytes. evidence for an autocrine role of tumor necrosis factor and IL-1β in mediating the production of IL-8 triggered by lipopolysaccharide. *J Exp Med* 178:2207-2211.

Centre for Biomolecular modelling:Herman T (1998). Elastase [Online]. Available at http://www.msoe.edu. *Milwaukee School of Engineering, Milwaukee, U S A.*

Chavakis T, Hussain M, Kanse SM, Peters G, Breizwel RG, Flock J-I *et al.* (2002). *Staphylococcus aureus* extracellular adherence protein serves as anti-inflammatory factor by inhibiting the recruitment of host leukocytes. *Nat Med* 8:687-693.

Chen J, Kasper M, Heck T, Nakagawa K, Humpert PM, Bai L *et al.* (2005). Tissue factor as a link between wounding and tissue repair. *Diabetes* 54:2143-2154.

Choy EH, Isenberg DA, Garrood T, Farrow S, Ioannou Y, Bird H *et al.* (2002). Therapeutic benefit of blocking interleukin-6 activity with an anti-interleukin-6 receptor monoclonal antibody in rheumatoid arthritis: a randomised, double-blind,placebo-controlled, dose-escalation trail. *Arthritis Rheum* 46:3143-3150.

Chua F, Laurent GJ (2006). Neutrophil elastase. *Proc Am Thor Soc* 3:424-427.

Cicco NA, Lindemann A, Content J, Vendenbussche P, Lubbert M, Gauss J *et al.* (1990). Inducible production of interleukin-6 by human polymorphonuclear neutrophils: Role of granulocyte-macrophage colony-stimulating factor and tumor necrosis factor-alpha. *Blood* 75:2049-2052.

Clark R, Kupper T (2005). Old meets new: the interaction between innate and adaptive immunity. *J Invest Dermatol* 125:629-637.

Clark RAF (1998). Wound Repair: Overview and general considerations. In: The molecular and cellular biology of wound repair. Clark RAF, editor. New York: Plenum Press, pp. 3-50.

Clinical trials.gov (2003). Effects of topical SLPI on skin wounds [Online]. Available at http://www.clinicaltrials.gov identifier NCT00005569. *National Institute of Dental and Craniofacial Research.*

Coakley RJ, Taggart CC, O'Neill SJ, McElvaney NG (2001). Alpha-1-antitrypsin deficiency: biological answers to clinical questions. *Am J Med Sci* 321:33-41.

Cockeran R, Durandt C, Feldman C, Mitchell TJ, Anderson R (2002). Pneumolysin activates the synthesis and release of interleukin-8 by human neutrophils *in vitro*. *J Infect Dis* 186:562-565.

Coeshott C, Ohnemus C, Pilyavskaya A, Ross S, Weiczorek M, Kroona H *et al.* (1999). Converting enzyme-independent release of tumor necrosis factor α and IL-1β from a stimulated human monocytic cell line in the presence of activated neutrophils or purified proteinase 3. *Proc Natl Acad Sci U S A* 96:6261-6266.

Collier M (2001). A ten-point assessment plan for wound management. *J Comm Nurs.* 16:22-26.

Collin M, Olsen A (2003). Extracellular enzymes with immunomodulating activities: Variations on a theme in *Streptococcus pyogenes*. *Infect Immun* 71:2983-2992.

Collins CH, Lyne P (2004). Collins and Lyne's microbial methods. 8th edition ed. London: Hodder Arnold.

Colotta F, Re F, Polentarutti N, Sozzani S, Mantovani A (1992). Modulation of granulocyte survival and programmed cell death by cytokines and bacterial products. *Blood* 80:2012-2020.

Colsky AS, Kirsner RS, Kerdel FA (1998). Microbiologic evaluation of cutaneous wounds in hospitalised dermatology patients. *Ostomy Wound Manage* 44:40-46.

Cook H, Stephens P, Davies KJ, Harding KG, Thomas DW (2000). Defective extracellular matrix reorganisation by chronic wound fibroblasts is associated with alterations in TIMP-1, TIMP-2, and MMP-2 activity. *J Invest Dermatol* 115:225-233.

Cooper DM, Yu EZ, Hennessey P, Ko F, Robson MC (1994). Determination of endogenous cytokines in chronic wounds. *Ann Surg* 219:688-691.

Cooper R, Lawrence J (1996). The prevalence of bacteria and implications for infection control. *J Wound Care* 5:291-295.

Costerton JW, Stewart PS, Greenberg EP (1999). Bacterial biofilms: a common cause of persistant infections. *Science* 284:1318-1322.

Cui W, Morrison DC, Silverstein R (2000). Differential tumor necrosis factor alpha expression and release from peritoneal mouse macrophages *in vitro* in response to proliferating Gram-positive verses Gram-negative bacteria. *Infect Immun* 68:4422-4429.

Cullen B, Smith R, McCulloch E, Silcock D, Morrison L (2002). Mechanism of action of PROMOGRAN, a protease modulating matrix, for the treatment of diabetic foot ulcers. *Wound Repair Regen* 10:16-25.

Cusumano V, Tufano MA, Mancuso G, Carbone M, Rossano F, Fera MT *et al.* (1997). Porins of *Pseudomonas aeruginosa* induce release of tumour necrosis factor alpha and interleukin-6 by human leukocytes. *Infect Immun* 65:1683-1687.

Cutting KF, Harding KG (1994). Criteria for identifying wound infection. *J Wound Care* 3:198-201.

Dallegri F, Ottonello L (1997). Tissue injury in neutrophilic inflammation. *Inflamm Res* 46:382-391.

Danielsen L, Balsey E, Doring G, Hoiby N, Madsen SM, Agren M *et al.* (1998). Ulcer bed infection. Report of a case of enlarging venous leg ulcer colonised by *Pseudomonas aeruginosa*. *APMIS* 106:721-726.

Davies CE, Hill KE, Newcombe RG, Stephens P, Wilson MJ, Harding KG *et al.* (2007). A prospective study of the microbiology of chronic venous leg ulcers to reevaluate the clinical predictive value of tissue biopsies and swabs. *Wound Repair Regen* 15:17-22.

Davies CE, Hill KE, Wilson MJ, Stephens P, Hill CM, Harding KG *et al.* (2004). Use of 16S ribosomal DNA PCR and denaturing gradient gel electrophoresis for analysis of the microfloras of healing and nonhealing chronic venous leg ulcers. *J Clin Microbiol* 42:3549-3557.

Davies E (1998). Education, microbiology and chronic wounds. *J Wound Care* 7:272-274.

Davis BH (2005). Improved diagnostic approaches to infection/sepsis detection. *Expert Rev Mol Diagn* 5:193-207.

Debats IBJG, Booi D, Deutz NEP, Buurman WA, Boechx WD, van der Hulst RRWJ (2006). Infected chornic wounds show different local and systemic arginine conversion compared with acute wounds. *J Surg Res*.

Degre M (1996). Cytokines and bacterial infections. *Biotherapy* 8:219-228.

Delclaux C, Delacourt C, d'Ortho M-P, Boyer V, Lafuma C, Harf A (1996). Role of gelatinase B and elastase in human polymorphonuclear neutrophil migration across basement membrane. *Am J Respir Cell Mol Biol* 14:288-295.

Dennison DK, Van Dyke TE (2000). The acute inflammatory response and the role of phagocytic cells in periodontal health and disease. *Periodontol 2000* 14:54-78.

Desrochers PE, Mookhtiar K, Van Wart HE, Hasty KA, Weiss SJ (1992). Proteolytic inactivation of α1-proteinase inhibitor and α1-antichymotrypsin by oxidatively activated human neutrophil metalloproteinases. *J Biol Chem* 267:5005-5012.

Dinarello CA (2003). Anti-cytokine therapeutics and infections. *Vaccine* 21:S24-S34.

DiPietro LA (1995). Wound healing: The role of the macrophage and other immune cells. *Shock* 4:233-240.

Dittmer H, Jochum M, Schmit-Neuerburg KP (1985). The PMN elastase plasma level, a biochemical parameter of the severity of trauma. *Chirurg* 56:723-727.

Dolman KM, van de Wiel BA, Kam C-M, Abbink JJ, Hack CE, Sonnenberg A *et al.* (1992). Determination of proteinase 3-α_1-antitrypsin complexes in inflammatory fluids. *FEBS Lett* 314:117-121.

Dong Z, Ghabrial M, Katar M, Fridman R, Berk RS (2000). Membrane-type matrix metalloproteinases in mice intracorneally infected with *Pseudomonas aeruginosa. Invest Ophthalmol Vis Sci* 41:4189-4194.

Doring G (1994). The role of neutrophil elastase in chronic inflammation. *Am J Respir Crit Care Med* 150:S114-S117.

Doxey DL, Nares S, Park B, Trieu C, Cutler CW, Iacopino AM (1998). Diabetes-induced impairment of macrophage cytokine release in a rat model: Potential role of serum lipids. *Life Sci* 63:1127-1136.

Drinkwater SL, Burnand KG, Ding A, Smith A (2003). Increased but ineffectual angiogenic drive in nonhealing venous leg ulcers. *J Vasc Surg* 38:1106-1112.

Drinkwater SL, Smith A, Sawyer BM, Burnand KG (2002). Effect of venous ulcer exudates on angiogenesis *in vitro*. *Br J Surg* 89:709-713.

Driver VR (2004). Silver dressings in clinical practice. *Ostomy Wound Manage* 50 (9A Suppl):11S-15S.

Dubin G (2002). Extracellular proteases of *Staphylococcus* spp. *Biol Chem* 383:1075-1086.

Dumitru CD, Ceci JD, Tsatsanis C, Kontoyiannis D, Stamatakis K, Lin JH *et al.* (2000). TNF-alpha induction by LPS is regulated posttranscriptionally via a Tpl2/ERK-dependent pathway. *Cell* 103:1071-1083.

Duncan MR, Berman B (1985). Gamma interferon is the lymphokine and beta interferon the monokine responsible for inhibition of fibroblast collagen production and late but not early fibroblast proliferation. *J Exp Med* 162:516-527.

Duranton J, Bieth JG (2003). Inhibition of proteinase 3 by α1-antitrypsin *in vitro* predicts very fast inhibition *in vivo*. *Am J Respir Cell Mol Biol* 29:57-61.

Duswald KH, Jochum M, Schramm W, Fritz H (1985). Released granulocytic elastase: an indicator of pathobiochemical alterations in septicemia after abdominal surgery. *Surgery* 98:892-899.

Edwards JV, Bopp AF, Batiste S, Ullah AJ, Cohen IK, Diegelmann RF *et al.* (1999). Inhibition of elastase by a synthetic cotton-bound serine protease inhibitor: in vitro kinetics and inhibitor release. *Wound Repair Regen* 7:106-118.

Edwards JV, Howley P, Cohen IK (2004). In vitro inhibition of human neutrophil elastase by oleic acid albumin formulations from derivatized cotton wound dressings. *Int J Pharm* 284:1-12.

Elek SD (1956). Experimental staphylococci infections in the skin of man. *Ann N Y Acad Sci* 65:85.

Ellmerich S, Djouder N, Scholler M, Klein J-P (2000). Production of cytokines by monocytes,epithelial and endothelial cells activated by *Streptococcus bovis*. *Cytokine* 12:26-31.

Engelhardt E, Toksoy A, Goebeler M, Debus S, Brocker E-V, Gillitzer R (1998). Chemokines IL-8, GROα, MCP-1, IP-10 and Mig are sequentially and differentially expressed during phase-specific infiltration of leukocyte subsets in human wound healing. *Am J Pathol* 153:1849-1860.

Ennis WJ, Formann P, Mozen N, Massey J, Conner-Kerr T, Meneses P (2005). Ultrasound therapy for recalcitrant diabetic foot ulcers: Results of a randomized, double-blind, controlled, multicenter study. *Ostomy Wound Manage* 51:24-39.

Epelman S, Bruno TF, Neely GG, Woods DE, Mody CH (2000). *Pseudomonas aeruginosa* exoennzyme S induces transcriptional expression of proinflammatory cytokines and chemokines. *Infect Immun* 68:4811-4814.

Eriksson G, Eklund A-E, Kallings LO (1984). The clinical significance of bacterial growth in venous leg ulcers. *Scand J Infect Dis* 16:175-180.

Ethuin F, Gerard B, Benna JE, Boutten A, Gougerot-Pocidalo MA, Jacob L *et al.* (2004). Human neutrophils produce interferon gamma upon stimulation by interleukin-12. *Lab Invest* 84:1363-1371.

Euler GJ, Miller GA, Hutter JW, D'Alsandro MM (1998). Interleukin-6 in neutrophils from peripheral blood and inflammatory periradicular tissues. *J Endod* 24:480-484.

Falanga V (2004). The chronic wound: impaired healing and solutions in the context of wound bed preparation. *Blood Cells Mol Dis* 32:88-94.

Faurschou M, Borregaard N (2003). Neutrophil granules and secretory vesicles in inflammation. *Microbes and Infection* 5:1317-1327.

Feghali CA, Wright TM (1997). Cytokines in acute and chronic inflammation. *Front Biosci* 2:12-27.

Fernandes Abbade LP, Lastoria S (2005). Venous ulcer: epidemiology, physiopathology, diagnosis and treatment. *Int J Dermatol* 44:449-456.

Ferrante JV, Huang ZH, Nandoskar M, Hii CST, Robinson BS, Rathjen DA *et al.* (1997). Altered responses of human macrophages to lipopolysaccharide by hydroperoxy eicosatetraenoic acid, hydroxy eicosatetraenoic acid, and arachidonic acid inhhibition of tumour necrosis factor production. *J Clin Invest* 99:1445-1452.

Finch R (1988). Skin and soft-tissue infections. *Lancet* Jan 23:164-167.

Fitch PM, Roghanian A, Howie SEM, Sallenave JM (2006). Human neutrophil elastase inhibitors in innate and adaptive immunity. *Biochem Soc Trans* 34:279-282.

Folkman J (1997). Angiogenesis and angiogenesis inhibition: An overview. *Regulation of Angiogenesis*:1-8.

Fox DA (2000). Cytokine blockade as a new strategy to treat rheumatoid arthritis: Inhibition of tumor necrosis factor. *Arch Intern Med* 160:437-444.

Frick I-M, Akesson P, Rasmussen M, Schmidtchen A, Bjorck L (2003). SIC, a secreted protein of *Streptococcus pyogenes* that inactivates antibacterial peptides. *J Biol Chem* 278:16561-16566.

Friedman SJ, Su WP (1984). Management of leg ulcers with hydrocolloid occlusive dressing. *Arch Dermatol* 120:1329-1336.

Fritz H, Jochum M, Geiger R, Duswald KH, Dittmer H, Kortmann H *et al.* (1986). Granulocyte proteinases as mediators of unspecific proteolysis in inflammation: A review. *Folia Histochem Cytobiol* 24:99-116.

Fujihara M, Muroi M, Tanamoto K-I, Suzuki T, Azuma H, Ikeda H (2003). Molecular mechanisms of macrophage activation and deactivation by lipopolysaccharide: roles of the receptor complex. *Pharmacol Ther* 100:171-194.

Gabriel P, Cakman I, Rink L (2002). Overproduction of monokines by leukocytes after stimulation with lipopolysaccharide in the elderly. *Exp Gerontol* 37:235-247.

Galkowska H, Wojewodzka U, Olszewski WL (2005). Low recruitment of immune cells with increased expression of endothelial adhesion molecules in margins of the chronic diabetic foot ulcers. *Wound Repair Regen* 13:248-254.

Gardner SE, Frantz RA, Doebbeling BN (2001a). The validity of the clinical signs and symptoms used to identify localised chronic wound infection. *Wound Repair Regen* 9:178-186.

Gardner SE, Frantz RA, Saltzman CL, Dodgson KJ (2004). *Staphylococcus aureus* is associated with high microbial load in chronic wounds. *Wounds* 16:251-257.

Gardner SE, Frantz RA, Saltzman CL, Hillis SL, Park H, Scherubel M (2006). Diganostic validity of three swab techniques for identifying chronic wound infection. *Wound Repair Regen* 14:548-557.

Gardner SE, Frantz RA, Troia C, Eastman S, MacDonald M, Buresh K *et al.* (2001b). A tool to assess clinical signs and symptoms of localised infection in chronic wounds: Development and reliability. *Ostomy Wound Manage* 47:40-47.

Garrett B (1998). Re-epithelialisation. *J Wound Care* 7:358-360.

Gasser O, Hess C, Miot S, Deon C, Sanchez J-C, Schifferli JA (2003). Characterisation and properties of ectosomes released by human polymorphonuclear neutrophils. *Exp Cell Res* 285:243-257.

George-Gay B, Parker K (2003). Understanding the complete blood count with differential. *J Perianesth Nurs* 18:96-117.

Gerold G, Zychlinsky A, de Diego JL (2007). What is the role of Toll-like receptors in bacterial infections? *Semin Immunol* 19:41-47.

Ghosh AK, Yuan W, Mori Y, Chen Sj, Varga J (2001). Antagonistic regulation of type 1 collagen gene expression by interferon-gamma and transforming growth factor-beta. Integration at the level of p300/CBP transciptional coactivators. *J Biol Chem* 276:11041-11048.

Giacometti A, Cirioni O, Schimizzi AM, Del Prete MS, Barchiesi F, D'Errico MM *et al.* (2000). Epidemiology and microbiology of surgical wound infections. *J Clin Microbiol* 38:918-922.

Gillepsie S, Bamford K (2000). Medical microbiology and infection at a glance. 1st Edition ed. Williston: Blackwell Publishing.

Gillitzer R, Goebeler M (2001). Chemokines in cutaneous wound healing. *J Leukoc Biol* 69:513-521.

Goldman R, Salcido R (2002). More than one way to measure a wound: an overview of tools and techniques. *Adv Skin Wound Care* 12:236-245.

Gomez Navalon LA, Salido Valle JA, Lopez Alonso A (2000). Alpha-1-antitrypsin in early infections after hip prosthesis surgery. *Rev Chir Orthop Reparatrice Appar Mot* 86:794-800.

Gomez DE, Alonso DF, Yoshiji H, Thorgeirsson UP (1997). Tissue inhibitors of metalloproteinases:structure, regulation and biological functions. *Eur J Cell Biol* 74:111-122.

Grenier D (1996). Degradation of host protease inhibitors and activation of plasminogen by proteolytic enzymes from *Porphyromonas gingivalis* and *Treponema denticola*. *Microbiology* 142:955-961.

Grey J (1998). Cellulitis associated with wounds. *J Wound Care* 7:338-339.

Grimble SA, Magee TR, Galland RB (2001). Methicillin resistant *Staphylococcus aureus* in patients undergoing major amputation. *Eur J Vasc Endovasc Surg* 22:215-218.

Grinnell F, Ho CH, Wysocki AB (1992). Degradation of fibronectin and vitronectin in chronic wound fluid: analysis by cell blotting, immunoblotting and cell adhesion assays. *J Invest Dermatol* 98:410-416.

Grinnell F, Zhu M (1994). Identification of neutrophil elastase as the proteinase in burn wound fluid responsible for degradation of fibronectin. *J Invest Dermatol* 103:155-161.

Guhu M, Mackman N (2001). LPS induction of gene expression in human monocytes. *Cell Signal* 13:85-94.

Hachicha M, Rathanaswami P, Naccache PH, McColl SR (1998). Regulation of chemokine gene expression in human peripheral blood neutrophils phagocytosing microbial pathogens. *J Immunol* 160:449-454.

Hahn J, Friedrich JB, Zuder D, Steins A, Hahn M, Klyscz T (1997). Cutaneous inflammation limited to the region of the ulcer in chronic venous insufficiency. *Vasa* 26:277-281.

Halbwachs-Mecarelli L, Bessou G, Lesavre P, Lopez S, Witko-Sarat V (1995). Bimodal distribution of proteinase 3 (PR3) surface expression reflects a constitutive heterogeneity in the polymorphonuclear neutrophil pool. *FEBS Lett* 374:29-33.

Hancock EW, Diamond G (2000). The role of cationic antimicrobial peptides in innate host defences. *Trends Microbiol* 8:402-410.

Hansson C, Hoburn J, Moller A, Swanbeck G (1995). The microbial flora in venous leg ulcers without clinical signs of infection. Repeated culture using a validated standardised microbiological technique. *Acta Derm Venereol* 75:24-30.

Harding CV, Ramachandra L, Wick MJ (2003). Interaction of bacteria with antigen presenting cells: influences on antigen presentation and antibacterial immunity. *Curr Opin Immunol* 15:112-119.

Harding KG (1996). Managing wound infection. *J Wound Care* 5:39-40.

Harris IR, Yee KC, Walters CE, Cunliffe WJ, Kearney JN, Wood EJ *et al.* (1995). Cytokine and protease levels in healing and non-healing chronic venous leg ulcers. *Exp Dermatol* 4:342-349.

Harrison-Balestra C, Cazzaniga AL, Davies SC, Mertz PM (2003). A wound-isolated *Pseudomonas aeruginosa* grows a biofilm *in vitro* within 10 hours and is visualised by light microscopy. *Dermatol Surg* 29:631-635.

Hasan A, Murata H, Falabella A, Ochoa S, Zhou E, Badiavas E *et al.* (1997). Dermal fibroblasts from venous ulcers are unresponsive to the action of transforming growth factor-beta-1. *J Dermatol Sci* 16:59-66.

Hatanaka E, Monteagudo PT, Marrocos MS, Campa A (2006). Neutophils and monocytes as potentially important sources of proinflammatory cytokines in diabetes. *Clin Exp Immunology* 146:443-447.

Hattar K, Grandel U, Moeller A, Fink L, Iglhaut J, Hartung T *et al.* (2006). Lipoteichoic acid (LTA) from Staphylococcus aureus stimulates human neutrophil cytokine release by a CD14-dependent, Toll-like-receptor-independent mechanism: Autocrine role of tumor necrosis factor-α in mediating LTA-induced interleukin-8 generation. *Crit Care Med* 34:835-841.

Hauschildt S, Kleine B (1995). Bacterial stimulators of macrophages. *Int Rev Cytol* 161:263-331.

He Y, Young PK, Grinnell F (1998). Identification of proteinase 3 as the major caseinolytic activity in acute human wound fluid. *J Invest Dermatol* 110:67-71.

Heggars JP (1998). Defining infection in chronic wounds: does it matter? *J Wound Care* 7:389-392.

Heggars JP, Haydon S, Ko F, Hayward PG, Carp S, Robson MC (1992). *Pseudomonas aeruginosa* Exotoxin A: Its role in retardation of wound healing: The 1992 lindberg award. *J Burn Care Rehabil* 13:512-518.

Heinzelmann M, Scott M, Lam T (2002). Factors predisposing to bacterial invasion and infection. *Am J Surg* 183:179-190.

Henderson B, Poole S, Wilson M (1996). Microbial/host interactions in health and disease: Who controls the cytokine network? *Immunopharmacology* 35:1-21.

Henry G, Garner WL (2003). Inflammatory mediators in wound healing. *Surg Clin North Am* 83:483-507.

Herouy Y (2001). Matrix metalloproteinases in skin pathology. *Int J Mol Med* 7:3-12.

Herouy Y, Trefzer D, Zimpfer U, Schopf E, Wanscheidt W, Norgauer J (2000). Matrix metalloproteinases and venous leg ulceration. *Eur J Dermatol* 10:173-180.

Herrick S, Ashcroft GS, Ireland G, Horan M, McCollum C, Ferguson M (1997). Up-regulation of elastase in acute wounds of healthy aged humans and chronic venous leg ulcers are associated with matrix degradation. *Lab Invest* 77:281-288.

Hessle C, Andersson B, Wold AE (2000). Gram-positive bacteria are potent inducers of monocytic interleukin-12 (IL-12) while Gram-negative bacteria preferentially stimulate IL-10 production. *Infect Immun* 68:3581-3586.

Heumann D, Barras C, Severin A, Glauser MP, Tomasz A (1994). Gram-positive cell walls stimulate synthesis of tumor necrosis factor alpha and interleukin-6 by human monocytes. *Infect Immun* 62:2715-2721.

Heumann D, Roger T (2002). Initial responses to endotoxins and Gram-negative bacteria. *Clin Chim Acta* 323:59-72.

Hiemstra PS (2002). Novel roles of protease inhibitors in infection and inflammation. *Biochem Soc Trans* 30:116-120.

Hill KE, Davies CE, Wilson MJ, Stephens P, Harding KG, Thomas DW (2003). Molecular analysis of the microflora in chronic venous leg ulceration. *J Med Microbiol* 52:365-369.

Hlasta DA, Pagani ED (1994). Human leukocyte elastase inhibitors. *Annual Reports in Medicinal Chemistry* 29:195-204.

Hofer HP, Kukovetz E, Petek W, Schweighofer F, Wildburger R, Schaur RJ (1995). Released PMN elastase: an indicator of postsurgical uneventful wound healing and early

inflammatory complications. A contribution to the search for objective criteria in wound healing monitoring. *Injury* 26:103-106.

Horvat R, Clabaugh M, Duval-Jobe C, Parmely M (1989). Inactivation of human gamma interferon by *Pseudomonas aeruginosa* proteases: Elastase augments the effects of alkaline protease despite the presence of α_2-macroglobulin. *Infect Immun* 57:1668-1674.

Howell-Jones RS, Wilson M, Hill KE, Howard AJ, Price P, Thomas DW (2005). A review of the microbiology, antibiotic usage and resistance in chronic skin wounds. *J Antimicrob Chemother* 55:143-149.

Hrabec E, Strek M, Zieba M, Kwiatkowska S, Hrabec Z (2002). Circulation level of matrix metalloproteinase-9 is correlated with disease severity in tuberculosis patients. *Int J Tuberc Lung Dis* 6:713-719.

Hu G-F (1997). Limited proteolysis of angiogenin by elastase is regulated by plasminogen. *J Protein Chem* 16:669-679.

Hutchinson JJ, Lawrence JC (1991). Wound infection under occlusive dressings. *J Hosp Infect* 17:83-94.

Iocono JA, Colleran KR, Remick DG, Gillepsie BW, Ehrlich HP, Garner WL (2000). Interleukin-8 levels and activity in delayed-healing human thermal wounds. *Wound Repair Regen* 8:216-225.

Iwadou H, Morimoto Y, Iwagaki H, Sinoura S, Chouda Y, Kodama M *et al.* (2002). Differential cytokine response in host defence mechanisms triggered by gram-negative and gram-positive bacteria, and the roles of gabexate mesilate, a synthetic protease inhibitor. *J Int Med Res* 30:99-108.

Jablonska E, Jablonski J (2002). Effect of IL-18 on the release of IL-6 and its soluble receptor: sIL-6Ralpha and sgp130 by human neutrophils. *Immunol Invest* 31:159-167.

Jablonska E, Kliuk M, Markiewicz W, Piotrowski L, Grabowska Z, Jablonski J (2001). TNF-alpha, IL-6 and their soluble receptor serum levels and secretion by neutrophils in cancer patients. *Arch Immunol Ther Exp (Warsz)* 49:63-69.

James TJ, Hughes MA, Cherry GW, Taylor RP (2000). Simple biochemical markers to assess chronic wounds. *Wound Repair Regen* 8:264-269.

Janciauskiene S, Zelvyte I, Jansson L, Stevens T (2004). Divergent effects of α1-antitrypsin on neutrophil activation, *in vitro*. *Biochem Biophys Res Commun* 315:288-296.

Janoff A (1985). Elastases and emphysema. Current assessment of the protease-antiprotease hypothesis. *Am Rev Respir Dis* 132:417-433.

Jenner RG, Young RA (2005). Insights into host responses against pathogens from transcriptional profiling. *Nature Reviews Microbiology* 3:281-294.

Jiang Y, Magli L, Russo M (1999). Bacterium-dependent induction of cytokines in mononuclear cells and their pathologic consequences *in vivo*. *Infect Immun* 67:2125-2130.

Johnson LL, Dyer R, Hupe DJ (1998). Matrix metalloproteinases. *Curr Opin Cell Biol* 2:466-471.

Jones EW, Edwards R, Finch R, Jeffcoate WJ (1985). A microbiological study of diabetic foot lesions. *Diabet Med* 2:213-215.

Kahari V-M, Saarialho-Kere UK (1997). Matrix metalloproteinases in skin. *Exp Dermatol* 6:199-213.

Kaminishi H, Miyagushi H, Tamaki T, Suenaga N, Hisamatsu M, Mihashi I *et al.* (1995). Degradation of humoral host defense by *Candida albicans* proteinase. *Infect Immun* 63:984-988.

Kantor J, Margolis DJ (2000). A multicentre study of percentage change in venous leg ulcer area as a prognostic index of healing at 24 weeks. *Br J Dermatol* 142:960-964.

Karlsson A, Arvidson S (2002). Variation in extracellular protease production among clinical isolates of *Staphylococcus aureus* due to different levels of expression of the protease repressor *sarA*. *Infect Immun* 70:4239-4246.

Keane MP, Strieter RM (2002). The importance of balanced pro-inflammatory and anti-inflammatory mechanisms in diffuse lung disease. *Respiratory Research* 3:5-12.

Kernacki KA, Fridman R, Hazlett LD, Lande MA, Berk RS (1997). *In vivo* characterisation of host and bacterial protease expression during *Pseudomonas aeruginosa* corneal infections in naive and immunised mice. *Curr Eye Res* 16:289-297.

Kielian TL, Blecha F (1995). CD14 and other recognition molecules for lipopolysaccharide: a review. *Immunopharmacology* 29:187-205.

Kingsley A (1992). Assessment allows action on risk factors: infection control and surgical wounds. *Prof Nurse* 7:644-648.

Kingsley A (2001). A proactive approach to wound infection. *Nurs Stand* 15:50-58.

Kishimoto T, Akira S, Narazaki M, Taga T (1995). Interleukin-6 family of cytokines and gp130. *Blood* 86:1243-1254.

Kodelja V, Muller C, Tenorio S, Schebesch C, Orfanos CE, Goerdt S (1997). Differences in angiogenic potential of classically vs alternatively activated macrophages. *Immunobiology* 197:478-493.

Korkmaz B, Attucci S, Hazouard E, Ferrandiere M, Jourrdan ML, Brillard-Bourdet M *et al.* (2002). Discriminating between the activities of human neutrophil elastase and proteinase 3 using serpin-derived fluorogenic substrates. *J Biol Chem* 277:39074-39081.

Kramer SA (1999). Effect of povidone-iodine on wound healing: a review. *J Vasc Nurs* 17:17-23.

Kreutz M, Ackermann U, Hauschildt S, Krause SW, Riedel D, Bessler W *et al.* (1997). A comparitive analysis of cytokine production and tolerance induction by bacterial lipopeptides, lipopolysaccharides and *Staphylococcus aureus* in human monocytes. *Immunology* 92:396-401.

Krishna C, Puppula A, Liu YY, Azim J, Khan LD, Cook BC *et al.* (1997). Serine protease inhibitors profiles distinguish chronic from acute wounds. *J Invest Dermatol* 108(4):617.

Krishnamoorthy L, Morris HL, Harding KG (2001). Specific growth factors and the healing of chronic wounds. *J Wound Care* 10:173-178.

Kusunoki T, Hailman E, Juan TS-C, Lichenstein HS, Wright SD (1995). Molecules from *Staphyloccocus aureus* that bind CD14 and stimulate innate immune responses. *J Exp Med* 182:1673-1682.

Kuzmic P, Sideris S, Cregar LM, Elrod KC, Rice KD, Janc JW (2000). High-throughput screening of enzyme inhibitors: automatic determination of tight-binding inhibition constraints. *Anal Biochem* 15:62-67.

Ladwig GP, Robson MC, Liu R, Kuhn MA, Muir DF, Shultz GS (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* 10:26-37.

Lamster IB, Novak MJ (1992). Host mediators in gingival crevicular fluid: implications for the pathogenesis of periodontal disease. *Crit Rev Oral Biol Med* 361:31-60.

Laplante AF, Germain L, Auger FA, Moulin V (2001). Mechanisms of wound reepithelisation: hints from a tissue-engineered reconstructive skin to long-standing questions. *FASEB J* 15:2377-2389.

Lauer G, Sollberg S, Cole M, Flamme I, Sturzebecher J, Mann K *et al.* (2000). Expression and proteolysis of vascular endothelial growth factos is increased in chronic wounds. *J Invest Dermatol* 115:12-18.

Laurell CB, Eriksson S (1963). The serum alpha-1-antitrypsin in families with hypo-alpha-1-antitrysinemia. *Clin Chim Acta* 11:395-398.

Lawrence JC, Ameen H (1998). Swabs and other sampling techniques. *J Wound Care* 7:232-233.

Leber TM, Balkwill FR (1997). Zymography: A single step staining method for quantification of proteolytic activity on substrate gels. *Anal Biochem* 249:24-28.

Ledur A, Fitting C, David B, Hamberger C, Cavaillon J-M (1995). Variable estimates of cytokine levels produced by commercial ELISA kits: results using international cytokine standards. *J Immunol Methods* 186:171-179.

Lee MM, Yoon BJ, Osiewicz K, Preston M, Bundy B, van Heeckeren AM *et al.* (2005). Tissue inhibitor of metalloproteinase 1 regulates resistance to infection. *Infect Immun* 73:661-665.

Lee VT, Schneewind O (2001). Protein secretion and the pathogenesis of bacterial infections. *Genes Dev* 15:1725-1752.

Levy JH (1996). The human inflammatory response. *J Cardiovasc Pharmacol* 27:S31-S37.

Li Y-Q, Doyle JW, Roth TP, Dunn RM, Lawrence T (1998). IL-10 and GM-CSF expression and the presence of antigen-presenting cells in chronic venous ulcers. *J Surg Res* 79:128-135.

Li Y, Karlin A, Loike JD, Silverstein SC (2002). A critical concentration of neutrophils is required from effective bacterial killing in suspension. *PNAS* 99:8289-8294.

Lin E, Calvano SE, Lowry SF (2000). Inflammatory cytokines and cell response in surgery. *Surgery* 127:117-126.

Lind L (2003). Circulating markers of inflammation and atherosclerosis. *Atherosclerosis* 169:203-214.

Liu J, Li X, Yue Y, Li J, He T, He Y (2005). The inhibitory effect of quercetin on IL-6 production by LPS stimulated neutrophils. *Cell Mol Immunol* 2:455-460.

Liu M, Sun H, Wang X, Koike T, Mishima H, Ikeda K *et al.* (2004). Association of increased expression of macrophage elastase (matrix metalloproteinase 12) with rheumatoid arthritis. *Arthritis Rheum* 50:3112-3117.

Liz-Grana M, Gomez-Reino Carnota JJ (2001). Tumour Necrosis Factor. Genetics, cell action mechanism and involvement in inflammation. *Allergol Immunol Clin* 16:140-149.

Loots MA, Lamme EN, Zeegelaar J, Mekkes JR, Bos JD, Middelkoop E (1998). Differences in the cellular infiltrate and extracellular matrix of chronic diabetic and venous ulcers verses the acute wound. *J Invest Dermatol* 111:850-857.

Lopez-Bojorquez LN, Dehesa AZ, Reyes-Teran G (2004). Molecular mechanisms involved in the pathogenesis of septic shock. *Arch Med Res* 35:465-479.

Lorentzen HF, Gottrup F (2006). Clinical assessment of infection in nonhealing ulcers analysed by latent class analysis. *Wound Repair Regen* 14:350-353.

Lowy FD (2003). *Staphylococcus aureus* infections. *N Engl J Med* 339:520-532.

Lundberg JE, Roth TP, Dunn RM, Doyle JW (1998). Comparison of IL-10 levels in chronic venous insufficiency ulcers and autologous donor tissue. *Arch Dermatol Res* 290:669-673.

Lynn WA, Liu Y, Golenbock DT (1993). Neither CD14 nor serum is absolutely necessary for activation of mononuclear phagocytes by bacterial lipopolysaccharide. *Infect Immun*(61):4452-4461.

Madsen SM, Westh H, Danielsen L, Rosdahl VT (1996). Bacterial colonisation and healing of venous leg ulcers. *APMIS* 104:895-899.

Majewski W, Cybulski Z, Napierala M (1995). The value of quantitative bacteriological investigations in the monitoring of treatment of ischemic ulcerations of lower legs. *Int Angiol* 14:381-384.

Majka SM, Kasimos J, Izzo L, Izzo AA (2002). *Cyrptococcus neoformans* pulmonary granuloma formation is associated with matrix metalloproteinase-2 expression. *Med Mycol* 40:323-328.

Makowski GS, Ramsby ML (2003). Zymographic analysis of latent and activated forms of matrix metalloproteinase-2 and -9 in synovial fluid: correlation to polymorphonuclear leukocyte infiltration and in response to infection. *Clin Chim Acta* 329:77-81.

Mani R (1999). Science of measurements in wound healing. *Wound Repair Regen* 7:330-334.

Marra MN, Thornton MB, Snable JL, Leong S, Lane J, Wilde CG *et al.* (1993). Regulation of the response to bacterial lipopolysaccharide by endogenous and exogenous lipopolysacharide binding proteins. *Blood Purif* 11:134-140.

Marra MN, Wilde CG, Collins MS, Snable JL, Thornton MB, Scott RW (1992). The role of bactericidal/permeability-increasing protein as a natural inhibitor of bacterial endotoxin. *J Immunol* 148:532-537.

Martin P (1997). Wound healing - aiming for perfect skin regeneration. *Science* 276:75-81.

Martinez FO, Sironi M, Vecchi A, Colotta F, Mantovani A, Locati M (2004). IL-8 induces a specific transcriptional profile in human neutrophils: synergism with LPS for IL-1 production. *Eur J Immunol* 34:2286-2292.

Marty KB, Williams CL, Guynn LJ, Benedik MJ, Blanke SR (2002). Characterization of a cytotoxic factor in culture filtrates of *Serratia marcescens*. *Infect Immun* 70:1121-1128.

Mast BA, Shultz GS (1996). Interactions of cytokines, growth factors, and proteases in acute and chronic wounds. *Wound Repair Regen* 4:411-420.

Matsuda Y, Marungama H, Kuraya K (1995). Relationship between granulocyte elastase levels and perinatal infections. *Gynecol Obstet Invest* 39:162-166.

Matsumoto T (1999). Diagnosis of sepsis based on the host response. *Rinsho Byori* 47:494-500.

Matsuzawa K, Fukuyama K, Dirks PB, Hubbard S, Murakami M, Becker LE *et al.* (1996). Expression of stromelysin 1 in human astrocytoma cell lines. *J Neurooncol* 30:181-188.

Mayer-Scholl A, Averhoff P, Zychlinsky A (2004). How do neutrophils and pathogens interact? *Curr Opin Microbiol* 7:62-66.

McBride JD, Freeman HNM, Leatherbarrow RJ (1999). Selection of human elastase inhibitors from a conformationally constrained combinatorial peptide library. *Eur J Biochem* 266:403-412.

Medina A, Scott PG, Ghahary A, Tredget EE (2005). The pathophysiology of chronic nonhealing wounds. *J Burn Care Rehabil* 26:306-319.

Meduri GU (2002). Clinical review: a paradigm shift: the bidirectional effect of inflammation on bacterial growth. Clinical implications for patients with acute respiratory distress syndrome. *Critical Care* 6:24-29.

Meduri GU, Kanangat S, Stefan J, Tolley E, Schaberg D (1999). Cytokines IL-1β, IL-6, and TNF-α enhance *in vitro* growth of bacteria. *Am J Respir Crit Care Med* 160:961-967.

Medvedez AE, Flo T, Ingalls RR, Golenbock DT, Teti G, Vogel SN *et al.* (1998). Involvement of CD14 and complement receptors CR3 and CR4 in nuclear factor-κB activation and TNF production induced by lipopolysaccharide and group B streptococcal cell walls. *J Immunol* 160:4535-4542.

Mendez MV, Raffetto JD, Phillips T, Menzoian JO, Park HY (1999). The proliferative capacity of neaonatal skin fibroblasts is reduced after exposure to venous ulcer wound fluid: A potential mechanism for senescence in venous ulcers. *J Vasc Surg* 30:734-743.

Mendez MV, Stanley A, Park HY, Shon K, Phillips T, Menzoian JO (1998). Fibroblasts cultured from venous ulcers display cellular characteristics of senescence. *J Vasc Surg* 28:876-883.

Mertz PM (2003). Cutaneous biofilms: Friend or foe? *Wounds* 15:129-132.

Mertz PM, Ovington LG (1993). Wound healing microbiology. *Dermatol Clin* 11:739-747.

Meszaros AJ, Reichner JS, Albina JE (2000). Macrophage-induced neutrophil apoptosis. *J Immunol* 165:435-441.

Michaud D (1998). Gel electrophoresis of proteolytic enzymes. *Anal Chim Acta* 372:173-185.

Micic S, Macura M, Lalic N, Dotlic R (1989). Elastase is an indicator of silent genital tract infection in infertile men. *Int J Androl* 12:423-429.

Miyajima S, Akaike T, Matsumoto T, Okamoto T, Yoshitake J, Hayashida K *et al.* (2001). Matrix metalloproteinases induction by pseudomonal virulence factors and inflammatory cytokines *in vitro*. *Microb Pathog*(31):271-281.

Miyasaki KT (1991). The neutrophil: mechanisms of controlling periodontal bacteria. *J Periodontol* 62:761-774.

Miyasaki KT (2006). Phagocytes-neutrophils. *[Online] http:/www dent ucla edu Last modified 2/3/2006.*

Mocellin S, Marincola FM, Rossi CR, Nitti D, Lise M (2004). The multifaceted relationship between IL-10 and adaptive immunity: putting together the pieces of a puzzle. *Cytokine Growth Factor Rev* 15:61-76.

Moore K (1999). Cell biology of chronic wounds: the role of inflammation. *J Wound Care* 8:345-348.

Moore K, Ruge F, Harding KG (1997). T lymphocytes and the lack of activated macrophages in wound margin biopsies from chronic leg ulcers. *Br J Dermatol* 137:188-194.

Moseby CB (1997). Mosebys medical dictionary. Fifth Edition ed. Harvard: C.B.Moseby.

Moseley R, Hilton J, Waddington RJ, Harding KG, Stephens P, Thomas DW (2004a). Comparison of oxidative stress marker profiles between acute and chronic wound environments. *Wound Repair Regen* 12:419-429.

Moseley R, Stewart JE, Stephens P, Waddington RJ, Thomas DW (2004b). Extracellular matrix metabolites as potential biomarkers of disease activity in wound fluid: lessons learned from other inflammatory diseases? *Br J Dermatol* 150:401-413.

Moseley R, Walker M, Waddington RJ, Chen WY (2003). Comparison of the antioxidant properties of wound dressing materials- carboxymethylcellulose, hyaluronan benzyl ester and hyaluronan, towards polymorphonuclear leukocyte-derived reactive oxygen species. *Biomaterials* 24:1549-1557.

Moser B, Wolf M, Walz A, Loetscher P (2004). Chemokines: multiple levels of leukocyte migration control. *Trends in Immunology* 25:75-84.

Muller G, Winkler Y, Kramer A (2003). Antibacterial activity and endotoxin-binding capacity of Actisorb Silver 220. *J Hosp Infect* 53:211-214.

Mullins RE, Miller RL, Hunter RL, Bennett B (1984). Standardised automated assay for functional alpha 1-antitrypsin. *Clin Chem* 30:1857-1860.

Murphy G (1995). Matrix metalloproteinases and their inhibitors. *Acta Orthop Scand* 66:55-60.

Murphy MA, Joyce WP, Condron C, Bouchier-Hayes D (2002). A reduction in serum cytokine levels parallels healing of venous ulcers in patients undergoing compression therapy. *Eur J Vasc Endovasc Surg* 23:349-352.

Nagase H, Woessner FFJr (1997). Matrix metalloproteinases. *J Biol Chem* 274:21491-21494.

Nakajima K, Matsuda T, Fujitana Y, Kojima H, Yamanaka Y, Nakae K *et al.* (1995). Signal transduction through IL-6 receptor: involvement of multiple protein kinases, stat factors, and a novel H7-sensitive pathway. *Ann N Y Acad Sci* 21:55-70.

Nanney LB, Skeel A, Luan J, Polis S, Richmond A, Wang MH *et al.* (1998). Proteolytic cleavage and activation of pro-macrophage-stimulating protein and upregulation of its receptor in tissue injury. *J Invest Dermatol* 111:573-578.

Nathan C, Sporn M (1991). Cytokines in context. *J Cell Biol* 113:81-86.

Neal MS (2001). Angiogenesis: is it the key to controlling the healing process. *J Wound Care* 10:281-287.

Nglia TV, Xue M, Castelnoble LA, Jackson J (2007). The dual personalities of matrix metalloproteinases in inflammation. *Front Biosci* 12:1475-1487.

Nicas TI, Iglewski BH (1985). The contribution of exoproducts to virulence of *Pseudomonas aeruginosa*. *Can J Microbiol* 31:387-392.

Nie J, Pei D (2004). Rapid Inactivation of alpha-1-proteinase inhibitor by neutrophil specific leukolysin/membrance-type matrix metalloproteinase 6. *Exp Cell Res* 296:145-150.

Normark S, Normark BH, Hornef M (2001). How neutrophils recognize bacteria and move toward infection. *Nat Med* 7:1182-1184.

Nussler AK, Wittel UA, Nussler NC, Beger HG (1999). Leukocytes, the Janus cells in inflammatory disease. *Langenbecks Arch Surg* 384:222-232.

Nwomeh BC, Hui-Xiu L, Cohen IK, Yager DR (1999). MMP-8 Is the predominant collagenase in healing wounds and nonhealing ulcers. *J Surg Res* 81:189-195.

O'Toole EA (2001). Extracellular matrix and keratinocyte migration. *Clin Exp Dermatol* 26:525-530.

Oikarinen A, Kylmaniemi M, Autio-Harmainen H, Autio P, Salo T (1993). Demonstration of 72-kDa and 92-kDa forms of type IV collagenase in human skin: Variable expression in various blistering diseases, induction during re-epithelialization, and decrease by topical glucocorticoids. *J Invest Dermatol* 101:205-210.

Okamoto T, Akaike T, Suga M, Tanase S, Horie H, Miyajima S *et al.* (1997). Activation of human matrix metalloproteinases by various bacterial proteinases. *J Biol Chem* 272:6059-6066.

Onuffer JJ, Horuk R (2002). Chemokines, chemokine receptors and small-molecule antagonists: recent developments. *Trends Pharmacol Sci* 23:459-467.

Opal SM, Palardy JE, Marra MN, Fisher CJ, McKelligon BM, Scott RW (1994). Relative concentrations of endotoxin-binding proteins in body fluids during infections. *Lancet* 344:429-431.

Owen CA, Campbell EJ (1995). Neutrophil proteinases and matrix degradation. The cell biology of pericellular proteolysis. *Semin Cell Biol* 6:367-376.

Pagano M (1999). Application of electrophoresis and related methods, such as Western blotting and zymography to the study of some proteins and enzymes. *Anal Chim Acta* 383:119-125.

Palma C, Cassone A, Serbousek D, Pearson CA, Djeu JY (1992). Lactoferrin release and interleukin-1, interleukin-6, and tumor necrosis factor production by human polymorphonuclear cells stimulated by various lipopolysaccharides: Relationship to growth inhibition of *Candida albicans*. *Infect Immun* 60:4604-4611.

Palolahti M, Lauharanta J, Stephens RW, Kuusela P, Vaheri A (1993). Proteolytic activity in leg ulcer exudate. *Exp Dermatol* 2:29-37.

Parks WC (1999). Matrix metalloproteinases in repair. *Wound Repair Regen* 7:423-432.

Parmely M, Gale A, Clabaugh M, Horvat R, Zhou WW (1990). Proteolytic inactivation of cytokines by *Pseudomonas aeruginosa*. *Infect Immun* 58:3009-3014.

Patel GK, Wilson CH, Harding KG, Finlay AY, Bowden PE (2006). Numerous keratinocyte subtypes involved in wound re-epithelisation. *J Invest Dermatol* 126:497-502.

Peleg AY, Weerarathna T, McCarthy JS, Davies TME (2007). Common infections in diabetes: pathogenesis, management and relationship to glycaemic control. *Diabetes Metab Res Rev* 23:3-13.

Percival SL, Bowler PG (2004). Biofilms and their potential role in wound healing. *Wounds* 16:234-240.

Perlino E, Cortese R, Ciliberto G (1987). The human α1–antitrypsin gene is transcribed from two different promoters in macrophages and hepatocytes. *EMBO J* 6:2767-2771.

Peschel A (2002). How do bacteria resist human antimicrobial peptides. *Trends Microbiol* 10:179-186.

Peschen M, Grenz H, Brand-Saberi B, Bunaes M, Simon JC, Schopf E (1998). Increased expression of platelet-derived growth factor receptor alpha and beta and vascular endothelial growth factor in the skin of patients with chronic venous insufficiency. *Arch Dermatol Res* 290:291-297.

Petropoulou P, Zhang Z, Curtis MA, Johnson NW, Hughes FJ, Winyard PG (2003). Measurement of both native and inactivated forms of α1 proteinase inhibitor in human inflammatory extracellular fluids. *J Clin Periodontol* 30:795-801.

Pierce GF (2001). Inflammation in nonhealing diabetic wounds. *Am J Pathol* 159:399-403.

Pieters J (2001). Evasion of host cell defense mechanisms by pathogenic bacteria. *Curr Opin Immunol* 13:37-44.

Pirila E, Korpi JT, Korkiamaki T, Jahkola T, Gutierrez-Fernandez A, Lopez-Otin C *et al.* (2007). Collagenase-2 (MMP-8) and matrilysin-2 (MMP-26) expression in human wounds of different etiolgies. *Wound Repair Regen* 15:47-57.

Playfair JHL, Chain BM (2001). Immunology at a glance. 7th Edition ed. Oxford: Blackwell Science Ltd.

Potempa J, Dubin A, Korzus G, Travis J (1988). Degradation of elastin by a cysteine proteinase from *Staphylococcus aureus*. *J Biol Chem* 263:2664-2667.

Pradella M, Memetz L, Zappala G, Ossani M, Plebani M (1995). Quantitative cytochemistry of human leukocyte elastase compared with plasma elastase and acute phase proteins in inflammatory disease. *Clin Chim Acta* 239:91-101.

Proctor RA, Peters G (1998). Small colony variants in staphylococcal infections: Diagnostic and therapeutic implications. *Clin Infect Dis* 27:419-423.

Proost MA, Van Damme J, Opdenakker G (1991). Purification and identification of 91-kDa neutrophil gelatinase. Release by the activating peptide interleukin-8. *Eur J Biochem* 198:391-398.

Pugin J, Widmer MC, Kossodo S, Liang CM, Preas HL, Suffredini AF (1999). Human neutrophils secrete gelatinase B *in vitro* and *in vivo* in response to endotoxin and proinflammatory mediators. *Am J Respir Cell Mol Biol* 20:458-464.

Quatresooz P, Henry F, Paquet P, Pierard-Franchimont C, Harding KG, Pierard GE (2003). Deciphering the impaired cytokine cascades in chronic leg ulcers (Review). *Int J Mol Med* 11:411-418.

Quiding-Jarbrink M, Smith DA, Bancroft GJ (2001). Production of matrix metalloproteinases in response to mycobacterial infection. *Infect Immun* 69:5661-5670.

Rabehi L, Irinopoulou T, Cholley B, Haeffner-Cavaillon N, Carreno M-P (2001). Gram-positive and Gram-negative bacteria do not trigger monocytic cytokine production through similar intracellular pathways. *Infect Immun* 69:4590-4599.

Rao CN, Ladin DA, Liu YY, Chilukuri K, Hou Z, Woodley DT (1995). Alpha 1-antitrypsin is degraded and non-functional in chronic wounds but intact and functional in acute wounds: the inhibitor protects fibronectin from degradation by chronic wound fluid enzymes. *J Invest Dermatol* 4:572-578.

Rappuoli R (2000). Pushing the limits of cellular microbiology: Microarrays to study bacteria-host cell intimate contacts. *PNAS* 97:13467-13469.

Raser KJ, Posner A, Wang KK (1995). Casein zymography: a method to study mu-calpain, m-calpain, and their inhibitory agents. *Arch Biochem Biophys* 1995:211-216.

Ratcliff CR, Rodeheaver GT (2002). Correlation of semi-quantitative swab cultures to quantitative swab cultures from chronic wounds. *Wounds* 14:329-333.

Ravanti L, Kahari V-M (2000). Matrix metalloproteinases in wound repair (Review). *Int J Mol Med* 6:391-407.

Reddi K, Wilson M, Nair S, Poole S, Henderson B (1996). Comparison of the pro-inflammatory cytokine-stimulating activity of the surface-associated proteins of periodontopathic bacteria. *J Periodontal Res* 31:120-130.

Reed SB, Wesson CA, Liou LE, Trumble WR, Schlievert PK, Bohach GA *et al.* (2001). Molecular charaterisation of a novel *Staphylococcus aureus* serine protease operon. *Infect Immun* 69:1521-1527.

Reeves EP, Lu H, Jacobs HL, Messina CGM, Bolsover S, Gabella G *et al.* (2002). Killing activity of neutrophils is mediated through activation of proteases by K^+ flux. *Nature* 416:291-297.

Reglier H, Arce-Vicioso M, Fay M, Gougerot-Pocidalo MA, Chollet-Martin S (1998). Lack of IL-10 and IL-13 production by human polymorphonuclear neutrophils. *Cytokine* 10:192-198.

Remer KA, Brcic M, Jungi TW (2003). Toll-like receptor-4 is involved in eliciting an LPS-induced oxidative burst in neutrophils. *Immunol Lett* 85:75-80.

Rennekampff H-A, Hansbrough JF, Kiessig BS, Dore C, Sticherling M, Schroder J-M (2000). Bioactive IL-8 is expressed in wounds and enhances wound healing. *J Surg Res* 93:41-54.

Robson MC (1997). Wound infection. A failure of wound healing caused by an imbalance of bacteria. *Surg Clin North Am* 77:637-650.

Robson MC, Stenberg BD, Heggars JP (1990). Wound healing alterations caused by infection. *Clin Plast Surg* 17:485-492.

Romagnani P, Lasagni L, Annunziato F, Serio M, Romagnani S (2004). CXC chemokines: the regulatory link between inflammation and angiogenesis. *Trends in Immunology* 25:201-209.

Rosenkilde MM, Schwartz TW (2004). The chemokine system - a major regulator of angiogenesis in health and disease. *APMIS* 112:481-495.

Rubinstein A, Pierce CE, Bloomgarden Z (1983). Rapid healing of diabetic foot ulcers with subcutaneous insulin infusion. *Am J Med* 75:161-166.

Sakai A, Ohsima M, Sugano N, Otsuka K, Ito K (2006). Profiling the cytokines in gingival crevicular fluid using a cytokine antibody array. *J Periodontol* 77:856-864.

Salo T, Makela M, Kylmaniemi M, Autio-Harmainen H, Larjava H (1994). Expression of matrix metalloproteinase-2 and -9 during early human wound healing. *Lab Invest* 70:176-182.

Sandler NG, Mentink-Kane MM, Cheever AW, Wynn TA (2003). Global gene expression profiles during acute pathogen-induced pulmonary inflammation reveal divergent roles for Th1 and Th2 responses in tissue repair. *J Immunol* 171:3655-3667.

Santala A, Saarinen J, Kovanen P, Kuusela P (1999). Activation of interstitial collagenase, MMP-1, by *Staphylococcus aureus* cells having surface-bound plasmin: a novel role of plasminogen receptors of bacteria. *FEBS Lett* 461:153-156.

Sautner T, Fugger R, Gotzinger P, Mittlbock M, Winkler S, Roth E *et al.* (1995). Tumour necrosis factor-alpha and interleukin-6: early indicator of bacterial infection after human orthotopic liver transplantation. *Eur J Surg* 161:97-101.

Scapini P, Calzetti F, Cassatella MA (1999). On the detection of neutrophil-derived vascular endothelial growth factor (VEGF). *J Immunol Methods* 232:121-129.

Schaechter M, Medoff G, Eisenstein BI (1993). Mechanisms of microbial disease. 2nd Edition ed. Baltimore: Williams and Wilkins.

Schlindler R, Mancilla J, Endres S, Ghorbani R, Clark SC, Dinarello CA (1990). Correlations and interactions in the production of interleukin-6 (IL-6), IL-1, and tumor necrosis factor (TNF) in human blood mononuclear cells: IL-6 surpresses IL-1 and TNF. *Blood* 75:40-47.

Schmidt K, St Jeβberger ESD, Ziegler U, Thiede A (2000). Bacterial population of chronic crural ulcers: is there a difference between the diabetic, the venous, and the arterial ulcer. *Vasa* 29:62-70.

Schmidtchen A (2000). Degradation of antiproteinases, complement and fibronectin in chronic leg ulcers. *Acta Derm Venereol* 80:179-184.

Schmidtchen A, Frick I-M, Andersson E, Tapper H, Bjorck L (2002). Proteinases of common pathogenic bacteria degrade and inactivate the antibacterial peptide LL-37. *Mol Microbiol* 46:157-168.

Schmidtchen A, Holst E, Tapper H, Bjorck L (2003). Elastase-producing *Pseudomonas aeruginosa* degrade plasma proteins and extracellular products of human skin and fibroblasts, and inhibit fibroblast growth. *Microb Pathog* 34:47-55.

Schmidtchen A, Wolff H, Hansson C (2001). Differential proteinase expression by *Pseudomonas aeruginosa* derived from chronic leg ulcers. *Acta Derm Venereol* 81:406-409.

Schonfelder U, Abel M, Wiegand C, Klemm D, Elsner P, Hipler U-C (2005). Influence of selected wound dressings on PMN elastase in chronic wound fluid and their antioxidative potential *in vitro*. *Biomaterials* 26:6664-6673.

Schroder AK, Rink L (2003). Neutrophil immunity of the elderly. *Mech Ageing Dev* 124:419-425.

Schroder AK, von der Ohe M, Kolling U, Altstaedt J, Uciechowski P, Fleischer D *et al.* (2006). Polymorphonuclear leukocytes selectively produce anti-inflammatory interleukin-1 receptor antagonist and chemokines, but fail to produce pro-inflammatory mediators. *Immunology* 119:317-327.

Segal AW (2005). How neutrophils kill microbes. *Annu Rev Immunol* 23:197-223.

Selvaggi G, Monstrey S, van Landuyt K, Hamdi M, Blondeel P (2003). The role of iodine in antisepsis and wound management: a reappraisal. *Acta Chir Belg* 103:241-247.

Shapiro S, Miller A, Lahat N, Sobel E, Lerner A (2003). Expression of matrix metalloproteinases, sICAM and IL-8 in CSF form children with meningitis. *J Neurol Sci* 15:43-48.

Shapiro SD (1999). The macrophage in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 160:S29-S32.

Sharpira L, Takashiba S, Amar S, Van Dyke TE (1994). Porphyromonas gingivalis lipopolysaccharide stimulation of human monocytes; dependence of serum and CD14 receptor. *Oral Microbiol Immunol* 9:112-117.

Sharpiro SD (1998). Matrix metalloproteinase degradation of extracellular matrix: biological consequences. *Curr Opin Cell Biol* 10:602-608.

Shoab SS, Scurr JH, ColeridgeSmith PD (1998). Increased plasma vascular endothelial growth factor amoung patients with chronic venous disease. *J Vasc Surg* 28:535-540.

Sibbald RG (2003). Topical antimicrobials. *Ostomy Wound Manage* 49:14-18.

Simon D, Simon H-U (2007). Eosinophilic disorders. *J Allergy Clin Immunol* 119:1291-1300.

Simpson DM, Ross R (1972). The neutrophilic leukocyte in wound repair: A study with antineutrophil serum. *J Clin Invest* 51:2009-2023.

Singer AJ, Clark RAF (1999). Cutaneous wound healing. *N Engl J Med* 341:738-746.

Singh PK, Parsek MR, Greenberg EP, Welsh MJ (2002). A component of innate immunity prevents bacterial biofilm develpoment. *Nature* 417:552-555.

Snoek-van Beurden PA, Von den Hoff JW (2005). Zymographic techniques for the analysis of matrix metalloproteinases and their inhibitors. *Biotechniques* 38:73-83.

Socransky SS, Haffajee AD (1991). Microbial mechanisms in the pathogenesis of destructive periodontal diseases: a critical assessment. *J Periodontal Res* 26:195-212.

Soo C, Shaw WW, Zhang X, Longaker MT, Howard EW, Ting K (2000). Differential expression of matrix metalloproteinases and their tissue-derived inhibitors in cutaneous wound repair. *Plast Reconstr Surg* 105:638-647.

Specks U (2000). What you should know about PR3-ANCA Conformational requirements of proteinase 3 (PR3) for enzymatic activity and recognition by PR3-ANCA. *Arthritis Res* 2:263-267.

Stacey MC, Lainez S, Skender-Kalnenas T, Morrison B (1995). Alterations in immune cells in human chronic leg ulcers. *Phlebologie* Suppl.1.:793-795.

Stadelmann WK, Digenis AG, Tobin GR (1998a). Impediments to wound healing. *Am J Surg* 176:39S-47S.

Stadelmann WK, Digenis AG, Tobin GR (1998b). Physiology and healing dymanics of chronic cutaneous wounds. *Am J Surg* 176:26S-38S.

Staiano-Coico L, Higgins PJ, Schwartz SB, Zimm MD, Goncalves G (2000). Wound fluids: A reflection of the state of healing. *Ostomy Wound Manage* 46(Suppl 1A):85S-93S.

Stavitsky AB (2007). The innate immune response to infection, toxins and trauma evolved into networks of interactive, defensive, reparative regulatory, injurous and pathogenic pathways. *Mol Immunol* 44:2787-2799.

Steinbeck MJ, Roth JA (1989). Neutrophil actviation by recombination cytokines. *Rev Infect Dis* 11:549-568.

Stephen-Haynes J, Toner L (2007). Assessment and management of wound infection: the role of silver. *Br J Community Nurs* 12:S6-S12.

Stephens P, Cook H, Hilton J, Jones CJ, Haughton MF, Wyllie FS *et al.* (2003). An analysis of replicative senescence in dermal fibroblasts derived from chronic leg wounds predicts that telomerase therapy would fail to reverse their disease-specific cellular and proteolytic phenotype. *Exp Cell Res* 283:22-35.

Stoller JK, Aboussouan LS (2005). α1-antitrypsin deficiency. *Lancet* 365:2225-2236.

Stover CK, Pham XQ, Erwin AL, Mizoguchi SD, Warrener P (2000). Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen. *Nature* 406:959-964.

Stvrtinova V, Jahnova E, Weissova S, Horvathova M, Ferencik M (2001). Inflammatory mechanisms involving neutrophils in chronic venous insufficiency of lower limbs. *Bratisl Lek Listy* 102:235-239.

Subrahmanyam YVBK, Yamaga S, Prashar Y, Lee HH, Hoe NP, Kluger Y *et al.* (2001). RNA expression patterns change dramatically in human neutrophils exposed to bacteria. *Blood* 97:2457-2468.

Sugawara S (2005). Immune functions of proteinase 3. *Crit Rev Immunol* 25:343-360.

Takatsu K, Moon B, Itakure A, Tsukamoto Y, Horikawa K, Ikutani M *et al.* (2005). Role of IL-5 in the innate immune system and disease control. *International Congress Series* 1285:145-154.

Takeichi O, Saito I, Tsurumachi T, Saito T, Moro I (1994). Human polymorphonuclear leukocytes derived from chronic inflammed tissue express inflammatory cytokines *in vivo*. *Cell Immunol* 156:296-309.

Tarlton JF, Vickery CJ, Leaper DJ, Bailey AJ (1997). Postsurgical wound progression monitored by temporal changes in the expression of matrix metalloproteinase-9. *Br J Dermatol* 137:506-516.

Tarnuzzer RW, Shultz GS (1996). Biochemical analysis of acute and chronic wound environments. *Wound Repair Regen* 4:321-325.

Tato CM, Hunter CA (2002). Host-pathogen interactions: Subversion and utilization of the NF-κB pathway during Infection. *Infect Immun* 70:3311-3317.

Tennant JR (1964). Evaluation of the trypan blue technique for determination of cell viability. *Transplanation* 2:685-694.

Theilgaard-Monch K, Knudsen S, Follin P, Borregaard N (2004). The transcriptional activation program of human neutrophils in skin lesions supports their important role in wound healing. *J Immunol* 172:7684-7693.

Thompson PD (2000). Immunology, microbiology, and the recalcitrant wound. *Ostomy Wound Manage* 46:77S-82S.

Todar K (2002). Textbook of Bacteriology [Online]. http://www.textbookofbacteriology.net/BSRP.hmtl. University of Wisconsin, Madison, U.S.A.

Todar K (2004). Textbook of Bacteriology. [Online]. http:www/textbookofbacteriology.net/BSRP.hmtl. University of Wisconsin, Madison, U.S.A.

Topham MK, Carveth HJ, McIntyre TM, Prescott SM, Zimmerman GA (1998). Human endothelial cells regulate polymorphonuclear leukocyte degranulation. *FASEB J* 12:733-746.

Towbin H, Staehelin T, Gordon J (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A* 76:4340-4354.

Travis J, Potempa J (2000). Bacterial proteinases as targets for the development of second-generation antibiotics. *Biochim Biophys Acta* 1477:35-50.

Trengrove NJ, Bielefeldt-Ohmann H, Stacey MC, Fracs DS (2000). Mitogenic activity and cytokine levels in non-healing and healing chronic leg ulcers. *Wound Repair Regen* 8:13-25.

Trengrove NJ, Stacey MC, Macauley S, Bennett N, Gibson J, Burslem F *et al.* (1999). Analysis of the acute and chronic wound environments: the role of proteases and their inhibitors. *Wound Repair Regen* 7:442-452.

Tschesche H, Kopp C, Horl WH, Hempelmann U (1994). Inhibition of degranulation of polymorphonuclear leukocytes by angiogenin and its tryptic fragment. *J Biol Chem* 269:30274-30280.

Tsunawaki S, Sporn M, Ding A, Nathan C (1988). Deactivation of macrophages by transforming growth factor-β. *Nature* 334:260-262.

Vaalamo M, Weckroth M, Puolakkainen P, Kere J, Saarinen P, Lauharanta J *et al.* (1996). Patterns of matrix metalloproteinase and TIMP-1 expression in chronic and normally healing human cutaneous wounds. *Br J Dermatol* 135:52-59.

Valencia IC, Falabella A, Kirsner RS, Eglstein WH (2001). Chronic venous insufficiency and venous leg ulceration. *J Am Acad Dermatol* 44:401-421.

Van Delden C, Iglewski BH (1998). Cell-to-cell signalling and *Pseudomonas aeruginosa* infections. *Emerg Infect Dis* 4:551-560.

Van Eeden SF, Klut ME, Walker BAM, Hogg JC (1999). The use of flow cytometry to measure neutrophil function. *J Immunol Methods* 232:23-43.

Van Gent D, Sharp P, Morgan K, Kalsheker N (2003). Serpins:structure,function and molecular evolution. *The Inter J. of Biochem & Cell Biol* 35:1536-1547.

Van Wynsberghe D, Noback CR, Carola R (1995). Human anatomy and physiology. Third Edition ed. New York: McGraw-Hill, Inc.

Vande Berg BJ, Rudolph R, Hollan C, Haywood-Reid PL (1998). FIbroblast senescence in pressure ulcers. *Wound Repair Regen* 6:38-49.

VanDelden C, Iglewski BH (1998). Cell-to-cell signalling and *Pseudomonas aeruginosa* infections. *Emerg Infect Dis* 4:551-558.

Varghese JN, Moritz RL, Lou M-Z, van Donkelaar A, Ji H, Ivacic N *et al.* (2002). Structure of the extracellular domains of the human interleukin-6 receptor α-chain. *PNAS* 99:15959-15964.

Veldkamp KE, Heezius HCJM, Verhoef J, van Strijp JAG, van Kessel KPM (2000). Modulation of neutrophil chemokine receptors by *Staphylococcus aureus* supernatant. *Infect Immun* 68:5908-5913.

Veldkamp KE, van Kessel KPM, Verhoef J, van Strijp JAG (1997). Staphylococcal culture supernates stimulate human phagocytes. *Inflammation* 21:541-551.

Venaille TJ, Misso NL, Phillips MJ, Robinsob BW, Thompson PJ (1994). Effects of different density gradient separation techniques on neutrophil function. *Scand J Clin Lab Invest* 54:385-391.

Vercellotti GM, McCarthy JB, Lindholm P, Peterson PK, Jacob HS, Furcht LT (1985). Extracellular matrix proteins (fibronectin, laminin, and type IV collagen) bind and aggregate bacteria. *Am J Pathol* 120:13-21.

Villavicencio RT, Wall MJ (1996). The pathogenesis of *Staphylococcus aureus* in the trauma patient and potential future therapies. *Am J Surg* 172:291-296.

Vissers MCM, George PM, Bathurst IC, Brennan SO, Winterbourne CC (1988). Cleavage and inactivation of α-1 antitrypsin by metalloproteinases released from neutrophils. *J Clin Invest* 82:706-711.

Waage A, Steinshamn S (1993). Cytokine mediators of septic infections in the normal and granulocytopenic host. *Eur J Haematol* 50:243-249.

Wadstrom T (1987). Molecular aspects on pathogenesis of wound and foreign body infections due to Staphylococci. *Zentralbl Bakteriol Mikrobiol Hyg [A]* 266:191-211.

Wagner S, Coerper S, Fricke J, Hunt TK, Hussain Z, Elmlinger MW *et al.* (2003). Comparison of inflammatory and systemic sources of growth factors in acute and chronic human wounds. *Wound Repair Regen* 11:253-260.

Waite RD, Paccanaro A, Papakonstantinopoulou A, Hurst JM, Saqi M, Littler E *et al.* (2006). Clustering of *Pseudomonas aeruginosa* transcriptomes from planktonic cultures, developing and mature biofilms reveal distinct expression profiles. *BMC Genomics* 7:162-176.

Wall IB, Davies CE, Hill KE, Wilson M, Stephens P, Harding KG *et al.* (2002). Potential role of anaerobic cocci in impaired human wound healing. *Wound Repair Regen* 10:346-353.

Wall SJ, Sampson MJ, Levell N, Murphy G (2003). Elevated matrix metalloproteinase-2 and -3 production from human diabetic dermal fibroblasts. *Br J Dermatol* 149:13-16.

Wallace HJ, Stacey MC (1998). Levels of tumor necrosis factor-α (TNF-α) and soluble TNF receptors in chronic venous leg ulcers - Correlations to healing status. *J Invest Dermatol* 110:292-296.

Weckroth M, Vaheri A, Lauharanta J, Sorsa T, Konttinen YT (1996). Matrix metalloproteinases, gelatinase and collagenase, in chronic leg ulcers. *J Invest Dermatol* 106:1119-1124.

Weiss J (2003). Bactericidal/permeability-increasing protein (BPI) and lipopolysaccharide-binding protein (LBP): structure, function and regulation in host defence against gram-negative bacteria. *Biochem Soc Trans* 31:785-790.

Werner S, Grose R (2003). Regulation of wound healing by growth factor and cytokines. *Physiol Rev* 83:835-870.

Werthen M, Davoudi M, Sonesson A, Nitsche DP, Morgelin M, Blom K *et al.* (2004). *Pseudomonas aeruginosa*-induced infection and degradation of human wound fluid and skin proteins *ex vivo* are eradicated by a synthetic cationic polymer. *J Antimicrob Chemother* 54:772-779.

Wesson CA, Liou LE, Todd KM, Bohach GA, Trumble WR, Bayles KW (1998). *Staphylococcus aureus* Agr and Sar global regulators influence internalisation and induction of apoptosis. *Infect Immun* 66:5238-5243.

Westfall LW, Luna AM, Francisco MS, Diggle SP, Worrall KE, Williams P *et al.* (2004). The *Pseudomonas aeruginosa* global regulator MvaT specficially bind to the ptxS upstream region and enhances ptxS expression. *Microbiology* 150:3797-3806.

Whiston RJ, Hallett MB, Davies EV, Harding KG, Lane IF (1994). Inappropriate neutrophil activation in venous disease. *Br J Surg* 81:695-698.

White RJ, Cutting KF, Kingsley A (2006). Topical antimicrobials in the control of wound bioburden. *Ostomy Wound Manage* 52:26-58.

Wilkinson LS, Bunker C, Edwards JCW, Scurr JH, ColeridgeSmith PD (1993). Leukocytes: Their role in the etiopathogenesis of skin damage in venous disease. *J Vasc Surg* 17:669-675.

Wilson AP, Weavill C, Burridge J, Kelsey MC (1990). The use of the wound scoring method 'ASEPSIS' in postoperative wound surveillance. *J Hosp Infect* 16:297-309.

Witko-Sarat V, Rieu P, Deschamps-Latscha B, Lesavre P, Halbwachs-Mecarelli L (2000). Neutrophils: Molecules, functions and pathophysiological aspects. *Lab Invest* 80:617-653.

Witte MB, Barbul A (1997). General principles of wound healing. *Wounds* 77.

Wojtowicz-Praga SM, Dickson RB, Hawkins MJ (1997). Matrix metalloproteinase inhibitors. *Invest New Drugs* 15:61-75.

Wong MM, Fish EN (2003). Chemokines: attractive mediators of the immune response. *Semin Immunol* 15:5-14.

Woodley DT, Chen JD, Kim JP, Sarrat Y, Iwasaki T, Kim Y-H *et al.* (1993). Re-epithelialisation. Human keratinocyte locomotion. *Dermatol Clin* 11:641-646.

Woods DE, Schaffer MS, Rabin HR, Campbell GD, Sokol PA (1986). Phenotypic comparison of *Pseudomonas aeruginosa* strains isolated from a variety of clinical sites. *J Clin Microbiol* 24:260-264.

Wretlind B, Pavlocskis OR (1983). *Pseudomonas aeruginosa* elastase and its role in pseudomonas infections. *Rev Infect Dis* 5:S998-S1004.

Wysocki AB (1996). Wound fluids and the pathogenesis of chronic wounds. *J Wound Ostomy Continence Nurs* 23:283-290.

Wysocki AB, Kusakabe AO, Chang S, Tuan T-L (1999). Temperal 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* 7:154-165.

Wysocki AB, Staiano-Coico L, Grinnell F (1993). Wound Fluid from chronic leg ulcers contains elevated levels of metalloproteinases MMP-2 and MMP-9. *J Invest Dermatol* 101:64-68.

Xing L, Remick DG (2003). Relative cytokine and cytokine inhibitor production by mononuclear cells and neutrophils. *Shock* 20:10-16.

Xu X, Hakansson L (2002). Degranulation of primary and secondary granules in adherent human neutrophils. *Scand J Immunol* 55:178-188.

Xue M, Le NTV, Jackson CJ (2006). Targeting matrix metalloproteases to improve cutaneous wound healing. *Expert Opin Ther Targets* 10:143-155.

Yager DR, Chen SM, Ward SI, Olutoye OO, Diegelmann RF, Cohen IK (1997). Ability of chronic wound fluids to degrade peptide growth factors is associated with increased levels of elastase activity and diminshed levels of proteinase inhibitors. *Wound Repair Regen* 5:23-32.

Yager DR, Nwomeh BC (1999). The proteolytic environment of chronic wounds. *Wound Repair Regen* 7:433-441.

Yager DR, Zhang L-Y, Liang H-X, Diegelmann RF, Cohen IK (1996). Wound fluids from human pressure ulcers contain elevated matrix metalloproteinase levels and activity compared to surgical wound fluids. *J Invest Dermatol* 107:743-748.

Yamamoto M, Akira S (2005). Mechanisms of innate immune responses mediated by Toll-like receptors. *Immunol Rev* 5:167-183.

Yanagisawa N, Geironson L, Al-Soud WA, Ljungh S (2005). Expression of matrix metalloprotease-2,-7 and -9 on human colon, liver and bile duct cell lines by enteric and gastric helicobacter species. *FEMS Immunol Med Microbiol* 44:197-204.

Yao YM, Redl H, Bahrami S, Schlag G (1998). The inflammatory basis of trauma/shock-associated multiple organ failure. *Inflamm Res* 47:201-210.

Yates GT, Smotzer T (2007). On the lag phase and initial decline of microbial growth curves. *J Theor Biol* 244:511-517.

Yerramilli VBK, Subrahmanyam YVBK, Yamaga S, Prashar Y, Lee HH, Hoe NP *et al.* (2001). RNA expression patterns change dramatically in human neutrophils exposed to bacteria. *Blood* 97:2457-2468.

Yoshimura A, Hara Y, Kaneko T, Kato I (1997). Secretion of IL-1β, TNF-α, IL-8 and IL-1ra by human polymorphonuclear leukocytes in response to lipopolysacchaide from periodontopathic bacteria. *J Periodontal Res* 32:279-286.

Yoshimura A, Lien E, Ingalls RR, Tuomanen E, Dziarski R, Golenbock DT (1999). Cutting Edge: Recognition of gram-positive bacterial cell wall components by the innate system occurs via toll-like receptor 2. *J Immunol* 163:1-5.

Zhang Z, Winyard PG, Chidwick K, Farrell A, Pemberton P, Carrell RW *et al.* (1990). Increased proteolytic cleavage of α-1-antitrypsin in knee synovial fluid from patients with rheumatoid arthritis. *Biochem Soc Trans* 18:898-899.

Ziegler-Heitbrock HWL, Blumenstein M, Kafferlein E, Kieper D, Petersmann IES, Legel A *et al.* (1992). *In vitro* sensitisation to lipopolysaccharide suppresses tumour necrosis factor, interleukin-1 and interleukin-6 gene expression in a similar fashion. *Immunology* 75:264-268.