UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE, HEALTH & LIFE SCIENCES

School of Health Sciences

A Preliminary Investigation into the Risk Factors Associated with Cellulitis of the Lower Limb

by

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ABSTRACT

FACULTY OF MEDICINE, HEALTH
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A PRELIMINARY INVESTIGATION INTO THE RISK FACTORS ASSOCIATED WITH CELLULITIS OF THE LOWER LIMB

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Cellulitis is an infective/inflammatory skin condition costing 426,000 bed days per year. The legs are most frequently affected and 18-20% of patients suffer from recurrent attacks. Bacteria are thought to be causal but are rarely identified, therefore antibiotic treatment is empirical and currently the only means of prophylaxis.

Previous studies have cited athlete's foot , skin vulnerability and oedema as risk factors for cellulitis and there may also be a relationship with the immune response. This preliminary study was designed to evaluate these risk factors and identify areas for further investigation.

Patients were matched with controls (N = 12 + 12 controls) by age, sex and mobility. Participants attended twice (during treatment and again 6 weeks after clinical resolution). Foot scrapings were cultured to examine the flora and blood samples taken to determine white cell types and numbers, cytokine levels and markers specific to fungal infection. Physiological measurement techniques were used to assess skin function. Psychological stress levels were evaluated and medical history recorded.

Fewer dermatophytes were grown from the feet of patients as compared to matched controls. Amongst patients blood profiling showed evidence of increased neutrophil count post episode and levels of IL-12 and IL-8 also reached near significance in this group. Physiological tests for skin blood flow, water loss and pH produced results consistent with cellulitic skin but persisting oedema was significantly higher in the patient group and characterised by loss of structure in the dermal tissues. Ipsilateral injuries, allergies, history of other bacterial infections, excessive life time prescription of antibiotics and levels of psychological stress, evaluated by a questionnaire, were also found to be significantly higher in the patient group. No evidence was produced to show any differences between acute and recurrent populations.

This preliminary study into the potential risk factors for cellulitis indicates that some factors merit further investigation. Larger studies are required to substantiate results.

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I, ……CAROL COLLINS……,
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ABBREVIATIONS

The abbreviations used throughout this thesis have been defined when they appear for the first time. Whenever possible the standard abbreviation has been used.

ACU	Ambulatory Care Unit
AOI	Area of investigation
APC	Antigen presenting cell
B cell	B lymphocyte
CABG	Coronary Artery Bypass Graft
CD	Cluster of Differentiation
CNS	Central nervous system
CRH	Corticotrophin-releasing hormone
CRP	C-reactive protein
CT	Computerised tomography
CVI	Chronic venous insufficiency
DTH	Delayed hypersensitivity reaction
DMT	InTray Dermatophyte Medium
DVT	Deep vein thrombosis
EDTA	Ethylenediaminetratraacetic acid
ELISA	Enzyme-linked immunosorbant assay
ELISPOT	Enzyme-linked immunospot
FACS	Fluorescence Activated Cell Sorting
GP	General Practitioner
HLA	Human leucocyte antigen system
HRUS	High resolution ultrasound

ABBREVIATIONS (cont)

ICD-9	International Classification of Diseases version 9
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IH	Immediate hypersensitivity reaction
IL-1	Interleukin 1
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-5	Interleukin 5
IL-6	Interleukin 6
IL-8	Interleukin 8
IL-10	Interleukin 10
IL-12	Interleukin 12
IV	Intravenous
LCs	Langerhans Cells
LDF	Laser Doppler Flowmeter
LDI	Laser Doppler Imager
LPS	Lipopolysaccharide
MHC	Major Histocompatibility Complex
MRI	Magnetic Resonance Imaging
MSD	Meso Scale Discovery
MWU	Mann-Whitney U test
OPAT	Out Patient Antibiotic Therapy
PATCH	Prophylactic Antibiotics for the Treatment of Cellulitis at Home
PC	Personal computer

ABBREVIATIONS (cont)

pH	potenz Hydrogen
PT	Paired T test
RCT	Randomised Controlled Trial
SA	Staphylococcus aureus
SAB	Sabouraud Dextrose Agar
SC	Stratum Corneum
SD	Standard deviation
SGH	Southampton General Hospital
SSTI	Skin and soft tissue infections
Tc^{99}	Technetium 99
T cell	T lymphocyte
T_C cell	Cytotoxic T cell
T_H cell	T helper cell
T_CPO_2	Transcutaneous partial pressure of oxygen
TcR	T cell receptor
TEWL	Transepidermal water loss
TGF-β	Transforming growth factor β
TNFα	Tumour necrosis factor alpha
TI	Trichophyton Interdigitale
TR	Trichophyton Rubrum
US	Ultrasound
WBC	White cell count
WSR	Wilcoxon Signed Ranks Test
X^{133}	Xenon 133

CHAPTER 1

INTRODUCTION

1 INTRODUCTION

Cellulitis is an infective/inflammatory skin condition that can affect any part of the body but the extremities and the face are most commonly involved with the majority of cases occurring in the lower limbs (Choo 2004, Ronnen et al 1985). Symptoms include fever and malaise accompanied by swelling and redness of the affected part; large areas of skin become inflamed and patients experience extreme pain, often for several weeks. While some cellulitic episodes resolve with minimal medication others require intensive antibiotic therapy. Cellulitis is classed as a skin and soft tissue infection (SSTI) within class 681 and 682 of the International Classification of Diseases (ICD) version 10; the number of UK recorded episodes in 2005/2006 was 74,000 and the percentage of cases requiring hospital admission in the UK (2005/6) was 73% with an overall total of 426,000 bed days (HES online 2007). At a local level the number referred to the Ambulatory Care Unit (ACU) within Southampton General Hospital (SGH) is approx 350 lower limb cases per annum. Recurrence rates are estimated to be between 18% and 30% and some patients experience several attacks within a year (Dermatology Clinical Trials Network ONLINE 2007, Choo 2004, Sjöblom et al 1993, Jorop-Rönstöm and Britton 1987, Baddour and Bisno 1984). Currently, the reason for predisposition to recurrent attacks remains unclear.

Locally, the introduction of new referral procedures and protocols by a combined programme of hospital and community care, has decreased the risk of more serious infection and reduced the number of bed days. Despite this the percentage of patients with recurrent infection leaves cause for concern. The clinical problems are difficulties with identifying the causal pathogens, the enduring prescription of antibiotic medication whose continual use may render subsequent courses ineffective and the concomitant burden placed upon the NHS resource. From the patient perspective the episodes are not only painful and debilitating but provoke considerable stress generated by the uncertain cause and the unpredictable timing of subsequent episodes. Many feel particularly vulnerable because no proven preventable measures have yet been identified and prophylactic antibiosis, with its attendant risk, remains the only currently available option.

1.1 Cellulitis the Problem

Cellulitis is a diffuse spreading skin infection characterised by swelling and erythema and accompanied by high fever. Although skin involvement is progressive the infection is usually confined to one anatomic region and distal foci are uncommon. Cellulitis involves the deep dermal and subcutaneous layers of the skin, frequently it is confused with erysipelas, which is confined to the upper dermal layers and characterised by well defined borders and attendant blisters; the contrast is well illustrated by Ashton and Leppard (2005) in figure 1 but in practice differentiation is often difficult and the two conditions are invariably treated as a single entity (Hay and Adrianns 2004). The red swollen appearance can be deceptive and it is important to exclude deep vein thrombosis (DVT) as well as to differentiate from other inflammatory skin conditions such as varicose eczema Quartey-Papafio (1999) and necrotizing fasciitis (Schmid at al 1998).

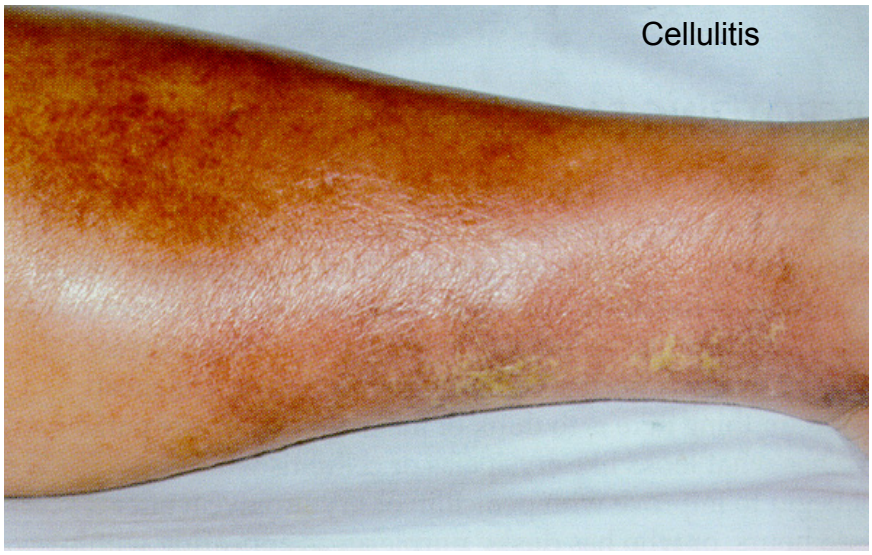
Cellulitis is thought to result when bacteria gain access to the dermal skin layers through a puncture or wound and the most likely causal agents are streptococci (Ashton and Leppard 2005) or commensal organisms such as staphylococcus aureas (SA) (Fung et al 2003, Hook et al 1986). This is difficult to confirm because swabs/fluid aspirations taken from the affected site frequently produce negative results (Mills and Chen 2005, Perl et al 1999, Leppard et al 1985), so the diagnosis is largely empirical, based on clinical appearance and confirmed by increased levels of inflammatory markers present in peripheral blood.

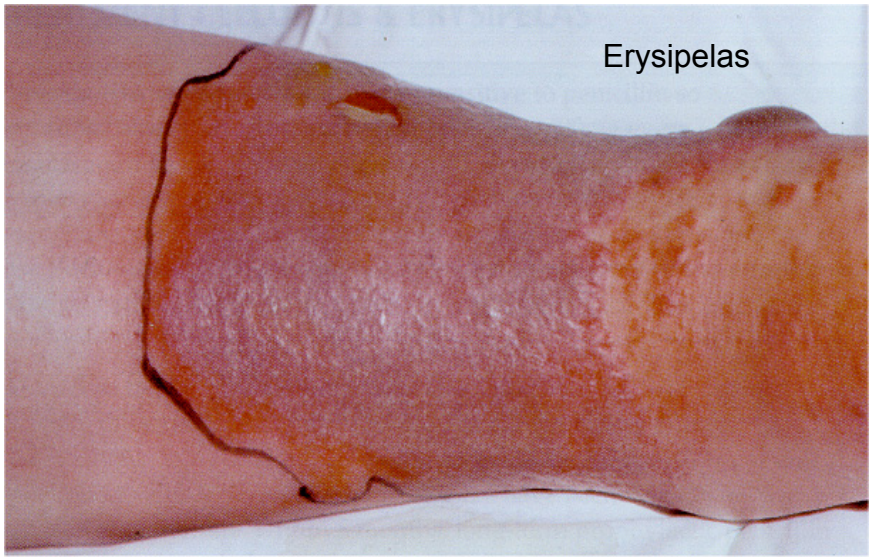
Compression ultrasonography can be used to rule out DVT by confirming vein patency (Mani et al 1995) and additionally to demonstrate fast virtually uniphasic venous flow and concomitant enlarged lymph nodes which are frequently present in patients suffering from infective/inflammatory episodes. Where necrotizing fasciitis is suspected, an MRI scan can provide the differential diagnosis (Schmid at al 1998).

The mainstay of the treatment for cellulitis is antibiotic therapy. The choice and duration of the treatment relies mainly on the empirical judgement of the clinician although it is generally accepted that the pharmacological agents should be active against both streptococci and staphylococcus aureus (SA) (Fung et al 2003). General Practitioners (GPs) normally commence treatment with oral medication and assess patients frequently to monitor progress, but slow or negative

Figure 1 - Presentation of cellulitis and erysipelas

(© Ashton, Richard and Leppard, Barbara, Differential Diagnosis in Dermatology 3rd edition. Oxford: Radcliffe Publishing; 2005. Reproduced with the permission of the copyright holder.)





responses may lead to the prescription of many and various antibiotic courses and failure to improve ultimately results in hospital referral and a switch to intravenous (IV) administration. Ongoing assessment includes clinical evaluation, blood markers such as C Reactive protein (CRP) (Isaacman and Burke 2002), WBC and differential count (Sachs 1990) and the time to when the cellulitis fails to advance (Corwin et al 2005). Monitored improvement informs the change from IV back to oral therapy. In addition to antibiosis, treatment for cellulitis includes pain control, reduction of oedema and care of the skin. Analgesia is administered as required and this is important not only to ensure patient comfort but also to encourage mobilisation of the limb, which assists in the reduction of oedema.

Estimated recurrence rates range from 18% at SGH to 29% (Sjöblom et al 1993, Jorop-Rönstöm and Britton 1987) and the use of prophylactic antibiotics is usually considered for patients with predisposing factors such as chronic co-morbidities or frequent attacks. There is a paucity of evidence to support the effectiveness of prophylactic therapy; studies are few and the numbers recruited are small. Jorop-Rönstöm and Britton (1987) evaluated 90 patients with recurrences and reported that in the short term prophylactic treatment was probably cost effective, Kremer et al (1991) and Sjöblom et al (1993) conducted Randomised Controlled Trials (RCTs) involving 40 patients and concluded that prophylactic antibiotic treatment appeared to reduce or prevent recurrent episodes but that more and larger trials were needed to provide a sufficient evidence base. As the problems surrounding the use of antibiotic therapy become apparent (Goossens et al 2005, Little 2005) the understanding behind the use of such prophylaxis requires a firm foundation. An RCT co-ordinated by the UK Dermatology Clinical Trials Network commenced in April 2006. Two studies (PATCH 1 and 2) are running concurrently to evaluate the impact of 6 and 12 months prophylactic antibiotic therapy on subsequent episodes of cellulitis of the legs. (Dermatology Clinical Trials Network ONLINE). The co-ordinators hope to recruit around 700 patients from hospitals within the UK before 2009. Results from this study should inform future management but the ongoing effects associated with long-term antibiotic use must also be considered.

The origin of any infective/inflammatory condition is questionable if no causal pathogen or pathway can be positively identified. This together with the unpredictable course of some episodes and the frequency of recurrence are major

problems for the management and prophylaxis of cellulitis. Under normal conditions commensal flora and pathogens are unable to breech the intact barrier or progress far in broken skin if the immune system is functioning properly. In cellulitis, it appears that this mechanism is disrupted causing an enhanced infective response and/or prolonged period of inflammation. Patients with cellulitis frequently display deviations from the expected infective/inflammatory responses to localised skin insult. Those most commonly encountered are listed below:

- Swabs/aspiration from the infected site rarely produce positive cultures (Karakas et al 2002, Sachs 1991)
- Although extensive areas of skin are involved the number of organisms obtained from positive cultures is often very low (Shelley 1995)
- A portal of entry is not always identified (Choo 2004, Bernard et al 1989)
- Although blood markers provide proof of systemic inflammation the skin infection is usually confined to one anatomical site (Stryjewski et al 2007)
- Recurrences predominantly occur at the same site (Badour and Bisno 1984)
- Some episodes require many courses of antibiotic therapy to achieve resolution (Stulberg 2002)
- The time lapse between recurrent episodes is variable; can be days, weeks, months or years (Karakas et al 2002, Badour and Bisno 1984)

Identification of the causal pathogens and entry sites is key to successful treatment and some research has already been done in this area. Sachs (1991) showed that aspiration at the affected site only produced positive culture results when performed within the first 24 hours of the infective event. Bernard et al (1989) and Shelley et al (1995) used punch biopsies and immunofluorescence in order to demonstrate the presence of bacteria deep within the reticular dermis after negative results from surface cultures. In a more recent study of 111 patients by Choo (2004) only 12% of cultured surface samples were positive, less that 1% were identified as streptococci and the remainder were all commensal flora: furthermore 39% of patients are unable to recall any predisposing factors or provide evidence of skin breech. Negative results may simply be due to low density of micro organisms (Semel et al 1996) but alternative theories suggest that

causal factors other than bacteria could be responsible for the inflammatory response (Hook et al 1986).

Seventy years ago Traub and Tolmach (1937) and Sulzberger et al (1937) identified a group of patients who were unable to associate an episode of skin breech with their cellulitic-like symptoms. Lesions in these patients were found to be in close proximity to areas of microscopically demonstrated fungal skin disease (dermatophytosis). Many initially experienced frequent recurrent episodes but application of topical fungicides appeared to alleviate the problem without further recourse to antibiotic therapy. More recently, studies by Dupuy et al (1999) and Roujeau (2004) have reported dermatophytosis as a risk factor for cellulitis and Björnsdóttir et al (2005) found a strong association between cellulitis of the lower limb and the presence dermatophytes in the toe web spaces. Similar associations have been implied by others (Roldan et al 2000, Koutia et al 1999, Day et al 1996, Kremer 1991) and are supported by local findings from SGH where recurrent cellulitic attacks appear to be more frequent in patients with dermatophytosis who fail to adhere to topical antifungal treatment.

Infective/inflammatory skin conditions such as cellulitis, may occur and recur because the skin and/or immune system fail to function effectively (Maleszka et al 2001, Woodfolk et al 1998) in response to one or more stressors arising from co-morbidities or other physiological, psychological or environmental factors (O'Sullivan et al 1998). Dermatophytosis represents one potential stressor, which is not only ubiquitous in its presence (Broughton 1955, Sugimoto1955) but also has an affinity for skin and an ability to elicit diverse responses from the immune system. Links with skin physiology have been suggested by Cox (2006), Björnsdóttir et al (2005), Roujeau (2004) and Dupuy et al (1999) who report associations between cellulitis and oedema. In addition Cox (2006) and Naguib et al (2004) implicate vascular disorders, in particular venous incompetence. As venous incompetence is strongly associated with oedema this particular aspect of skin physiology would appear to be important. These same studies also propose associations with immune related co-morbidities such as diabetes and malignancy suggesting implications for immunological mechanisms although as yet there is no evidence to support this theory.

One common feature links the proposed causal factors described above; their presence adds a stress burden to the system providing the potential to challenge

homeostasis and affect the course and outcome of other disease processes (Tracey 2002, O'Sullivan et al 1998). It may be in their role as stressors and by mechanisms as yet unknown that they contribute to the cellulitic process.
In the next chapter the literature concerning the relationship between cellulitis, skin /immune physiology and stress factors will be reviewed. It begins with detailed accounts of skin structure and function and an explanation of the expected infective/inflammatory response to skin insult. The evidence to support links between causal factors, skin function and immune responses will be discussed. It concludes by identifying the proposed areas of research, which could contribute to a better understanding of the problem.

CHAPTER 2

LITERATURE REVIEW

2 LITERATURE REVIEW

2.1 The Skin

Skin forms the interface between the external environment and the internal body structures; together with the external appendages (hair, nails, sebaceous and sweat glands) it forms the integumentary system which is the largest organ of the body. Its functions are diverse and vital and include roles in protection, homeostasis, immunoregulation, sensation and display. Despite being composed of structurally distinct areas the skin is co-ordinated and integrated so that it operates as a single functional unit.

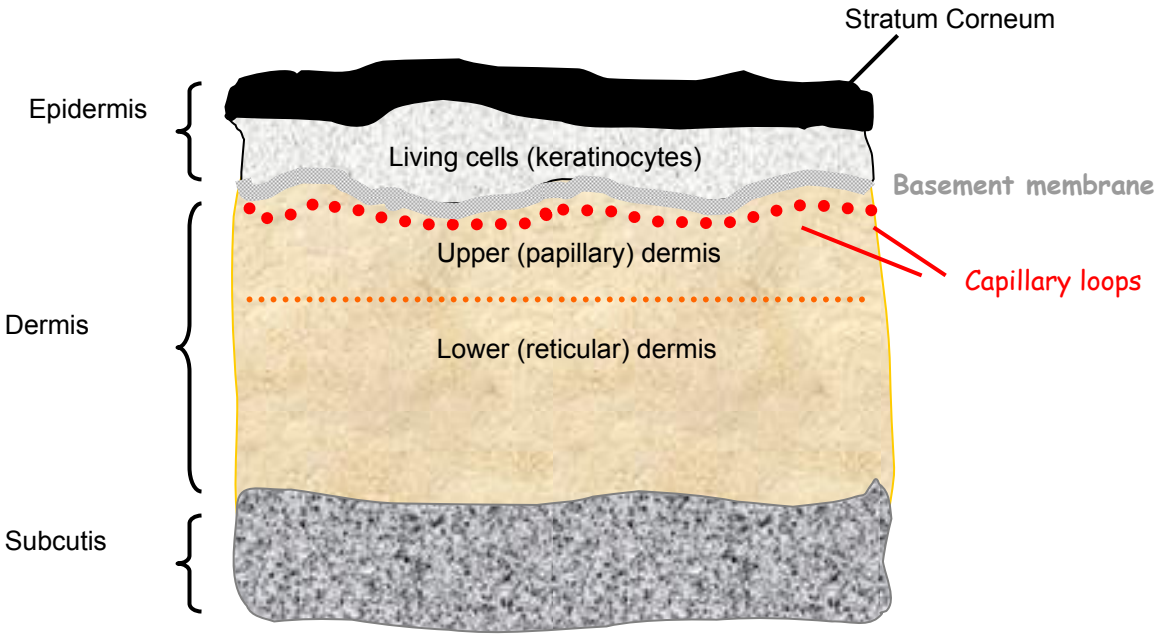
2.1.1 Skin structure

Structurally skin consists of two regions; the epidermis or outer layer, in contact with the environment, and beneath this a thicker supportive layer known as the dermis. Although structurally separate the epidermis and the dermis communicate via a semi permeable basement membrane, which forms the interface between a system of downward projecting epidermal ridges and upward projecting dermal counterparts. The dermis interfaces with the underlying fascia via a layer of loose connective tissue and fat known as the hypodermis.

The epidermis consists of several layers of cells in various processes of differentiation and derived from a basal layer, which is continually engaged in mitosis (Figure 2a). Cells generated by the basal layer are known as keratinocytes, they are living, nucleated and form columns which move upwards, synthesising keratin and lipids and differentiating as they pass through the spinous and granular layers (Fuchs 2007). As they progress and approach the skin surface they become enucleated, flattened and tightly packed and consist mainly of insoluble keratin. These dead cells, now known as corneocytes, occupy the uppermost layer of the epidermis known as the Stratum Corneum (SC). Although tough this outer layer remains supple due to the presence of lipids synthesised in the granular layer (Madison 2003) which together with secretions from the sebaceous and sweat glands form an emulsion permeating the intercellular spaces (McEwan Jenkinson 1993).

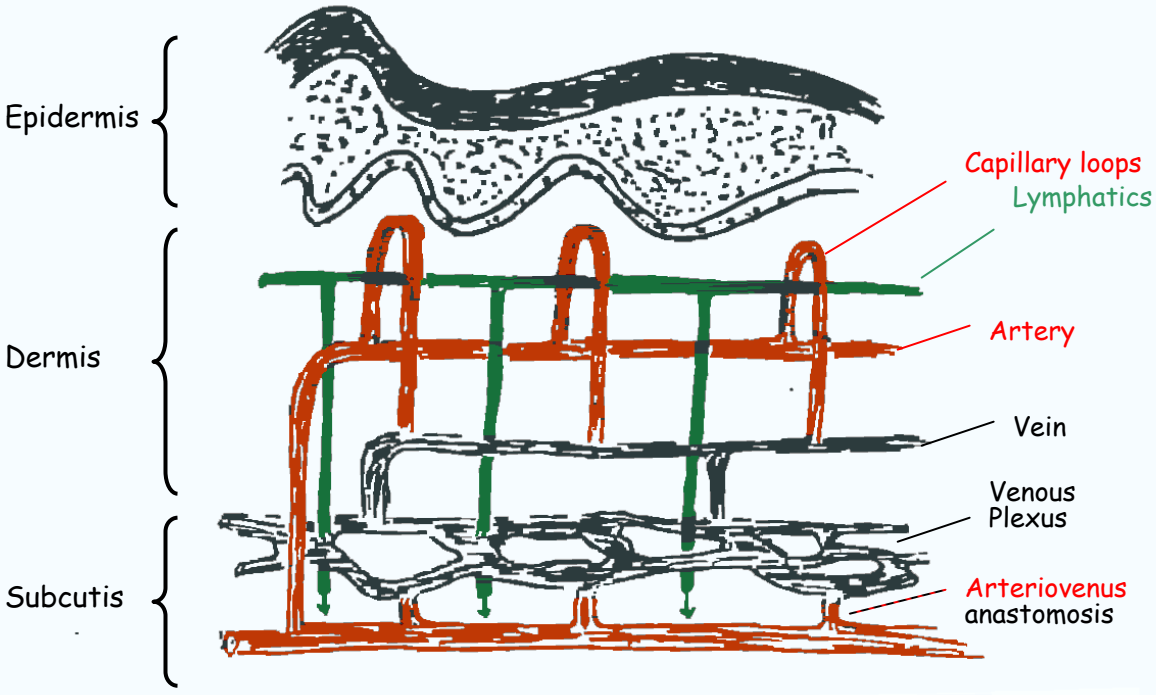
The skin appendages all derive from the ectodermal embryological germ layer and are therefore epidermal structures (Moore and Persaud 1998). Most sebaceous

Figure 2a - Structure of the skin



(*Derived from Ashton and Leppard 2004)*

Figure 2b - Blood distribution to the skin



(*Derived from Guyton page 823*)

glands arise from an associated hair follicle and form a pilosebacious unit. Sweat glands are coiled epidermal downgrowths and nails are keratinised epidermal invaginations situated on the distal dorsal surface of each finger and toe.
The dermis consists mainly of collagen and elastin fibres secreted by the predominant cells known as fibroblasts. Fibres and cells are embedded in a matrix of glycosaminoglycans composed largely of hyaluoronic acid and dermatan sulphate forming a connective tissue mesh subdivided into two strata; the upper loosely packed papillary dermis and the lower reticular dermis which is thicker and denser.
Vascular input for both epidermal and dermal layers of the skin is supplied by vessels within the papillary and reticular dermis. The circulatory arrangement with respect to the skin regions is shown in figure 2b. Dermal papillary loops follow the outline of the epidermal ridges thus increasing the diffusible surface area and providing closer contact with the cells of the avascular epidermis. The lymphatic plexus lies deep to the arteriovenous microcirculation, it is similarly arranged but devoid of papillary loops.
The skin as a whole supports an active immune and surveillance system. Within the epidermis this is provided by immune system activators (Langerhans' and mast cells) and interspersed T lymphocytes (T cells). Protective pigment cells (melanocytes) and sensory co-ordinating (Merkel) cells are also found adjacent to the basement membrane. Within the dermis nerve fibres and immune cells are found in close association with the microcirculation. Macrophages and phagocytic cells (similar to epidermal Langerhans cells) are abundant in the papillary dermis and large numbers of mast cells and T cells are present throughout (Spellberg 2000).

2.1.2 Skin function

In its protective function skin provides a barrier between the internal organs and the environment minimising damage resulting from insults such as trauma, chemicals and ultraviolet light and providing protection from desiccation and the intrusion of pathogenic microbes which compromise health and obstruct healing. As a first line of defence the stratum corneum (SC) is tough and resilient and offers a continuous and substantial shield against insult and invasion. Integration with the other skin layers, particularly the tough reticular dermis, creates an effective cushion which absorbs initial impact and disseminates transmitted force

by distribution through the sliding action of the loosely attached hypodermis. Skin mechanical properties vary with anatomical site. There are differences in thickness, elasticity and distensibility, which relate to functional and gravitational adaptations. Thus the skin in the gaiter region of the ankle is stiffened to withstand gravitational stress (Gniadecka et al 1994), and the stratum corneum of the heel is much thickened to endure the forces applied to the weight bearing lower extremity. In many mammals hair plays a major part in the protective process. In some, such as rodents, dense hair covering is important to protect from trauma and consequently the thickness of the underlying epidermis is much reduced. In humans, dense hair cover is restricted to a small proportion of total body area; the remainder is devoid of hair (glaborous) or only sparsely covered. The role of human hair in mechanical protection is therefore minimal but it does assist in protection against pathogens by acting as a foil to prevent microbes reaching the skin surface (McEwan Jenkinson 1993).

In order to maintain skin health and provide good protective function it is important that the skin layers should be adequately hydrated while the surface remains dry. The skin organ as a whole acts as a fluid reservoir for the body. Water is stored in the dermis by attraction to proteoglycans (hyaluronic acid in particular), which are present in the matrix and can be utilised if fluid levels become low.

Regulation of skin water content is crucial for efficient and effective skin function. Water constitutes an essential component of interstitial fluid; part of which is immobile or 'bound' and part of which is free. The quantity of free fluid is variable but is normally maintained close to homeostasis by the lymphatic system (Courtice 1984). In the inflammatory state fluid content between skin layers is greatly increased due to an influx of water and plasma proteins, which exit the small blood vessels and pool in the extravascular space. Clearance of this protein laden fluid or exudate depends on the efficiency of the lymphatic system and 10 times the normal lymph flow may be measured (Hurley 1984). If the lymphatic system becomes obstructed or overloaded, fluid builds up within the tissues and results in oedema. The type of oedema which resolves is regarded as acute; other types of long term fluid retention and associated persistent swelling are classified as chronic oedema and lymphoedema. The former is associated with changes in posture or pressure, vascular and/or endocrine dysfunction and certain metabolic disorders. The latter, linked with congenital defects, filariasis, cancer, radiotherapy

and surgery, results from anomalies/damage to lymphatic pathways (Browse and Stewart 1985) and requires specific physiological tests to establish a differential diagnosis.

Changes in water content are therefore partially responsible for variations in skin thickness (Eisenbiss et al 2001). Water entry from the surface and water loss from underlying layers is controlled by the intercellular lipid matrix within the SC, which maintains the external surface in a dry but pliable state (Tagami et al 2001). The rate at which water is transferred between the skin and the atmosphere is known as transepidermal water loss (TEWL). Disruption of the lipid barrier creates increases in TEWL causing skin to become cracked and brittle, conversely, occlusion or hyperhydration impede TEWL with the effect that the skin surface may become macerated.

All microbes possess the ability to become pathogenic if they are allowed to proliferate without control and/or infiltrate the tissues beyond their normal range (Sprunt et al 1971). Changes in skin physiology together with trauma or disease states can affect the morphology of the skin surface facilitating access, offering sites for colonisation and favourable conditions for growth. To counter this, the intercellular epidermal matrix contains antimicrobial peptides known as cathelidicins and β-defensins which have been shown to confer a degree of natural prophylaxis against bacteria and fungi (Nizet 2007, Nizet et al 2001, Ricketts at al 1951). An additional antibiotic peptide has also been identified in sweat (Schittek at al 2001) and the constituents of sweat and sebum together with the products of lipid degradation (Huraib et al 1994) help to maintain an acidic pH at the skin surface. Despite these defences healthy skin is colonised by an indigenous population of commensal bacteria, which play a major role in maintaining the ecological balance that exists at the skin surface (Sprunt et al 1971). Commensal bacteria have the ability to thrive under varying conditions of pH, temperature and humidity and this favours numerical dominance over transient pathological species, which tend to be more sensitive (Holland 1993, Amonette and Rosenburg 1973). Further advantages are achieved by secretion of toxins and bacteriocins (Selwyn 1975), lipid degradation and maintaining the immune system in a constantly primed state (Scheffel 1979).

The balance is delicate and easily upset. Disruption may arise from localised changes such as the use of detergents, which alter factors such as pH and lipid

control (Korting and Braun-Falco 1996) or more generalised changes concomitant with co-morbid disease. Added to this is the increasing and continuous use of antibiotic therapy which has been shown to disrupt the normal pattern of bacterial flora providing the potential for overgrowth of normally subordinate pathogens and possibly contributing to antibiotic resistance in later years (Weinstein 1947).
Desquamation (shedding of cells from the SC) is an ongoing process and is the final step and an integral part in the differentiation of keratinocytes that have travelled upwards through the layers of the epidermis. The turnover of cells varies with anatomical site (on average 12-14 days) and abnormalities in the shedding of skin scales occur as a result of disruptive skin physiology (Millstone 2004, Madison 2003); this has been demonstrated in patients with exfoliative dermatitis and related conditions (Palmer et al 2006, Thody and Friedmann 1986).
Desquamation is thought to have evolved to facilitate repair and regeneration, evict toxins and maintain the balance of microbes at the skin surface; it is also considered to be part of the innate (inherited) mechanism for clearing pathogens including fungal organisms from the epidermis (Millstone 2004). Exfoliation is an integral part of infective/inflammatory diseases such as scalded skin syndrome (Chiller et al 2001) and has also been shown to feature in some patients recovering from episodes of cellulitis (Badour and Bisno 1984).
In order to function effectively the skin requires and adequate and efficient blood supply; this is provided by a complex network of anastomosing arterioles and venules, which constitute the microcirculation. Oxygen and nutrients travel to the reticular dermis and to the papillary loops of the dermis where they diffuse through the thin capillary walls to supply the living cells of the epidermis. The resting rate of flow is controlled by minute intermittent contractions known as vasomotion, governed by the opening and closing of sphincters at the entrance to the arterioles and acting in response to the oxygen concentration in the adjacent tissues (Guyton and Hall 2000). When demand for oxygen is high the arterioles dilate and blood flow to the capillaries increases; this mechanism, which also serves to facilitate the dissipation of body heat through the skin, is under the control of the autonomic nervous system. The arterio-venus microcirculation works in conjunction with the lymphatic plexus to maintain a delicate pressure balance controlling the amount of fluid within the tissues, which would otherwise lead to the formation of oedema.

Arguably the most important part of the skin defence mechanism is the immune response; a complex system incorporating primitive mechanisms and therefore capable of raising responses to a catalogue of antigens (molecules and organisms regarded as non self by the body) both evolutionary and novel (Alberts et al 1983). There are two systems of defence which work in concert to combat antigens: firstly the local or innate system which consists of body membranes and their unique populations of immune cells; the second or adaptive system includes cells which mature in specialised immunological tissues and which can mobilise to specific body sites via the vasculature.

2.1.3 The skin immune system

All immune cells originate in the bone marrow from either myeloid and lymphoid progenitor cells. Myeloid precursors give rise to erythrocytes, platelets, dendritic cells, monocytes and polymorphonuclear leukocytes (eosinophils, neutrophils, basophils and mast cells). Lymphoid precursors differentiate into Natural Killer (NK) cells and lymphocytes. Lymphocytes further undergo a maturation phase either in bone marrow or thymus and are then referred to as B and T cells (Benjamini et al 1996). Some cells are predominantly resident in skin, these include dendritic (Langerhans) cells, Natural Killer Cells, Macrophages, memory T cells and mast cells while others, leukocytes and T and B lymphocytes, are recruited from circulating peripheral blood and lymph organs when required. Immune cells have two major functions, the first is to ingest or phagocytose alien cells and destroy them with toxins; this process, which is a key first line of defence, is enhanced by substances present in blood serum collectively known as complement. The second role is to denature antigenic material and express it at the cell surface for recognition by T and B cells; a process known as antigen presentation.

Mast cells facilitate vascular permeability, release histamine and initiate allergic responses. They also participate in phagocytosis but their role in this is minimal.

2.1.3.1 T and B lymphocytes

T and B lymphocytes are the only cells responsible for specific cell mediated antigenic immunity. Some T lymphocytes can interact directly with the protein components of intracellular antigen, others have a role in regulating the immune response. B cells interact with antigen via antibodies called immunoglobulins and

in association with T cells. Both T and B lymphocytes must be matured and then primed to elicit the appropriate responses.

T cells

T cells mature in the thymus gland and may be broadly categorised as either CD8 or CD4 cells by reference to adhesion and signal transduction glycoproteins present on the cell surface. CD8 cells are also known as cytotoxic (T_C) cells whereas CD4 cells may also be called helper (T_H) cells. Each mature T cell possesses a unique T-cell receptor (TcR) capable of interaction with a specific antigenic determinant or epitope (Benjamini et al 1996, Male et al 1996) but needs to be primed before it becomes fully functional. Priming takes place in peripheral lymphoid tissue (spleen, lymph nodes, Peyers patches etc) and requires that each T cell must simultaneously encounter its specific antigen together with a discrete body cell marker which is individually unique and recognised as 'self'. Self markers are a group of glycoproteins known as Major Histocompatibility Complex 1 and 2 (MHC1, MHC2). CD4 cells must engage in conjunction with MHC2 while CD8 cells require MHC1.

Following activation some T cells enter the circulation and become available to move to sites of injury in a process known as trafficking. Once inside the injured tissues T_H (CD4) cells are able to stimulate other immune cells to proliferate and mobilise while T_C (CD8) cells can attack infected cells directly and destroy them along with the infective agents that they contain. As the immune reaction proceeds chemical messengers (cytokines and chemokines) produced by the participating cells bring about changes in the milieu, which influence both the secreting cells (autocrine effect) and also those in close proximity (paracrine effect). These changes are responsible for the further differential development of T helper cells and for variations in immune responses, which subsequently ensue (Marieb 2004, Male et al 1996).

T_H cells may differentiate into either a T_H1 or a T_H2 phenotype. The T_H1 subset is associated with the stimulus for acute inflammation by also has a role the differentiation of cytotoxic CD8 cells, delayed type (DTH) hypersensitivity reactions and chronic inflammation (Male et al 1996). Cytokines such as Interleukin 12 (IL-12) and Interferon Gamma (IFN-γ) promote this process, which results in the synthesis of Interleukin 2 (IL-2) and IFN-γ. The T_H2 subset promotes antibody

responses from B cells and may be associated with immediate type (IH) allergic reactions. It is driven by Interleukin 4 (IL-4) and produces a predominance of IL-4 and IL-5 (Benjamini 1996, Romagnani 1996).

An additional T cell subset (the regulatory T_H3 cell or Treg) is produced by negative selection in the thymus. Treg cells control the amplitude of the immune response and later curtail the episode to restore homeostasis. They require the stimulation of IL-2 and are thought to suppress by action of anti-inflammatory cytokines IL-10 and TGF-β (Robertson 2007, Taylor et al 2007).

Following an inflammatory episode a pool of activated T cells creates a memory bank. These cells make their way to designated tissues by a process similar to trafficking known as homing. Large numbers of such memory cells are resident in skin and play an important role in the prompt initiation of future inflammatory responses.

B cells

B cells pass through a series of maturation stages in the bone marrow. They produce antibodies called immunoglobulins whose structure is similar to the TcR and co-receptors CD4 and CD8 expressed on T cells. Immunoglobulins are bound to the surface of B cells and are also present in plasma and some secretory fluids. Their purpose is to fix and bind complement and cross link receptor sites on soluble molecules, pathogens and toxins thus facilitating and enhancing phagocytosis and inflammation. There are five major classes of immunoglobulin (Ig) designated IgM, IgA, IgD, IgG, and IgE. The structure of the Ig molecule allows for genetic variation of the antigen binding region allowing progeny B cells to be antigen specific within their Ig class.

Immature B cells inherently express IgM and IgD. Activation takes place in the secondary lymphoid tissues where encounters with antigen take place and further differentiation then becomes milieu dependant. B cells may clone and become plasma cells or, under the influence of T cell derived cytokines, switch isotypes and begin to synthesise IgG, IgA or IgE.

IgD and IgM in their monomer forms act as antigen receptors. IgM in its pentamer form circulates in plasma where it acts as an efficient agglutinator and activator of complement. IgG represents the largest proportion of plasma Ig with the longest half life and has major and diverse roles including agglutination, opsonization, activation of complement, neutralisation of toxins and immobilisation of bacteria.

IgA is present in body secretions and helps to prevent the attachment of pathogens to epithelial surfaces. IgE is secreted by plasma cells in epithelial tissues, including skin, and some lymph organs. It has the ability to adhere and illicit a reaction from cells such as basophils, eosinophils and mast cells, participating in the allergic reaction and causing excessive release of histamine and concomitant increased vascular permeability. Serum levels of IgE in normal healthy individuals are very low < 300 iu/mL but in severe allergic reactions readings in excess of 10,000 iu/mL may be obtained (Ownby 1998, Rihoux 1993, Grundbacher 1975)

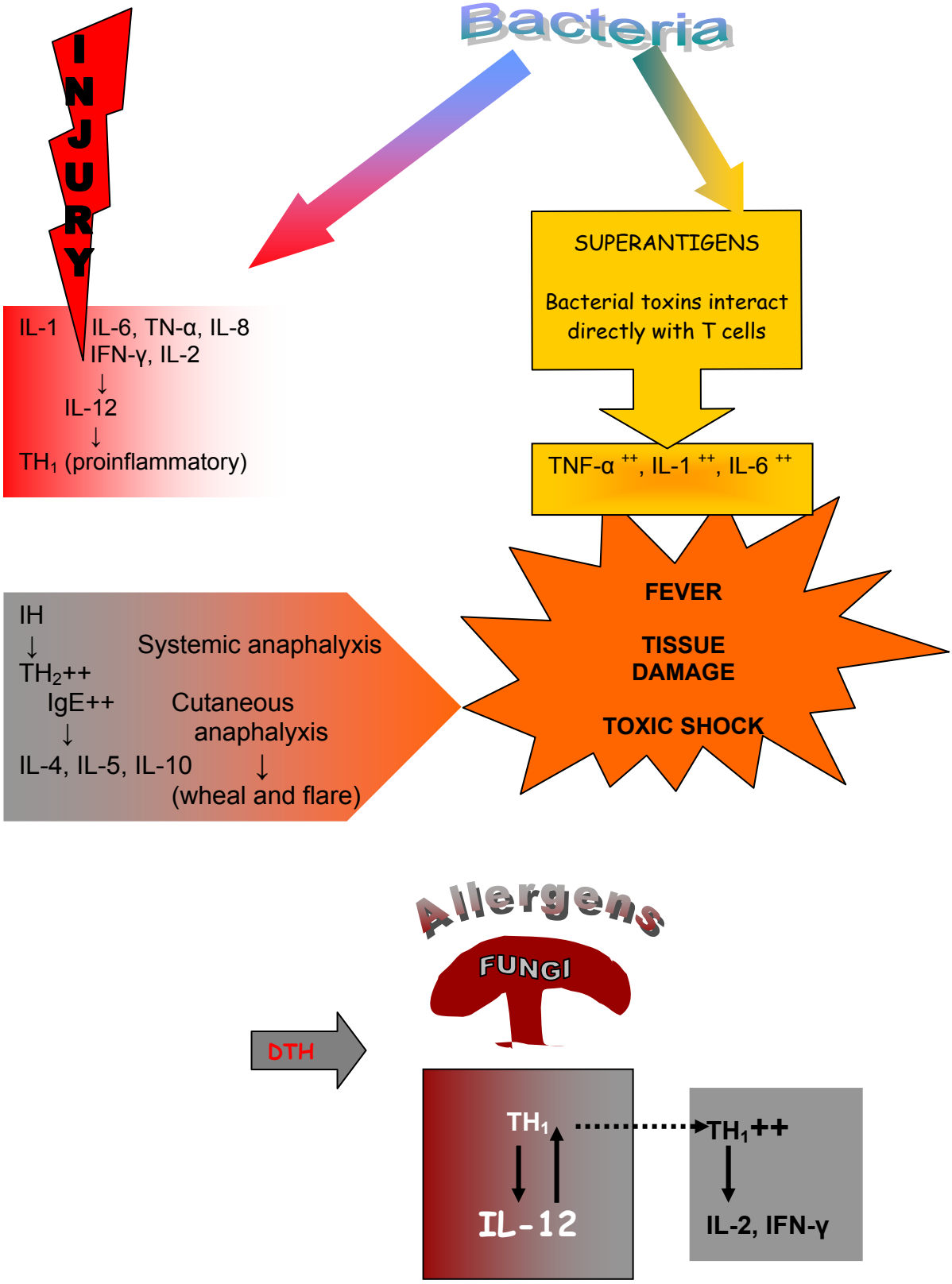
Initial exposure to infection produces a primary response with a lag time of several days during which B cells proliferate and levels of first IgM and then IgG rise sharply. Some of the B cells produced in this primary response are selected and retained. Because these cells are already primed subsequent identical challenges result in shorter reaction times and amplified responses.

2.1.3. 2 Infective/inflammatory responses in the skin

When skin is attacked by insult or infection the innate response is instigated by keratinocytes and the inflammatory process begins. On the skin surface this is characterised by swelling, redness, heat and pain with concomitant decrease in local mobility function. Acute phase proteins C-Reactive protein (CRP) in particular are released by the liver, the complement system is activated. Initially leukocytes are recruited and the WBC rises as large numbers of neutrophils arrive; lysis and phagocytosis of microorganisms begins. Immune system residents relay and amplify the response and achieve co-operation and co-ordination between cells by the production of cytokines and chemokines particularly IL-1, IL-6 and TNF-α (See figure 3). Both cellular activity and chemical production operate within feed back loops through links between local nerve fibres and the central nervous system (CNS), which is therefore intelligent and involved in the direction and control of the episode (Tracey 2002).

If the episode fails to resolve chemokines induce the onset of the adaptive immune response. Influx of T and B cells from the peripheral circulation is mediated by the vascular endothelium whose cell receptors bind leukocytes which then pass through the vessel wall by a process known as diapedesis and follow the chemokine gradient toward the site of injury (Spellburg 2000). The chemical milieu

Figure 3 - Simple graphical representations to identify the different TH_1 and TH_2 type cytokines associated with proinflammatory, IH, DTH and anaphylactic reactions.
(IL-6 has been included for completeness to highlight its important role in the proinflammatory response)



from which lymphocytes are recruited and to which they are introduced strongly influences the ensuing immune response and has important consequences for the outcome. The processes involved should be aggressive enough to combat the pathogens but also controlled so that the system shuts down when the desired effect has been achieved. Failures in this mechanism can lead to exaggerated or inappropriate responses with the potential for damaging effects upon the tissues.

2.1.3.3 Hypersensitivities

The various types of hypersensitivity (allergy) are examples of exaggerated or inappropriate responses and are thought to have evolved as protection against parasites or other organisms, too large to be destroyed by phagocytosis alone, and where extreme responses may be required to prevent infestation (Benjamini et al 1996). Basically hypersensitivities can be divided into two groups depending on their time course and whether antibodies or T cells are involved.

Immediate type hypersensitivities (IH) commence within seconds of allergen contact and involve a predominance of the T_H2 subset (figure 3) and concomitant increased levels of IgE antibody (Al Hansan at al 2004, Sato and Tagami 2003, Leung 1999, Blake et al 1991, Jones et al 1973) with subsequent mast cell degranulation, leading to an excessive histamine induced inflammation which typically manifests in skin as a 'wheal and flare' response (Benjamini et al 1996, Church et al 1999). Extreme episodes may result in systemic allergic reaction (anaphylaxis) with attendant risk of mortality (Simons et al 2007).

Delayed type hypersensitivities (DTH) have longer onset and are regulated by the T_H1 subset of helper T cells with the co-operation of the cell mediated effect of macrophages (figure 3). This type of hypersensitivity occurs in response to antigen such as allografts, intracellular parasites or fungi; manifesting in skin as erythema and induration (raised thickening) appearing around 48 hours after challenge. If the reaction succeeds in combating the antigen no untoward effects are observed; in many cases however the antigen is able to persist provoking prolonged macrophage activation with attendant tissue destruction. Moreover antigenic material may never be completely eliminated allowing the possibility that further challenge may reactivate the process.

Superantigens

Exaggerated responses to antigens are not confined to hypersensitivity reactions. Unprimed T cells can sometimes be activated by antigens without involving

antigen presenting cells (APCs). Such antigens, including some bacterial toxins, are able to bypass the normal binding constraints and simultaneously activate large numbers of T cells leading to the production of huge quantities of cytokines especially, IL-6, IlL-1 and TNF-α (figure 3). Otherwise harmless commensal bacteria such as Staphylococcus Aureas (SA) are capable of producing such responses in certain circumstances; the toxins responsible are categorised as 'superantigens'. The effects can initiate an immediate hypersensitivity (IH) reaction, which in extreme cases can result in anaphylaxis (Manders 1998).

It is well accepted that many infective/inflammatory diseases have both a toxic and an allergic component (Sulzberger et al 1937). The toxin is a metabolite produced by the pathogen while the allergic component arises from its physiological constituents eg; lipopolysaccharide (LPS) from the cell wall. Conditions such as diphtheria, tuberculosis and dermatophytosis are examples where varying contributions may be made by both infective and allergic components. In patients with dermatophytosis, an allergic skin reaction resembling cellulitis or erysipelas, was sometimes observed at the site of infection (Sultzberger et al 1937, Traub Tolmach 1937). In addition, isolated foci with similar appearances were noted at other anatomical locations. Because associations between these findings are plausible but as yet unproven, such reactions are described as idiopathic or by the shortened form "id ", (Rippon 1974, Sultzberger et al 1937, Traub Tolmach 1937).

With respect to the toxic component a study by Carratalà (2003) analysed the outcomes of 332 patients hospitalised for episodes of cellulitis. No studies had previously analysed the risk factors for mortality resulting from cellulitic episodes. It was found that toxic shock at presentation caused mortality in seven of the eight patients who died within the first 72 hours of admission. Various pathogenic bacteria including pseudomonas were assumed to be responsible, but in two cases staphylococcus aureas (SA), a skin commensal, was thought to be the cause.

2.2 Dermatophytes

Dermatophyte fungi have evolved from soil dwelling species that have adapted to use the keratinised layers of the skin together with its appendages (hair, fur and nails) for their nutrition (Hay and Baran 2004). Infections affecting the superficial layers of the epidermis are known as Dermatomycoses while those that have penetrated more deeply are described as Dermatophytoses. The term

onycomycosis encompasses all fungal nail disease. Dermatomycoses include Trichophyton Nigra (Cladosporium), and other opportunistic moulds such as Penicillium, Paecillomyces, Scopulariopsis and Acremonium. The Dermatophyte group is divided into three genera (Microsporum, Trichophyton and Epidermophyton); within these groups the most clinically relevant species are Trichophyton Rubrum, Trichophyton Mentagrophytes (Interdigitale), Trichophyton Tonsurans and Microsporium Canis. Other frequently used terms for these conditions are ringworm and tinea (Rippon, 1974) frequently with the addition of the body part eg, tinea pedis (tinea affecting the foot), tinea capitis (tinea affecting the head). Athlete's foot is commonly used as an alternative to tinea pedis.

Fungi are ecologically important as they form the basis for all biological recycling. Although their life form remains primitive their life cycles are complicated and their morphological appearances diverse, in addition they can proliferate/survive in sexual/asexual forms, combination types or as resistant spores. Microscopically they present in tissues as string like collections of cells (hyphae), which combine to form dense mats or mycelia. Reproduction commonly involves fragmentation of individual hyphae to produce satellites (arthroconidia) from which new colonies arise, and the production of spores (conidia), which are able to exist in a dormant state before germinating into new organisms. Classification is based on the morphology of these asexual spores (Clayton and Midgley 1989), but spores are only produced if the chosen culture medium and the growing environment (heat, humidity etc) meet species requirements. Confident identification therefore requires long periods of observation to assess the macroscopic shape and colour of the colony, both in obverse and reverse view, together with the microscopic appearance of the hyphae and the shape and arrangement of the spores (Rippon, 1974).

Fungal infections of the skin may involve any part of the body but the majority of cases occur in moist enclosed areas and the feet are particularly at risk, especially for those who predominantly wear boots or other enclosed footwear (Ananthakrishnan et al 2004). Fungal presence in skin is often unremarkable and organisms may reside undetected in areas such as the foot sole and the nails so the prevalence is unknown but is thought to be between 30-70% within the total population (Woodfolk and Platts-Mills 1998, Fitzpatrick 1994, Jones at al 1973). This is unsurprising as the proportion of shed skin scales with potential microbial

attachment, in airborne particles, could be as high as 10% (Mc Ewan Jenkinson 1993) and studies have shown that dermatophytes can be cultured from footwear, laundered socks, floors and the contents of vacuum cleaners, even after elapse of months or years (Broughton 1955, Sugimoto1955).

Dermatomycoses are opportunistic organisms and their presence is most relevant in the mucosae where application or inhalation of significant numbers may lead to serious disease (eg Farmers Lung). In most other cases the opportunists only become clinically relevant in the immunocompromised patient and skin is sometimes involved (Rippon 1974)

Dermatophytes are the major perpetrators of fungal skin disease and capable of populating the skin of both vulnerable and healthy individuals alike. Mild infections are considered benign but excessive fungal proliferation leads to changes that threaten the skin environment. If fungi access the deep skin through open wounds they may disseminate via the blood stream and subsequent related disease has been demonstrated particularly in immunocompromised patients (transplants etc) and in guinea pigs (Seçkin 2004, Sulzburger 1928). Normally the numbers of dermatophytes residing in the superficial skin layers is controlled by the process of desquamation, which eliminates the fungus as the dead skin scales are shed (Blake et al 1991) however if fungal proliferation exceeds the rate of skin turnover the immune system intervenes. Dermatophytes signal their presence to the immune system by diffusion of toxins produced from their sloughed cell wall eliciting a delayed type hypersensitive skin response (DTH) and concomitant increase in the desquamation process. There is no innate immune response mechanism Rippon 1974), the DTH (cell mediated response) is the only designated pathway known to confer immunity; this can be confirmed by the presence of significant titres of specific antibodies in the serum. Abnormality or absence of the DTH response allows the fungus to proliferate (Al Hassan et al 2004, Woodfolk and Platts-Mills 1998, McGregor et al 1992) altering the skin environment in favour of bacteria. Subsequently bacteria in turn become dominant, the numbers of active fungi decline, produce spores and enter a dormant phase (Hay 1993).

2.2.1 Cellulitis and dermatophytosis

Research by Day et al (1996) has shown that uncontrolled dermatophytosis results in increased alkalinity at the skin surface, concomitant progressive skin debilitation

and a gradual change from a preponderance of gram positive to gram negative bacterial colonisation. This change, which assists the overgrowth of organisms such as pseudomonas and klebsiella, is clinically recognised as gram-negative cellulitis, and is particularly important in immunocompromised patients and those with neuropathy because delay in averting its rapid progress can lead to necrotizing faciitis or gas gangrene with subsequent loss of limb. These observations suggest that dermatophytes promote cellulitis by creating changes in the skin surface environment, which favour transient pathological bacterial species. Other studies however, suggest that their primary role is to provide a portal of entry by which any bacteria may gain access to the deeper layers of the skin. Dupuy et al (1999) studied 167 patients with clinically diagnosed erysipelas of the leg and 294 controls. Their results showed toe web intertrigo (fissures between the toes) to be a risk factor for developing cellulitis that increased with the number of toe spaces involved, however the presence of dermatophytosis was only assumed as no cultures were performed. In (2004) Roujeau et al studied 243 cases of acute clinically diagnosed cellulitis and 467 controls. In this study dermatophytosis was confirmed by culture and found to be present in 42.5% of cases compared with 24.1% of controls. Björnsdóttir et al (2005) used both culture and microscopy to demonstrate dermatophytes and types of pathogenic bacteria in the toe web spaces of 100 hospitalised patients with cellulitis and 200 hospitalised controls. They used odds ratios to calculate the risk factors associated with various attributes and concluded that dermatophytosis was high risk (OR,3.86; 95% CI, 1.32-11.27) but only in the absence of proven bacterial infection. As it was impossible to confirm either fungi or bacteria as causal they recommended improved management of foot care particularly toe web intertrigo, as a means of reducing the incidence of cellulitic episodes.

In all these studies acute and recurrent cases were analysed together and although previous history was recorded and cited as a risk factor for future cellulitic attacks there is a paucity of data relating to long term outcomes in such patients. Only one study was found which specifically addressed this issue (Baddour and Bisno 1984). It relates to a group of patients who were recalled for surveillance following coronary artery bypass grafts (CABG); some of which suffered repeated attacks of cellulitis at the donor site after saphenous vein harvesting. The attacks were unpredictable and irregular and ranged from hours to weeks or months post

operation. Within this group seven patients suffered from concomitant dermatophytosis of the feet and it was found that in two cases the recurrent attacks ceased following antifungal medication.

The above studies suggest a link between cellulitis and dermatophytosis and favour the theory that dermatophytes provide a portal of entry through which bacteria may enter and infect the skin, however this does not explain why dermatophytes breech the stratum corneum when their presence should be contained within the uppermost layers of cornified cells.

It has been shown that a DTH reaction speeds the desquamation process eliminating fungal bodies and priming the immune system to confer long lasting protection. However it has also been shown that some individuals produce an IH response to dermatophyte invasion, which results in an initial exaggerated reaction, absent or incomplete skin shedding and allows fungi and spores to be retained (Woodfolk and Platts-Mills1998). There are also a group who fail to mount any type of response and these are classed as anergic (McGregor 1992). In addition individuals have been shown to produce varying immunological responses to the same fungal species on subsequent occasions (Woodfolk et al 1998) despite displaying the expected immunological outcomes in response to other non fungal antigenic material (Svejgaard 1985).

Attempts have been made to understand the mechanisms responsible for these differing responses. Walters (1974) recruited patients suffering with acute and chronic dermatophyte infections and demonstrated that serum taken from patients with chronic Trichophyton Rubrum infection had the ability to block their own cell mediated responses. Maleszka (2001) studied 35 patients with onychomycosis (fungal nail disease) and 20 controls and showed decreased lymphocyte trafficking, lymphoproliferation and differentiation during dermatophyte infestation. In vitro experiments conducted by Baldo (1997) demonstrated precipitative reactions between fungal 'C' substances and C-reactive protein with the potential to cause thrombus like formations within the vessels of the microcirculation. Such observations are endorsed by Cermak et al (1993) who postulate that changes in the microcirculation, during and subsequent to inflammatory events, may occur because of interactions between CRP and lipopolysaccharide (LPS) and manifest from modification to the coagulation cascade. These findings suggest that immune responses elicited by dermatophytes could have implications for the

immunological control of other infective organisms. The mechanisms are as yet unknown but have been variously attributed to factors within the milieu at the infection site or serum (Maleszka 2001), the severity of dermatophyte infection (Woodfolk and Platts-Mills 1998), host factors (Hay 1993) and cross reactivity between fungal species (Hopfer 1975).

It is also possible that changes in skin structure and/or physiology favour fungal proliferation and assist their progress into the deeper layers of the skin or that defects in the normal desquamation process favour retention of fungi or spores. The evidence therefore suggests that dermatophytes could play a more major part in the infective process than just providing a portal for bacterial to enter (Kremer et al 1991). Spore production assures a substantial source of successive dermatophytes and this together with inhibition of lymphoproliferation, conferred by their continuing presence, may predispose vulnerable individuals to repeated episodes of bacterial infection (Blake 1991).

2.3 Stressors

Stress may be defined as force acting against resistance. When stress is imposed a reaction is provoked; this reaction is described as strain. In a medical context stress can be defined as any disruptive factor that threatens the homeostasis of the body (Boon and Christensen 1997).

Factors including injury, disease or worry as well as environmental effects such as temperature, pollution and malnutrition are all therefore potential stressors. It is now well accepted that the effect of stressors both physiological and psychological affect the course of disease by links with the endocrine and immune systems (Tracey 2002, (Maleszka et al 2001) and in conjunction with attributes which result from differences in genotype (Korte et al 2005, Cheng et al 2004, Kustima et al 2004, Leung 1999). While acute stress has been shown to be beneficial by priming, testing and effecting responses, deleterious effects are produced when the stressor is exerted for long periods resulting in a cumulative stress burden known as allostatic load (Korte et al 2005, McEwen 2003, Schulkin et al 1998). The effects are initially manifested indirectly through events that manipulate the chemical environment, promoting changes in cellular function, which then translate into disturbances within more integrated processes (blood pressure etc). The disease process emerges as a consequence of these precursors and thereafter

plays a major role in this self perpetuating progression (Choi et al 2005, Dhabhar 2003, Vanitallie 2002, Denda et al 2000, Harris 1996).

2.3.1 Effect of stressors on the skin

Because the skin is responsible for protecting the entire body surface against the hazards of the outside world any detrimental change to its morphology or physiology is likely to have an effect upon its function. Small changes are important (Hay 1993) as they may cause subtle differences in host defence that over time render the skin more vulnerable to invasion by pathogens from either external or internal sources (Tagami et al 2001, Finch 1988). Implications associated with the ubiquitous presence of dermatophytosis have already been discussed. Conditions such as psoriasis and eczema are thought to be associated with psychological stress (O'Sullivan et al 1998), manifested through manipulation of the cytokine environment, modulation of the skin immune function and delays in recovery in the skin barrier (Nakano 2004, O'Sullivan et al 1998). Other stressors such as trauma, surgery, diabetes, smoking and systemic vascular disorders together with the ageing process have all been shown to affect skin function presenting as changes in skin thickness, decreased skin vascularity and modification to the surface environment (Ryan 2005, Clark et al 2003, Harrison et al 1993, Tur et al 1992). Aside from abnormalities resulting from arterial delivery, malfunctions in the venous system resulting from faulty venous valves, may initiate microcirculatory defects, which are later displayed in skin variations such as oedema and lipodermatosclerosis (Topham and Mortimer 2002, Ryan 2002).
The above findings suggest that a wide range of potential stressors have the ability to affect skin structure and physiology and increase its vulnerability. With respect to cellulitis and the potential links with dermatophytosis, some factors are particularly important; these include the skin environment, pH at the skin surface, changes to the microcirculation and variations in skin structure.

2.3.1.1 The skin environment

In order to maintain skin health and provide good protective function it is important that the skin layers should be adequately hydrated while the surface remains in a dry but pliable state. Many factors including skin lipid content, transepidermal water loss (TEWL) and sweating affect this balance and the environment that exists close to the skin surface is a major influencing factor with implications for infection and inflammation. Dehydration encourages cracks and fissures to form

allowing microbes to penetrate below the surface; hyperhydration promotes maceration, which breaks down the protective barrier dissuading the growth of natural flora in favour of more pathogenic forms (Day 1996, Mc Bride 1993). In 1978 Leyden and Kligman used plastic bags to simulate a moisture laden environment around the feet of persons suffering from dermatophytosis and controls. Their experiments showed that in the presence of hyperhydrosis, dermatophytosis simplex was transformed to dermatophytosis complex with concomitant change from the normal gram positive flora to their more pathogenic gram negative counterparts. At the start of the experiment, skin colonisation in the patient group showed a bacterial/fungal mix with gram negative dominance dependant on the severity of the dermatophyte infection. After 2-3 days the enclosed skin became macerated and malodorous with greatly increased numbers of gram negative bacteria and biopsies were required to demonstrate the fungal presence, which had migrated to the lowest level of the SC. A return to the original floral mix was observed once again when the experimental hyperhydrosis had resolved. By contrast, the flora of control individuals was normal at the beginning, increased with accompanying mild maceration during the experiment, and rapidly returned to the original state post episode. These findings suggest that fungi and bacteria are able to coexist in equilibrium on the skin if the surface humidity is within the normal range. Excessive moisture appears to facilitate/accelerate the overgrowth of pathogenic bacteria but the presence of dermatophytes also seems to be required. This theory is supported by evidence from studies of populations (soldiers, construction workers etc), whose feet are predominantly enclosed inside boots or substantial shoes (Ananthakrishnan et al 2004, Fitzpatrick 1994), where the presence of untreated dermatophytosis has been shown to lead to serious bacterial complications. It therefore appears likely that dermatophytosis, skin humidity and wearing confined footwear have implications for patients with cellulitis, although at present the mechanisms are unclear.

2.3.1.2 Skin pH

The symbol pH is used to indicate the concentration of hydrogen (H^+) ions in solution and is normally recorded as a logarithm. A recorded pH of 7 is regarded as neutral; values below this are acidic and those above are alkaline. The reported pH range for normal human skin varies from 3 – 7.0 (Matousek and Campbell 2002), this falls within the acid range and the skin surface is often referred to as

the 'acid mantle'. Maintenance of acidity is attributed to contributions made by the constituents of sweat and sebum together with the products of lipid degradation (Huraib et al 1994).

Chikakane and Kenichiro (1995) demonstrated that in skin diseases such as eczema and atopic dermatitis pH values were alkaline (pH above 7.0); in healing ulcer tissue high initial pH values were shown to fall as the wound volume reduced (Roberts et al 2002). Except for babies and the very elderly, age does not seem to affect skin pH neither does anatomical site appear to influence pH values except in areas of moist occlusion such as the toe web (Parra and Paye 2003).

Authors such as Spellberg (2000) and Sprunt et al (1971) suggest that an acidic pH is important because it provides optimal conditions to maintain growth of the commensal organisms responsible for discouraging the expansion of transient pathological species. However a different view is expressed by McBride (1993) who maintains that the change in electrostatic charge generated on the skin surface, due to changes in pH, may be the effector mechanism for either attracting, releasing, or mobilising the prevailing flora. Variations in pH are also considered to have an impact on the rate of desquamation, which in turn may affect the colonisation at the skin surface (Rippke et al 2004).

Disruption of pH may arise from localised changes resulting from the use of detergents (Korting and Braun-Falco 1996), germicidal soaps (Amonette and Rosenburg 1973) or antibiotics (Sprunt et al 1971) but the presence of dermatophytes can also produce the same effect. In (1956) Goldfarb and Herrmann cultured dermatophytes and other saphrophytes on Sabouraud`s medium and recorded levels of pH by observing colour changes in the medium around the colonies. All eleven species of dermatophyte produced pH changes in the alkaline range while the saprophytes produced varying degrees of acidity. Ryall (1980) reports similar observations in vitro emphasising that although dermatophytes may grow freely on contact with an acidic medium, the substrate rapidly becomes alkaline due to release of ammonia by their metabolism. In 1995 Chikakane and Takahashi studied pH values on the hands and feet of 68 patients with athletes foot as compared to 19 control subjects. They found that the mean skin surface pH values recorded on the feet of the patient group was significantly higher than those of controls (Patients = 6.1 ± 0.6, controls 5.2 ± 0.5 respectively) but no significant differences were demonstrated on the hands.

These findings taken together suggest that the pH value at the skin surface may have implications for microbial colonisation and may therefore have a role in cellulitic episodes.

2.3.1.3 Skin microcirculation

One important function of the microcirculation is to elicit an effective response during episodes of infection and inflammation. This is achieved by communication/co-ordination between cells of the immune and microvascular systems. The start of an infective/inflammatory response generates cytokines and neuropeptides, which diffuse through the skin layers to alert the cells of the dermal endothelium and initiate the feed back system via the brain. Interaction between endothelial cells and neuropeptides cause microvascular vessels to dilate, while further action by mast cell induced histamine, causes them to become more porous. Increasing dilation and porosity allows serum to leak into the interstitium, decreasing vascular pressure, lowering shear forces, and permitting blood flow to slow and facilitate diapedesis of immune cells into the tissues. These changes promote the well recognised signs of inflammation; erythema, heat, swelling and pain.

The ability to mobilise immune cells and effect immune responses is therefore highly dependant upon both afferent and efferent physiology of the skin microvascular system but an efficient, effective lymphatic system is also required to carry away excess interstitial fluid and convey antigen and antigen presenting cells to the lymphatic tissues. In conjunction with lymphatic drainage the role of the deep and superficial venous systems must also be considered.

2.3.1.4 Venous function

Venous function has been shown to be an important factor in the aetiology of diseases affecting the lower limbs (Wollina et al 2006, Khan and Newton 2003, del Mar Sāez et al 2001) and chronic venous insufficiency (CVI) is a key factor in the initial decline in skin health leading to conditions such as lipodermatosclerosis, oedema and ulceration (Cox 2006, Naguib et al 2004, Jones et al 2004). CVI results from defects in venous return to the right atrium. The most important cause of venous insufficiency is damage to the venous valves, either in the deep or superficial venous systems and commonly as a result of thrombophlebitis or DVT (Haneke 1991, Ciocon 1993, Thomas 1988, Killewich et al 1985). Impaired venous flow results in venous reflux, sending pressure back through the venous system to

the capillaries, disturbing the microcirculation and leading to oedema (see section 2.3.1.5), which impedes the diffusion of nutrients and gases and disrupts normal cellular functioning.

The microcirculation plays a vital role in the effectiveness of immunological outcomes. Its responses must be prompt at the outset, efficient in delivery and curtailed at the appropriate time. Defects in this sequence have the potential to hinder or prolong the inflammatory process and this may have important implications with respect to the extended course of some cellulitic episodes. No studies were found which specifically address the role of the microcirculation in cellulitic attacks but CVI was cited as a risk factor in the study by Roujeau et al (2004). It was found to be significant when entered into conditional logistic regression analysis ($p = < 0.1$), however this finding is difficult to evaluate as the defining criteria for CVI was based on clinical findings alone and no laboratory investigations were performed.

2.3.1.5 Skin properties

Skin varies in thickness and mechanical properties according to anatomical site and in conjunction with functional and gravitational adaptations. Variations in thickness also reflect the influence of age, sex, hormones and circadian rhythms. The thickness of the epidermis is maintained by spontaneous desquamation (Milstone 2004) a process, which normally increases in response to a variety of insults including dermatophytosis (Jones 1974). In the study by Badour and Bisno (1984) desquamation was observed at the affected cellulitic site in the days following cessation of antibiotic treatment but no studies were carried out to measure the changes in skin thickness that occurred.

The thickness of the dermal layers varies in response to the amount of free fluid transitional in the tissues (Eisenbiss et al 2001); in health this fluid is maintained close to homeostasis by the lymphatic system (Courtice 1984). In the inflammatory state fluid content between skin layers is greatly increased due to an influx of water and plasma proteins, which exit the small blood vessels and pool in the extravascular space. The increasing volume of fluid soon exceeds the capacity of the lymphatic vessels and the tissues begin to swell, a condition known as acute oedema (Browse and Stewart 1985). This type of oedema usually resolves as the inflammation subsides but if excess fluid production persists the lymphatic system becomes permanently overloaded and the oedema becomes chronic. Such

chronic oedema is a ubiquitous complaint and the consequence of diverse clinical conditions including heart failure, immobility, dependency and endocrine dysfunctions.

Lymphoedema is another form of oedema which results in tissue swelling but the underlying cause in this case is disease/anomaly of the lymphatic vessels brought about by changes initiated by parasites, cancer, surgery or Radiotherapy (Topham and Mortimer 2002, Browse and Stewart 1985), thus fluid retention, in this instance, results from failure in the transport system by which it is removed. Because most oedema contains an element of lymphoedema and the clinical presentation is often similar, the terms are often used synonymously. Physiological tests and invasive imaging modalities are required to establish a differential diagnosis (Burnand et al 2002, Topham and Mortimer 2002, Duewell et al 1992, Mortimer 1990, Vaughan 1990, Partsch 1988, Mortimer 1986), these are expensive, time consuming and not without risk but may be necessary because the differing underlying physiologies should be understood when considering the role of oedema in conjunction with other medical conditions.

Studies by Depuy et al (1999) and Roujeau et al (2004) assessed swollen legs as risk factors for cellulitic episodes by reference to lymphoedema and oedema. In a case controlled study of 167 patients and 294 controls Dupuy et al showed lymphoedema to be a major risk factor for cellulitis while oedema was found to be insignificant. In a similar but later study (243 patients and 467 controls) Roujeau el al (2004) found significance for the presence of oedema per se. Cox (2006) performed a retrospective study on 143 patients with assumed streptococcal cellulitis of the lower leg. Medical history and questionnaire was used to record complications and identifiable risk factors for further episodes and to establish the frequency of post cellulitic oedema. Oedema (defined only as persistent leg asymmetry), was found to be strongly correlated with recurrent cellulitis ($P = 0.0002$) but onset of asymmetry and its temporal relationship with the original cellulitic episode was not established.

In all these studies oedema and lymphoedema were assessed clinically; linear measurements, physiological tests, scoring systems and imaging modalities were not used. Although persistent oedema appears to be associated with cellulitic attacks, more thorough investigations are required to provide conclusive proof.

It is not known whether residual oedema is retained once the cellulitic episode has resolved, whether the epidermal thickness remains stable or whether differences in these parameters exist between those who suffer acute as opposed to recurrent attacks. Such information may be significant as it may substantiate the evidence for the role of oedema in the cellulitic process and/or implicate abnormalities in the normal desquamation process.

2.4 Rationale and aims of study

Cellulitis is a debilitating infective/inflammatory skin disorder with a high rate of recurrence. Definitive diagnosis is difficult and treatment is therefore largely empirical. Doubts as to the aetiology of any infective/inflammatory condition must always exist if no causal pathogen can be positively identified and this, together with the unpredictable course of some episodes and the frequency of recurrence, are major problems for the management and prophylaxis of cellulitis. The diversity of individual response suggests that although pathogens play a major role, other influencing factors may also be important.

Previous research suggests that infective/inflammatory skin conditions such as cellulitis may occur and recur because the skin and/or immune system fail to function properly and an association with dermatophytosis could be implicated. There is some evidence that stressors (physiological/co-morbidity/psychological/ environmental) may be involved. The relationships are complex but represented in a simplified form in figure 4. Much work has been done, but some aspects, such as the characterisation of the immune response and quantification of vulnerability factors within the skin, have not been fully investigated in this context. There is a need to determine the frequency of dermatophytosis in patients with cellulitis, to understand the effects that this might have upon skin function and the immune response, and to examine the role that might be played by other stressors. The methodology for this study has been devised to examine these associations.

2.4.1 Study aims

This study was intended to provide a preliminary investigation into the risk factors associated with cellulitis of the lower limb. The following aims were identified as important :

- To investigate the frequency of dermatophytosis amongst patients with clinically diagnosed cellulitis of the lower limb as compared with matched controls.

- To investigate the risk factors relating to skin structure and physiology in patients with clinically diagnosed cellulitis of the lower limb as compared with matched controls.
- To compare blood profiles and types of immunological responses in patients with clinically diagnosed cellulitis of the lower limb and matched controls.
- To consider the role of other potential stressors within the context of these findings.
- To compare the above with respect to acute and recurrent cases

In order to address these aims a case controlled pilot study was designed and suitable methodology and testing procedures selected. The subsidiary aim was

- To establish the suitability, feasibility and reliability of the proposed tests and to provide data on which to base a realistic sample size for a subsequent larger scale study.

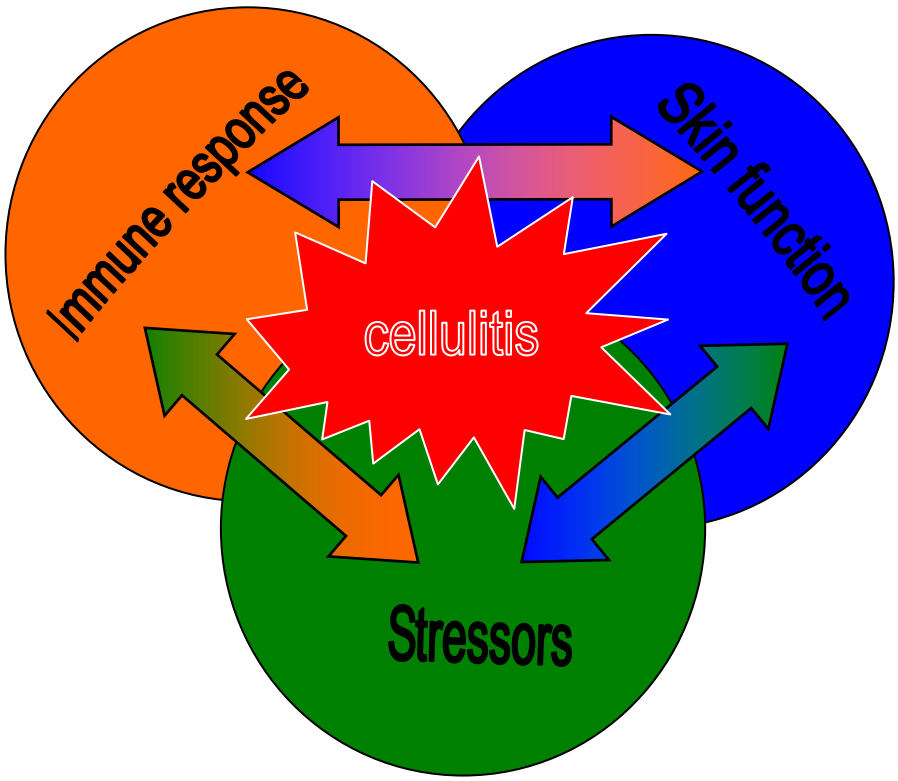


Figure 4
Possible relationships between stress factors, skin function and the immune response

CHAPTER 3

STUDY DESIGN

3 STUDY DESIGN

This pilot study was designed to investigate the risk factors associated with cellulitis of the lower limb and consider the possible relationships. The subsidiary aim was to establish the suitability, feasibility and reliability of the proposed tests and to provide data on which to base a realistic sample size for a subsequent larger scale study. Those tests to be included in the main study were therefore dependent upon the outcomes of the pilot study and those found to be unsuitable, unfeasible or unreliable would be removed.

The risk factors may be regarded as stressors acting to predispose or in conjunction with the principle cause of pathology ie: bacterial infection. They are divided into four groups as follows:

Microorganisms other than bacteria - specifically dermatophytes

Factors relating to skin structure and physiology

Immunological outcomes

Additional stress factors

The risk factors to be assessed and the related tests are outlined in table 1.

Initially it was decided to recruit patients with cellulitis confined to one leg so that within patient comparisons could be made between legs. However, this made no provision for control comparisons with respect to frequency of microorganisms or immunological outcomes and a case control study design was therefore adopted. Within patient comparisons were made between the affected (cellulitic leg) and the unaffected (contralateral leg) and patient control comparisons were made between the cellulitic leg and the control cellulitic leg (matched by left or right). Where appropriate, comparisons were also made between the patient contralateral leg and the control contralateral leg (eg: numbers of dermatophytes cultured). All tests were repeated on a second occasion approximately one month after the cessation of antibiotic therapy. This second visit was crucial to establish whether results from cellulitic patients returned to normal values post episode as no previous studies had been identified which presented such data. The timing selected for this second visit is explained in section 4.2.2.

Because comparisons were required between patients and controls at each visit, between visits and also between patients suffering from acute and recurrent

Table 1 – Risk factors and related tests

Category	Risk factor	Mechanism	Test selected for use in the study
Microorganism	Dermatophytosis	Portal for bacterial entry Changes in skin morphology Defects in desquamation Allergic reaction →	Specimen collection, culture and microscopy Serum immunoglobulin to Trichophyton Rubrum (IgE to TR)
Skin structure and physiology	Changes in skin water loss	Changes in skin surface environment, vulnerability to bacterial/fungal proliferation	Measurement of Transepidermal water loss (TEWL)
Skin structure and physiology	Changes in pH balance at the skin surface	Changes in skin surface environment, bacterial/fungal proliferation	Measurement of skin pH
Skin structure and physiology	Oedema	Skin vulnerability Impeded immune response	Leg circumference measurements Ultrasound tissue measurements
Skin structure and physiology	Changes in skin blood flow	Changes in skin nutrition, function and immune response	Measurement of skin blood flow Laser Doppler Imager (LDI)
Skin structure and physiology	Venous incompetence	Precursor to oedema	Photoplethysmography Laser Doppler flowmeter (LDF)
Immunological outcomes	Abnormal haematological response to inflammation/infection	Deviation from the expected patterns/sequences of cell responses to inflammation	Blood profiles
Immunological outcomes	Exaggerated immune response	Predominance of T-helper 2 (TH_2) subset ↓ > serum IgE	Serum cytokine profiles
Immunological outcomes	Allergic response		Serum immunoglobulin (IgE)
Stress factors	Previous episode Co-morbidity Previous surgery etc	Skin vulnerability Immune defects	Patient history
Stress factors	Psychological stress	Skin vulnerability Immune defects	Stress questionnaire

episodes, the statistical analysis was complicated. A systematic approach was therefore adopted and this is explained in section 5.2.

3.1 Choice of methods

Patients suffering from cellulitis present with high fever, attendant malaise and large areas of infected skin. Investigative methods should therefore be quick, minimally invasive with low risk of infection and tolerable to sick individuals. In addition they should be appropriate, reliable, reproducible and validated. All of the tests chosen for use in this study are currently in use in either a clinical or a research setting; in some cases the methodology has been refined in order to more closely fit the intended purpose.

3.1.1 Investigation for dermatophytosis.

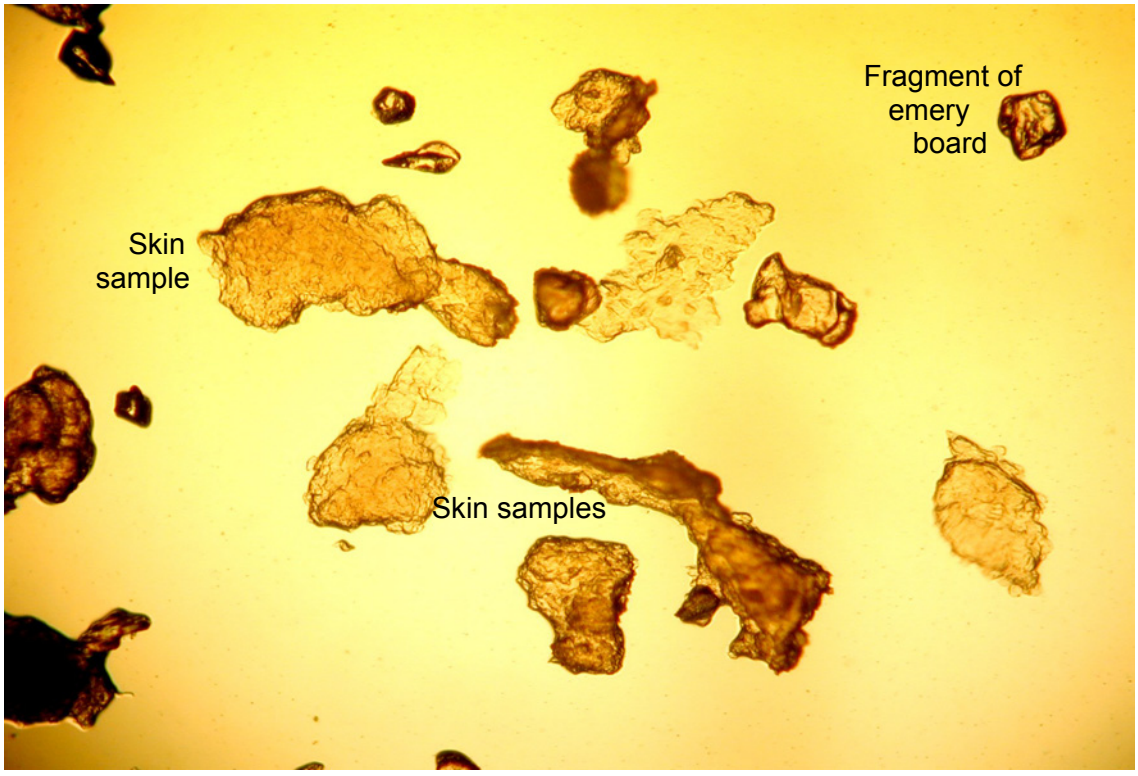
The basis of mycological diagnostic tests is the identification of fungal elements present in samples taken from the skin at the affected site. Diagnosis may be difficult because the samples obtained may lack hyphae or spores, fungal concentration may be too small to support viable colonies or similarities in appearance may make it impossible to interpret between species (Lawry 2000, Taplin 1969). For these reasons and because individuals may be asymptomatic, cases often remain undiagnosed.

Foot scraping or tape stripping are the methods currently used by podiatrists to obtain skin samples from the feet but these were unsuitable for use in this study because they were considered to be too invasive in the presence of infection. A sample collection method was required which would ensure a good success rate whilst being fairly quick and well tolerated by those whose cellulitis included their feet. Initially samples were obtained by applying pressure to the foot sole and interdigital spaces using a soft toothbrush and pressing the bristles into an agar medium, however microscopic observation revealed that few skin scales had been collected by this method indicating that it was not sufficiently aggressive. Subsequently emery boards were applied with a firm rubbing motion and the fine dust generated was collected onto coloured paper and evenly distributed on the surface of the agar. When viewed microscopically this process revealed large numbers of skin scales (including aggregates), together with crystalline deposits from the emery board (figure 5). As this method was consistently found to produce adequate quantities of skin scales from both the foot sole and the interdigital

spaces and was also well tolerated by the patients, it was adopted as the method of choice for the duration of the study.

Culture mediums for dermatophytes and bacteria can be made in house or purchased ready made to suit specific organisms. After considering the options, a commercially available dextrose agar system (IN TRAY ^{TM} DM (Biomed Diagnostics, USA) was obtained. This system consists of individual agar coated trays with transparent lids, which are used to seal the tray when the sample has been inserted and thereafter provide an inspection window. The sealed tray is low risk for infection and is designed for use at room temperature and without reference to culture and microscopy departments. The medium changes from yellow to pink in response to changes in pH, denoting the presence of

Figure 5 – Skin samples on DM x 10



dermatophytes (Pariser and Opper 2006, Taplin et al 1969). This system was tested using identified dermatophyte cultures from the Microbiology Department within Southampton General Hospital. It was found to be highly sensitive but low on specificity as other organisms (moulds, bacteria and yeasts) produced similar colour changes.

Further experimentation proved that it was possible to discriminate between organisms when morphological observations of tray sample cultures were evaluated in conjunction with microscopic appearance. When these methods proved inconclusive organisms were recultured onto Sabouraud dextrose agar (SAB) to encourage further growth (Evans and Richardson 1989); observations were then compared with documented morphology for that media. In difficult cases advice was sought from a mycologist from the Microbiology Department within Southampton General Hospital.

3.1.2 Investigations of skin structure and physiology

Changes in morphology and/or physiology of the skin may have important implications for diseases such as cellulitis and dermatophytosis. Initially these diseases may occur as a result of insult or injury but subsequent attacks may arise because the skin remains vulnerable. Morphological changes provide access for pathogenic and commensal organisms allowing them to penetrate beyond the stratum corneum and infiltrate the dermal layers. Physiological changes may affect homeostasis and/or alter the microenvironment favouring growth and prolonging the presence of microorganisms within the skin. Defects in the microcirculatory system may contribute to these processes but its role in the delivery of the immune response must also be considered.

In this study non invasive tests were selected to assess some skin attributes which might favour growth of microorganisms, facilitate their entry or compromise the skin immune response. Measurement of skin water loss, skin pH, oedema, skin thickness, venous incompetence and skin blood flow were selected. Comparisons between these functions in normal as opposed to cellulitic skin could indicate factors that may be associated with increased vulnerability.

3.1.2.1 Skin water loss

Hydration at the skin surface depends on external humidity, internal water loss and whether the area is directly exposed to the atmosphere or confined within an occlusive cover such as a bandage or a shoe. The temperature gradient is also an important factor. The water content of the stratum corneum at any one time affects the skin surface humidity and visa versa and this has implications for both microbial colonisation and skin surface physiology. The rate at which water is transferred between the skin and the atmosphere is known as transepidermal water loss (TEWL). Under controlled conditions (avoiding excessive humidity

which causes sweating or extremes of cold which cause skin circulation to decrease) the rate of water loss through the stratum corneum is estimated at 6-11 g/hr/cm^2 (Nilsson 1977) in most body parts including the legs, while in inflamed skin much higher rates are recorded (Tagami et al 2001).

The universally accepted method for the measurement of TEWL is the measurement of evaporative water loss, calculated by determining the vapour pressure gradient between two sensors positioned within a cylinder in a vertical line above the skin surface. The gradient between the two readings is calculated with reference to temperature and the result is proportional to the amount of water vapour evaporating from the skin surface in unit time (Nilsson 1977). This holds true providing that the cylinder is open ended and exposed to the atmosphere and that measurements take place in a draught free environment within the stated temperature range. TEWL measurements have been shown to increase as the outer layers of the SC are stripped away (Morgan et al 2003, Kalia et al 1996), for this reason and in order effect good comparisons as well as minimise infection, measurement sites were confined to intact skin.

3.1.2.2 Skin pH

The symbol pH is used to indicate the concentration of H^+ ions in a solution, expressed as the logarithmic reciprocal and reflecting its relative alkaline, neutral or acidic attributes. A recorded pH of 7 is regarded as neutral; values below this are acidic and those above are alkaline. Repeated studies on healthy human skin reveal pH levels maintained below 7 pH (between 3 - 6.5) and therefore in the acid range (Matousek and Campbell 2002, Chikakane and Takahashi1995, McBride 1993). This has been attributed to contributions made by the constituents of sweat and sebum together with the products of lipid degradation. The only study found to specifically address the calf skin was conducted on school children (11 – 16 years) where the mean pH was found to be 5.6 (Noble 1968). With respect to dermatophytosis the pH value on the foot sole has been shown to be significantly higher in sufferers as opposed to healthy controls (Chikakane and Takahashi1995); for those with cellulitis, variations in pH values are thought to have relevance for the balance of the microflora at the skin surface (McBride 1993).

Early experimenters measured pH by colorimetric methods (Anderson 1951) but latterly a technique employing a hand held glass probe, containing a combined

active and reference pH electrode, has been universally adopted (Parra and Paye 2003, Korting and Braun-Falco 1996). The pH value can be read directly from an integral pH meter or recorded continuously via a PC interface. Because the skin is not a solution measurements must be made with a damp electrode tip and time must be allowed for the reading to settle in order to record a reliable measurement. Care must be taken with the protocol and the results interpreted with caution because pH values are greatly affected by changes in temperature and any applications made to the skin surface. Comparisons must also consider that pH is a logarithmic scale so that small unit changes actually represent large differences (Matousek and Campbell 2002).

In this study calibration was carried out at regular intervals over the expected pH range (4 and 7pH values). As alcohol wipes were required to disinfect the probe between readings and there was some concern that this would affect the response times to subsequent readings, experiments were conducted in fluids of known pH with alternating disinfection to evaluate the effects. Neither sharp variations in pH or disinfection made any effect on the accuracy of the readings or the time taken to stabilise the results.

3.1.2.3 Oedema

Skin thickness varies with different areas of the body together with the influence of age, sex, hormones and circadian rhythms. Variations in epidermal thickness may be due to functional adaptation but has also been shown to relate to defects in the desquamation process with consequent effects for the microfloral ecosystems at the skin surface (Milstone 2004, McEwan Jenkinson 1993). In the dermis transient or permanent changes in thickness primarily result from variation in the amount of fluid present in the tissue spaces (Eisenbiss et al 2001) and manifest as tissue swelling defined as oedema. Infection/inflammation of the skin promotes acute oedema which normally resolves post episode, chronic dermal oedema is long lasting and associated with impedances in healing, oxygen delivery and elimination processes (Cox 2006, Koutia 1999) and therefore has implications for recurring skin infections such as cellulitis. Epidermal and dermal thickness measurements therefore provide one parameter by which to assess changes to the stratum corneum and evaluate the presence and persistence of dermal oedema.

Circumferential limb measurements were used to compare swelling between legs, between visits and between participants. Measurements were recorded for the lower leg and taken just below the knee, at mid calf level and at the ankle. Precise circumferential measurements can be obtained by various modalities using light alignment techniques but for this study the disposable tape measure provided a quick and easy method with low risk of infection.

Several methodologies are available for visualising and measuring the layers of the skin. The biopsy is the most comprehensive as it has the potential to provide information on both measurement and histology (Whitton and Everall 1973) however excised tissue is subject to shrinkage artefact, which makes comparisons with in vivo measurements subject to bias. Other techniques include the various types of light microscopy (Abramovits and Stevenson 2003), computerised tomography (CT) (Vaughan 1990), magnetic resonance imaging (MRI) (Schmid et al 1998) and ultrasound (Rallan and Harland 2003). Because ultrasound imaging is a non invasive modality which is generally quicker, less expensive and more easily accessible than these other techniques and because measurements of skin thickness taken from ultrasound images have been shown to correlate well with in vivo confocal microscopy (Nouveau-Richard et al 2004) as well as the gold standard xeroradiography (Alexander and Miller 1979) it was selected as the method of choice for use in this study.

Ultrasound has been used as an imaging modality for many years and the advent of high frequency (20MHz+) scanners now allows the outermost layers of the skin to be studied in some detail (Serup 1992, Gniadecka 1996, Altmeyer et al 1991). Interface measurements can be performed and images analysed by the application of mathematical transforms. The advantage over conventional lower frequency ultrasound is the greater definition that can be obtained for surface structures by positioning the focal zone to coincide with the superficial layers of the skin; this is achieved by placing a membrane behind the ultrasound window and filling the space between with water.

A high frequency 20MHz ultrasound scanner interfaced to a PC (Dermascan C, Cortex Technology Denmark) was available for use in this study. The system contains an inherent measurement system based on A-scan (amplitude scan) presentation and allows distances between structures to be calculated by placing a cursor at each end of the relevant section of the image (figure 6b). However

measurements are only valid if the scanning plane intersects the skin surface at right angles (Collins 1996); the correct orientation has been achieved if the image shows the scanner membrane lying parallel to the skin surface (figure 6a). A stand was designed which would hold the scan head in the correct alignment and allow it to be raised and lowered to make contact with the skin surface (figure 7).

Thickness measurements

The first interface visualised on an ultrasound image of skin relates to the echo created between contact jelly and the outer surface of the stratum corneum (SC). The first bright echogenic band (figure 8) represents the entire epidermis extending from the skin surface to the papillae adjacent to the basement membrane (Nouveau-Richard et al 2004); it is not possible to distinguish between the layers of dead corneocytes and the living keratinocytes below. Beneath this the papillary dermis is identified as a lower echogenic band extending into the reticular dermis, which is thicker and more highly echogenic due to increases and orientation of the fibrous bundles which predominantly lie parallel to the skin surface and therefore provide maximum reflection (Querleux et al 1988).

An A-scan is a line graph representing the amplitude of the signals derived from a single line of grey scale values contained within an image. A-scans derived from different images can be compared providing that the image acquisition settings, most importantly the time gain compensation, remain unchanged. In order to obtain the grey scale values the signals must be digitised allowing each pixel to be allocated a value between 0 and 256. Values around 0 represent low levels of returning echoes, such areas are perceived as dark grey and black and termed hypoechoic, examples would be fluid filled spaces and water interfaces. High level echoes reach maximum at 256 when virtually all the incident beam is reflected backwards. These are perceived as bright (hyperechoic) areas, examples include the SC and fibrous bundles within the skin. Most tissues contain a mixture of components with high and low reflective qualities whose density and arrangement affect their interaction with the ultrasound beam and therefore produce a variety of imaging patterns.

The Dermascan C software digitises pixel values and presents a line selected from an image as an A-scan superimposed upon the image so that the amplitude values can be related to the visual components. Measurements can then be obtained by manipulating the cursors so that they intercept the entry and exit

Figure 6a - High frequency ultrasound image of normal skin

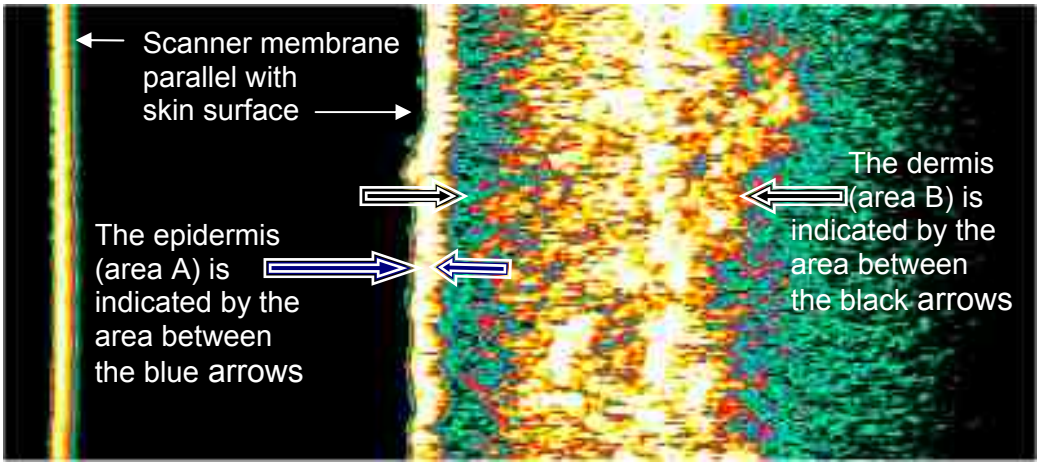
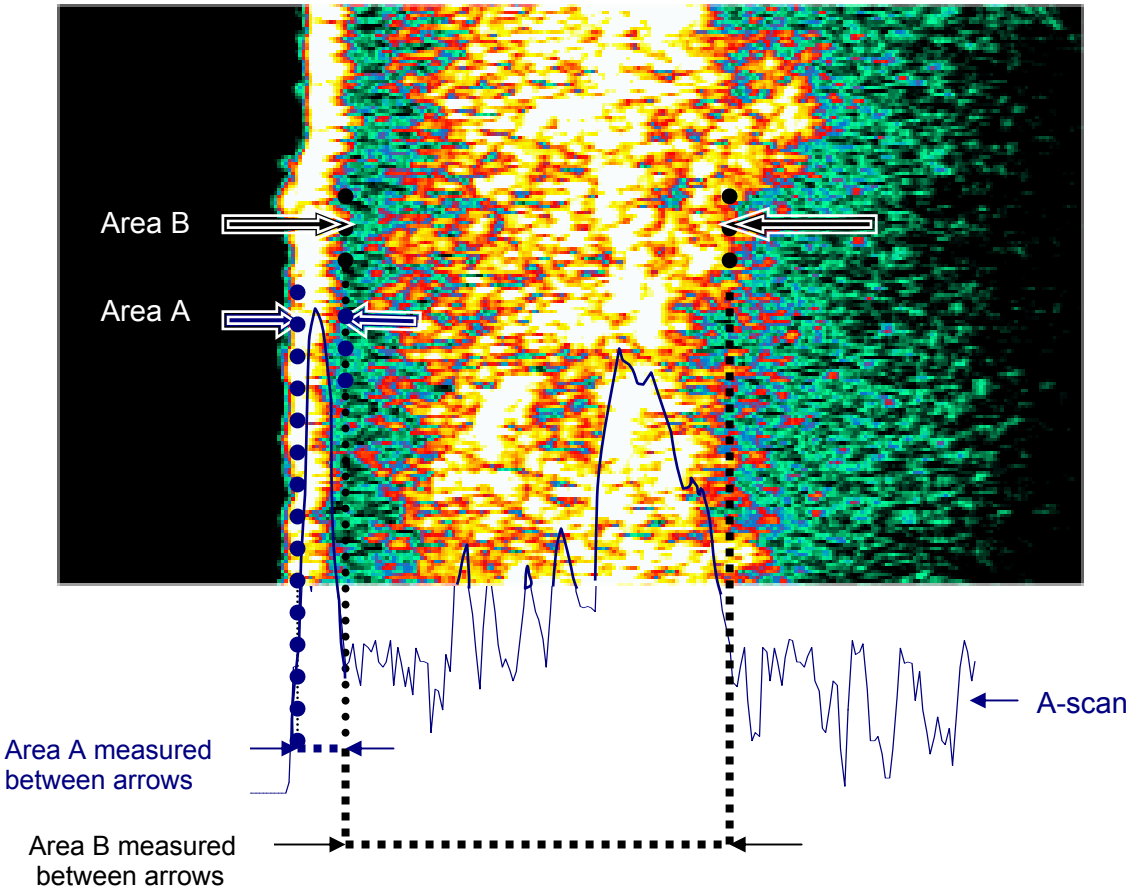
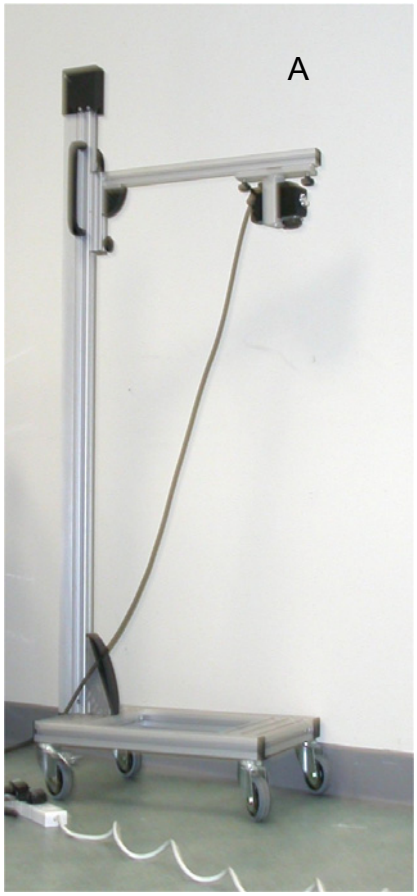


Figure 6b - Measurement of area A and area B from A-scan profile



The Dermascan C software digitises pixel values and presents a line selected from an image as an A-scan superimposed upon the image so that the amplitude values can be related to the visual components. Measurements can then be obtained by manipulating the cursors so that they intercept the entry and exit points of selected regions of interest. To measure the thickness of the epidermis (area A) the vertical measurement cursors are positioned at the interfaces (between arrows. Dermal measurements (area B) can be obtained by repositioning the cursors to include both the papillary and reticular dermis.



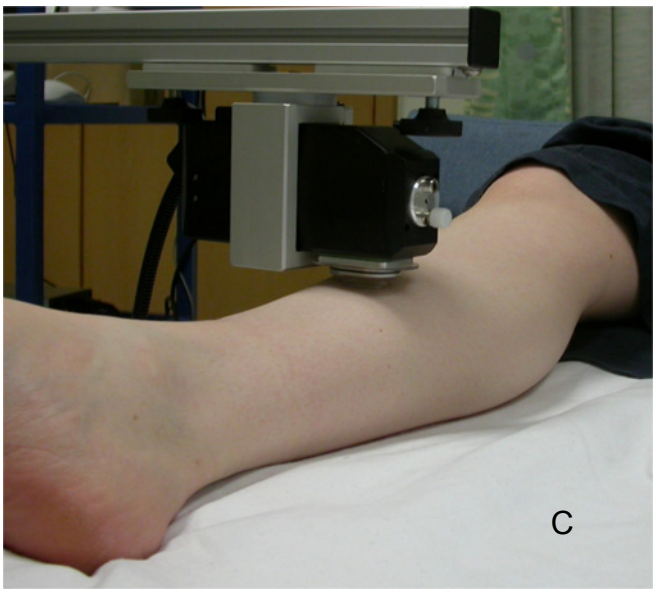


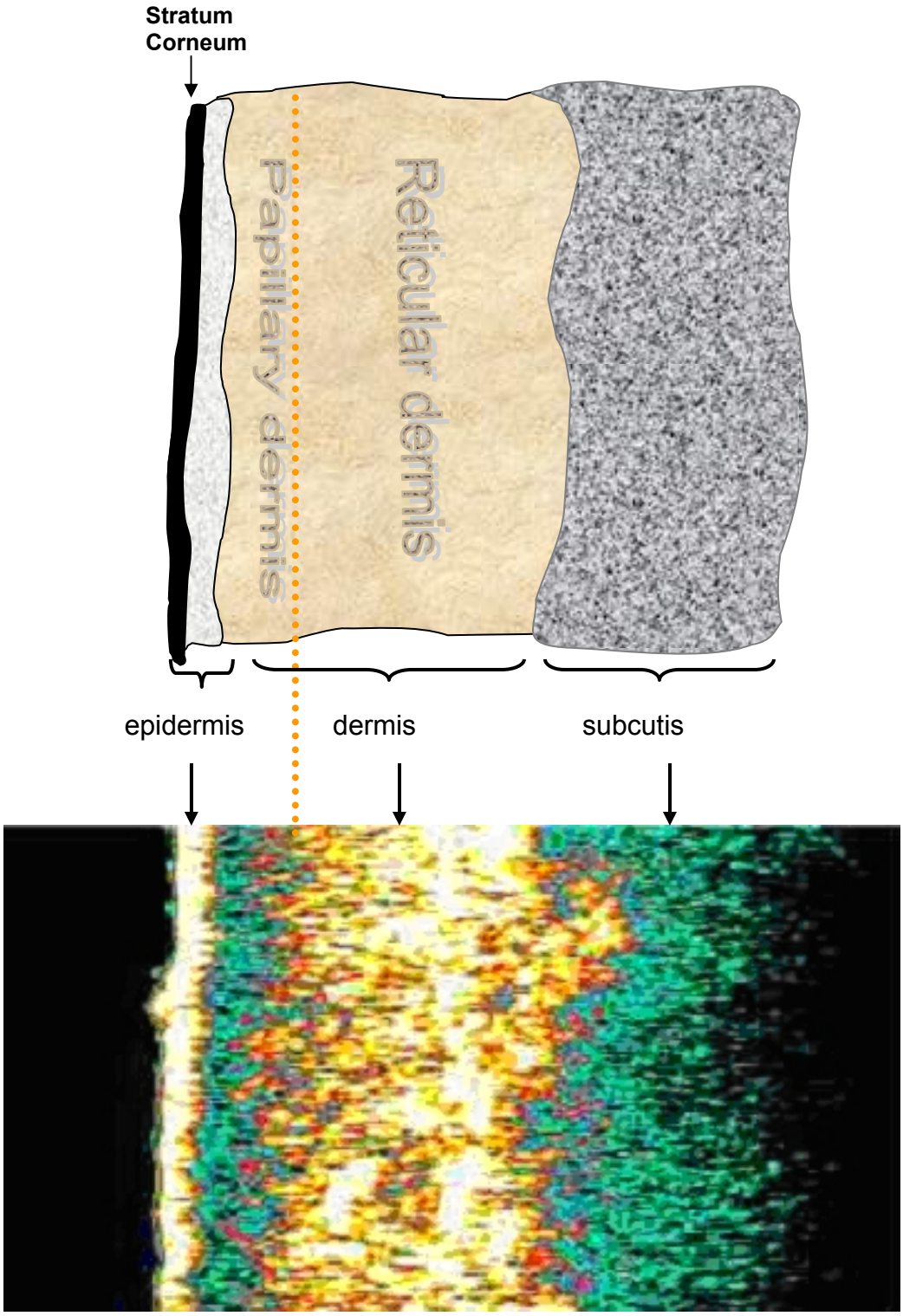
Figure 7

A - Stand designed to hold ultrasound scan head in a horizontal position

B - Close up view of scan head and support mechanism

C - Scan head positioned to capture a transverse image of skin from the right calf

Figure 8 – Ultrasound appearances of the epidermis, papillary and reticular dermis and subcutaneous layers



points of selected regions of interest. To measure the thickness of the epidermis the vertical measurement cursors are positioned at the interfaces (between arrows) as demonstrated in figure 6b. The result is presented in an on screen results table. Dermal measurements can be obtained by repositioning the cursors to include both the papillary and reticular dermis (figure 6b).

Validation of the Dermascan measurement system

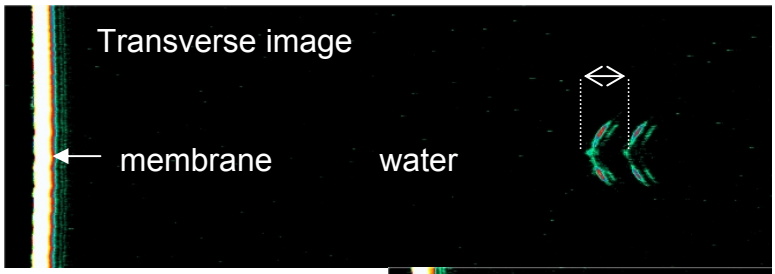
The measurement system was tested to confirm reliability over the expected range of epidermal and dermal thicknesses (0.5 – 4mms). Two items, a microdialysis tube and a drinking straw, were chosen to simulate well defined highly reflective structures such as the SC and fibrous bundles; the decreased reflectivity and less well defined borders of the dermal matrix was simulated by a length of soft twine. All objects were placed in a water bath and aligned with their long surfaces parallel to the transducer. The twine was suspended between upright supports and held firmly but without tension. The speed of ultrasound was adjusted to account for transmission in water (1480 m/s) and images were obtained in both longitudinal and transverse planes. System measurements were performed as described above and external diameter measurements recorded in both planes (figure 9). Comparisons were then made with the corresponding dimensions obtained by measuring the external diameters of all items using a micrometer. The results are compared in table 2. Results from the highly reflective microdialysis tube were identical. The micrometer measurements for the straw were slightly higher and this

Table 2 – Comparison between Dermascan measurements and results obtained using a micrometer

Item	Scan plane	Micrometer measurement	Dermascan measurement
Microdialysis tube	Transverse	0.6 mms	0.6 mms
	Longitudinal	0.6 mms	0.6 mms
Drinking straw	Transverse	3.65 mms	3.6 mms
	Longitudinal	3.65 mms	3.6 mms
Twine	Transverse	0.35 mms	0.4mms
	Longitudinal	0.35 mms	0.4 mms

may be because the membrane and the long axis of the straw were not quite parallel. Micrometer measurements of the twine were slightly lower than system dimensions; this was most likely due to difficulties in placing the cursors because

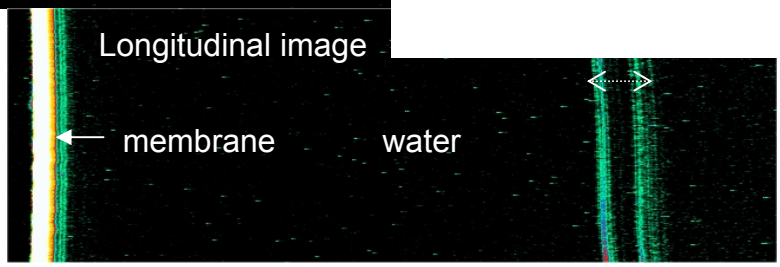
Figure 9 – Validation of Ultrasound Measurements

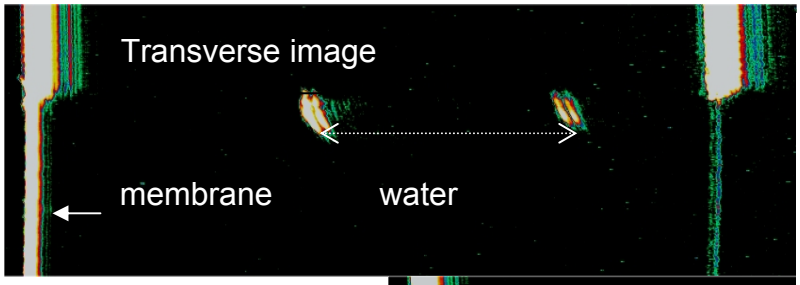


20MHz Ultrasound images of microdialysis tube

Micrometer measurement of external diameter = 0.6 mms

Ultrasound system measurements between arrows = 0.6 mms (both planes)

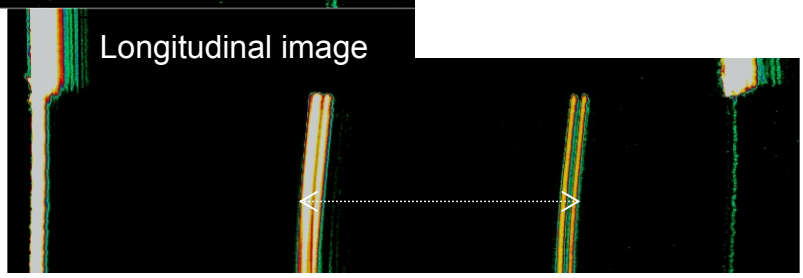


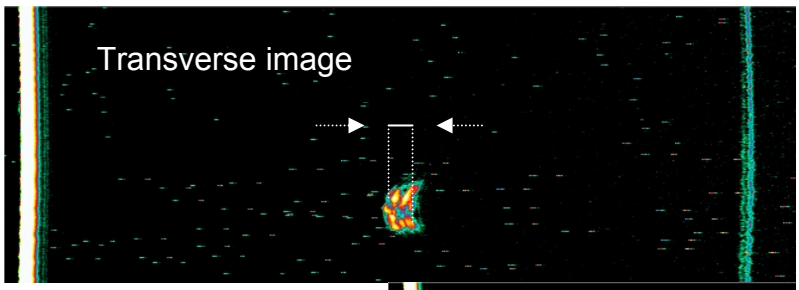


20MHz Ultrasound images of drinking straw

Micrometer measurement of external diameter = 3.65 mms

Ultrasound system measurements between arrows = 3.6 mms (both planes)

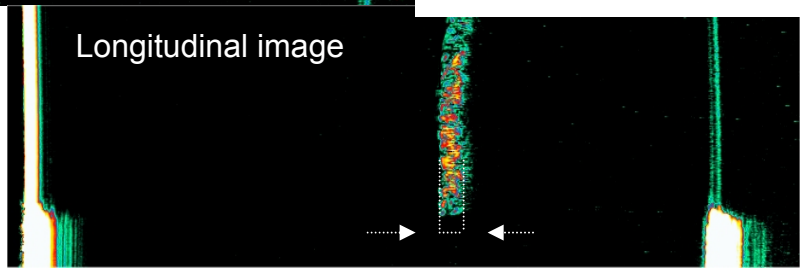




20MHz Ultrasound images of twine

Micrometer measurement of external diameter = 0.35 mms

Ultrasound system measurements between arrows = 0.4 mms (both planes)



the outline of the object was less sharp. Such errors are likely to occur when capturing and measuring skin images in vivo. In order to minimise these errors it was decided to record the average of three measurements obtained from different parts of the image.

Image analysis

Ultrasound image interpretation requires pattern recognition and depends on the experience of the operator to distinguish pathology from normal appearances. Image analysis is an extension of this concept but allows digitised data to be compared mathematically thus facilitating statistical analysis. Transformation programmes can also be used to identify subtle variations of patterns or texture not always obvious to the naked eye.

The normal high frequency ultrasonic (HRUS) appearance of the various layers of human skin has already been described and is shown in figure 8. The literature suggests that various types of oedema can also be characterised with confidence. Histamine induced oedema, characterised as a band of low echogenic pixels in the papillary dermis, has been demonstrated by HRUS and verified by Magnetic Resonance Imaging (MRI) (Gniadecka and Quistorf 1996). Similarly bands associated with lipodermatosclerosis occur at the same depth while those attributable to chronic heart failure manifest in the deep dermis (Gniadecka 1996). Oedema is important in this study, its location, extent and persistence with respect to cellulitis need to be assessed. A technique for comparing areas of low echogenic pixels presented in an A-scan format was selected for this purpose. Coloured images used to obtain thickness measurements were transformed into greyscale equivalents, saved as lossless TIF files and transferred to a PC. A digitising programme (S.W.Computing 1999) was used to obtain numerical data for each image. A line through each image was chosen according to the following criteria:

- The skin surface was parallel to the ultrasound membrane
- The line chosen was separate from any appendages or artefacts (eg: hair follicles or acoustic shadowing from surface hairs)
- The SC was clearly defined and represented by at least 3 highly reflective values (256)

The selected data lines were graphed (Microsoft Excel) to produce A-scans. By comparing A-scans, differences between visits and between subjects, could be shown. High value pixels representing the SC (256) were used as a marker to align A-scans so that the skin depths for comparison could be correctly superimposed.

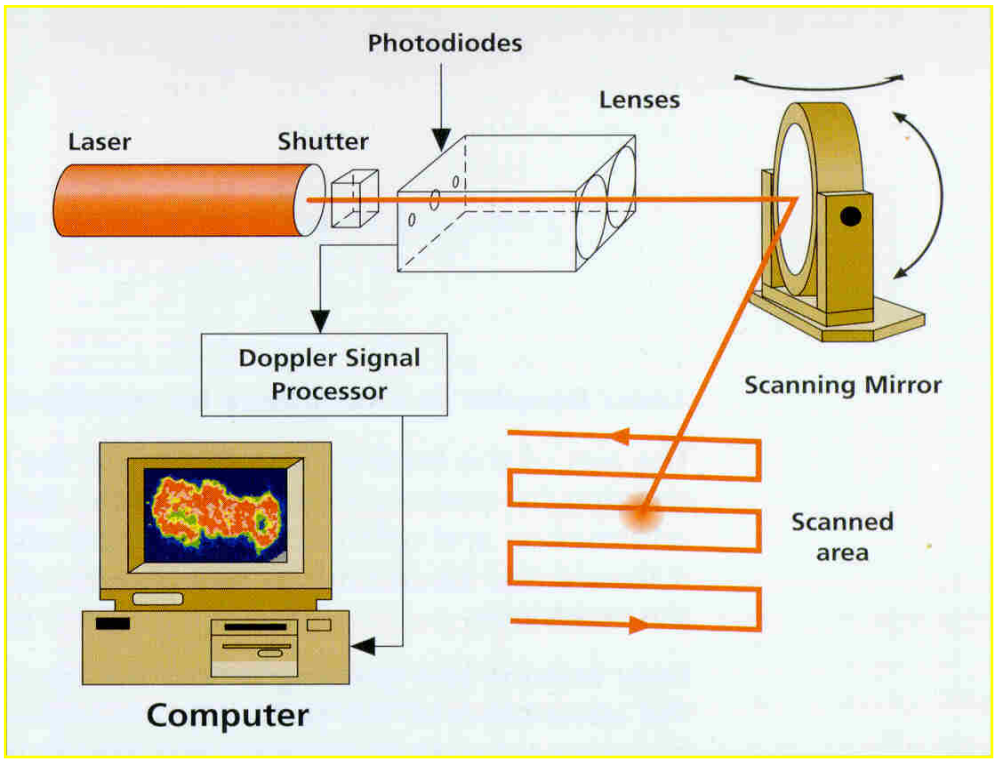
3.1.2.4 Skin blood flow

The skin microcirculation consists of a complex of arterioles, capillaries and venules, which collectively regulate tissue perfusion. One important function is to act as a vehicle to deliver large numbers of specialised blood cells to the tissues in response to episodes of infection and/or inflammation. Delivery must reflect the scale of the episode and endure for the length of the episode before shutting down at the appropriate time. If this mechanism is impaired, infective/inflammatory events may be prolonged or incompletely resolved and this could have implications for episodes of cellulitis. It was therefore decided to assess the functional effectiveness of the microcirculation as part of this study.

Because skin blood flow is so complex it is difficult to measure all the various parameters by one technique. For instance erythema measurements depend on reflection by chromophores within the sample, which only partially reflect the haemoglobin content (Piérard 1998, Fullerton et al 1996). Likewise evaluation of transcutaneous partial pressure of oxygen (T_CPO_2) is an indirect assessment of vascular delivery (Khan and Newton 2003, Barnes et al 1991). Other modalities, capillaroscopy, videomicroscopy and spectrophotometry (Simonen et al 1997, Chul Eun 1995) demonstrate vascular distribution but fail to quantify the rate or volume of blood flow. The only comprehensive method for evaluating the microcirculation is to measure the uptake and clearance of radioactive isotopes (X^{133} or Tc^{99}) that have been injected into the dermal vasculature (Sejrsen 1968). Progress is tracked with a gamma camera or scintillation counter and the clearance rate calculated from documented decay rates. Although thorough, this process is not without risk, and only used after some deliberation. An alternative is the use of Laser flowmetry which has been shown to closely approximate microcirculatory blood flow when tested against a fluid model (Nilsson 1980); this technique has further advanced to produce perfusion images without skin contact making it ideal for use in the presence of infection. For these reasons it has been selected as the technique of choice for use in this study.

Laser flow measurement and imaging techniques are able to demonstrate vascular perfusion in skin by measuring the frequency shift obtained when laser light is deflected from moving particulate matter beneath the skin surface (Fullerton et al 2002, Harrison et al 1993, Stern 1975). The phenomenon is known as the Doppler effect (White 1982). The laser Doppler imager (LDI) employs a system of mirrors which direct the laser beam to a depth of between 0.3 and 0.6 mms below the skin surface as the machine scans the region of interest in a raster pattern (figure 10). The signals received are converted to colour coded images representing vascular perfusion, which can then be analysed and compared.
In this study images were used to assess microculatory blood flow within the skin of cellulitic legs during and post episode and to effect comparisons with contralateral legs as well as the normal legs of control subjects.

Figure 10



Line Diagram of the Principle of the LDI Imager
(Courtesy of Moor Instruments UK and Newcastle General Hospital)

3.1.2.5 Venous incompetence

Abnormalities in venous function have been shown to be an important factor in the aetiology of diseases affecting the lower limbs (Wollina et al 2006, Khan and Newton 2003, del Mar Sãez et al 2001). Chronic venous insufficiency (CVI) is a key factor in a decline in skin health leading to conditions such as oedema and ulceration (Ryan 2002), but because progress to these conditions is often slow, changes in skin appearance such as lipodermatosclerosis frequently provide the first evidence of underlying disease. CVI has been cited as a risk factor for cellulitis (Roujeau et al 2004, Dupuy et al 1999); this finding was based on clinical appearance and the underlying causes were not investigated. The most common cause of CVI is damage to the venous valves, a condition known as venous incompetence whose sequelae include oedema and through this possible links with cellulitis that have already been explained. For these reasons, the testing of venous competence was included in this study and placed within this section.

Venous incompetence can be assessed by direct pressure measurements (vein cannulation), changes in leg volume (plethysmography) or methods involving the Doppler shifting of laser light or ultrasound. Vein cannulation and volume plethysmography were unsuitable for this study as the former is invasive and the latter requires meticulous measurements taken over several hours.

Laser Doppler flowmetry is a quick, easy, non invasive technique which has been validated against direct in vivo pressure measurements (Abromowitz 1979). Small probes are fixed to the skin surface and signals are obtained when laser light is shifted from moving blood cells according to the Doppler principle. The signals represent an arbitrary unit known as ‘flux’ relating to the velocity and cell volume within the sampled area (Braverman et al 1990), and can be used to assess changes in microcirculatory blood flow in response to exercise with reference to a baseline determined during the resting state. Following muscular exercise venous volume and pressure drop and it takes time for arterial inflow to refill the capillary bed. If venous valves are working well the time to return to baseline will be relatively long, if they are damaged backflow will quickly refill the capillary bed and shorted times will be recorded. Readings of >25 secs represent adequate venous function, readings below this figure are regarded as consistent with venous incompetence (Stücker et al 2005).

3.1.3 Immunological outcomes

Cellulitis and dermatophytosis are both conditions that should evoke immune responses. The immune pathway during cellulitis is initiated as an innate response but latterly extends to involving the adaptive immune mechanism and is therefore cell mediated. Dermatophytes should be expelled by a delayed type hypersensitivity (DTH) response, which is an allergic reaction.

This part of the study was designed to use haematological methods to plot the course of the immune response during and after the cellulitic event, observe its severity and duration, and identify unexpected variations in cell types and numbers. Evidence to support the presence of either a proinflammatory TH_1 or antibody mediated TH_2 cytokine environment was sought, together with indicators of exaggerated or unexpected responses. Presence of dermatophyte specific antibodies was included to corroborate evidence obtained by culture and microscopy. The spectrum of cytokines chosen to identify T_H1 or T_H2 responses could also assist in evaluating any immune effects likely to have been manipulated by fungal presence.

Biopsy specimens taken at the cellulitic site were considered because they could provide histological information, evaluate cell types and numbers and also investigate the milieu at a local level. However, this method was considered to be unacceptable because of its invasive nature, the risk of infection, and poor tolerability by sick patients.

The following alternative methods were therefore chosen:

- Continuous assessment of blood profiles. Types and numbers of specialised cells circulating in peripheral blood were assessed with reference to levels of CRP, which was used as an indicator for the severity and time scale of the cell mediated response.
- Indirect analysis of T cell status by reference to cytokines present in peripheral blood. This was included to assess detection levels of cytokines associated with T_H1 (inflammatory) and T_H2 (antibody mediated) phenotype and also to evaluate the practicality of this method to differentiate between IH, DTH and anergic reactions in patients/controls where dermatophytosis had been demonstrated.

- Measurements of levels of total serum IgE as an additional marker for antibody mediated (T_H2) phenotype and as an indicator of possible allergy.
- Measurements of levels of serum specific IgE to Trichophyton Rubrum (TR) as an adjunct to the fungal investigations. (TR is the most frequently occurring dermatophyte grown from human feet, specific IgE to TR was therefore chosen because this dermatophyte was the most likely to be cultured).

3.1.3.1 Blood profiles

Blood profiles taken serially through the course of an infective/inflammatory episode provide evidence of its severity and duration. CRP is an acute phase protein produced by the liver in response to increases of acute phase cytokines, predominantly IL-6. Levels of CRP are normally low (< 6mg/L) but rise steeply in infective/inflammatory episodes and may remain elevated for some considerable time. CRP values of > 500mg/L are typical in the acute phase, but the half life is short (approx 9 hours), so that levels plummet once the initiating stimulus has been removed (Gozzard 1986). Because the response curve of CRP in inflammation assumes the same shape as that of IL-6 a surrogate relationship has been proposed (Bataille 1992). Total white cell expansion during the episode follows a similar but less steep curve at the outset although contraction is slower and levels may still be falling when CRP has already returned to normal. Conversely, during the inflammatory response leucocytes traffic to the tissues and therefore numbers in the peripheral circulation fall. Plotting numbers of these various cell groups in conjunction with CRP therefore provides a means by which to evaluate the severity and duration of an episode as well as assess variations from the sequence just described.

3.1.3.2 Serum cytokine profiles

The number of the various types of T cell present in a sample can be measured accurately by a technique known as Flow Cytometry (Hill and Martin 2006). With fluorescent tagging, the various protein modulators (cytokines) produced by each cell type, can also be determined by an extension of this technique known as Fluorescence Activated Cell Sorting (FACS). FACS analysis can determine T_H status and identify the cytokines produced by these cells but does not provide an overall picture of the prevailing milieu in peripheral blood or suggest the stimulus

for the differential T_H1 and T_H2 phenotype. Cytokine production is complicated because many cells secrete the cytokines contributing to the milieu, which in turn influences the differentiation and proliferation of the participating cells. Cell cytokine secretion affects the cells themselves (autocrine effect), neighbouring cells (paracrine effect), all cells in a given tissue (endocrine effect) or adjacent cells by receptor activation (juxtacrine effect). One aim of this part of the study was to attempt to categorise the immune stimulus causing predisposition to the exaggerated inflammatory skin effects. Alternatives to the FACS technique were considered.

Western blotting involves protein separation by gel electrophoresis and subsequent transfer to a membrane where it can be detected by antibodies (Kurien and Scofield 2006). An alternative is the immunoassay test based on the formation of antibody-antigen complexes; the former being fixed or solid state and the latter contained within the supernatant undergoing analysis. When the antibody-antigen complex has been formed an enzyme is added which links and labels the antigen so that amounts can be quantified using a suitable detection technique such as radioisotope detection, spectrophotometry, fluorescence or luminescence (de Jegar and Rijkers 2006). Such tests are known as Enzyme - Linked ImmunoSorbent Assays or (ELISAs) and measure the amount of specific biomarker(s) in the unit volume of supernatant under test by reference to a dose response curve obtained from a standard analyte solution. The method described is known as a 'sandwich' immunoassay but many other variations exist; one such is the enzyme-linked immunospot (ELISPOT), which allows the measurement of cytokines associated with a specific cell population (Cox et al 2006).

With strict adherence to the protocol such tests are accurate and reproducible and able to accommodate a wide range of biological fluids including serum, urine and saliva. They are however purely quantitative, furthermore results may be attributed to a large amount of biomarker being released by a few cells or to a small contribution made by many. The most recent immunoassay technology has provided the ability to detect several different biomarkers from within a single sample allowing best use of small quantities of supernatant and drastically reducing costs. Meso Scale Discovery (MSD) has developed a sandwich immunoassay for the specific analysis of cytokines relating to T_H1 and T_H2 type cells. The solid state consists of 10 captured antibodies presented in a patterned

Table 3 – Some of the origins and effects of the cytokines/chemokines included in the TH_1/TH_2 MSD assay

Cytokine	Production - effect
IL-2	Produced by TH_1 cells amongst others, differentiates TH_1 cells (autocrine effect), promotes T cell expansion
IL-1β	Produced by macrophages in epithelium (amongst others) in response to bacteria and toxins. First phase response induces fever with > CRP , promotes proliferation of TH_2 cells
IL-4	Produced by T cells and mast cells, differentiates TH_2 cells, promotes TH_2 cells and IgE, inhibits TH_1 cells
IL-5	Produced by activated TH_2 cells , stimulates B cell growth and Ig secretion, promotes generation of eosinophils. Associated with parasites.
IL-10	Produced by TH_2 cells and macrophages (amongst others), inhibits cytokine synthesis by TH_1 cells, Inhibits macrophage function, is inhibited by IFN-γ
IL-12	Produced by B cells, monocytes and macrophages, Acts with IL-2 to produce IFN-γ, differentiates TH_1 cells.
IL-13	Produced by activated TH_2 cells, promotes isotype switching to IgE synthesis, prevents synthesis of IL-1α IL1- β IL-6 IL-8 and TNF-α in response to LPS.
TNF-α	Produced by macrophages, T cells and B cells and monocytes in response to bacteria and viruses, diverse roles including inflammation, activating neutrophils, fever and septic shock.
IFN-γ	Produced by TH_1 cells and NK cells, differentiates TH_1 cells, inhibits TH_2, activates macrophages inducing NO synthesis, TNF-α and IL-1.
Chemokine	
IL-8	Produced by monocytes, lymphocytes, endothelial cells and keratinocytes, chemotactic for neutrophils,< IL-4 induced IgE.

Differentiates TH_1 cells	Differentiates TH_2 cells	Promotes TH_2 cells or is produced by TH_2 cells

array at the bottom of each well; the detection antibody is labelled with a reagent that quantifies the results by electrochemiluminescence. The range of cytokines together with their possible origins and some of their effects are summarised in table 3. The technique was selected for preliminary testing in this study because of the range of cytokines offered. In addition it was cost effective, able to accommodate serum or plasma and also faster and easier than processing a conventional ELISA. The technology is currently in use by other groups within our hospital and the specialist hard and software is available on site. Results are reported to compare well with conventional ELISA techniques although some of the cytokines included in the T_H1 /T_H2 kit have not previously been evaluated by

our researchers and therefore the levels of detection in peripheral blood are unconfirmed. An important part of the preliminary testing was to generate this data.

3.1.4 Stress factors

The final aim of this study was to consider the role of stressors, other than dermatophytosis, that might predispose patients to attacks of cellulitis through changes in skin function and/or immune responses. Such stressors could arise from co-morbidities or other physiological, psychological or environmental factors. It was not possible to evaluate all of these categories but the following were considered to be the most relevant in this context:

- Detailed chronological history of previous trauma, surgery, co-morbidities (including psychological disease) and genetic factors.
- Evaluation of levels of perceived psychological stress relating to the time period directly preceding the cellulitic attack.

3.1.4.1 Patient history

Patient history was obtained from the hospital or primary care notes together with patient interview, subsequent to written consent. A chronological order of events was compiled to create a framework allowing systematic searching for interrelated links between cellulitic episodes and other medical events. A six month follow up review was conducted to document further episodes and other relevant medical occurrences.

3.1.4.2 Stress questionnaire

Stress results when demand on the individual exceeds the available resources (Boone and Christensen 1997). Differences in outcome are known to exist between stress in its acute and chronic forms: the former may be beneficial particularly to the immune system by enhancing priming, testing and effecting mechanisms while the latter may produce opposite and deleterious effects (Malarkey et al 2001, Bartrop et al 1977). In addition the genetic variation between proactive and reactive personality types may produce different coping strategies to the same disease process (Korte et al 2004).

The diagnosis of stress may be assessed by either physiological measures, biochemical measures or by questionnaire (Noble 2002). The role of some of the physiological stressors have been discussed in the literature review and some of

their effects are investigated by the skin and immunological tests already described.

The hypothalamic-pituatory-adrenal (HPA) axis governs the biochemical reaction in response to stress through the release of corticotrophin-releasing hormone (CRH). The effect of CRH on the adrenal cortex induces increased secretion of corticosteroid hormones that are distributed via the circulation and can be measured directly in blood or other body fluids such as saliva and urine (Shimomitsu and Odagiri 2001). Although appealing by the apparent simplicity such measurements are problematic since levels of these hormones are subject to circadian rhythms and variations in physiological status (Posener et al 1996, Yehuda et al 1995, Sandin and Chorot 1985). They require successive or continuous measurements to establish meaningful reference points by which to assess results (Noble 2002, Kelly and Hertzman 2001). Alternatively a specifically tailored stress questionnaire may be used, this has the added advantage of being quick and convenient.

Stress questionnaires may relate to subjective evaluation of life events (Clements and Turpin 2000, Holmes and Rahe 1967) or measurement confined to acute stress (Sandin 1985). For the purpose of this study evaluation by the number and severity of stressors alone was unsuitable because it assumed a universal response to the same stimulus. In order to investigate whether stress may have an effect on cellulitic attacks a questionnaire was required that would relate to the time period preceding the onset of the episode and subsequent hospital admission. The Perceived Stress Scale designed by Cohen at al (1983) is currently in use for evaluating stress in many contexts, assesses individual stress responses without reference to particular life events and has been shown to match well with clinical findings (Noble 2002). The questionnaire (appendix 1) consists of 14 questions relating to levels of perceived stress as experienced over the preceding 30 days and was chosen as it was felt to most closely represent the investigative parameters required from the study. Each question offers 5 options ranging from never (0) to very often (5). 7 of the questions are scored in a positive direction and 7 in the opposite or negative direction; positive and negative questions are randomly arranged. Scoring methods are defined in Cohen et al (1983 p 387).

Table 4 – Risk factors, associated tests and expected results

Risk factor/test/investigation	Expected results
Dermatophytosis *Tests : Specimen collection, culture and microscopy Serum immunoglobulin to Trichophyton Rubrum (TR)*	Expected frequency of dermatophytosis in the patient group was higher than for controls (Roujeau et al 2004) however reported frequency within the general population is variable (between 30 and 70%). ID spaces were expected to produce more cultures than the foot sole (Björnsdóttir et al 2005). Presence of IgE specific to TR would confirm current or previous exposure. (Woodfolk and Platts-Mills 1998, Fitzpatrick 1994).
Changes in skin water loss *Test: Measurement of Transepidermal water loss (TEWL*)	Higher values of TEWL were expected in the patient group during the cellulitic event. Enduring high or abnormally low values post event in this group might have implications for skin vulnerability (Nilsson 1977).
Changes in pH balance at the skin surface *Test: Measurement of skin pH*	Skin pH measurement might be expected to be higher (more alkaline) in the patient group both during and post episode. This would be consistent with altered skin physiology and frequency of bacterial/fungal infection (McBride 1993)
Oedema *Tests: Leg circumference measurements + Ultrasound tissue measurements*	Compared to contralateral and control legs cellulitic legs were expected to be greater in circumference and display greater dermal thickness during the episode as a consequence of oedema. This physiology might be expected to endure post episode particularly in those with recurrence (Cox 2006).
Changes in skin blood flow *Test: Measurement of skin blood flow Laser Doppler Imager (LDI)*	Hot erythematous skin would be expected to display increases in skin blood flow relative to normal (Ryan 2005) therefore higher values were expected in the patient group during the cellulitic event. Variations from the accepted normal values post event in this group would have implications for skin vulnerability.
Venous incompetence *Test: Photoplethysmography Laser Doppler flowmeter (LDF)*	Venous incompetence is a ubiquitous physiology, however because of associations with oedema high frequencies were expected in patients with enduring oedema particularly recurrent cases (Mortimer 2000, Johnson 1996).
Abnormal haematological response to inflammation/infection *Test: Blood profiles*	> CRP, > WBC, > neutrophil and < lymphocyte counts would be expected in patients during the cellulitic event. Deviation from this or failure to return to normal ranges post event could indicate immunological abnormalities.
Exaggerated immune response *Test: Serum cytokine profiles*	> levels of IL-12 would support a TH_1 cytokine profile and acute inflammation. > levels of IL-4 would support a TH_2 cytokine profile and DTH reaction. In the latter case > serum levels of IgE would also be expected.
Allergic response *Test: Serum (IgE)*	
Previous episode Co-morbidity Previous surgery etc *Investigation: Patient history*	No studies were identified that had systematically searched the patient history for predisposing factors although the literature suggests that previous episode may be a risk factor for future episodes (Cox 2006).
Psychological stress *Investigation: questionnaire*	High total scores from the stress questionnaire would indicate > levels of psychological stress (Cohen1983)

3.2 Expected results

In order to fully investigate the risk factors associated with cellulitis of the lower limb it was necessary to conduct a diverse range of tests (see table 1) and the rationale for those chosen has been discussed in this section. In each case a considered outcome, which would lend support to the theory, was anticipated. The expected results are set out in table 4.

CHAPTER 4
MATERIALS AND METHODS

4 MATERIALS AND METHODS

4.1 List of materials used in this study

Equipment: IN TRAY ^{TM} DM (Biomed Diagnostics, USA)
SABOURAUD DEXTROSE AGAR (Fisher Scientific, UK)
MSD Human TH1/TH2 Cytokine Assay (Meso Scale Discovery, USA)
Cotton Blue stain
Sellotape (Guilbert , UK)
Large emery boards, blister pack 12 (Tesco Ltd, UK)
Small emery boards, blister pack 10 (Murrays Key Creations, UK)
Disposable tape measures (NHS logistics, UK)
10mm green sticky dots (Guilbert, UK)

4.2 Plan of investigation

The study was designed to address the study aims stated in section. 2.4.1.
As part of this it was necessary to assess the logistics of recruiting from an acute clinic and conducting tests within a research facility situated in a distant part of the hospital. Ethics approval was obtained allowing patients to be recruited from the Ambulatory Care Unit (ACU) and for the study to be conducted within the Wellcome Clinical Research Facility at Southampton General Hospital (SGH). Patient consent included venesection, access to hospital notes and blood results, photographic imaging of the affected site and letter to the GP.
The ACU within SGH accepts GP referred patients with suspected cellulitis, assesses them and initiates their therapy. Its purpose is to provide patient management on an out patient basis whenever possible and it is therefore described as an OPAT (Out Patient Antibiotic Therapy) unit. Current treatment policy for cellulitis within the ACU at SGH is as follows:

- Simple cases (no previous history, no confounding co-morbidities and with pain management under control) - 3 days of intravenous (IV) antibiotic therapy (commencing on day of referral) followed by 7 days of oral therapy.

- Complicated cases (those with confounding co-mobidities such as cancer, those who have been on antibiotic treatment for some time, or whose condition demands additional nursing care) – 4 to 5 days of IV antibiotics (commencing on day of referral) followed by 14 days of oral therapy.

Objective measures include clinical evaluation, blood markers CRP (Isaacman and Burke 2002) and WBC (Sachs 1990) and the time to when the cellulitis fails to advance (Corwin et al 2005); thus monitored improvement informs the change from IV back to oral therapy.

4.2.1 Participants

Potential recruits were identified by the medical team and nursing staff within the ACU. Suitable candidates included those with clinically diagnosed cellulitis of the lower limb confined to one leg (including calf) which were unresolved in primary care.

- Cellulitis was defined as an area of erythematous skin, exuding heat, advancing in all or any direction and accompanied by swelling of the affected limb. Presence of fever and malaise and /or evidence of > white cell count and levels of CRP were also required. The differential diagnosis was determined by a member of the medical team and confirmation of this was sought by the chief investigator with reference to the clinical notes.

The following exclusion criteria were used:

- Age < 16 years, cellulitis in both legs, presence of comorbidities likely to have dominant and overriding systemic effects (eg Rheumatoid Arthritis, Cancer), systemic antifungals within the last 4 weeks or application of topical antifungal medication within the last 7 days, patient unable to comply or give written consent.

The definition of acute and recurrent cases was defined as follows:

- Acute cases were classified as those without recall or medical reference to a previous cellulitic event
- Recurrent cases were classified as those who had experienced a least one previous event displaying the same symptoms at a similar anatomical location.

Healthy controls matched for age, sex and mobility were recruited by poster from the general staff and student populations of the University of Southampton and

Southampton University Hospitals NHS Trust. The following exclusion criteria were used:

- Age < 16 years, previous proven episodes of cellulitis, presence of comorbidities likely to have dominant and overriding systemic effects (eg Rheumatoid Arthritis, Cancer), systemic antifungals within the last 4 weeks, or application of topical antifungal medication within the last 7 days, person unable to comply or give written consent.

4.2.2 Sample size

It was necessary to recruit sufficient numbers to provide an indication of differences in the proposed test results between groups of patients and control subjects as well as between acute and recurrent cases within the patient group. It was difficult to calculate a sample size with confidence because two of the proposed tests, namely TEWL values for calf skin and levels of serum cytokines, did not have well confirmed normal ranges. Advice from a statistician was sought and it was decided that equal numbers of acute and recurrent cases should be recruited along with matched controls. A total of 60 individuals composed of 30 patients n = 15 (acute) and n = 15 (recurrent) together with their respective matched controls was considered to be adequate to provide the preliminary data, as well as achievable within the available time frame.

4.2.3 Recruitment and attendance

Potential participants were approached by the chief investigator who explained the purpose of the study, the broad outline and purpose of the tests and the commitment required. Participants who expressed interest were given an information sheet and allowed a period of 24 hours for consideration before written consent was obtained.

Patients attended the Wellcome Clinical Research Facility on two occasions:

Visit 1 – during the course of intravenous therapy, as soon after referral as permitted by OPAT treatment protocol and obtaining consent.

Visit 2 – approximately 4 weeks after completion of the final course of oral antibiotics.

OPAT treatment continues until the treatment criteria (beginning of section 4.2.) are fulfilled and patients are then discharged into the care of the GP who may prescribe additional courses of oral antibiotic treatment in cases where the skin appearances are assessed as not yet ‘normal’. For the purpose of this study a

period of four weeks following completion of the last antibiotic treatment was allowed between visits. No data was found which would provide guidance with this time interval, which was assumed to be adequate for two reasons:

1 – The switch from IV to oral antibiotics is informed by near normal levels of inflammatory markers in peripheral blood indicating that systemic effects are reaching resolution. The time period between this switch and the second visit would therefore amount to at least six weeks.

2 – Antibiotic treatment continues until visual skin appearances are considered to be normal which then allows a further four weeks to clear antibiotics from the system and for cellulitic skin regain its baseline physiological characteristics.

The final appointment date was set after the relevant information had been obtained through discussion with the patient and the time interval for control participants was set to correspond with their respectively matched recruit.

4.2.4 Data

Data and samples relating to each patient and control were labelled with a unique code number known only to the chief investigator. All data was stored in compliance with the data protection act.

4.2.5 The protocol

On each occasion the study was conducted according to a protocol; a flow diagram for investigations is presented in figure 11.

The investigation for dermatophytosis and skin physiological tests were conducted with patients semi-recling on a bed with legs elevated and the back supported by pillows. The calves of both legs and the feet were exposed. The physiological tests required a period of acclimatisation and 20 mins was allowed; during this time those measurements that were not affected by the resting state were conducted (see figure 11). Skin physiological measurements were performed with the leg abducted, within an area of interest (AOI) 4 x 4 cms, bounded by green sticky dots, on the medial aspect of each calf situated half way between the medial malleolus and the angle of the knee (figure 12 and figure 13) or at a closely related site within the area of erythema. In the patient group tests were conducted on the cellulitic leg and on the unaffected (contralateral) leg; matching legs ,either left or right , were chosen in the control group and referenced as either control or contralateral control respectively (see figure 12). Measurements of venous competence were performed with legs dependant (a full description is included in

the relevant section). All tests were conducted on intact skin to minimise the risk of infection.

Digital photography and recording of anatomical landmarks were performed on each leg and at each visit in order to compare changes in appearance and to provide a permanent record of the anatomical location. A digital camera NIKON COOLPIX 4500 was used to record a digital image that included the foot, the area of interest and the leg up to the level of the knee line. Images were coded, transferred to a PC and stored as jpeg files.

Figure 11 - Study Investigations

Tests were conducted within the Wellcome Trust Clinical Research Facility on two occasions:
Visit 1 – during the course of intravenous therapy, as soon after referral as permitted by OPAT treatment protocol and obtaining consent.
Visit 2 – approximately 4 weeks after completion of the final course of oral antibiotics.
The time interval for control participants was set to correspond with their respectively matched recruit.

15 to 20 minutes was allowed for acclimatisation to the resting state, this was important for the physiological tests shown in the lower box. The following tests, which did not require acclimatisation, were conducted during this period:

- *Digital photography (photographic record)
- *Investigation for dermatophytosis
- *Venesection to obtain blood for the analysis of immunological outcomes
- Stress questionnaire

Tests marked* were conducted at both visits. The questionnaire was presented and completed once, at the first visit.

The following skin structure and physiology tests were conducted after the 2 20 minute acclimatisation period.

- *Measurement of skin water loss
- *Measurement of skin blood flow
- *Measurement of skin pH
- Measurement of venous incompetence
- *Leg circumference measurements
- *Ultrasound skin images and tissue measurements

Tests marked* were conducted at both visits. Measurement of venous incompetence was performed once, at the second visit.

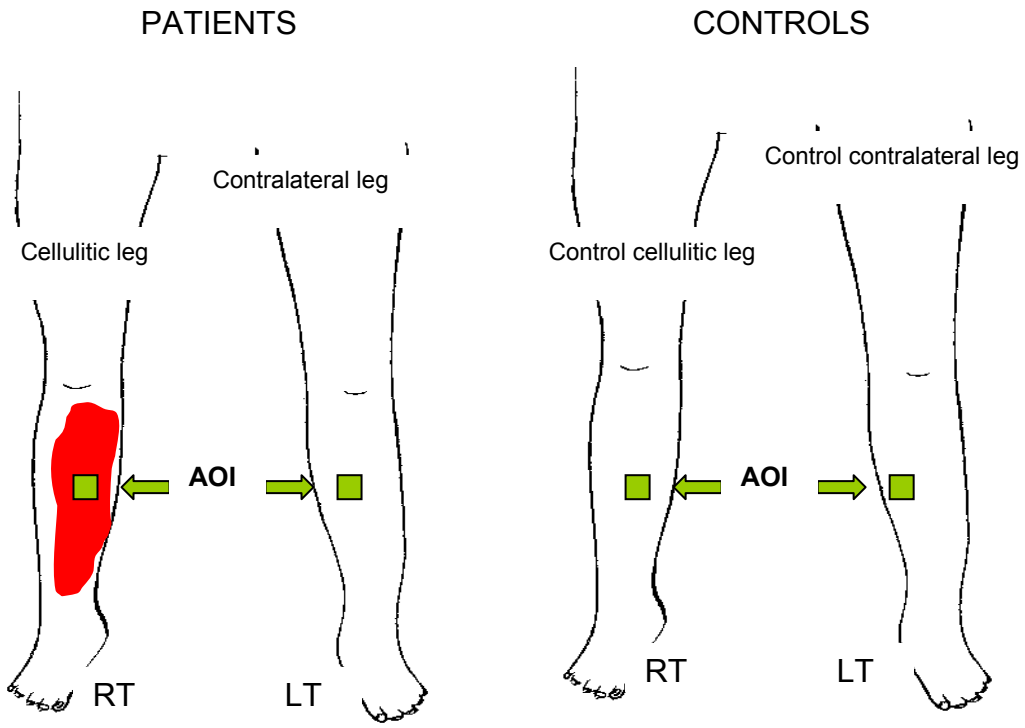


Figure 12 – Area of investigation (AOI) for cellulitic and control legs

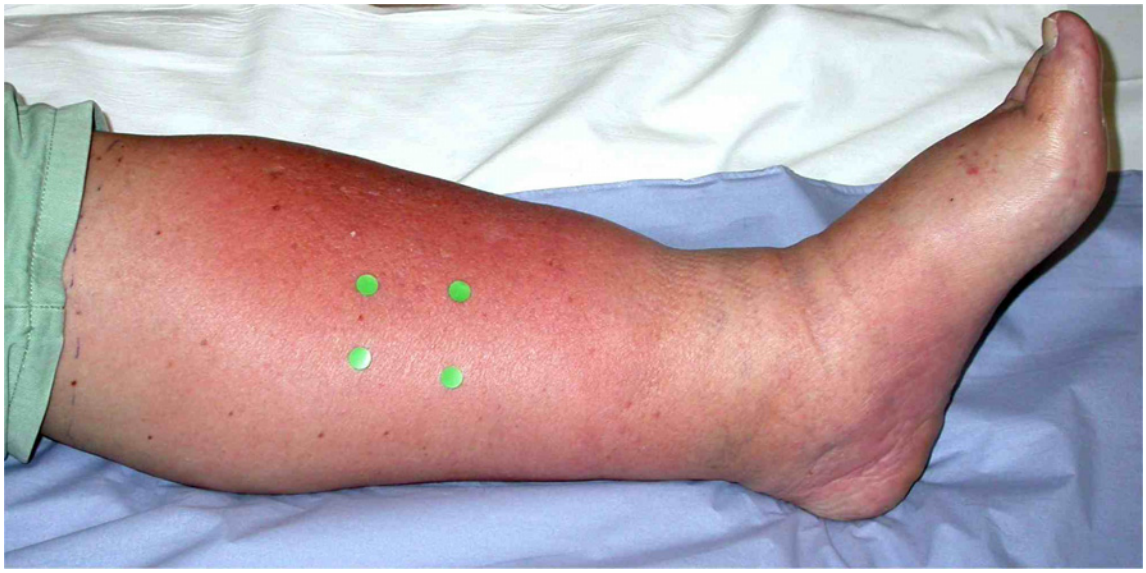


Figure 13 – Cellulitic leg to show AOI bounded by green dots

4.3 Methods

4.3.1 Investigation for Dermatophytosis

4.3.1.1 Initial culture

Skin samples were taken from each of the soles and the combined interdigital spaces of both right and left feet; the individual samples were cultured in four separate trays. The investigator wore disposable gloves, which were changed between obtaining samples from right and left feet.

Soles - A sheet of coloured paper was positioned under each foot. The foot sole was abraded with a large emery board producing a fine dust, which was collected on the paper. The transparent window of the dermatophyte culture medium (DM) was peeled back and the protective covering removed, the collected sample was distributed evenly over the agar surface and the window resealed around the edges of the plate (figure 14).

Interdigital spaces – A small emery board was used to abrade the skin between each of the toes in turn. The flat surface of the emery board was then pressed directly onto the surface of the agar plate.

The plates were labelled with patient code, date and anatomical site and incubated in racks on trays at 25°C (figure 15). Humidity was maintained by adding a small amount of tap water to the tray. Incubation time was variable (between 1-6 weeks). Cultures were observed through the transparent window (figure 16) and serial images were recorded with a digital camera (NIKON COOLPIX 4500) to plot progress. Cultures were also observed microscopically at a magnification of X 10. The depth of the tray limited the scope for obtaining higher magnification because the distance between tray and objective was too small; pictures on dissecting and inverted microscopes were poor because the incident light could only poorly penetrate the dense agar. In some cases it was necessary to produce slides to achieve the higher magnification required for positive identification.

4.3.1.2 Microscopy

Slides for microscopic examination were prepared as follows:

1 - Between 1 and 2 drops of cotton blue dye were placed on a microscope slide

2 - The transparent culture tray window was peeled back.

3 - A small piece of sellotape was cut and attached at one corner to the head of a pin.

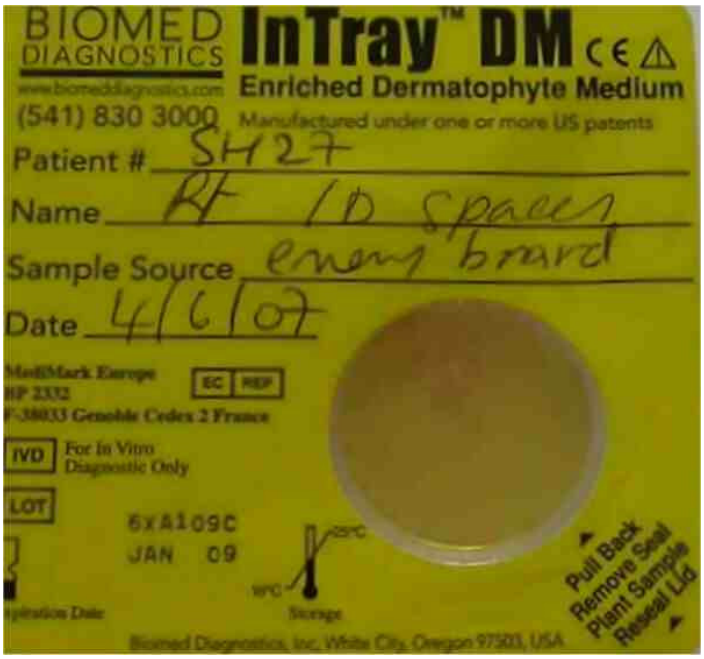


Figure 14 – DM Tray with sample

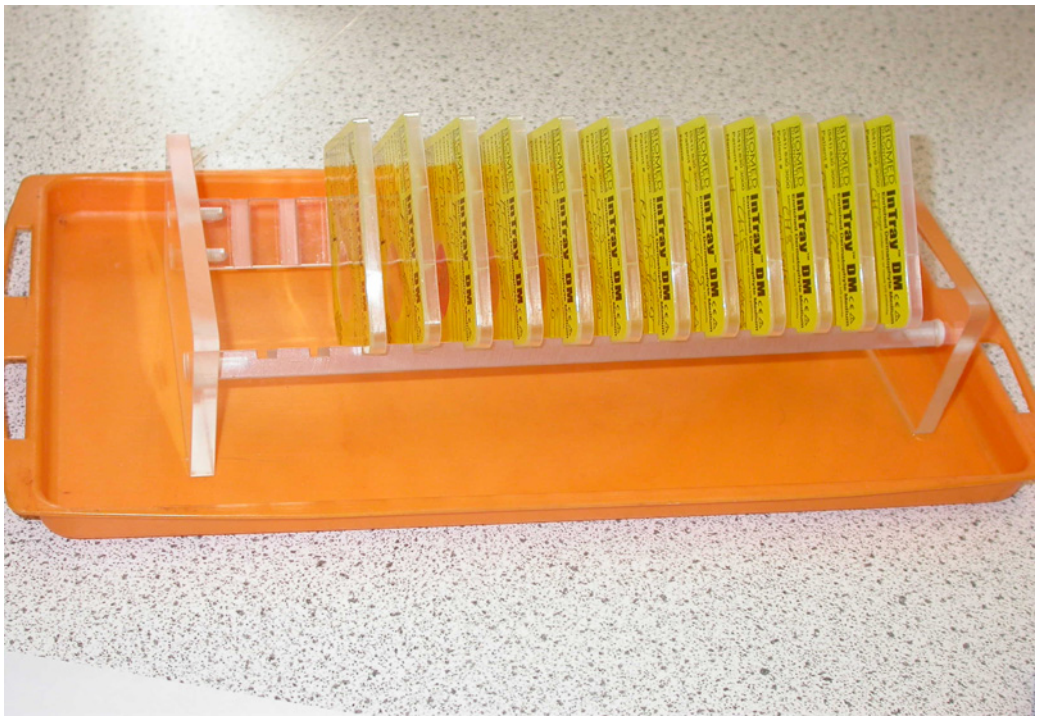


Figure 15 – Arrangement of DM Trays in the rack

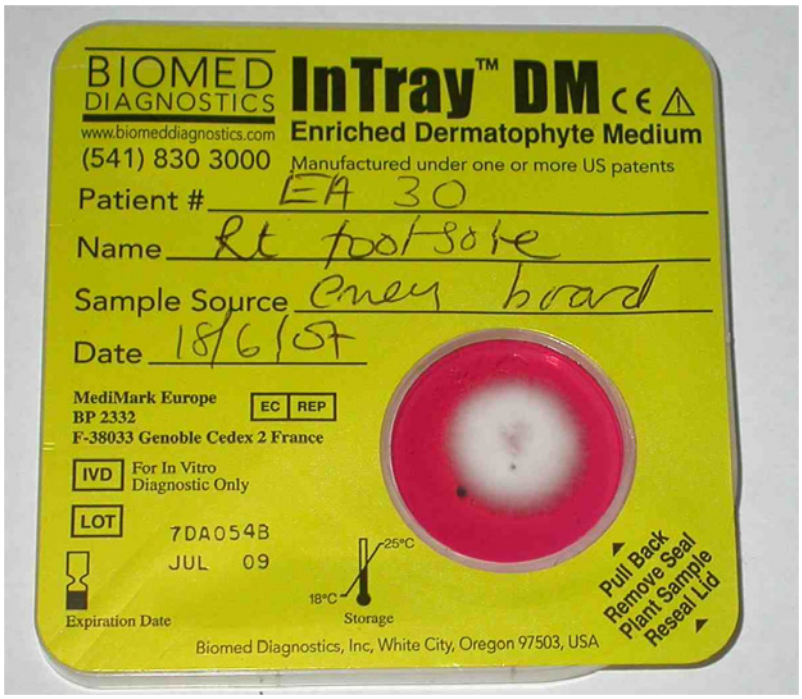


Figure 16
Trichophyton Rubrum (TR) growing on InTray DM

Figure 17
Sabouraud Dextrose Agar growing TR (white colonies) and penicillium (black colonies)
Note - penicillium appears to be inhibiting the growth of TR, this is particularly evident in one colony where the demarcation line between the two organisms can be clearly seen (arrows).

4 – The pin was lowered to allow the sticky side of the sellotape to be brought into contact with the surface of the colony.
5 – Gentle finger pressure was applied through the sellotape to obtain the sample.
6 - The sample was placed in contact with the cotton blue dye and the pin slid away allowing dye to be dispersed throughout the sample beneath the sellotape window.
7 – Another drop of cotton blue was placed over the sample area on top of the sellotape window.
8 – A cover slip was gently placed over the area of interest and pressed down to exclude the air and preserve the contents.
Slides were observed at magnifications of X 10, X 20, X 40 and X 80. Images were recorded via the microscope by interfacing with the digital camera.

4.3.1.3 Reculture

Positive identification of fungi requires visualisation of both hyphae and spore receptacles (conidia) and it is important to observe the pattern of attachment between the conidia and the hyphal elements. Production of conidia is dependant upon the culture conditions and it was often necessary to change the growing medium to encourage development of the identifying factors. Sabouraud dextrose agar (figure 17) is available ready plated and is universally used in mycology laboratories. Culture plates have clear transparent lids and growing conditions are similar to those required for DM. Samples were transferred between mediums using a clean pin head. SAB cultures were then grown on at 25°C to produce new colonies. Progress was recorded by digital photography and slides produced by the previous method where appropriate.

4.3.2 Investigation of skin structure and physiology

4.3.2.1 Skin water loss

A TEWAMETER 300 (Courage + Khazaka Electronic, Germany was used to measure the skin evaporative water loss (Pinnagoda et al 1990) within the area of interest on both legs. Calibration procedures were performed and the results recorded at regular intervals. For each session the equipment was switched on and allowed to stabilise for at least 1 hour prior to measurements taking place. Between readings the part of the cylinder used for skin contact was cleaned with an alcohol wipe and sufficient time was allowed for stabilization before the next reading took place.

The probe was applied to the skin of each leg, in the centre of the 4 x 4 square, and held in place while continuous measurements of evaporative water loss were recorded until the reading became stable (approx 60 secs). Simultaneous temperature and humidity values were also acquired and the results were stored as graphs onto an integrated portable PC. The final TEWL result was expressed as a single reading stated as g/hr/cm^2 recorded from the stable end of the curve.

4.3.2.2 Skin pH

A SKIN pH METER 905 (Courage + Khazaka electronic, was used to measure the pH of skin (Parra and Paye 2003, Roberts et al 2002). Calibration and stabilisation were performed as described in the previous section. The tip of the pH probe was rinsed in sterile distilled water and the probe shaken so that the surface was just damp. The tip was then applied to the skin of the contralateral leg, in the centre of the area of interest, and held in place until the reading became stable (approx 60 secs). The probe was disinfected and rinsed in sterile distilled water and shaken before being applied to the affected leg. Continuous pH values were recorded and stored as graphs onto an integrated portable PC. The final result for each leg was expressed as a single pH value recorded from the stable end of the curve.

4.3.2.3 Oedema

Limb volume

Circumferential leg measurements were taken in order to compare swelling between legs, between visits and between participants. A disposable tape measure was used and measurements were recorded at three levels: just below the knee, at mid calf level and at the ankle. In patients, the asymptomatic leg was measured first, in order to minimise the risk of infection spreading between legs.

Skin structure and thickness

A DERMASCAN C (Cortex Technology Denmark) 20 MHz ultrasound scanner was used to assess skin structure and measure the thickness of the epidermal and dermal layers (Serup 1992). The membrane was applied to the ultrasound window and secured with the water retaining sealing ring. Deionised water was injected into the transducer cavity to provide the stand off required to achieve focussed images within the skin. The transducer was positioned and secured on the scanning arm of the purpose-designed stand (section 3.1.2.3). The equipment was disinfected between scans by wiping the ultrasound window with an alcohol wipe and between sessions by removing and disposing of the water tight membrane

and disinfecting all other contact surfaces. The profile and level of the time gain compensation curve was preset and remained unchanged throughout the study. Each leg was abducted to present a parallel surface to the ultrasound beam. Ultrasound jelly was applied to the skin within the area of interest and the scan head lowered until even contact was made with the ultrasound window. Care was taken to apply firm but not excessive pressure (figure 7c). Images were obtained by successive frames and were considered to be satisfactory if the water membrane and skin surface were straight and parallel indicating that the scan/skin plane intersect approximated 90°. Images were captured in Dermascan format (dsc) and were labelled and recorded via a PC interface. Measurements were made using the inherent software measuring facility, which had previously been validated (3.1.2.3). Images were also saved as lossless greyscale TIF files for further image analysis. (Details of skin measurements and A-scan image analytical technique have been described in section 3.1.2.3)

4.3.2.4 Skin blood flow

A LASER DOPPLER IMAGER LDI-2 (Moor Instruments Ltd, UK.) was used to assess blood flow in the microcirculation of the skin (Fullerton et al 2002, Harrison et al 1993, Stern 1975). Calibration procedures were performed and the results recorded at regular intervals.

The scan head was positioned above the skin at a distance of 30 cms and centrally aligned to the area of interest. Images were obtained from each leg as the beam scanned the tissue surface in a raster pattern (figure 10). Colour coded images were obtained which represented vascular perfusion patterns within the region highlighted by the marker dots picked out by the scanner. The images were labelled and transferred to an integrated portable PC prior to analysis.

The dedicated LDI software was used to perform the following analysis on the LDI images:

An area of interest (AOI) was selected within the boundary of the green dots.

AOI: Area = 2.41 cm^2 (total pixels enclosed within AOI = 2809).

The mean flux value for the pixels within each AOI was recorded and the results compared by statistical analysis.

4.3.2.5 Venous incompetence

A LASER DOPPLER FLOWMETER DRT4 (Moor Instruments Ltd, UK) was used to assess venous refilling time in the lower extremity (Stücker et al 2005,

Abromawitz et al 1979). Calibration procedures were performed and the results recorded at regular intervals. The technique is non invasive but requires the sensors to come into contact with the skin so between tests the adhesive contact covering the sensors was removed and the sensors cleaned with an alcohol wipe. Subjects were asked to sit upright on the edge of the bed with legs dependant and the laser Doppler probes were positioned on the medial aspect of each calf half way between the medial malleolus and the angle of the knee and secured with designated double sided sticky O rings (figure 18).

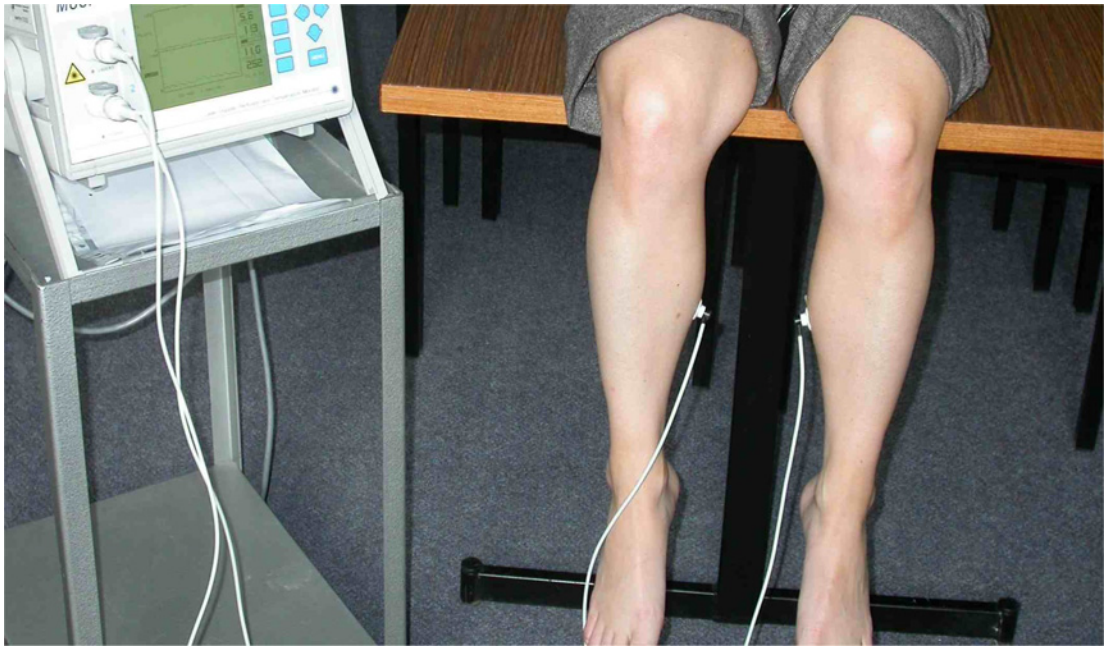


Figure 18
Measurement of venous refilling time (VRT)
Legs were dependant and the electrodes were positioned on the medial aspect of each calf

Both laser Doppler channels were switched on to allow simultaneous monitoring of right and left legs. Patients were asked to remain still for a period of 2-3 minutes to establish a base line measurement and then asked to perform 10 vigorous sequential dorsiflexions and plantarflexions of the foot, followed by immediate and complete relaxation of the foot. Continuous traces of ‘flux’ were recorded and transposed as txt files, which could be opened and graphed in an EXCEL spreadsheet. The point at which exercise ceased was recorded on the graph and

the time taken to return to the base line reading calculated. Times of < 25 secs were considered consistent with venous incompetence (Stūcker et al 2005). Skin quality, the presence of oedema and the discomfort experienced by the patients made this test unsuitable to use during the initial cellulitic episode. For these reasons and also because venous incompetence is a chronic condition and unlikely to fluctuate, a single evaluation conducted at the second session, was performed.

4.3.3 Immunological Outcomes

Routine blood investigations are performed on patients attending the ACU on alternate days and surveillance of the blood markers is used to monitor the course of cellulitic episodes. CRP, WBC, neutrophil, basophil and monocyte counts are always requested. In the acute stage levels of CRP are frequently in excess of 500^{+}mg/L and white blood cell counts, particularly those of neutrophils, are expected to be high. Decreasing CRP, WBC and neutrophil levels are signs that the acute phase is resolving and this information is an important part of the clinical evaluation and determines further management.

Results of routine blood analyses for patients were accessible with consent and retrieved via the hospital computerised E-Quest system. At the second visit samples were collected from patients within the Wellcome Clinical Research Facility so that all routine tests could be repeated and control participants donated blood at both visits to provide the full range of comparisons. A dedicated nurse performed the venesection at each occasion and samples were coded for identity. Initial analyses, follow up tests and tests from control subjects were conducted within the Haematology Department at SGH according to their standard protocol. Follow up reports and those from controls were sent to the chief investigator.

4.3.3.1 Blood profiles

Research samples were taken from patients in conjunction with the routine collection on the first available occasion after obtaining written consent and again at the follow up visit. The extra blood required for these tests was also taken from controls on both occasions. Samples were collected in red topped blood bottles (no additives) for serum and purple topped bottles (EDTA added) for plasma. Samples were spun, pipetted into 500 µL aliquots, labelled and stored at -20°C.

Analysis of IgE

A serum sample from each patient and each volunteer was used to determine IgE levels at each visit. As an adjunct to the fungal investigations levels of IgE to Trichophyton Rubrum (TR) from each visit were also assessed. TR was chosen as it is regarded to be the most frequently occurring dermatophyte to be found on human feet and therefore the most likely to present circulating antibodies.

All IgE tests were conducted within the Immunology Department at SGH where results were determined according to hospital protocol by enzyme-linked immunosorbant assay (ELISA). 500μL of serum was required for each separate test and frozen aliquots were stored until sufficient numbers were obtained to carry out a complete batch. All reports were sent to the chief investigator.

TH_1/TH_2 cytokine analysis

A 96 well multi array MSD Human TH1/TH2 Cytokine Assay was used (Meso Scale Discovery, USA). 40 wells were available for use in this study permitting analysis of plasma for five patients and five controls, from both initial and follow up visits, to be performed in duplicate. This number was considered to be sufficient to provide data on cytokine detection levels in peripheral blood and also evaluate the suitability of the method as a means of assessing the milieu and comparing levels of proinflammatory cytokines against leukocyte numbers as described in 4.3.3.

The solid state consists of 10 captured antibodies relating to the following cytokines (IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-8, IL-10, IL-12, IL-13, TNF-α) these are presented in a patterned array and correspond to the numbers as shown in figure 19. Samples were allocated to the plate as shown in figure 20. The numbers and pattern arrangement are fixed to create links with the software, plate packaging and data files permitting traceability of the data throughout the system. Details of reagent preparation and the protocol are set out in table 5.

Levels of each cytokine were determined by referencing the seven point standard curve, (derived from the average of duplicate samples), and ranging from 0-2500 pg/ml. The software calculates results and presents them as a series of detailed tables. The following raw data can also be accessed

- individual readings for each cytokine in each well
- accuracy of pipetting expressed as a coefficient of variation
- full calibration data
- levels of cytokine below the limit of detection for the assay

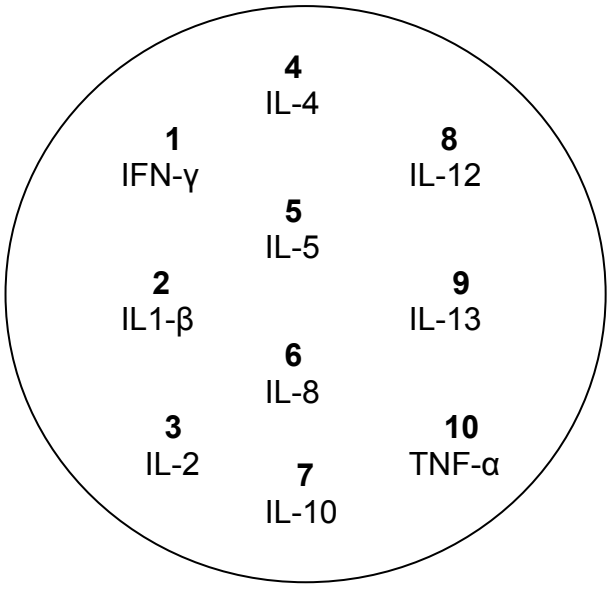


Figure 19

Spot numbers and position of related cytokines at the base of each well

	1	2	3	4	5	6	7	8	9	10	11	12
A	2500pg/mL		S1	S1	S9	S9	S17	S17	S25	S25	S33	S33
B	625pg/mL		S2	S2	S10	S10	S18	S18	S26	S26	S34	S34
C	156pg/mL		S3	S3	S11	S11	S19	S19	S27	S27	S35	S35
D	39pg/mL		S4	S4	S12	S12	S20	S20	S28	S28	S36	S36
E	9.8pg/ml		S5	S5	S13	S13	S21	S21	S29	S29	S37	S37
F	2.4pg/mL		S6	S6	S14	S14	S22	S22	S30	S30	S38	S38
G	0.6pg/mL		S7	S7	S15	S15	S23	S23	S31	S31	S39	S39
H	0pg/mL		S8	S8	S16	S16	S24	S24	S32	S32	S40	S40

Figure 20

Example of set up for 96 well MSD plate
Columns 1 – 2 contain the standard curve dilutions in duplicate
Columns 3 –12 contain duplicate samples in adjacent columns

Table 5 – MSD reagent preparation and protocol

Step	Method	
1	Calibration curve	A standard curve was generated between the ranges of 0.6pg/mL and 2500pg/mL
		1µg/mL of stock solution was added to 990uL of Human Serum Cytokine (HSC) Assay Diluent. This high calibrator was used to prepare the standard curve following a 1:4 dilution series as follows: 2500pg/mL 50µL of combined high calibrator was added to 150µL of HSC assay diluent 625 pg/mL 50µL of 2500pg/mL calibrator was added to 150µL of HSC assay diluent 156 pg/mL 50µL of 625pg/mL calibrator was added to 150µL of HSC assay diluent ↓ 0 pg/mL 150µL of HSC assay diluent
2	Detection Antibody Solution	60µL of the Stock Detection Antibody Mix was diluted into 2.94mL of HSC Antibody Diluent
3	Read buffer	12.5mL of Read Buffer was added to 25mL of deionised water
4	Addition of Human Serum Cytokine Assay Diluent	25µL of Human Serum Cytokine Assay Diluent was dispensed by pipette into the bottom of each well. The plate was sealed and incubated while being shaken (300-1000rpm) for 30 mins at room temperature
5	Addition of Sample or Calibrator	25µL of each calibrator or sample solution was pipetted into a separate well of the MSD plate (duplicate samples were pipetted into adjacent wells figure 18). The plate was sealed and incubated while being shaken (300-1000rpm) for 2 hours at room temperature
6	Wash	The plate was washed 3 times with a solution of PBS containing 0.05% Tween-20.
7	Addition of Detection Antibody Solution	25µL of the Detection Antibody Solution (step 2) was pipetted into each well of the MSD plate. The plate was sealed and incubated while being shaken (300-1000rpm) for 2 hours at room temperature
8	Wash	The plate was washed 3 times with a solution of PBS containing 0.05% Tween-20.
9	Read	150µL of of the Read Buffer (step 3) was pipetted into each well of the MSD plate.The plate was then analysed on the SECTOR PR ™ Reader. Quantification of cytokines present in each sample is derived from the levels of light measured from the tracer during electrochemicoluminescence with reference to the 7 point standard curve.

4.3 4 Stress factors

4.3.4.1 Medical History

This data was obtained verbally from the patient and from detailed searches of the medical history after obtaining consent. Information regarding causes of present episode, details of previous episodes, co-morbidities and medication were required for inclusion/exclusion criteria and for grouping. Clinical assessment with regard to fungal foot infection was documented and assessed in conjunction with observations from a foot inspection carried out as part of the study. Previous history was noted and evidence to support the presence of the risk factors identified in the literature or as previously discussed (chapter 2) was sought together with other medical events with possible relationship to the cellulitic attacks. For each patient and control the evidence was compiled in chronological order and tabulated to allow systematic searching. The categories chosen are listed in table 6. For each category scores of 0 were recorded if no supporting evidence was found and scores of 1 were recorded for positive evidence. Hospital records were recalled after a period of six months for update and review.

4.3.4.2 Stress questionnaire

Participants were asked to complete a stress questionnaire (Cohen et al 1983) appendix 1. The choice of questionnaire has been reviewed in section 3.1.4.2. Participants were asked to respond to each question by ticking one of 5 options ranging from never (0) to very often (5). The questionnaire was presented only on the first occasion since it was intended to relate to events directly preceding the cellulitic episode.

Table 6 - Medical history

Previous history of skin or mucosal infections assumed to be caused by bacteria
Recurrent skin rashes (not cellulitis)
Previous history of Athletes foot
Previous history of any fungal infection
Presence of fissures on feet
Powdery skin on the foot sole
Dry skin on legs
Previous abdominal surgery/ injury, trauma, surgery to cellulitic leg
Previous injury /trauma/surgery to cellulitic or contralateral leg
Previous abdominal surgery/ injury, trauma, surgery to cellulitic or contralateral leg
Persistent oedema (post study) in cellulitic leg
History of thrombophlebitis
Prescription antibiotics ++ (recall/record of numerous courses of antibiotic treatment in association with recurrent infections)
Asthma
Allergies including asthma
Overweight
History of cancer
Diabetes

Previous history was noted and evidence to support the presence of the risk factors identified in the literature was sought together with other medical events with possible relationship to the cellulitic attacks. For each patient and control the evidence was compiled in chronological order and tabulated to allow systematic searching. The categories chosen are shown in the table above. For each category scores of 0 were recorded if no supporting evidence was found and scores of 1 were recorded for positive evidence. Hospital records were recalled after a period of six months for update and review.

CHAPTER 5

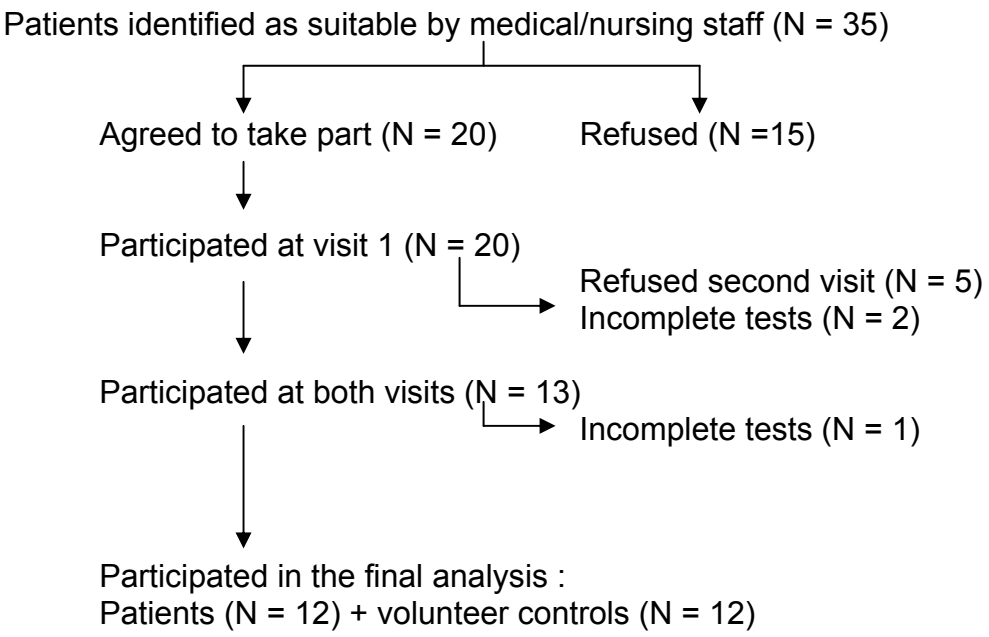
STUDY RESULTS

5 STUDY RESULTS

5.1 Recruitment

Patient recruitment began at the end of October 2006 and finished in mid August 2007. During this time thirty five patients were identified who matched the inclusion criteria (see section 4.2) and fifteen patients declined when approached with details of the study. When the recruitment of control participants was complete a total of thirty two individuals had taken part (recruitment details are shown in figure 21). Twelve patients and matched controls completed both visits, five patients completed the first visit but later refused a second appointment, one patient completed all tests but venesection had to be abandoned on both occasions, two patients donated blood samples but were unable to take part in other tests.

Figure 21 - Numbers of patients and controls recruited to the study



5.1.1 Participants

The twelve completed pairs consisted of 8 males and 4 females, age range 24-77 years. (Mean 55.6 ± 14.2). All patients were clinically diagnosed as suffering from cellulitis. Five were classified as a first episode and seven as recurrences. Patient 3 (P3) classed as recurrent, had been taking oral antibiotic treatment prescribed

by the GP for several months. Table 7 shows the age distribution of the twelve pairs together with frequencies for sex, acute and recurrent episodes and whether the right or left leg was affected.

Patient /control	Age (years)	Sex M/F	Cellulitic leg	Acute/Recurrent
P1/C1	55/54	F	Left	Acute
P2/C2	50/50	M	Right	Acute
P3/C3	61/60	F	Right	Recurrent
P4/C4	46/46	M	Right	Recurrent
P5/C5	24/25	M	Right	Recurrent
P6/C6	66/66	M	Left	Recurrent
P7/C7	77/75	M	Left	Recurrent
P8/C8	62/61	F	Left	Recurrent
P9/C9	61/60	M	Left	Acute
P10/C10	61/60	M	Left	Recurrent
P11/C11	38/38	M	Left	Acute
P12/C12	66/67	F	Left	Acute

Distribution by	Age	Sex	Right/Left	Acute/recurrent
	20-30 = 1 = 8.3%	M = 8 = 66.6%	Right = 4 = 33.3%	Acute = 5 = 42%
	30-40 = 1 = 8.3%			
	40-50 = 1 = 8.3%			
	50-60 = 2 = 16.6%	F = 4 = 33.3%	Left = 8 = 66.6%	Recurrent = 7 = 58%
	60-70 = 6 = 50%			
	70-80 = 1 = 8.3%			

Table 7 – Patient/control characteristics and distribution by age, sex, status (acute/recurrent) and leg affected by cellulitis (right/left).

5.2 Statistics

This was a case controlled study designed to make comparisons between cellulitic and normal legs, acute and recurrent cases and patients and controls. Within patient comparisons were made between the affected (cellulitic leg) and the unaffected (contralateral leg) and patient control comparisons were made between the cellulitic leg and the control cellulitic leg (matched by left or right) see figure 12. Where appropriate comparisons were also made between the patient contralateral leg and the control contralateral leg (eg: numbers of dermatophytes cultured). Differences between these comparisons at visit 1 and visit 2 were important to establish whether results from cellulitic patients returned to near normal values post episode. For this reason only the twelve matched pairs have been included in the analysis although occasional reference is made to those patients who only attended for a first visit. Even with few matched pairs a large amount of data was

generated. A statistician reviewed this data and the following statistical tests were selected to conduct the analysis:

- Normally distributed data from related samples describing continuous variables (eg. measurements of pH) - **paired t-test (PT)**
- Skewed data describing continuous variables from related samples **Wilcoxon Signed Ranks Test (WSR)**
- Normally distributed data describing discrete variables from related samples (eg. stress questionnaire) **Wilcoxon Signed Ranks Test (WSR)**
- Binary (nominal) data from related samples (eg. patient history) - **Chi-squared (McNemar)**
- Data from unrelated samples and data sets of unequal length (eg. Comparison between acute /recurrent cases – **Mann-Whitney U (MWU)**

The probability value (P value) for significance was set at 5% (P = 0.05)
Dot plots were constructed to demonstrate the distribution of each data set before deciding on the appropriate test. In some sections these dot plots are presented in the text in others they are referenced and appear as part of the appendix. The significance tests for results of each modality have been compiled into a table showing the number of results, the P value and the chosen test. In each table (1) and (2) indicate whether the results were obtained at visit 1 or at visit 2 and the number of results tested either patient or control is shown as N. The last three columns show the test used, the degrees of freedom and the P values obtained. Where appropriate the mean value, standard deviation (SD) and minimum to maximum values of the data together with the 95% Confidence Interval (95% CI) of the mean are also shown.
These tables appear either in the text or in the appendix. Dot plots were constructed in Minitab 15, statistical data was generated in SPSS 15. Graphs were produced in Minitab 15 and Microsoft Excel 2000.

5.3 Results by category

The results are presented by the same categories as set out in the methodology:
Results of culture and microscopy and IgE tests for Trichophyton Rubrum
Results relating to skin structure and physiology
Results of immunological tests
Results relating to additional stress factors

5.3.1 Results of culture, microscopy and IgE tests for Trichophyton Rubrum

The InTray Dermatophyte Medium chosen was convenient and low risk for health and safety; however it was not specific for Dermatophyte growth as moulds, bacteria and yeasts also grew and produced similar colour changes. Morphological observation and microscopic examination were always required for positive identification and it was sometimes necessary to subculture onto SAB. 4 weeks was the average time to obtain a confident positive identification of species using DTM and microscopy, plus a further 2-3 weeks if recultures onto SAB were required. The primary objective was to identify dermatophytes but during this process it was necessary to identify other microorganisms in order to exclude the rarer dermatophyte forms. These other organisms were categorised as either opportunists, bacteria or yeasts. Opportunists or opportunistic dermatomycoses, include penicillium, paecilomyces, aspergillas, scopulariopsis, cladosporium and alternaria. Most of these could only be positively identified by microscopy and some examples are shown in figure 22. Some colonies of yeasts and bacteria (eg Rhodotorula and Pseudomonas (figure 23) were easily identified by culture alone. Because of the diversity of organisms grown, once dermatophytes had been excluded, only the most obvious from the other categories were positively identified.

5.3.1.1. Culture results

Both patients and controls produced more cultures from the foot sole than from the ID spaces and larger and more diverse categories of organisms were obtained from the feet of control subjects. Table 8 shows the number of patients and controls who grew dermatophytes, opportunists, bacteria and yeasts from either site or either foot during the course of the study. Wilcoxon Signed Ranks Test (WSR) was performed on this data but no significance was found between categories. Three individuals, patient 7 (P7), patient 12 (P12) and Control 1 (C1) cultured no organisms although adequate amounts of skin scales were demonstrated on the DM surface. Distributions of the numbers of the four different categories of microorganisms grown at the various sites is shown in appendix 2-9.

Dermatophytes

The only patient included on the analysis who grew Dermatophytes was P11, Trichophyton Interdigitale (TI) was grown from both foot soles at the first visit and Trichophyton Rubrum (TR) was grown from the sole of the cellulitic (left) foot at

visit 2. The microscopic appearances of TR and TI are quite distinct and shown in figure 24. No other patients grew dermatophytes on either foot or at either visit

	Dermatophytes	Opportunists	Bacteria	Yeasts
Number of Patients	1	9	5	4
Number of Controls	6	11	4	3

Table 8 - Numbers of patients and controls who grew colonies of dermatophytes, opportunists, bacteria and yeasts.

although 3 of the patients who were unable to attend for follow up grew dermatophytes (TI in one case and TR in the other two).

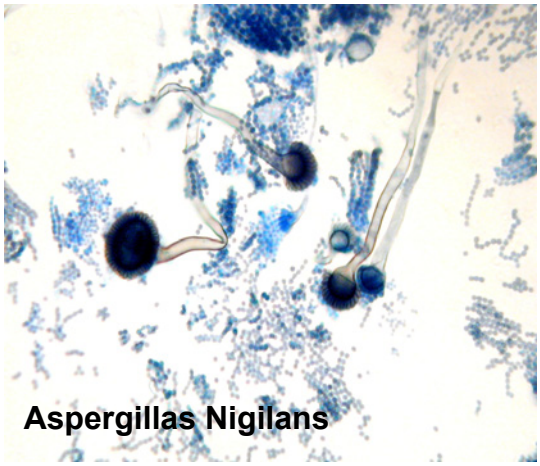
Dermatophytes were grown from 6 (50%) of the control group. Trichophyton Rubrum was grown from four individuals (C2, C6, C9 and C10) and Trichophyton Interdigitale was grown from two (C3 and C11). C2 and C6 grew dermatophytes from both feet at both visits, C3, C6, C9 and C10 produced one colony from one foot on either occasion.

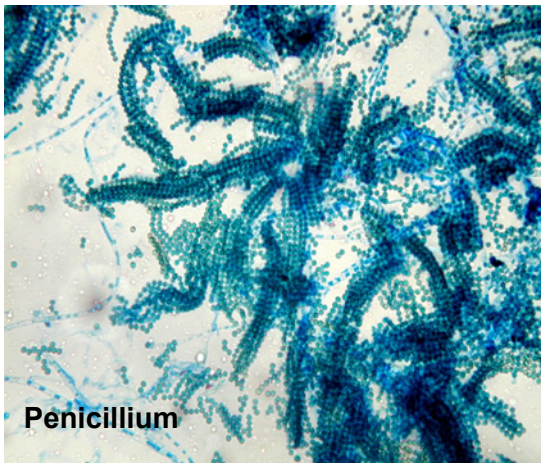
WSR was performed on the data to look for evidence of statistical significance between the cellulitic and the contralateral feet of patients and between corresponding feet of the matched controls at both visits. The data between acute and recurrent groups (acute = 5, recurrent = 7) was analysed using the Mann-Whitney U test because the data was unrelated and the data sets unequal. The results are presented in table 9 and show that no significance was found for any of the statistical testing carried out on the dermatophyte data.

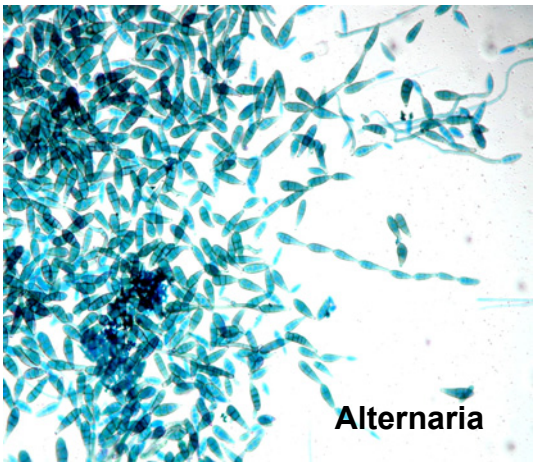
Opportunists

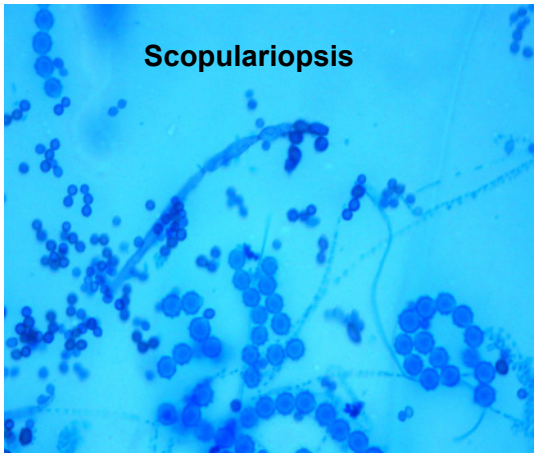
Large numbers of opportunistic dermatomycoses (section 2.2) were cultured from the feet of both patients and controls. In total for both visits patients grew more colonies (P= 20, C = 17) than controls on the first occasion and also (P = 23, C = 21) at the second visit but these results did not reach significance (Chi×). The most frequently occurring opportunist was Penicillium (patients = 7 = 58%, controls = 7 = 58%) and its closely related variant Aspergillas (P = 2 = 17%). Cladosporium Werneckii (Tinea Nigra) was identified in four patients (25%) and in five (42%) of controls (P2, P3, P5, P9, C3, C6, C7, C8 and C10). Other opportunists identified

Figure 22 – Opportunistic Dermatomycoses
Microscopic appearance x 60









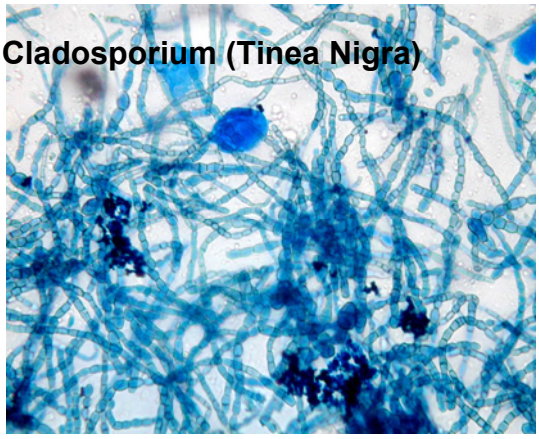
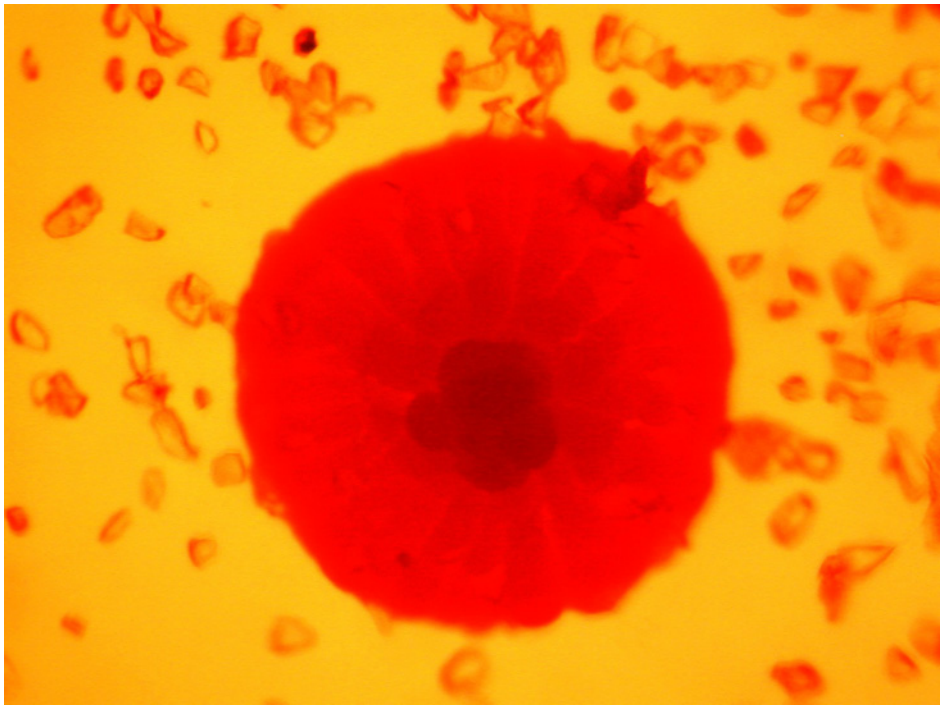
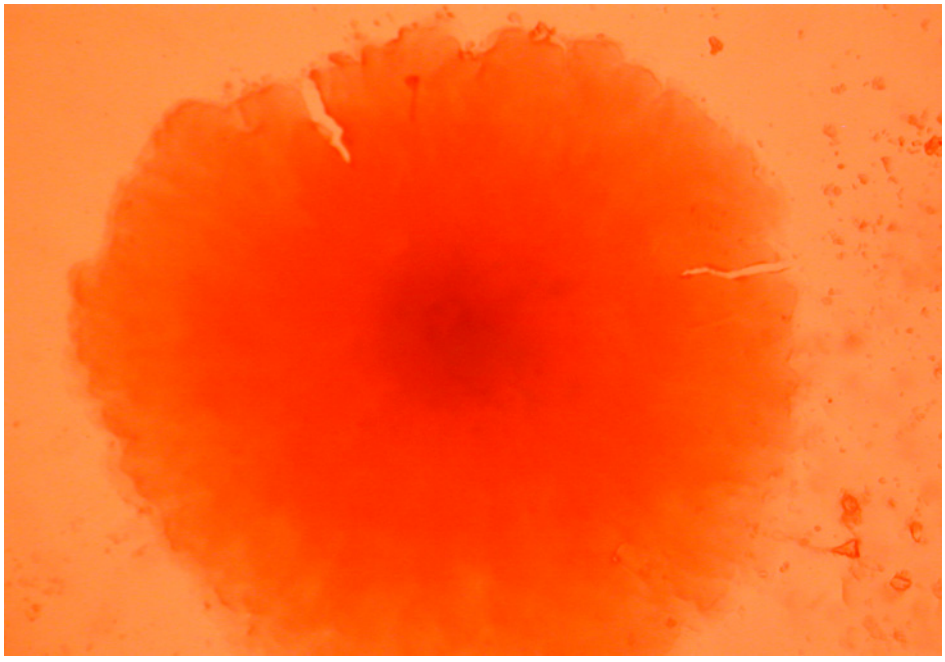


Figure 23

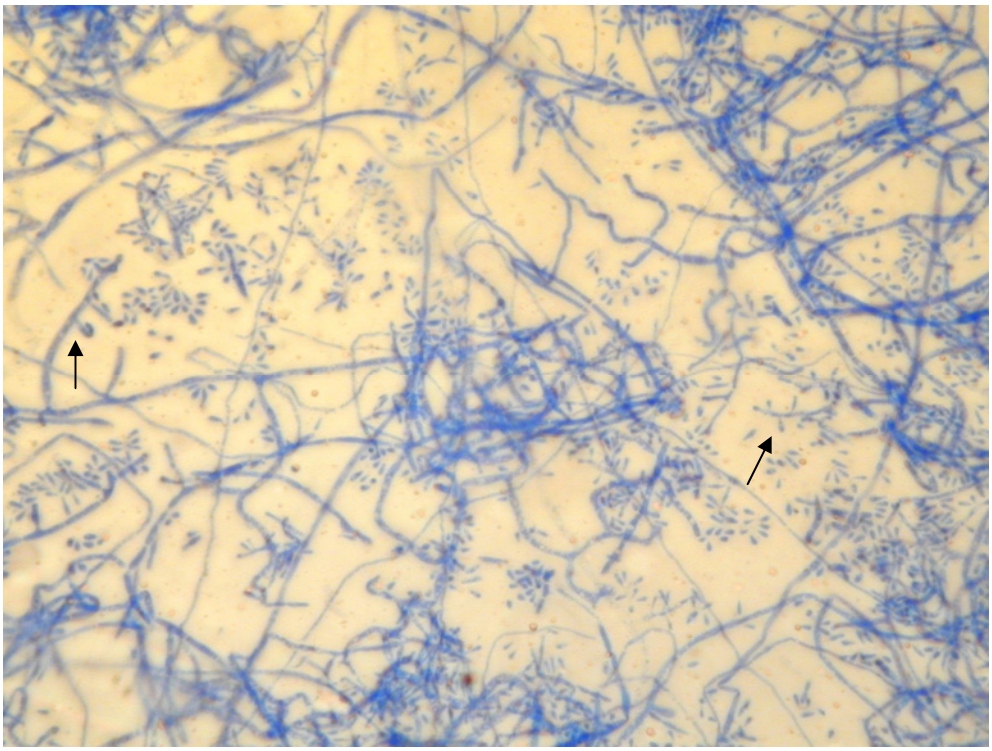


Morphological appearance Rhodotorula (yeast)

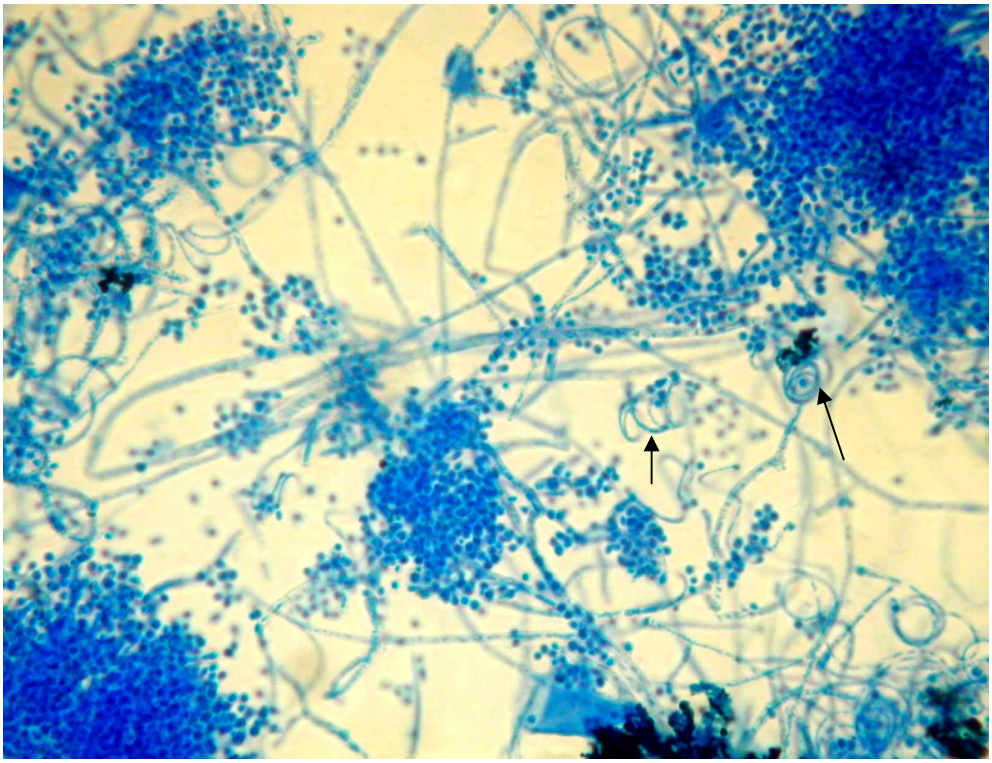


Morphological appearance Pseudomonas (bacteria)

Figure 24



Microscopic appearance of Trichophyton Rubrum x 60– conidia are club shaped and attached to hyphae by stalks (arrows)



Microscopic appearance of Trichophyton Interdigitale x 60 – conidia are round and form clumps. A distinguishing feature is the presence of coiled spirals (arrows).

were Acromium (P = 2, C = 1), Alternaria (C = 1), Exophiala (P = 1), Geomyces (P = 1), Scopulariopsis (P = 2) see figure 22. The statistical testing described in the previous section was repeated using this data, no significance was found and the results are shown in appendix 10.

Table 9 - Significance tests for results of Dermatophyte cultures

N = 12	Min-Max	N = 12	Min-Max	Test	P value
Cell foot (1)	0 - 1	Contra foot (1)	0 - 1	WSR	1.0
Cell foot (1)	0 - 1	Control foot (1)	0 - 1	WSR	0.188
Cell foot (1)	0 - 1	Cell foot (2)	0 - 1	WSR	1.0
Cell foot (2)	0 - 1	Contra foot (2)	0 - 0	WSR	1.0
Cell foot (2)	0 - 1	Control foot (2)	0 - 1	WSR	0.625
Contra foot (1)	0 - 1	Contra control foot (1)	0 - 2	WSR	0.500
Contra foot (1)	0 - 1	Contra foot (2)	0 - 0	WSR	1.0
Contra foot (2)	0 - 0	Contra control foot (2)	0 - 1	WSR	0.500
Control foot (1)	0 - 2	Control foot (2)	0 - 1	WSR	0.500

N = numbers compared (column 1 and column 3)
(1) = visit 1 (2) = visit 2

WSR = Wilcoxon Signed Ranks Test (exact 2 tailed test)

Table 9 shows the results of statistical testing for the results of the dermatophyte cultures.The first row shows the test results for cultures grown from cellulitic feet and contralateral feet. (1) and (2) indicate whether the results were obtained at visit 1 or at visit 2. N = the number of results tested (in this case a full data set of 12). The minimum and maximum (min-max) numbers of cultures obtained on an individual basis are recorded. The last two columns show the test used and the P values obtained.

Bacteria

Unidentified colonies of bacteria were grown from six participants (P1, P10, C5, C6, C7 and C11). Pseudomonas (figure 23) was positively identified from the foot soles of both feet in one patient (P6) and was grown on both occasions. No significance was found for this data and the results are shown in appendix 11.

Yeasts

Unidentified yeasts were cultured from four participants (P1, P11, C3 and C7). The yeast Rhodotorula (figure 23) was positively identified in two cases (P8 and C6) and Trico Beigelli in one (P10). No significance was found for this data and the results of the statistical tests are shown in appendix 12.

5.3.1.2 Results for specific IgE to Trichophyton Rubrum (TR).

The results of this immunological test have been included in this section because they relate directly to the identification of dermatophytosis.

Serum levels of antibody (IgE) to TR are reported as negative = 0 (< 35 kUA/L), low = 1 (0.4 – 0.7 kUA/L), moderate = 2 (0.7 – 3.5 kUA/L), high = 3 (3.5 – 17.5 kUA/L), and very high = 4 (17.5 - >100kUA/L) and values between 0 and 4 are commonly used to reflect the immunological status at the time of testing. Amongst the matched pairs, three patients (25%) (P2, P5 and P11), and three controls (25%) (C2, C6 and C9), recorded detectable levels of IgE to TR. All three control subjects and P11 produced TR on culture but although P2 and P5 produced other microorganisms including the opportunist Tinea Nigra neither produced TR from samples taken during the study. No antibodies to TR were recorded from C3 and C11 who both cultured TI, but this was as expected. One control C10, with an intermittent history of athletes foot, produced a culture with macroscopic appearances of TR but no antibodies were reported. Microscopy was equivocal and the culture may have been TI or another dermatophyte.

WSR was used to compare test results between patient and controls and between visits; MWU was performed between acute and recurrent cases but no statistical differences were found between any groups. Levels of IgE between the patient and control groups at both visits are shown in figure 25 and the statistical results and raw data appear in appendix 13 and 14.

5.3.2 Results relating to skin structure and physiology

Measurements of skin water loss, skin pH, skin blood flow and skin thickness were attempted on all participants at both sites and at both visits in order to establish normal values and to assess any changes, transient or permanent, that might affect skin physiology in the cellulitic legs.

5.3.2.1 Skin water loss

The Tewameter was easy to operate and well received by patients. There was no difficulty in obtaining measurements on normal or cellulitic skin. However it was difficult to calibrate and several attempts had to be made each time before acceptable calibration results were obtained. This may be because the Tewamater references a closed volume during calibration; in operation the cylinder is open to the air and the study readings were obtained according to the recommended instructions; in a draught free room, within the stated temperature and humidity values and after a suitable period had been allowed for acclimatisation to take place.

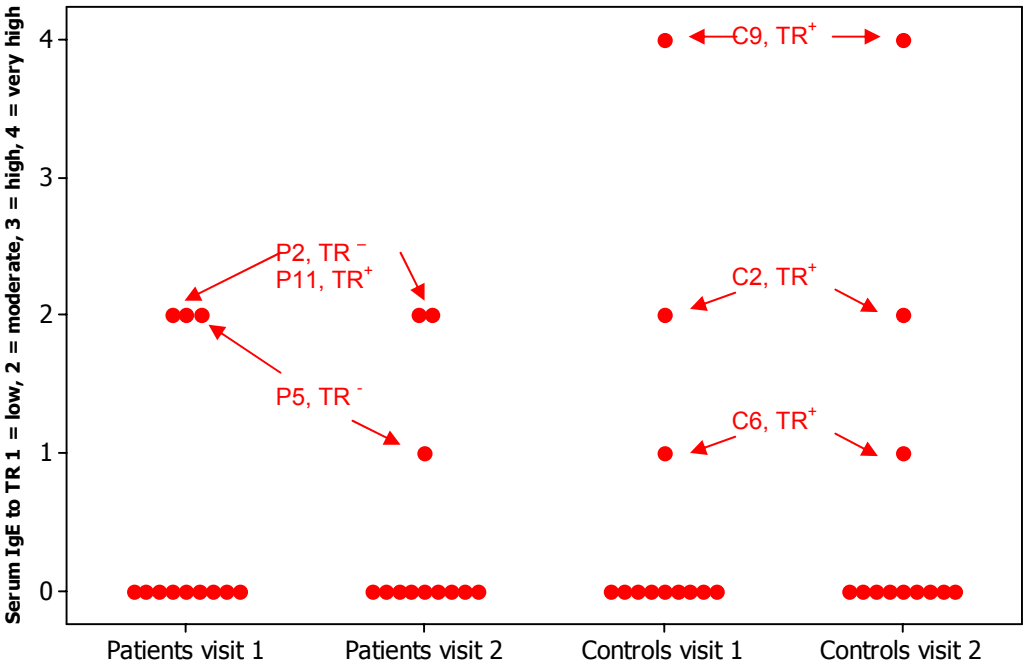


Figure 25 - Graph to show serum levels of IgE toTrichophyton Rubrum. Patients and controls (n = 12) visits1 and 2.
Patient (P11) and controls (C2, C6 and C9) who cultured TR are shown as TR^{+}. Patients P2 and P5 who did not culture TR are shown as TR $^{-}$

Results were obtained from all twelve pairs of participants at both visits. Measurements were initially higher and remained higher in cellulitic legs as compared to the contralateral legs and controls (figure 26). In the cellulitic legs the range at the first visit was 2.5 – 17.2 and at the second visit 6.1 – 20 g/hr/cm^2 respectively. The data were compared using paired T test or WSR depending on distribution (section 5.2). Raw data and distribution plots are set out in Appendix 15 and 16 and the results shown in table 10.

TEWL readings were found to be significantly higher in the cellulitic legs than in contralateral legs or control legs at visit two, P = 0.003 and 0.04 respectively. Comparisons between cellulitic legs, contralateral legs and control legs were close to but did not achieve significance at visit one see table 10. No significance was found between acute and recurrent cases on either occasion.

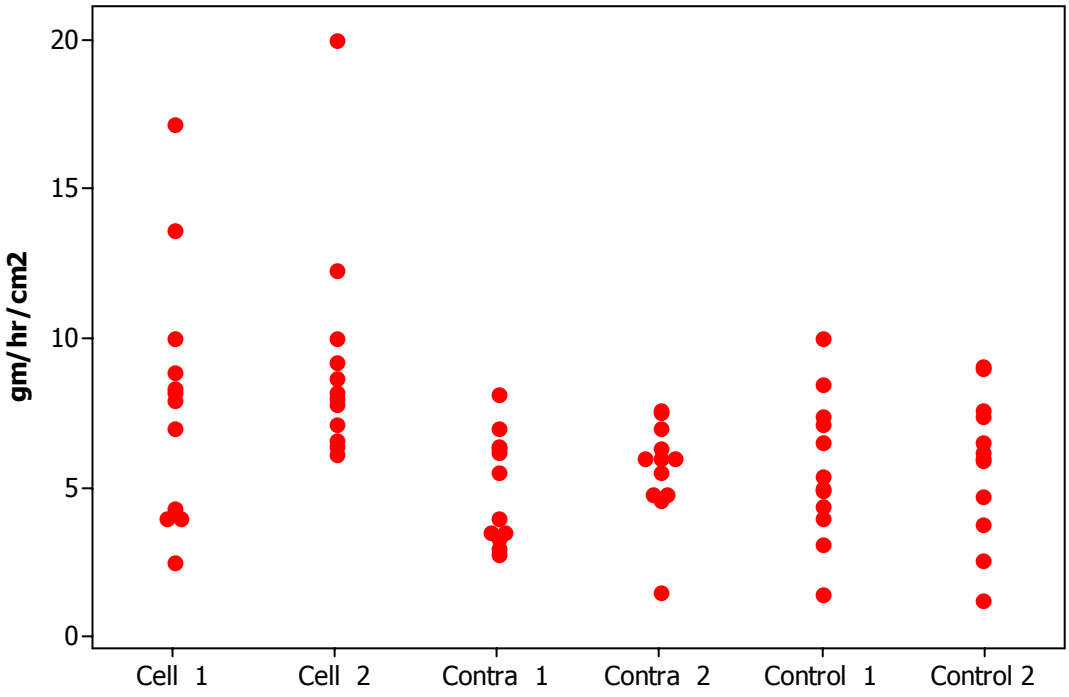


Figure 26 - Graph to show TEWL results (gm/hr/cm^2) for cellulitic legs visit 1 (cell 1), cellulitic legs visit 2 (cell 2), contralateral legs visit 1(contra 1), contralateral legs visit 2 (contra 2) and control legs at both visits (N = 12). TEWL readings were found to be significantly higher in the cellulitic legs than in contralateral legs or control legs at visit two, P = 0.003 and 0.04 respectively.

Normal ranges established from the twelve controls were between 1.2 and 10.0 g/hm^2. C2. C3, C4, C6, C7, C8, and C9 all recorded one or more readings below the lower limit of normal as defined by Nilsson (1977) who documented 6-11 g/hr/cm^2 as the normal range for TEWL measurements recorded from the legs. However, except for one control, results were comparably low for both right and left legs on each separate occasion. The contralateral legs in the patient group presented similar ranges ; 2.8 – 8.1 g/hr/cm^2 at visit 1 and 1.5 to 7.6 g/hr/cm^2 at visit 2. These findings suggest that the results obtained were reliable and that the accepted lower end of normal might need to be revised.

It was anticipated that TEWL readings would be higher in the cellulitic legs during the episode due to increased blood flow and skin temperature at the site of infection. Comparisons with control and contralateral legs did not quite achieve significance at visit 1 but this may have been because the numbers were small. The significance found at the second visit is interesting as it suggests changes in physiology. P1, P2, P8 and P10 all showed increases in the rate of TEWL at visit two as compared to visit one but these results were within the normal range. P3 however, produced a considerable increase from 7.9 – 20 g/hr/cm^2 and this is interesting as this patient had been on antibiotic therapy for several months and

Table 10 - Significance tests for results of Transepidermal Water Loss

N = 12	Mean (SD) Min-Max	N = 12	Mean (SD) Min-Max	95% mean CI (A - B)	Test	DF	P value
Cell legs (1)	2.5 – 17.2	Contra legs (1)	2.8 – 8.1		WSR		0.065
Cell legs (1)	2.5 – 17.2	Control legs (1)	1.4 – 10.0		WSR		0.092
Cell legs (1)	2.5 – 17.2	Cell legs (2)	6.1 – 20.0		WSR		0.583
Cell legs (2)	6.1 – 20.0	Contra legs (2)	1.5 – 7.6		WSR		0.003
Cell legs (2)	6.1 – 20.0	Control legs (2)	1.2 – 9.1		WSR		0.04
A = Contra legs (1)	4.96 (1.81) 2.8 – 8.1	B = Contra control legs (1)	4.9 (2.33) 1.4 – 9.0	-1.185 –1.918	PT	11	0.613
Contra legs (1)	2.8 – 8.1	Contra legs (2)	1.5 – 7.6		WSR		0.232
Contra legs (2)	1.5 – 7.6	Contra control legs (2)	2.5 – 9.4		WSR		0.807
A = Control legs (1)	5.6 (2.39) 1.4 – 10.0	B = Control legs (2)	5.8 (2.41) 1.2 – 9.1	-1.465 –0.773	PT	11	0.529
A = Control legs (1)	5.6 (2.39) 1.4 – 10.0	B = Control legs (2)	5.8 (2.41) 1.2 – 9.1	-1.465 –0.773	PT	11	0.529

N = numbers compared (column 1 and column 3)
(1) = visit 1 (2) = visit 2

CI = confidence interval (A = parameter column 1, B = compared parameter column 3)
PT = Paired T-test
WSR = Wilcoxon Signed Ranks Test (exact 2 tailed test)
DF = degrees of freedom

= significant (P = 0.05)

= P value close to 0.05

was clinically the least representative of cellulitis with respect to skin characteristics. With the exception of P5 all other patients recorded readings within the normal range on both occasions. Readings for P5 were 17.2 – 12.3 g/hr/cm^2 at visit 1 and 2 respectively. This patient had substantial longstanding oedema in the cellulitic leg and a history of frequent recurrence. Because increases in skin water content are likely to disturb the rate at which water leaves the skin this result is not unexpected.

5.3.2.2 Skin pH

pH measurements were performed at the centre of the area of interest of the cellulitic legs, contralateral legs and control legs at both visits. Calibrations were within range on each occasion and results were obtained from all twelve pairs of participants at both visits.

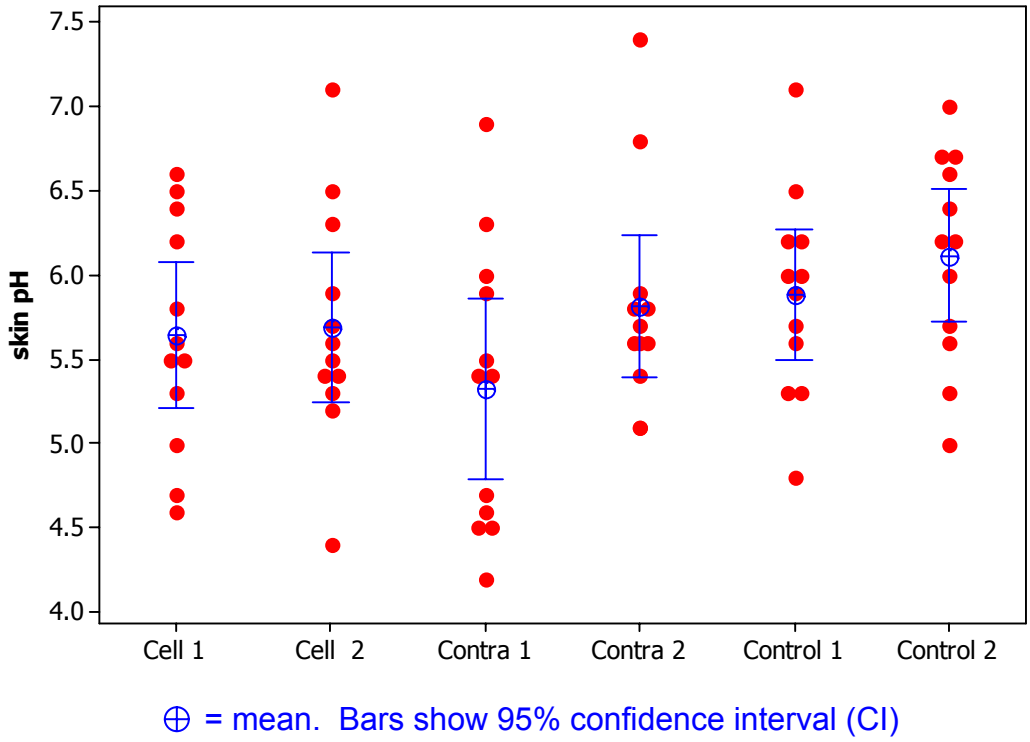


Figure 27 - Graph to show pH results for cellulitic legs visit 1 (cell 1), cellulitic legs visit 2 (cell 2), contralateral legs visit 1(contra 1), contralateral legs visit 2 (contra 2) and control legs at both visits (N = 12). (mean and 95% confidence intervals are shown). No significant differences were found between the groups

The reported pH range for normal unoccluded human skin varies from 3 - 6.5 (Matousek and Campbell 2002). Noble (1968) demonstrated the mean pH value

for calf skin of the legs to be 5.5. In this study the range of pH values in cellulitic legs at visit one was 5.6 – 6.6 (mean 5.6 ± 0.68) and within the limits generally accepted as normal. At visit two the highest values for cellulitic legs extended into the alkaline range; a similar pattern was obtained for contralateral, control and contralateral control legs at both visits (figure 27). Three out of twelve (25%) of patients (P3, P8 and P7 all recorded pH values above 6.5 in one or both legs at one or both visits. Six out of twelve 50% of controls (C1, C3, C5, C7, C10 and C11) also produced these high values.

Table 11 - Significance tests for pH results

N = 12	Mean (SD) Min-Max	N = 12	Mean (SD) Min-Max	95% mean CI (A - B)	Test	DF	P value
A = Cell legs (1)	5.642 (0.68) 4.6 – 6.6	B = Contra legs (1)	5.325 (0.84) 4.2 – 6.9	-0.124 – 0.758	PT	11	0.142
A = Cell legs (1)	5.642 (0.68) 4.6 – 6.6	B = Control legs (1)	5.883 (0.60) 4.8 – 7.1	-0.790 – 0.306	PT	11	0.370
A = Cell legs (1)	5.642 (0.68) 4.6 – 6.6	B = Cell legs (2)	5.692 (0.69) 4.4 – 7.1	-0.516 – 0.416	PT	11	0.818
A = Cell legs (2)	5.692 (0.69) 4.4 – 7.1	B = Contra legs (2)	5.817 (0.66) 5.1 – 7.4	-0.429 – 0.179	PT	11	0.385
A = Cell legs (2)	5.692 (0.69) 4.4 – 7.1	B = Control legs (2)	6.117 (0.61) 5.0 – 7.0	-0.982 – 0.132	PT	11	0.128
A = Contra legs (1)	5.325 (0.84) 4.2 – 6.9	B = Contra control legs (1)	6.033 (0.72) 4.6 – 7.2	-1.376 – 0.041	PT	11	0.039
Contra legs (1)	4.2 – 6.9	Contra legs (2)	5.1 – 7.4		WSR		0.095
Contra legs (2)	5.1 – 7.4	Contra control legs (2)	5.2 – 6.8		WSR		0.241
A = Control legs (1)	5.883 (0.61) 4.8 – 7.1	B = Control legs (2)	6.117 (0.61) 5.0 – 7.0	-0.574 – 0.108	PT	11	0.160

N = numbers compared (column 1 and column 3)
(1) = visit 1 (2) = visit 2

CI = confidence interval (A = parameter column 1, B = compared parameter column 3)
PT = Paired T-test
WSR = Wilcoxon Signed Ranks Test (exact 2 tailed test)
DF = degrees of freedom

= significant (P = 0.05)

The data were compared using PT or WSR. Descriptive statistics and significance results are set out in table 11. (Raw data and distribution plots are shown in appendices 17 and 18).

For differences between right and left legs within visit variability was less than between visit variability for both patients and controls. There was no statistically significant difference between the values for the cellulitic legs during the cellulitic episode and the follow up visit or between the cellulitic legs and the contralateral or control legs on either occasion or between acute and recurrent cases. However a statistically significant difference was found between contralateral legs and contralateral control legs at visit 1 ($p = 0.04$). This is likely due to the unusual distribution of values from the contralateral legs giving a cluster of values just above 6 and because of the low numbers of participants.

Although no statistical significance was found between the groups the overall pH values were higher than expected, there are many possible explanations and these will be discussed in the next chapter.

5.3.2.3 Oedema

This section contains the results of the leg circumference measurements, the skin thickness measurements and the ultrasound image analysis. These tests were performed to assess the oedema in the cellulitic leg in order to understand where it was located, whether it was acute or chronic and whether the skin structure appeared changed.

Leg circumference measurements

Circumferential limb measurements were used to compare swelling between legs, between visits and between groups. Measurements were recorded for the lower leg and taken just below the knee, at mid calf level and at the ankle. Data was obtained from eleven pairs of patients and controls . (P10 was heavily bandaged on the first occasion so measurements were not performed).

The data obtained was normally distributed throughout (see appendices 19 - 21) and paired t tests were used for statistical analysis.

Significant differences were found between patients and controls for both legs at the ankle and mid calf sites and on both occasions (appendices 22-23). Measurements at these sites were consistently higher in the patient group. The mean value for the cellulitic legs at the ankle was 29.5 ± 5.4 cms at visit 1(V1) and 28.2 ± 4.5 cms at visit 2 (V2) and for the mid calf 40.1 ± 7.2 cms (V1) and 40.3 ±

7.7 cms (V2). Mean values for the control cellulitic legs at the ankle were 22.2 ± 0.9 cms (V1) and 22.1 ± 0.8 cms (V2), and at the mid calf 33.2 ± 2.7 (V1), 33.6 ± 0.9 (V2). Significance was also found between these two groups at the upper calf level (see table 12). These findings are most likely the result of differences in body profile between patients and controls and occurred because the control group had thinner calves.

Within the patient group no significant differences were found for ankle or mid calf measurements taken from the cellulitic legs at either visit at any site, or between measurements taken from the cellulitic legs and contralateral legs at either visit. However circumferential measurements of the cellulitic upper calf were significantly higher (p = 0.013) than at the same site on the contralateral leg but only at the first visit (figure 28 and table 12). The raw data in appendix 24 shows that in four patients (P3, P6, P7 and P12) the upper calf circumference in the cellulitic leg was greater at the second visit and increases were also found in the contralateral leg in seven patients (P2, P3, P5, P6, P7, P9 and P11) also at visit 2.

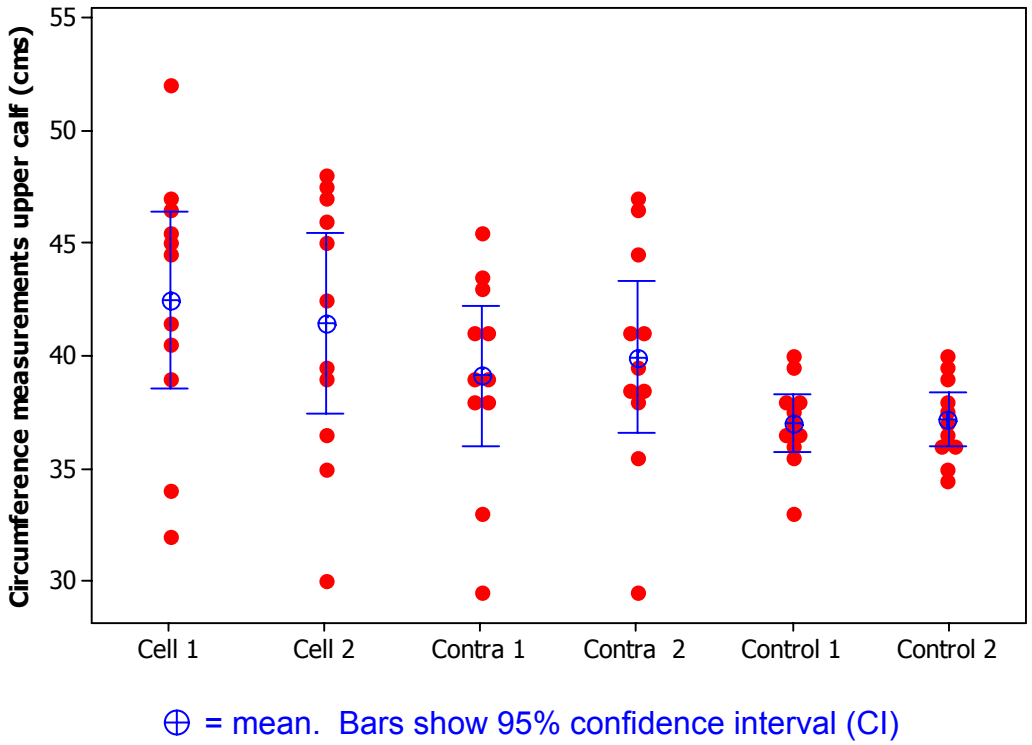


Figure 28 - Graph to show results for circumferential measurements (cms) taken at the upper calf. Cellulitic legs visit 1 (cell 1), cellulitic legs visit 2 (cell 2), contralateral legs visit 1(contra 1), contralateral legs visit 2 (contra 2) and control legs at both visits (N = 11). Circumferential measurements of the cellulitic upper calf were significantly higher (p = 0.013) than at the same site on the contralateral leg but only at the first visit.

Table 12 - Significance tests for leg measurements (cms) taken at upper calf level

N = 11	Mean (SD) Min-Max	N = 11	Mean (SD) Min-Max	95% mean CI (A - B)	Test	DF	P value
A = Cell legs (1)	42.5 (5.9) 32 - 52	B = Contra legs (1)	39.1 (4.6) 29 – 45.5	0.89 – 5.83	PT	10	0.013
A = Cell legs (1)	42.5 (5.9) 32 - 52	B = Control legs (1)	37.0 (1.9) 33 - 40	1.47 – 9.44	PT	10	0.012
A = Cell legs (1)	42.5 (5.9) 32 - 52	B = Cell legs (2)	41.4 (5.9) 30 - 48	-1.134 – 3.225	PT	10	0.310
A = Cell legs (2)	41.4 (5.9) 30 - 48	B = Contra legs (2)	39.9 (5.0) 29.5 - 47	-1.134 – 3.225	PT	10	0.310
A = Cell legs (2)	41.4 (5.9) 30 - 48	B = Control legs (2)	37.2 (1.8) 34.5 - 40	0.31 – 8.23	PT	10	0.037
A = Contra legs (1)	39.1 (4.6) 29 – 45.5	B = Contra control legs (1)	37.5 (2.0) 35 – 41.5	-1.63 – 4.90	PT	10	0.290
A = Contra legs (1)	39.1 (4.6) 29 – 45.5	B = Contra legs (2)	39.9 (5.0) 29.5 - 47	-1.819 – 0.181	PT	10	0.098
A = Contra legs (2)	39.9 (5.0) 29.5 - 47	B = Contra control legs (2)	37.5 (1.8) 34.5 - 41	-0.96 – 5.87	PT	10	0.140
A = Control legs (1)	37.0 (1.9) 33 - 40	B = Control legs (2)	37.2 (1.8) 34.5 - 40	-0.739 – 0.466	PT	10	0.625

N = numbers compared (column 1 and column 3)
(1) = visit 1 (2) = visit 2

CI = confidence interval (A = parameter column 1, B = compared parameter column 3)
PT = Paired T-test
DF = degrees of freedom

= significant (P = 0.05)

During the episode the legs were elevated to encourage a reduction in oedema; this would be marginally effective in the cellulitic leg but more effective in its counterpart. Following the episode increased dependency would encourage swelling in both legs and this is the likely cause of the significance between legs in the patient group.

Ultrasound

Ultrasound imaging was attempted on ten pairs of patients and controls (two pairs were not imaged because the equipment was unavailable). Image capture was conducted according to the protocol set out in 4.3.2.4, aiming to achieve the correct orientation between the scanner membrane and the skin surface in order to produce reliable measurements (see figures 6a and 7c).

It was difficult to obtain good ultrasound images on many of the participants. Some were unable to abduct their legs sufficiently to allow the scan to be conducted at the correct angle and some were unable to tolerate skin pressure. For these reasons there are gaps in the data (appendix 26) and only seven pairs produced images suitable for measuring and data analysis (P1, P2, P3, P5, P8, P10, P11 and matched controls). It was not possible to evaluate the extent of the dermis in P11 at visit one because of the diffuse oedema and poor images; comparisons for area B are therefore limited to six. The distances between the A-scan interfaces relating to the epidermis (area A) and the dermis (area B) were measured at three levels on each image and the average calculated (see section 3.1.2.3).

- Area A (epidermis)

The data were compared using paired t test or WSR according to the distribution (see appendix 27). No statistical differences were found between patients and controls or between cellulitic or normal legs at either visit. (appendix 28).

- Area B (dermis)

The data was all normally distributed and tested by paired t test (appendix 29) the results are shown in figure 29 and table 13. Dermal measurements were significantly greater in the cellulitic legs as compared to the cellulitic control legs at the first visit ($p = 0.029$). Comparison of the cellulitic legs between visits, and the cellulitic legs and contralateral legs at both visit 1 and visit 2 were almost significant ($p = 0.071$, $p = 0.081$ and $p = 0.087$ respectively).

The numbers entered into the analysis were low but the findings are consistent with those in the previous section and suggest that leg swelling in the cellulitic legs is persistent and that the difference may be partly or totally due to increases in thickness in the dermis. Figure 30 shows the images taken from the cellulitic leg of P8. In figure 30a the arrows indicate low echogenic pixels consistent with the presence of fluid (oedema) in the papillary dermis. In figure 30b the oedema can be seen to have reduced. It may be that the fluid remains within the papillary dermis, that it disperses deeper into the dermal tissue or that it resolves. In order to more fully investigate this some data was selected for image analysis.

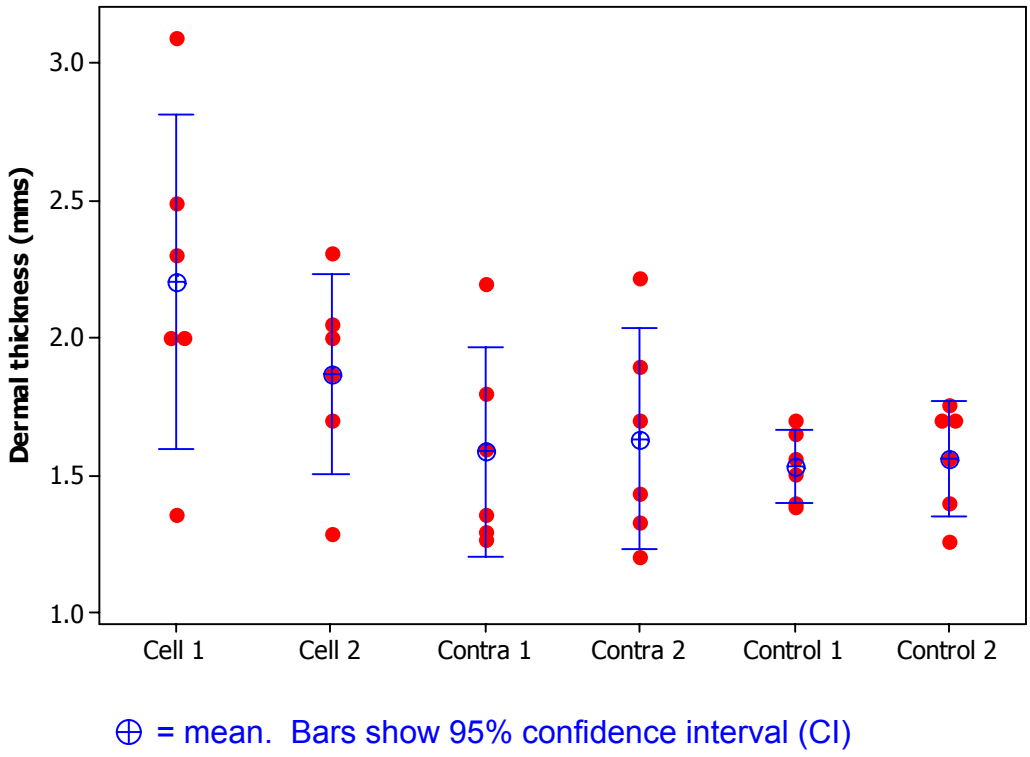


Figure 29
Graph to show ultrasound measurement results (area b dermis) for cellulitic legs visit 1 (cell 1), cellulitic legs visit 2 (cell 2), contralateral legs visit 1(contra 1), contralateral legs visit 2 (contra 2) and control legs at both visits (N = 6). Dermal measurements were significantly greater in the cellulitic legs as compared to the cellulitic control legs at the first visit (p = 0.029). Comparison of the cellulitic legs between visits, and the cellulitic legs and contralateral legs at both visit 1 and visit 2 were almost significant (p = 0.071, p = 0.081 and p = 0.087 respectively).

Table 13 - Significance tests for ultrasound measurements (area b) mms

N = 6	Mean (SD) Min-Max	N = 6	Mean (SD) Min-Max	95% mean CI (A - B)	Test	DF	P value
A = Cell legs (1)	2.20 (0.58) 1.36 – 3.09	B = Contra legs (1)	1.58 (0.36) 1.27 – 2.20	-0.076 – 1.313	PT	5	0.071
A = Cell legs (1)	2.20 (0.58) 1.36 – 3.09	B = Control legs (1)	1.53 (0.13) 1.39 – 1.70	0.104 – 1.239	PT	5	0.029
A = Cell legs (1)	2.20 (0.58) 1.36 – 3.09	B = Cell legs (2)	1.87 (0.34) 1.29 – 2.31	-0.060 – 0.733	PT	5	0.081
A = Cell legs (2)	1.87 (0.34) 1.29 – 2.31	B = Contra egs (2)	1.63 (0.38) 1.21 – 2.22	-0.119 – 0.592	PT	5	0.148
A = Cell legs (2)	1.87 (0.34) 1.29 – 2.31	B = Control legs (2)	1.56 (0.19) 1.26 – 1.76	-0.065 – 0.678	PT	5	0.087
A = Contra legs (1)	1.58 (0.36) 1.27 – 2.20	B = Contra control legs (1)	1.68 (0.19) 1.46 – 2.04	-0.482 – 0.282	PT	5	0.531
A = Contra legs (1)	1.58 (0.36) 1.27 – 2.20	B = Contra legs (2)	1.63 (0.38) 1.21 – 2.22	-0.109 – 0.019	PT	5	0.135
A = Contra legs (2)	1.63 (0.38) 1.21 – 2.22	B = Contra control legs (2)	1.62 (0.23) 1.37 – 2.01	-0.036 – 0.367	PT	5	0.973
A = Control legs (1)	1.53 (0.13) 1.39 – 1.70	B = Control legs (2)	1.56 (0.19) 1.26 – 1.76	-0.174 – 0.117	PT	5	0.638

N = numbers compared (column 1 and column 3)
(1) = visit 1 (2) = visit 2

CI = confidence interval (A = parameter column 1, B = compared parameter column 3)
PT = Paired T-test
DF = degrees of freedom

= significant (P = 0.05)

= P value close to 0.05

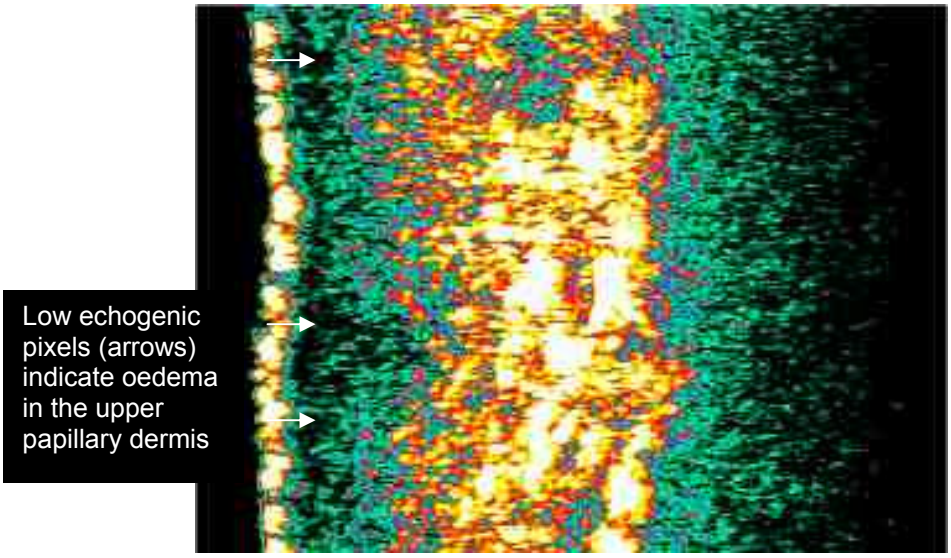


Figure 30a - Acute dermal oedema during the cellulitic episode (20MHz ultrasound scan)

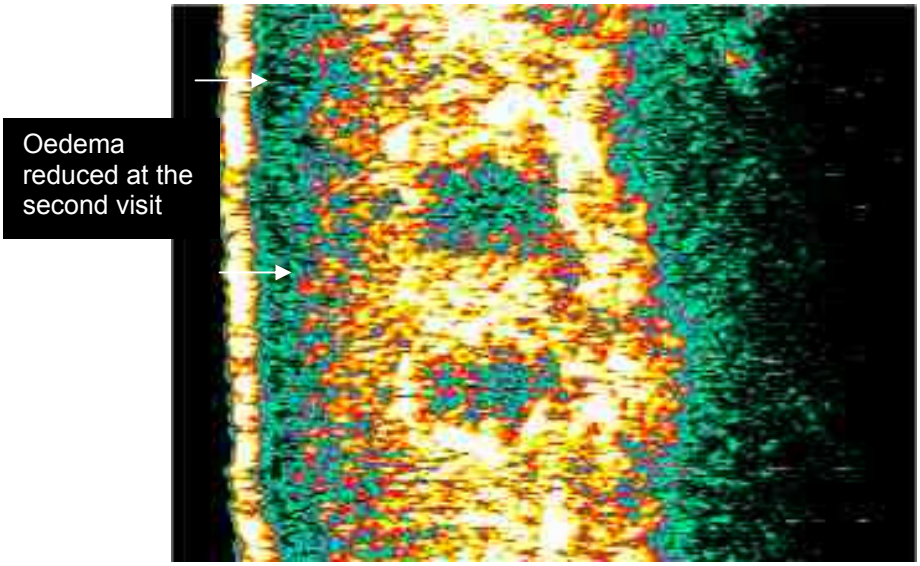
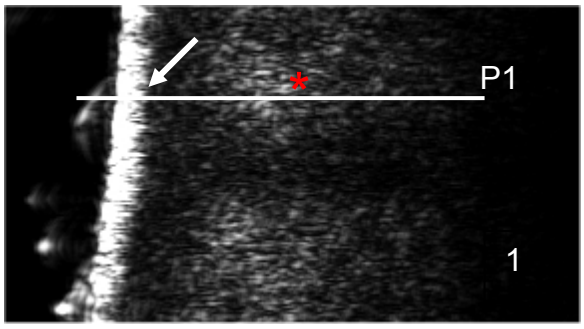
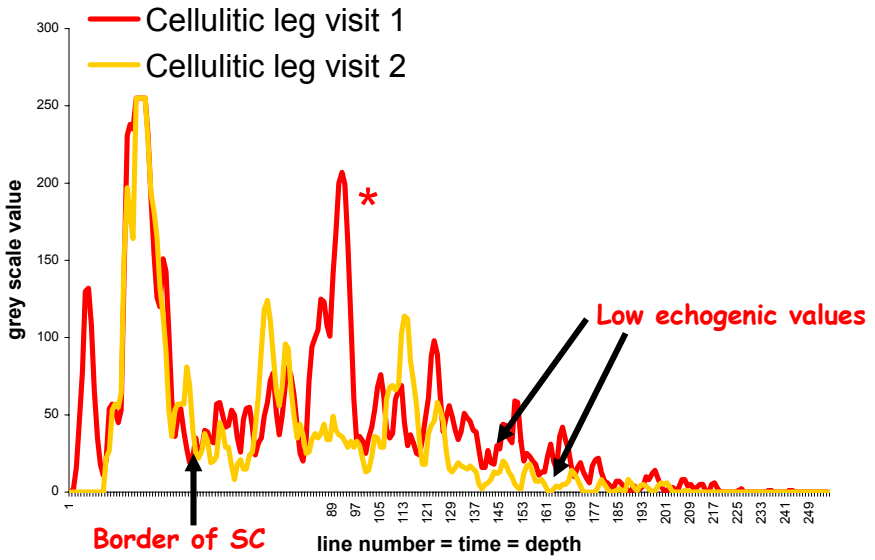


Figure 30b - Repeat scan of the same area 6 weeks post episode.

Figure 31 - Images taken from the cellulitic leg of P5 at visit 1 and visit 2 and A-scans derived from the data lines P1 and P2

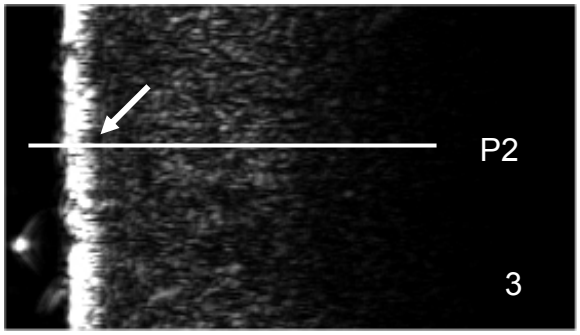


Greyscale image from visit 1. The A-scan is derived from line P1 and aligned with data from image 3 (Line P2) using the border of the SC as a guide (arrow)



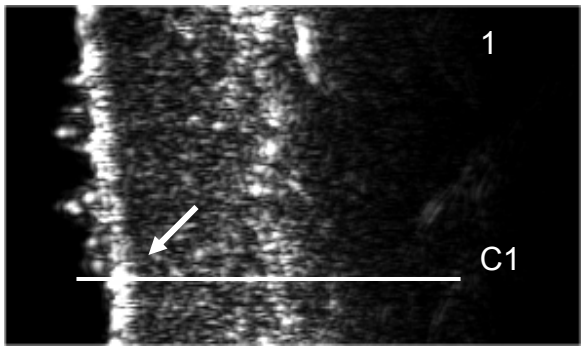
2

A-scan graphs of lines P1 and P2 matched at the SC border (arrow). The grey scale values deep to the SC are predominantly low (note some higher values from 1* produced by an area of high reflectivity within the dermis).

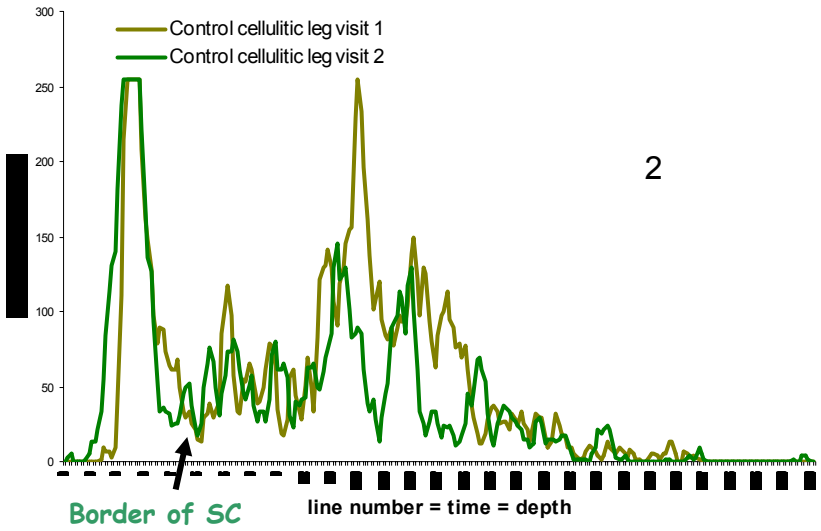


Greyscale image from visit 2. The A- scan is derived from line P2 and aligned with data from image 1 (Line P1) using the border of the SC as a guide (arrow).

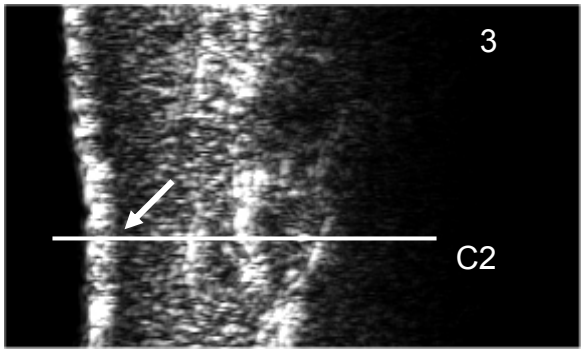
Figure 32 - Images taken from the control cellulitic leg of C5 at visit 1 and visit 2 and A-scans derived from the data lines C1 and C2



Greyscale image from visit 1. The A-scan is derived from line C1 and aligned with data from image 3 (Line C2) using the border of the SC as a guide (arrow)



A-scan graphs of lines C1 and C2 matched at the SC border (arrow). In both images the region deep to the SC contains much more structure. The grey scale values are higher than those in figure 31.



Greyscale image from visit 2. The A-scan is derived from line C2 and aligned with data from image 1 (Line C1) using the border of the SC as a guide (arrow).

Image analysis

The image analysis was performed as described in section 3.1.2.3 (*Image Analysis*). The colour images were also saved in a lossless grey scale format. Figure 31 shows images taken from the cellulitic leg of P5 at visit 1 and visit 2. A line of pixels was selected from each image (lines P1 and P2) and the data was digitised and graphed to produce A-scans. Graphs from both visits were matched together using the high values from the SC to position the skin entry points (figure 31). The corresponding images and graphs from the matched control C5 are shown in figure 32. P5 had long standing oedema which may have increased during the cellulitic episode but there is no obvious fluid collection in the papillary dermis as seen in figure 30. C5 shows structural differences in the strata deep to the SC and this is consistent with a fibrous content and reflected in the higher values obtained in the graphs. The lack of structure and the predominance of low echogenic pixels shown in images and A- scans from P5 suggest a higher fluid concentration and textural differences.

The results obtained from this part of the study lend support for the role of oedema in cellulitic episodes . A-scans are useful but are unable to quantify the nature of the textural differences that can be observed. More advanced analytical techniques need to be applied to images en block in order to advance the understanding and relate more exactly to the physiology.

5.3.2.4 Skin blood flow

Laser Doppler Imaging (LDI) was performed to compare changes in skin blood flow between cellulitic legs, contralateral legs and controls at both visits. This test was well tolerated by patients and controls because no skin contact was required. Only one patient (P7) had difficulty in remaining still during the scan, his involuntary movements are reflected in the results and will be discussed later.

The LDI software was used to calculate the mean flux value for the pixels within each ROI (see section 4.3.2.5). All twelve pairs were entered into the statistical analysis for cellulitic legs, contralateral legs and control legs, the data were evenly distributed and paired t tests were performed. Two data sets were missing for contralateral control legs and WSR tests were used because the numbers were uneven.

Mean flux values for the cellulitic legs were 371 ± 149 during the cellulitic episode and 176 ± 103 at the second visit. Values for the contralateral legs were 134 ± 39

(visit 1) and 173 ± 47 (visit 2). Values for control legs were 152 ± 36 (visit 1) and 149 ± 41 (visit 2). These results are presented in figure 33 and table14, raw data and distribution plots are shown in appendix 30 and 31.

Results from the cellulitic legs were significantly higher at visit 1 than at visit 2 (p = 0.0001) and also significantly higher than controls or contralateral legs at the first visit (p = 0.0001) in both cases. No other significance was found between acute and recurrent cases at either visit. These findings show that microcirculatory blood flow increased during the cellulitic episode and this is assumed to be in conjunction with the immune response. Decreased mean values at the second visit suggest that blood flow had by then returned to normal levels. However the

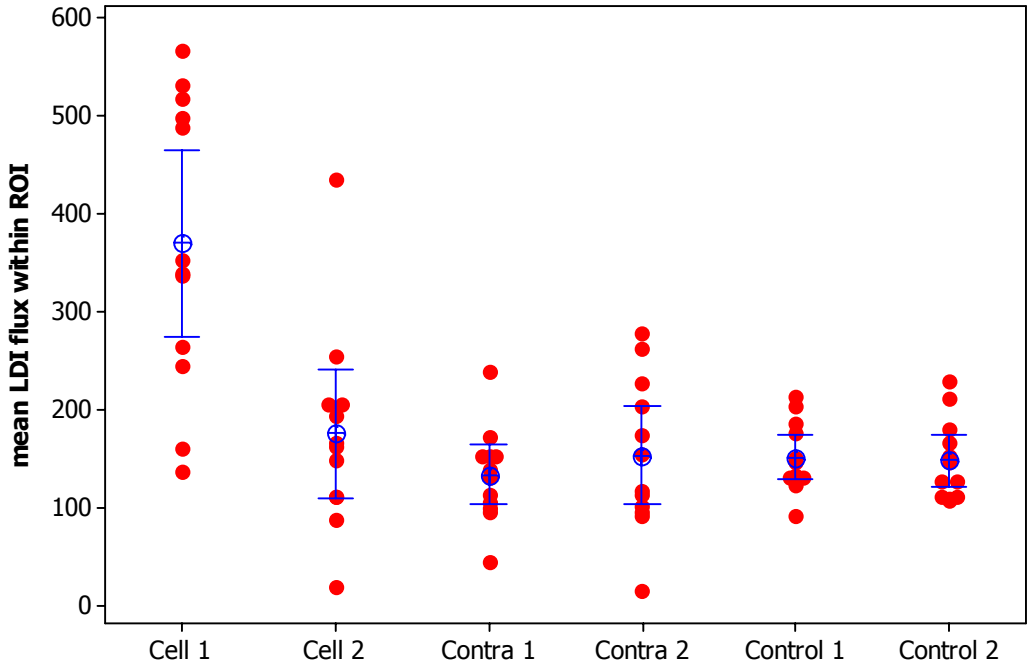


Figure 33
Graph to show results for LDI flux for cellulitic legs visit 1 (cell 1), cellulitic legs visit 2 (cell 2), contralateral legs visit 1(contra 1), contralateral legs visit 2 (contra 2) and control legs at both visits (N = 12).
Paired t tests were used to compare data (mean and 95% confidence intervals are shown.
Results from the cellulitic legs were significantly higher at visit 1 than at visit 2 (p = 0.0001). Results from the cellulitic legs were also significantly higher than controls or contralateral legs at visit 1 (p = 0.0001) in both cases.

raw data shows some very low mean flux values in the patient group (P4 and P5) where textural differences, thought to be related to chronic oedema, had also been demonstrated. Although in these patients blood flow was shown to increase during the cellulitic episode the increase in relation to normal resting flow for that individual was unknown.

Table 14 - Significance tests for results of mean flux Laser Doppler Imager (LDI)

N = 12 * N = 10	Mean (SD) Min-Max	N = 12 *N = 10	Mean (SD) Min-Max	95% mean CI (A - B)	Test	DF	P value
A = Cell legs (1)	371.0 (149.0) 138 - 567	B = Contra legs (1)	134.4 (48.1) 45 - 240	144 – 329.5	PT	11	0.0001
A = Cell legs (1)	371.0 (149.0) 138 - 567	B = Control legs (1)	152.2 (36.2) 93.0 - 214	128.6 – 309.1	PT	11	0.0001
A = Cell legs (1)	371.0 (149.0) 138 - 567	B = Cell legs (2)	176.4 (103.1) 21 - 436	106.6 – 282.6	PT	11	0.0001
A = Cell legs (2)	176.4 (103.1) 21 - 436	B = Contra legs (2)	153.8 (78.7) 16.0 – 280	-19.3 - 64.6	PT	11	0.259
A = Cell legs (2)	176.4 (103.1) 21 - 436	B = Control legs (2)	149.4 (41.2) 109 - 230	-56.1 – 110.1	PT	11	0.489
Contra legs (1)	45 - 240	*Contra control legs (1)	101 - 224		WSR		0.386
A = Contra legs (1)	134.4 (48.1) 45.0 – 240	B = Contra legs (2)	153.8 (78.7) 16.0 – 280	-51.8 – 12.8	PT	11	0.211
Contra legs (2)	16.0 – 280	*Contra control legs (2)	115 - 246		WSR		0.754
A = Control legs (1)	152.2 (36.2) 93.0 - 214	B = Control legs (2)	149.4 (41.2) 109 - 230	-26.6 – 32.1	PT	11	0.841

N = numbers compared (column 1 and column 3)
(1) = visit 1 (2) = visit 2

CI = confidence interval (A = parameter column 1, B = compared parameter column 3)
PT = Paired T-test
WSR = Wilcoxon Signed Ranks Test (exact 2 tailed test)
DF = degrees of freedom

= significant (P = 0.05)

Parameters for normal resting flow

In order to establish a set of parameters for normal resting flow the control images were compared with respect to the distribution of the histogram of flux values together with the mean, standard deviation and maximum flux values for the pixels within the AOI. A criterion representing normal was devised (value 2 in table 15). Using this as a reference AOIs with higher or lower overall flux values could then be defined (values 1 and 3). The higher values were further subdivided into high (value 3) and very high (value 4). The appearance of LDI images relating to these categories is shown in figure 34. Each AOI was then assigned to a value of 1, 2, 3 or 4 (see table 15). The results are shown in figure 35 and the raw data in appendix 32.

Value	Relative flux	Criteria
1	Low	80% of pixels < 75 relative flux units (RFU), mean = < 50 RFU, Max = < 250 RFU
2	Normal	80% of pixels < 250 RFU, mean = > 50 <240 RFU
3	High	80% 0f pixels > 200 RFU, mean = > 240 < 440 RFU
4	Very high	Absence of pixels below 100 RFU, 80% of pixels > 400 RFU, mean = > 440 RFU

Table 15 – Criteria for categorising resting flow by reference to mean/ maximum flux values and histogram distribution.

All controls showed resting blood results within category 2 for each leg on both occasions. All patients except three (P4, P7 and P8) exhibited a resting blood flow consistent with category 3 or category 4 during the cellulitic episode and normal values on the follow up visit. (P4) showed normal values (category 2) in the cellulitic leg at visit 1 and low (category 1) values in this leg at the second visit while in the contralateral leg category 1 values were recorded at both visits. By external appearance both legs were oedematous on both occasions and the ultrasound images revealed structural changes thought to be associated with oedema. Low flow in the microcirculation of both legs is clearly a permanent feature; despite this flow levels increased during the episode but only attained normal resting levels. (P8) displayed normal pallor in the calf skin of the affected

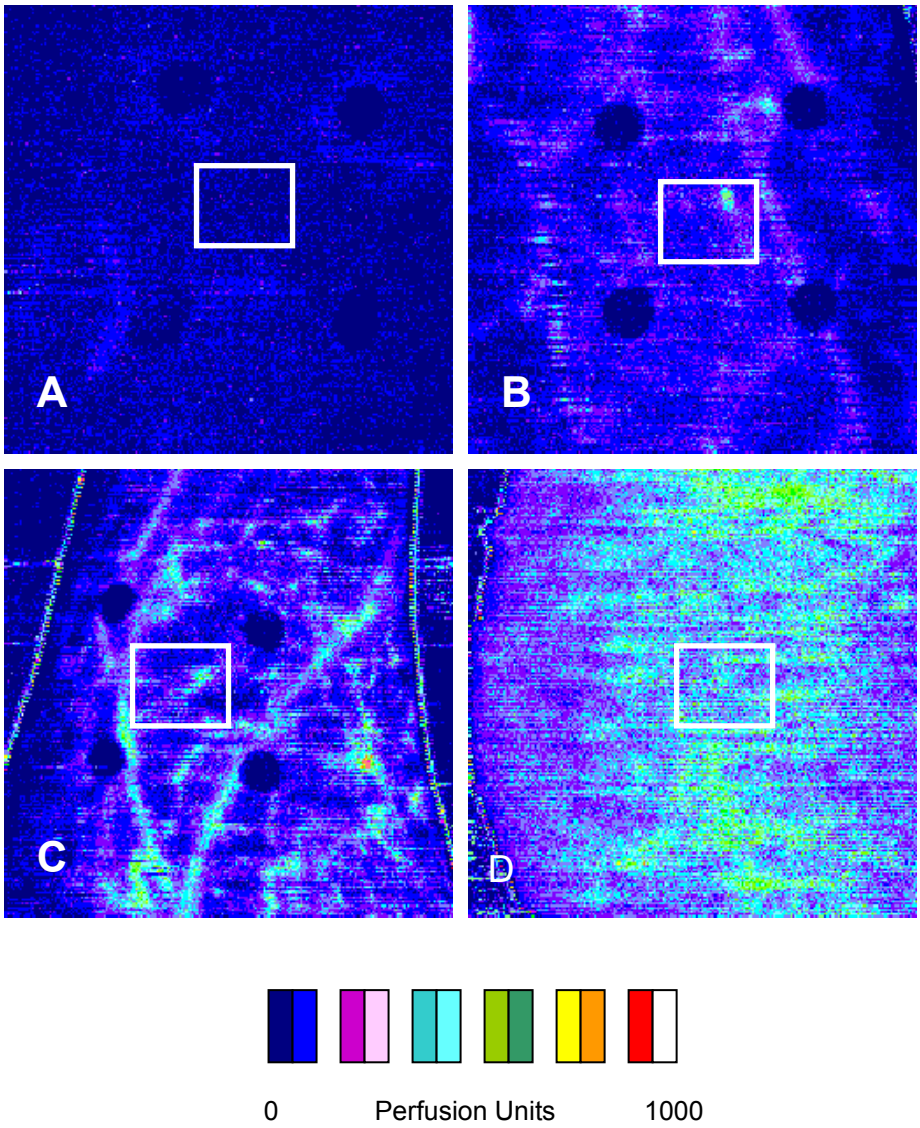
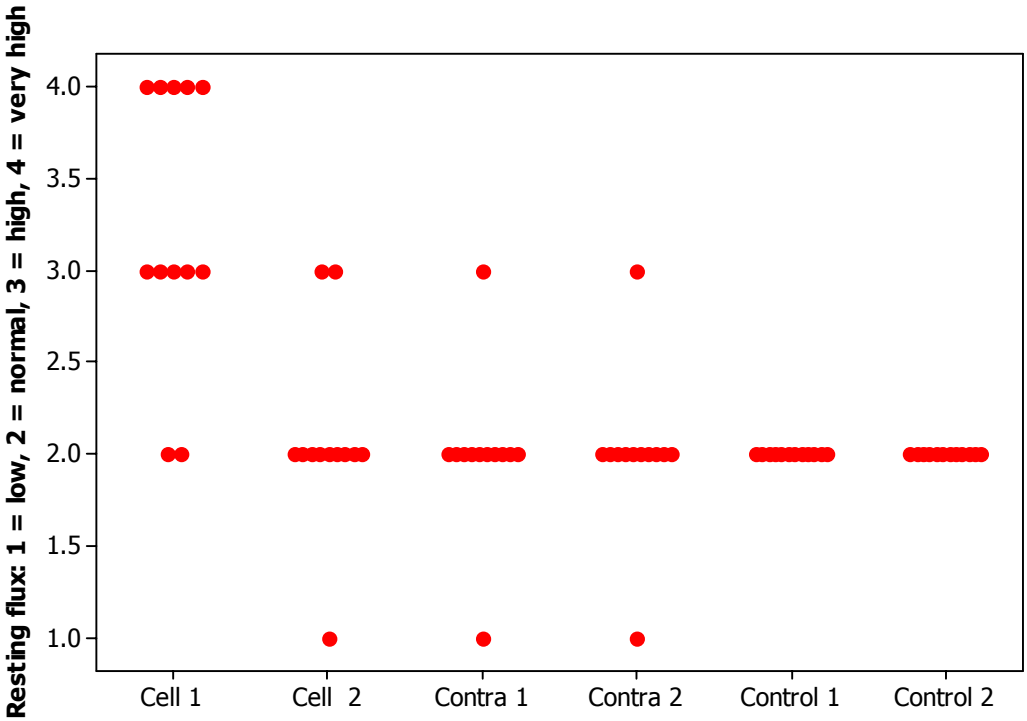


Figure 34 - LDI perfusion scans: skin of medial calf with participant in resting mode after 15 mins acclimatisation. Scan area (marked by black dots) and selected AOI (white box).
A = low perfusion
B = normal resting perfusion
C = high perfusion
D = very high perfusion (dots were not applied as this patient was unable to tolerate any contact)
(For classification criteria see table 15)

Figure 35 - Graph to show results for resting flow after categorisation according to table 15.



Cellulitic legs visit 1 (cell 1), cellulitic legs visit 2 (cell 2), contralateral legs visit 1(contra 1), contralateral legs visit 2 (contra 2) and control legs at both visits (N = 12).
All controls showed resting blood results within category 2 for each leg on both occasions. All patients except three (P4, P7 and P8) exhibited a resting blood flow consistent with category 3 or category 4 during the cellulitic episode and normal values on the follow up visit.

leg at the first visit. Six days had elapsed since the onset of the episode and although the skin had been red initially and the leg swollen there was a rapid response to treatment and swelling was thereafter confined to the foot. Resting relative flux measurements were found to be normal in both legs on both occasions. (P7) produced scores of 3 for both legs for both occasions. This patient was unable to remain still for the period of the scan and the flux patterns obtained were consistent with the presence of mild physical activity. It was impossible to estimate the contribution made by inflammation in the cellulitic leg although a consistent score of 3 on both legs suggests that it was low.

5.3.2.5 Assessment of venous incompetence

This assessment took place at the second visit and participants performed the exercise according to the protocol (see section 4.3.2.6). The data obtained was graphed in Microsoft EXCEL to produce a trace of the exercise routine (figure 36) and the time taken for the trace to return to baseline values (venous refilling time) was calculated. Times in excess of 25 secs were scored as competent and those less than 25 secs were scored as incompetent. Two patients were unable to comply with the calf exercise; P4 had oedema in the cellulitic leg and a dropped foot on the contralateral side, P6 was diabetic with neuropathy in both feet and was unable to coordinate his movements.

Statistical analysis was performed on the actual venous refilling times for the ten pairs of patients and controls (raw data is shown in appendix 33); this produced an uneven distribution and WSR tests were used (appendix 34). The raw data was also scored as competent and incompetent (competent = 1, incompetent = 2) this data is shown in appendix 35.

Incompetence was found to be statistically higher in the cellulitic legs as compared to the control cellulitic legs or the contralateral legs (P = 0.016 and 0.047) respectively (Table 16). Seven patients (70% of those tested) were found to be incompetent in the cellulitic leg (P3, P7, P8, P9, P10, P11 and P12) and six of these were also incompetent in the contralateral leg. The remaining patients (P1, P2 and P5) were found to be competent in both legs, although P5 recorded a much lower level of flux on exercise in the cellulitic leg, which was oedematous and had also been found to have a high rate of TEWL and textural skin changes. However he was young (24), otherwise physically fit and able to perform the test with ease.

Three members of the control group (30% of matched controls) were found to have incompetence. (C4 and C7) were incompetent in one leg and (C3) was incompetent in both.

These findings show a high rate of incompetence in the patient group involving both legs in most cases. In four cases incompetence was coincident with increases in leg circumference (P3, P7, P11 and P12) suggesting the link with oedema and altered skin physiology.

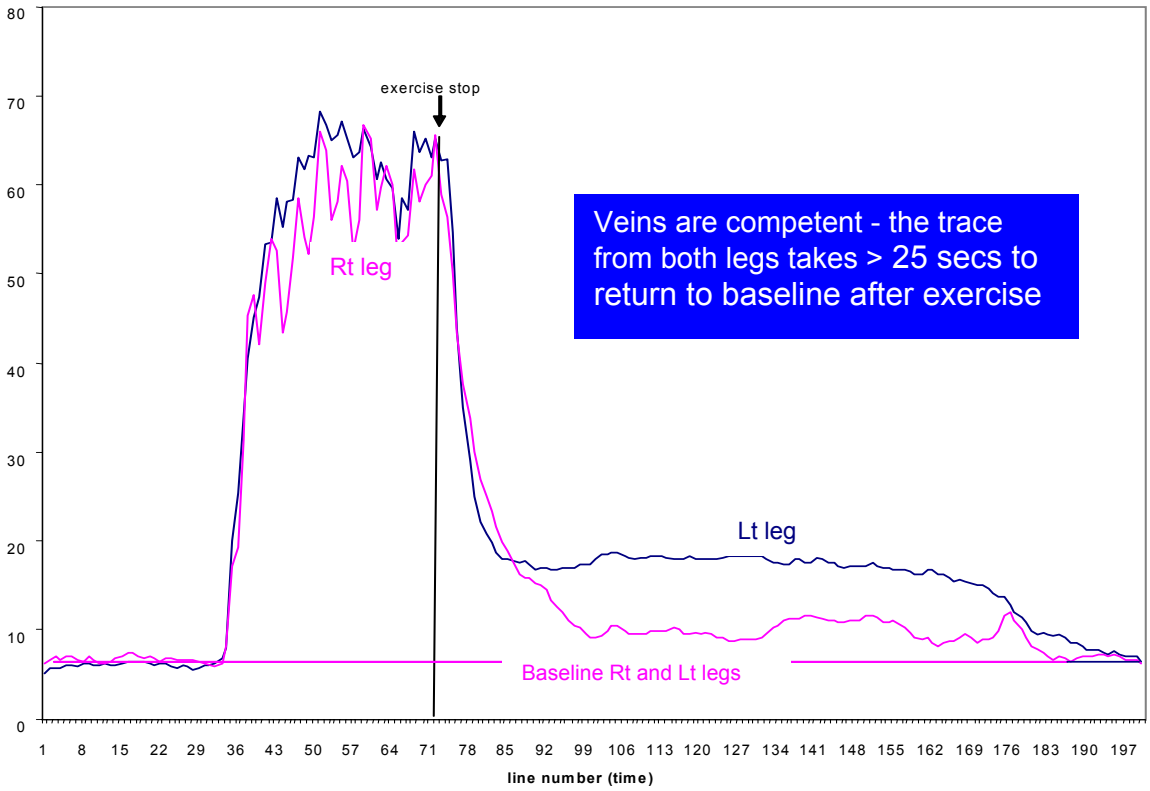


Figure 36a – LDF traces to show venous competence in both legs

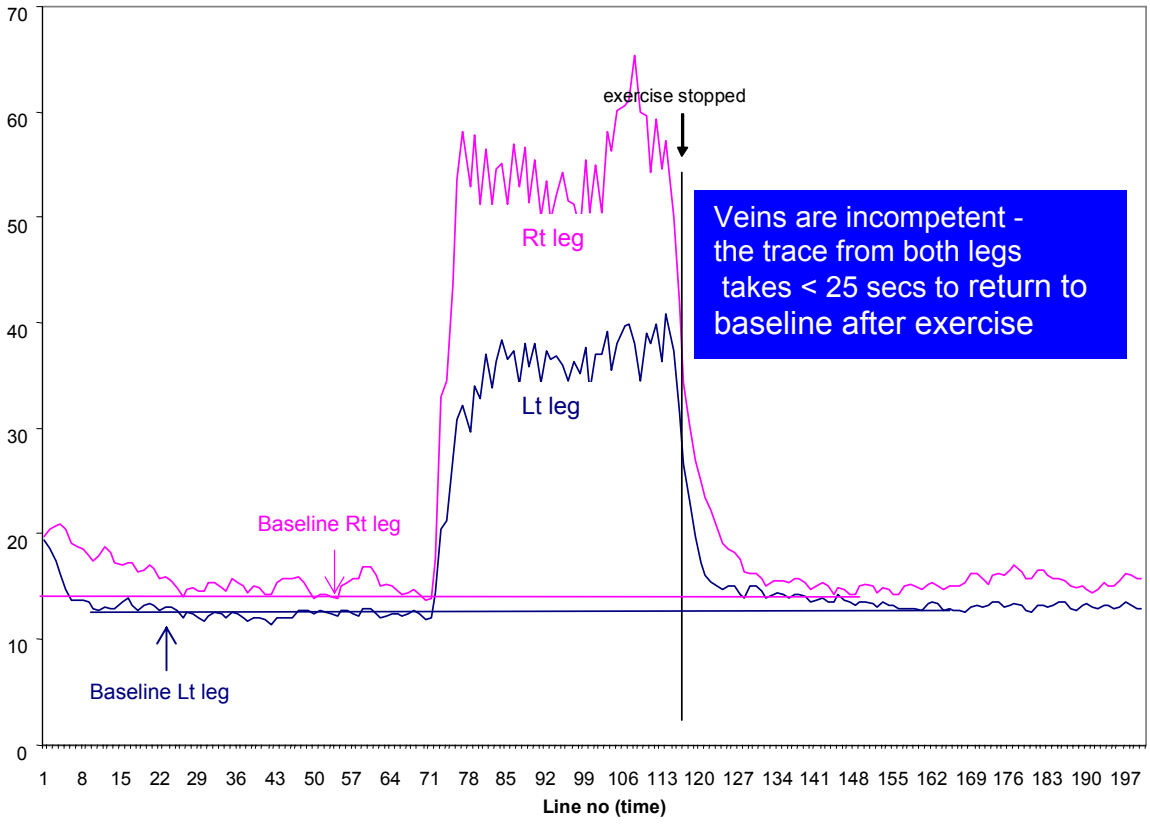


Figure 36b – LDF traces to show venous incompetence in both legs

Table 16 - Significance tests for venous refilling times (secs)

N = 10	Min-Max	N = 10	Mean	Test	P value
Cellulitic leg	5 - 30	Contralateral leg	5 - 30	WSR	0.469
Cellulitic leg	5 - 30	Control leg	18 - 30	WSR	0.016
Contralateral leg	5 - 30	Control contralateral leg	12 - 30	WSR	0.047

N = numbers compared (column 1 and column 3)

= significant (P = 0.05)

WSR = Wilcoxon Signed Ranks Test (exact 2 tailed test)

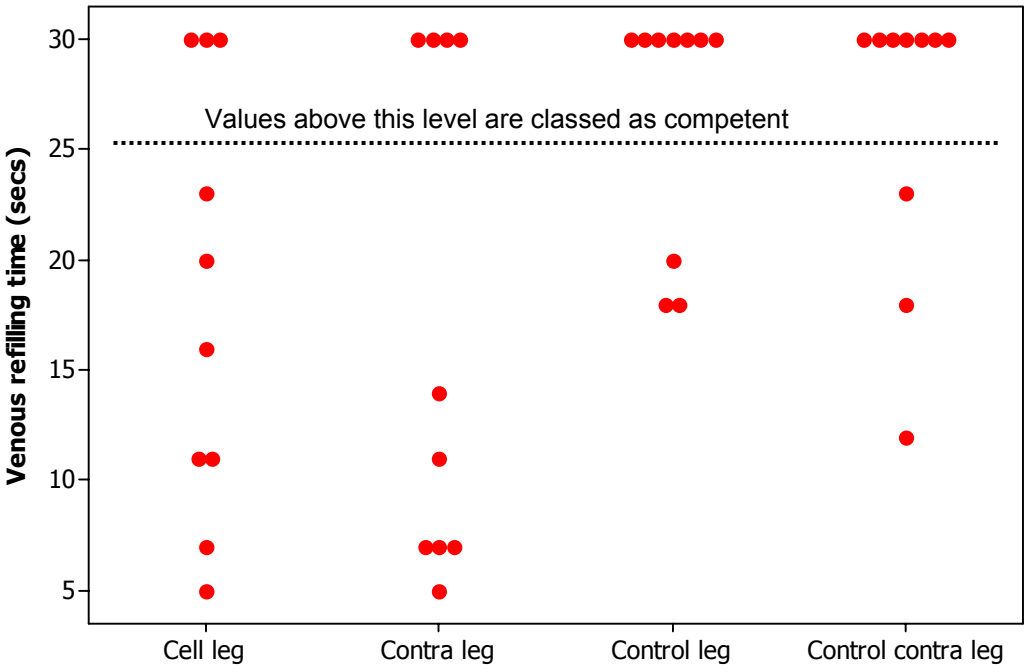


Figure 37
Graph to show venous refilling times for cellulitic legs (Cell leg), contralateral legs (Contra leg), control legs and contralateral control legs (N = 10). Values > 25 secs are classed as competent, values < 25 secs are classed as incompetent.
Incompetence was found to be statistically higher in the cellulitic legs as compared to the control cellulitic legs or the contralateral legs (P = 0.016 and 0.047).

5.3.3 Immunological Outcomes

5.3.3.1 Routine blood results

Full blood count (FBC) and CRP results for patients were obtained for alternate days throughout attendance at the ACU. All tests were repeated at the second visit and results were received, together with those for the controls, directly from the haematology department at SGH. Data sets were compared at two time points:

For patients, at the start of hospital treatment and at the follow up visit

For controls the results from the two consecutive visits.

The time interval between visits was matched between pairs to adjust for different lengths of antibiotic treatment. Statistical analysis was conducted for levels of CRP, numbers of white blood cells, neutrophils and lymphocytes and comparisons made between patients and controls as well as between visits (raw data are presented in appendix 36 and 37, distributions are shown in appendix 38 and 39). Levels of CRP for patients were statistically higher at visit 1 than at visit 2 ($p = 0.001$) and also statistically higher than controls between visits ($p = 0.001$). White cell count was also statistically higher in the patients between visits and between patients and controls at visit 1 ($p = 0.015$ and $p = 0.011$) respectively (see appendix 40). Lymphocyte levels only showed significance between visits in the patient group ($p = 0.034$) see table 17a. These results reflect the expected immune response to inflammation. Neutrophil counts were statistically higher in the patient group between visits ($p = 0.016$) and patient counts were also significantly higher than controls at the second visit ($p = 0.022$) and this was unexpected see table 17b. The mean value in the patient group was 4.31 ± 1.35 and in the control group 3.13 ± 0.73. Although these results all lie within the accepted range 2 -7.5 (10^9/L) the trend in the patient group is interesting and may be important.

Profiles of cell counts and CRP levels were constructed in order to examine the relevance of these findings. Examples are shown in figures 38a and 38b. 38a represents an acute case and plots the expected haematological response from day 1- day 25 of IV antibiotic therapy. High levels of CRP fall rapidly as the episode becomes controlled signalling a decline in first the neutrophil count and then the white cell count while lymphocyte levels gradually return to normal.

For most patients little data exists for the first few days of the episode when treatment is carried out in primary care. Figure 38b (P8) shows a limited blood

Table 17a
Significance tests for Lymphocytes (10^9/L)

N = 12	Mean (SD) Min-Max	N = 12	Mean (SD) Min-Max	95% mean CI (A - B)	Test	DF	P value
A = Patients (1)	1.433 (0.693) 0.6 – 3.3	B = Patients (2)	1.733 (0.667) 0.6 – 3.0	-0.574 - 0.026	PT	11	0.034
A = Controls (1)	1.642 (0.452) 0.9 – 2.5	B = Controls (2)	1.675 (0.494) 1.0 – 2.6	-0.187 – 0.121	PT	11	0.643
A = Patients (1)	1.433 (0.693) 0.6 – 3.3	B = Controls (1)	1.642 (0.452) 0.9 – 2.5	-0.759 – 0.342	PT	11	0.422
A = Patients (2)	1.733 (0.667) 0.6 – 3.0	B = Controls (2)	1.675 (0.494) 1.0 – 2.6	-0.458 – 0.575	PT	11	0.808

Table 17b
Significance tests for Neutrophils (10^9/L)

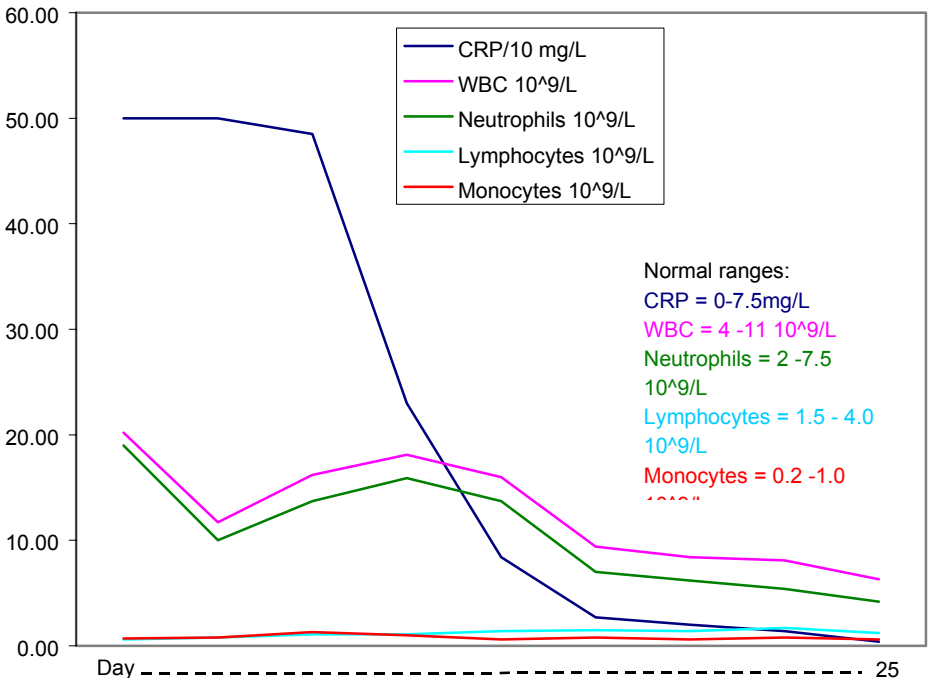
N = 12	Mean (SD) Min-Max	N = 12	Mean (SD) Min-Max	95% mean CI (A - B)	Test	DF	P value
A = Patients (1)	6.54 (3.175) 1.9 - 12	B = Patients (2)	4.31 (1.357) 1.9 – 6.9	0.494 – 3.972	PT	11	0.016
A = Controls (1)	3.233 (0.611) 2.4 – 4.3	B = Controls (2)	3.133 (0.73) 2.3 – 4.3	-0.268 – 0.468	PT	11	0.562
A = Patients (1)	6.54 (3.175) 1.9 - 12	B = Controls (1)	3.233 (0.611) 2.4 – 4.3	1.259 – 5.357	PT	11	0.005
A = Patients (2)	4.31 (1.357) 1.9 – 6.9	B = Controls (2)	3.133 (0.73) 2.3 – 4.3	0.209 – 2.141	PT	11	0.022

N = numbers compared (column 1 and column 3)
(1) = visit 1 (2) = visit 2

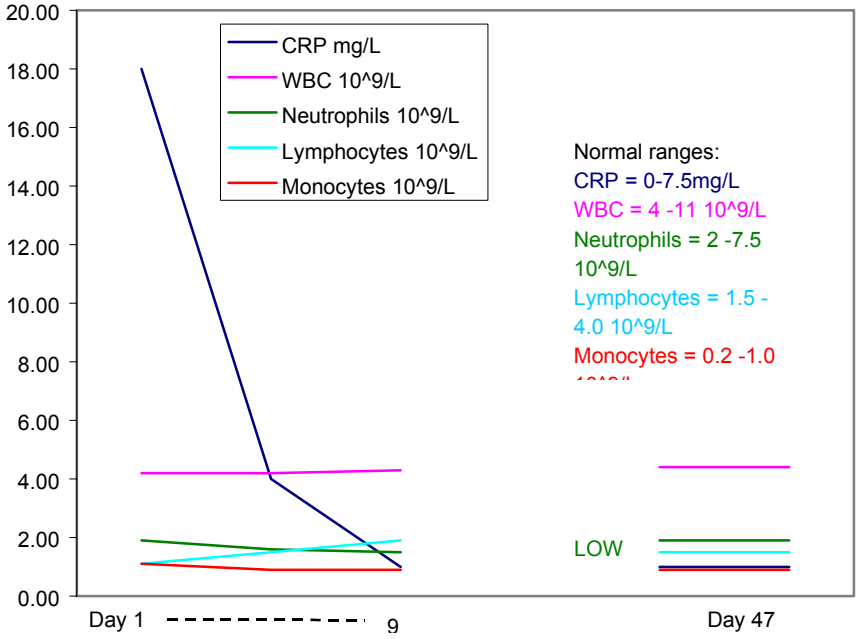
CI = confidence interval (A = parameter column 1, B = compared parameter column 3)
PT = Paired T-test
DF = degrees of freedom

= significant (P = 0.05)

Figure 38 – Blood profiles



38a - Blood profile acute episode day 1 – day 25 of IV antibiotics



38b - Blood profile of (P8) Day 1-9 of IV antibiotics and follow up visit day 47

profile starting at day 5 of the episode and suggests that both the white cell count and the neutrophil count remained low throughout the episode and failed to reach normal limits even by the time of the second visit. Other patients show individual variations from the normal blood cell and serum protein ranges, (P7) and (P12) exhibit enduring lymphopenia and (P6) shows a high final value for CRP and was found to culture pseudomonas at the second visit. P6 and P12 were both suffering from diabetes.

Although the numbers are small some interesting results have emerged in particular the difference in neutrophil counts between patients and controls at the second visit. These findings may be important and need to be considered in association with the immunology results. The data between acute and recurrent groups was analysed using the MWU but no significance was found.

5.3.3.2 Cytokine results

Cytokine assays were carried out on duplicate plasma samples from five pairs of patients and controls (P1/C1, P2/C2, P5/C5, P6/C6 and P7/C7) according to the protocol set out in 4.3.3.3. This was a preliminary experiment to ascertain detection levels for the ten cytokines predictive of T_H1 and T_H2 response (see table 3) and to ascertain possible links with the routine blood profiles already described.

Detectable levels of all 10 cytokines were found (see table 18).

	INF-γ	IL-1β	IL-2	IL-4	IL-5	IL-8	IL-10	IL-12	IL-13	TNF-α
Max	17.40	0.60	3.02	5.69	448.7	3.90	1559.0	4625.0	1213.0	7.09
Min	0.379	0.03	0.05	0.105	0.03	0.008	0.106	0.082	0.262	0.041

Table 18– Maximum and minimum values of detected cytokines (pg/mL) in plasma. Patients and matched controls (n = 5).

The standard curves for all cytokines produced good correlation throughout the range. The coefficient of variation (C of V) for pipetting into each well did not exceed 16%, and most were below 10%. The mean values of adjacent samples were calculated and the results for the five pairs of patients and controls are shown in appendix 41.

Because one patient (P2) produced very high results for some cytokines some distributions were abnormal (appendix 42– 46) and WSR tests were used to compare data. For normal distributions paired t tests were performed. Statistical

analysis was not performed between acute and recurrent cases because the numbers were too low.

No significant differences was found between the data at p = 0.05. However results between patients and controls at visit 1 for levels of IL-12 and IL-8 were close to significance (P = 0.063) see table 19 and 20 and appendix 47 – 50. This would suggest a pro inflammatory pattern consistent with the cellulitic episode.

Table 19 - Significance tests for serum IL-8 results (pg/ml)

N = 5	Min - Max	N = 5	Min - Max	Test	P value
Patients (1)	1.05 -3.9	Patients (2)	0.43 – 2.19	WSR	0.313
Controls (1)	0.35 – 0.81	Controls (2)	0.31 – 3.27	WSR	0.188
Patients (1)	1.05 -3.9	Controls (1)	0.35 – 0.81	WSR	0.063
Patients (2)	0.43 – 2.19	Controls (2)	0.31 – 3.27	WSR	0.625

Table 20 - Significance tests for serum IL-12p70 results (pg/ml)

N = 5	Min - Max	N = 5	Min - Max	Test	P value
Patients (1)	0 - 3511	Patients (2)	0 - 4625	WSR	0.188
Controls (1)	0 – 1.160	Controls (2)	0 – 1.380	WSR	0.625
Patients (1)	0 - 3511	Controls (1)	0 – 1.160	WSR	0.063
Patients (2)	0 - 4625	Controls (2)	0 – 1.380	WSR	0.125

N = numbers compared (column 1 and column 3)
(1) = visit 1 (2) = visit 2

WSR = Wilcoxon Signed Ranks Test (exact 2 tailed test)

= P value close to 0.05

P2 shows elevated levels of all ten cytokines at both visits. IL-10, IL-12 and IL-13 are exceptionally high and most of the results were higher at the second visit. Following antibiotic treatment this patient developed a diffuse skin rash with fluid filled vesicles covering his face, trunk, arms, face, head and ears. The medical history described an 'id' reaction thought to be secondary to a systemic fungal infection possibly caused by scopulariopsis (a dermatomycosis grown from both feet in this study). Swabs taken from the ears grew SA. The routine blood profile was unremarkable but levels of IgE were high (474iu/Ml at visit 1 and 373iu/mL at visit 2), moderate levels of IgE to TR were recorded but no dermatophytes were grown. Elevated levels of IgE, IL-4 and IL-5 would support a systemic fungal

infection while the high levels of pro inflammatory cytokines particularly IL-12 would suggest a superantigen response to SA. The patient was treated with systemic antifungals and topical antifungal cream. There was no recurrence within six months.

The MSD ELISA was able to detect high and low levels of all ten cytokines from the samples of peripheral plasma tested in this study. Unfortunately the number of samples tested was low so no definite comparisons can be made although the results for P2 are in accordance with the clinical evidence. Raised levels of IL-12 and IL-8 in the patient group confirm a proinflammatory response and might reach significance if the samples from all twelve pairs were tested. The higher numbers of neutrophils recorded in the routine blood results suggest that increased levels of IL-8 should be seen particularly at the second visit, this is not evident from the existing data but may be because the numbers are low. These results suggest a definite role for the TH_1/TH_2 ELISA and a need to complete the analysis in order to better understand the data.

5.3.3.3 IgE results

Serum samples to be tested for levels of IgE were processed by ELISA in batches of 16 within the Immunology Department at SGH. Results for the twelve pairs are presented in figure 39 and appendix 51. Values were higher in the patient group at both visits and the distribution was uneven (appendix 52) therefore WSR was used for the statistical analysis and the results are shown in appendix 53.

No statistical differences were found between the patient and control groups at either visit. No statistical differences were found between acute and recurrent patient groups.

Levels of serum IgE vary within the population and the range is wide. The cut off for normal value is generally taken as 81 iu/mL but 1 standard deviation only includes 60% of normals (Ownby 1998). Furthermore a condition known as hyper IgE syndrome exists where individuals consistently display IgE levels in excess of 1000 iu/mL sometimes with no other apparent abnormality (Desai et al 1996, Hochreutener et al 1991). Five patients and four controls produced elevated levels of serum IgE (P2, P5, P6, P7 and P11,C4, C5, C6 and C9). Although two patients (P2) and (P5) showed concomitant raised levels of IgE to TR others (P6) and (P7) did not. Similarly, in the control group raised levels in (C2, C5, C6 and C9) were accompanied by elevated levels of IgE to TR in only two (C6 and C9).

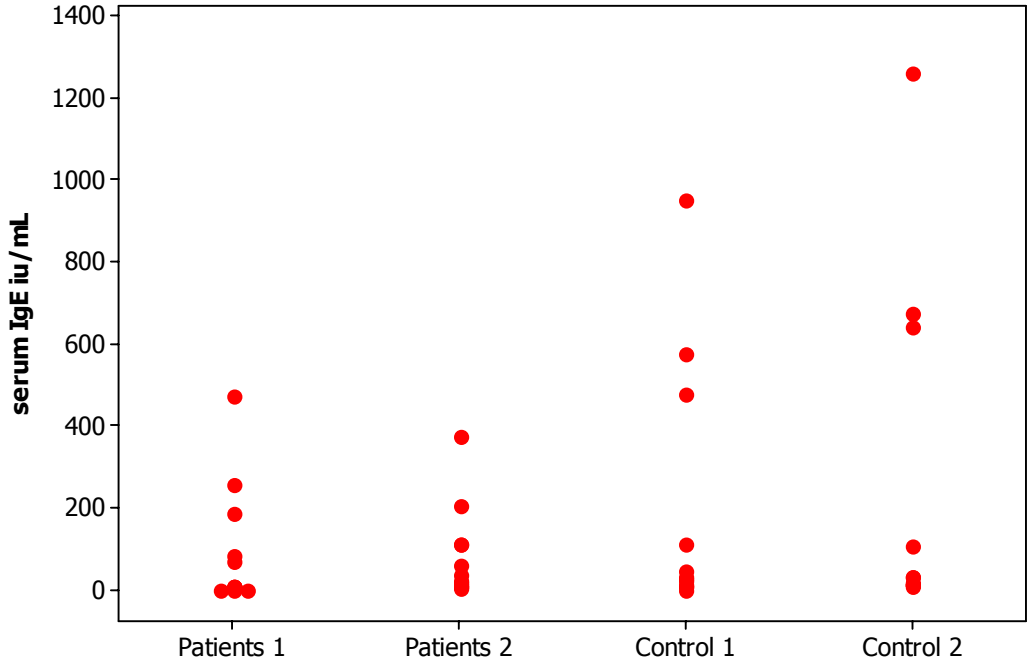


Figure 39
Graph to show IgE levels in peripheral blood. Patients and controls (N = 12) visits 1 and 2.
No statistical differences were found between the patient and control groups at either visit

If we consider the various possible allergic responses (immediate, delayed and anergy) interpretation of these results as a set may be unhelpful. It may more informative to review the cases separately and with reference to the results of other individualised tests.

5.3.4 Stress Investigations

5.3.4.1 Medical history

Details of medical history were obtained verbally from participants and also from the medical records and categories for data analysis were selected (see section 4.2.3.4 and table 6). For each variable scores of 0 (absent) or 1 (present) were recorded for each patient and matched control and McNemar's test was performed on the paired binary data sets. Comparison between acute and recurrent cases was made using MWU because of the small and unequal numbers in each group. In eight categories results were statistically higher in the patient group as compared with the control group (see table 21). No statistical differences were found in any category between acute and recurrent cases (data not shown).

The patient group recorded a much greater number of previous bacterial infections (p= 0.008) and recurrent skin rashes, which were not attributed to cellultiis (p = 0.016) they also recorded a greater prescription of antibiotics throughout their medical history aside from those prescribed for cellulitis (p = 0.031). Patients were found to have more allergies (food, drugs, contact sensitivities) than the control group (p = 0.008) when asthma was not included; asthma alone was not significant. Many more patients recorded fissuring on the feet and this was significant (p = 0.016); previous history of athletes foot, other fungal infections or powdery skin on the foot were however insignificant. History of surgery, trauma or injury to the cellulitic leg as opposed to the control cellulitic leg did not reach significance between the groups (p = 0.125) however when history of abdominal surgery was added the result was significantly higher in the patient group (p = 0.031) and became greater (p = 0.008) when surgery, trauma or injury to the contralateral leg was also included. Oedema, defined as persistent leg swelling perceived by the patients or as documented in the medical history and occurring after the cellulitic episode used in this study, was recorded in nine (75%) out of twelve patients but not in controls. The significance for this was p = 0.002. Thrombophlebitis, cancer, diabetes or being overweight did not achieve significance in this study; previously Cox (2006) and Naguib et al (2004) have found associations between cellulitis and both diabetes and cancer, in retrospective studies but with much larger numbers.

5.3.4.2 Results of the stress questionnaire

All participants completed the stress questionnaire at the first visit. Many also reported specific incidents or ongoing stress at that time although this was not confined to the patient group. The design of the questionnaire requires that responses should be rapid in order to gain the first impression of the participant without in depth analysis and therefore capture their perception without reserve. The subtlety is achieved by slight differences between the questions and by interspersing positive and negative concepts. The suggested time allowance to answer the fourteen questions was approximately 5 minutes and participants were urged to keep within this limit. Despite this most participants took much longer and consequently many expressed problems with in depth interpretation of the questions, commenting that many were ambiguous or repetitive.

The scoring for each line is shown in appendix 1. The minimum obtainable score

Table 21 - Significance tests for medical history (Patients and Controls)

Scores of 0 or 1 were recorded for each of the categories for each patient and matched control
0 = negative response or no evidence in medical history
1 = positive response or evidence in medical history
McNemar's test was performed between patients (N = 12) and controls (N = 12) for each variable.

Variable	Number and % of patients in which variable was present	Number and % of controls in which variable was present	P value
History of skin or mucosal infections assumed to be caused by bacteria	9/12 = (75%)	1/12 = (8%)	0.008
Recurrent skin rashes (not cellulitis)	8/12 = (66%)	0/12 = (0%)	0.008
Allergies including asthma	11/12 = (92%)	4/12 = (33%)	0.008
Presence of fissures on feet	7/12 = (58%)	0/12 = (0%)	0.016
Previous abdominal surgery/ injury, trauma, surgery to cellulitic leg	9/12 = (75%)	3/12 = (25%)	0.031
Previous abdominal surgery/ injury, trauma, surgery to cellulitic or contralateral leg	11/12 = (92%)	3/12 = (25%)	0.008
Persistent oedema (post study) in cellulitic leg	10/12 = (83%)	0/12 = (0%)	0.002
Prescription antibiotics ++	7/12 = (58%)	1/12 = (8%)	0.031
Previous history of Athletes foot	8/12 = (66%)	8/12 = (66%)	1.0
Previous history of any fungal infection	9/12 = (75%)	8/12 = (66%)	1.0
Powdery skin on the foot sole	5/12 = (42%)	1/12 = (8%)	0.125
Dry skin on legs	5/12 = (42%)	0/12 = (0%)	0.063
Previous injury /trauma/surgery to cellulitic or contralateral leg	7/12 = (58%)	2/12 = (16)	0.125
History of thrombophlebitis	3/12 = (25%)	1/12 = (8%)	0.500
Asthma	2/12 = (16%)	2/12 = (16%)	1.0
Overweight	5/12 = (42%)	1/12 = (8%)	0.125
History of cancer	2/12 = (16%)	2/12 = (16%)	1.0
Diabetes	2/12 = (16%)	0/12 = (0%)	0.5

= significant (P = 0.05)

= P value close to 0.05

was 0 and the maximum 56. The results were discrete data and WSR was used for analysis.

Min – Max values for the patients were 10 - 41 points and for the control group 11 – 27 points and the raw data is shown in appendix 54. Figure 40 shows the recorded scores relating to the two groups. Patient scores were statistically higher than those for controls (p = 0.052) see table 22, but no difference was found between the results for acute as opposed to recurrent cases (data not shown). Ten patients (83%) recorded higher scores than their matched controls. P11 was the only patient to record a lower score (10 points as compared to 23 points from the matched control). The oldest pair, aged between 70 and 80 years, were equal with the lowest scores (11 points each), apart from this no age related scoring patterns were found between the groups. The results are difficult to interpret because of the difficulties already mentioned. But these difficulties were experienced by most of the participants and the data may therefore be sound. Although the concept of stress evaluation by this means fits well with this study the choice of questionnaire needs to be reconsidered since the one selected proves to have been a lot less 'user friendly' than expected.

Table 22 - Significance test for the stress questionnaire

N = 12	Min - Max	N = 12	Min - Max	Test	P value
Patient scores	10 - 41	Control scores	11 - 27	WSR	0.052

N = numbers compared (column 1 and column 3)

WSR = Wilcoxon Signed Ranks Test (exact 2 tailed test)

= significant (P = 0.05)

5.4 Summary of results

It was difficult to recruit patients into this study and the expected numbers (30 patients divided equally into acute and recurrent cases) was not realised. In the event 12 patients (five acute and seven recurrent) completed both visits and were successfully matched with controls. Even with few matched pairs a large amount of data was generated, thirteen different tests or investigations were carried out and ten of these required data collection from both visits.

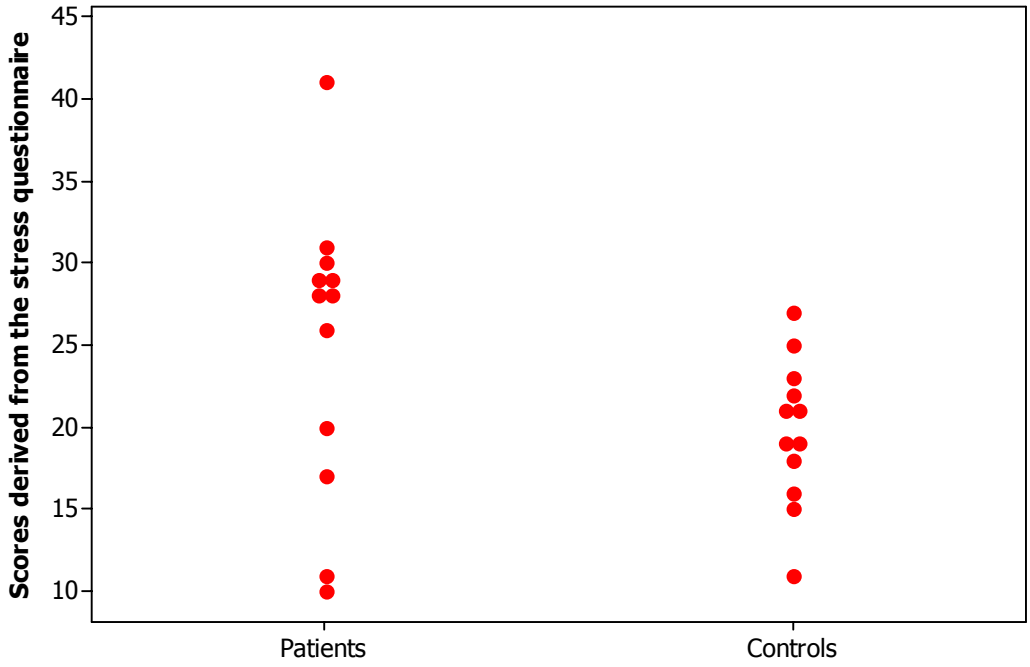


Figure 40
Graph to show results of scoring from the stress questionnaire (N = 12) Patient scores were statistically higher than those for controls (p = 0.52) see table 22. The oldest matched pair, aged between 70 and 80 years, produced the lowest scores (11 points each), apart from this no age related scoring patterns were found between the groups.

The results presented in this section have been subdivided into four sections, investigation for microorganisms (particularly dermatophytes), skin structure/ physiological tests, immunological outcomes and stress factors. A summary of these results is presented in table 23 and their implications will be discussed in the next chapter.

Table 23 – Summary of results

Risk factor test/investigation	Results
Dermatophytosis *Tests :Specimen collection, culture and microscopy Serum immunoglobulin to Trichophyton Rubrum*	The frequency of dermatophytosis and superficial dermatomycoses was lower in the patient group than the control group. No significance was found between the matched groups for any of the cultured organisms. Overall the study population produced more cultures from the foot sole than the ID spaces. Presence of IgE specific to TR was demonstrated in all subjects who cultured TR and also in 2 patients who did not thus providing proof of previous exposure.
Changes in skin water loss *Test: Measurement of Transepidermal water loss (TEWL)*	Both patients and controls produced some TEWL readings that were lower than the expected normal. No significant differences were demonstrated between patients and controls at visit 1 but TEWL was found to be significantly higher in the cellulitic legs as compared to contralateral legs and control legs at visit 2.
Changes in pH balance at the skin surface *Test: Measurement of skin pH*	A statistically significant difference was found between contralateral legs and contralateral control legs at visit 1, likely due to the unusual distribution of values from the contralateral legs and the low sample size. No other statistically significant differences were demonstrated between groups at either visit.
Oedema *Tests: Leg circumference measurements + Ultrasound tissue measurements*	Circumference measurements taken at the ankle and mid calf were significantly higher in patients as compared to controls on both occasions. Dermal measurements were significantly greater in the cellulitic legs as compared to the cellulitic control legs at visit 1. Comparison of the cellulitic legs between visits, and the cellulitic legs and contralateral legs at both visits almost reached significance but numbers tested were low. Image analysis supports the presence of dermal oedema in cellulitic legs and suggests a 'unique' fluid retention pattern in those with chronic oedema.
Changes in skin blood flow *Test: Measurement of skin blood flow Laser Doppler Imager (LDI)*	Mean flux values from the cellulitic legs were significantly higher at visit 1 than at visit 2 and also significantly higher than controls or contralateral legs at the first visit. Values returned to within normal ranges for all except two patients at visit 2. These patients produced lower than average scores on both occasions consistent with their enduring oedema.
Venous incompetence *Test: Photoplethysmography Laser Doppler flowmeter (LDF)*	Venous incompetence was found to be statistically higher in the cellulitic legs as compared to the control cellulitic legs or the contralateral legs. In four patients (66%) incompetence was coincident with increases in leg circumference suggesting the link with oedema and altered skin physiology.

Table 23 continued

Risk factor test/investigation	Results
Abnormal haematological response to inflammation/infection *Test: Blood profiles*	Levels of CRP and WBC for patients were statistically higher at visit 1 than at visit 2 and also statistically higher than controls between visits. WBC was also statistically higher between patients and controls at visit 1. Lymphocyte and neutrophil levels were significant between visits in the patient group. These results reflect the expected immune response to inflammation. Unexpectedly, neutrophil counts for patients were found to be significantly higher than controls at the second visit.
Exaggerated immune response *Test: Serum cytokine profiles*	5 paired samples were tested for cytokine data and detectable levels of all10 cytokines were obtained. No significant differences were found between the data however results between patients and controls for levels of IL-12 and IL-8 at visit 1 were close to significance suggesting a pro inflammatory pattern consistent with the cellulitic episode. Values of serum IgE were higher in the patient group at both visits but no statistical differences were found between the patient and control groups at either visit. Results from one patient showed > levels of IgE, IIL4 and IL-5 and > levels of pro inflammatory cytokines particularly IL-12.
Allergic response *Test: Serum (IgE)*	
Previous episode Co-morbidity Previous surgery etc *Investigation: Patient history*	The patient group recorded higher frequencies of the following as compared to controls • previous bacterial infections and recurrent skin rashes • prescription of antibiotics throughout their medical history • allergies (food, drugs, contact sensitivities) • fissuring on the feet (significant) • history of surgery/trauma or injury to the cellulitic leg/abdominal surgery (significant) • oedema (significant)
Psychological stress *Investigation: Stress questionnaire*	The total scores for the patient group were statistically higher than for the control group.

CHAPTER 6

DISCUSSION

6 DISCUSSION

There are two major problems affecting the management of patients with cellulitis. The first is the lack of proof concerning the nature of the infective organisms and the second is an uncertainty that exists in many cases relating to the mechanism by which the assumed causal pathogens access the skin. There is no doubt that new evidence concerning either of these would consolidate the treatment and advance the future management. In recent years several studies have attempted to address the former but the enigma surrounding the inability to culture the causal pathogens remains. The latter problem has received more attention and has mainly been directed towards the investigation of predisposing factors assuming that cellulitic skin is either more vulnerable to pathogens or that portals of entry must be created in order to provide assess to the skin. The proposed effector mechanisms include dermatophytosis, portals of entry on the feet, venous incompetence and its sequelae cutaneous oedema as well as other universally accepted risk factors such as obesity and diabetes (Cox 2006, Björnsdóttir et al 2005, Ryan 2005, Roujeau 2004, Clark et al 2003, Dupuy et al 1999, Harrison et al 1993). Although these causal factors are constantly put forward none of them seems to have been investigated in detail, for instance oedema has been defined only as persistent leg swelling and dermatophytosis has often been assumed by findings of toe web intertrigo but without reference to culture or microscopy. Moreover they are all ubiquitous which raises the need to establish their true relationship with episodes of cellulitis.

Under normal conditions commensal flora and pathogens breeching the skin remain confined to the affected area and are dealt with by localised immune skin residents. Visual evidence, such as pain and swelling, together with haematological markers, indicate that the expected infective/inflammatory response is in progress and after an accepted time course the event subsides without recourse to intervention. Cellulitic events appear to differ in some aspects and previous studies highlight the following unexplained deviations:

- Swabs/aspiration from the infected site rarely produce positive cultures (Karakas et al 2002, Sachs 1991)

- Although extensive areas of skin are involved the number of organisms obtained from positive cultures is often very low (Shelley 1995)
- A portal of entry is not always identified (Choo 2004, Bernard et al 1989)
- Although blood markers provide proof of systemic inflammation the skin infection is usually confined to one anatomical site (Stryjewski et al 2007)
- Recurrences predominantly occur at the same site (Badour and Bisno 1984)
- Some episodes require many courses of antibiotic therapy to achieve resolution (Stulberg 2002)
- The time lapse between recurrent episodes is variable; can be days, weeks, months or years (Karakas et al 2002, Badour and Bisno 1984)

Observations that the paucity of cultured organisms bears no relationship to the extent of skin involvement, that few patients have identifiable portals of entry and that recurrence often occurs at the previous site with unpredictable time lapses between events, have become the subject of much speculation and lack of conclusive proof for the accepted risk factors permit conjecture for alternative mechanisms. Implications for a prime role for immune involvement is appealing; concomitant with this is the inference that skin physiological defects such as oedema may be an effect rather than a cause. Despite this, no previous studies have investigated the relationship between cellulitis and the immune response, and this is surprising when associations between immunology and other clinical areas such as fungal disease and stress related skin conditions have been investigated in some detail and continue to provoke interest. However before any relationships can be established it is important to precisely define the features of individual factors in order to make meaningful advances upon the existing knowledge.

The risk factors fall into three main categories; stress factors (including microorganisms), skin physiology and immune responses (see figure 4).

This pilot study was designed to re-examine more precisely the accepted risk factors, contribute additional information regarding immunological outcomes and assess the possible relationships between these factors. The ultimate aim was to pursue areas worthy of further investigation in a subsequent larger scale study. As

part of this it was also necessary to evaluate the suitability, reliability and feasibility of the chosen investigations within an ambulatory care setting.

Aside from issues of debilitation, this work is clinically important because the rate of recurrence amongst cellulitis patients is high and the extensive prescription of antibiotic treatment appears to be the only available mainstay. It is now well known that the continuous usage of antibiotic treatment impairs the effectiveness of future courses (Del Mar 2007, Goossens 2005) and may also contribute to the current high rate of nosocomial infections; moreover patients are now well informed and most would prefer to choose alternative treatments which carry less long term risk, if these are effective. If such treatments could be found the benefit to both patients and NHS resource would be great.

6.1 The study

A case controlled study was selected and an ambulatory care setting chosen in order to access a study population providing a relatively straightforward comparison between patients with cellulitis and matched controls by creating groups with minimal other medical complications. Previous prospective case controlled studies have involved hospitalised patients with controls matched for age and sex selected from the in patient population undergoing treatment for various acute conditions. Southampton General Hospital provides the facility for ambulatory care available to those who are fit enough to travel and have the necessary home support. These patients tend to have less comorbidity and when matched with healthy control subjects provide a more straightforward comparison than patients hospitalised for other illnesses. In this study mobility along with age and sex was included in the matching because physical activity was considered important in relation to venous incompetence and oedema, and also because mobile patients and healthy controls were more likely to encounter a mixture of indoor and outdoor environmental conditions than those whose movements were restricted by disability.

It was important that the initial patient visit be repeated at a future date in order to compare results and establish a patient baseline for comparison with controls. The nature of cellulitic disease suggests that some normal physiological or immunological factors may be absent or impaired and that this predisposes certain individuals to attacks. Whether this would be a prerequisite or a consequence is one of the questions yet to be answered, unfortunately in such cases previous

baseline information is unavailable so subsequent or serial investigation is the only choice.

One aim of the pilot study was to assess the logistics of recruiting patients from an acute ambulatory unit and concurrently conduct a study in a research facility in a separate part of the hospital; as anticipated it proved to be quite difficult to coordinate patient treatment and study requirements. Patients recruited to the Ambulatory Care Unit (ACU) are mobile and attend from home each day. Preliminary assessment and haematological results are required to inform the intravenous antibiotic treatment, which may take 2-3 hours. The clinic can be very busy as new referrals are accepted on the day and fitted in amongst previous bookings. Because of limited bed/cubicle space and fixed levels of staffing waiting times are often long and the study protocol added an extra 1–1½ hours to time spent at the hospital; many patients were experiencing fever and pain and felt unable to cope with the protocol or with the extra time required. Many were willing but could not guarantee a second visit often because of family commitments or because self employment meant loss of earnings for a subsequent visit. Some who consented were lost because it was not possible to use the Wellcome Research Facility at weekends or on bank holidays, this meant that patients on short courses of IV therapy (3 days) who were recruited on Fridays and consented at the weekend, were discharged with oral therapy before a preliminary visit could be undertaken.

From the investigators point of view coordination between the units was problematic. The research facility is situated some distance from the treatment unit and requires to be prebooked, in addition the time needed to set up and test the equipment is around 45 mins. On many occasions rooms were booked and equipment set up in anticipation of patients who subsequently failed to arrive. Return visits and appointments for controls were however easier to organise as these could be prebooked well in advance with some flexibility to fit in with the requirements of the participants and research unit alike.

It was therefore difficult to recruit patients into this study and the numbers who succeeded in completing both visits were low, producing only twelve pairs by the end of the time period set aside for recruitment. These numbers would have been larger but data obtained from a single visit was not included in the analysis, neither were single visits matched with controls because interpretation of results was very

dependant on comparisons obtained between visits one and two. For instance TEWL values recorded from cellulitic legs would be expected to decrease significantly between visits because of decreased blood flow and heat dissipation, they would also be expected to be significantly higher than controls at visit one for the same reason. Because they should essentially be normal at visit two no differences should be seen with comparison between any group at that time. A finding of significant differences between patients and controls would therefore be more important in relation the second visit.

The value of the physiological and immunological evidence depends on the establishment of baseline reference points for which it is important to have at least two measurements, this is required to aid in deciding whether a result is normal and also detect changes in the measurement system. This would apply to all physiological and immunological tests conducted in this study, where comparison between results from two points is minimal although serial recordings taken over time would be more advantageous.

Although only twelve matched pairs completed both visits there were many comparisons and a large amount of data was generated. It was hoped to recruit equal numbers of acute and recurrent cases but this was not the case and when recruitment ended only five acute and seven recurrent cases had taken part. No statistical differences were demonstrated between these groups possibly as a result of the small numbers. Most acute patients felt too ill to cope with study protocol in addition to treatment although many expressed interest. In contrast recurrent cases were much more willing to take part although many had to be refused because they did not meet the inclusion criteria often because of comorbidities such as rheumatoid arthritis or because both legs were affected. However a large proportion of patients expressed a willingness to assist with the study offering commitment to tests such as venesection that could be conducted within the ACU while treatment was ongoing. In order to recruit sufficient numbers into a subsequent study it would be necessary to consider tailoring the protocol to more closely fit this patient requirement. It is accepted that this study covered many aspects and included many tests all of which have added to the knowledge base, but there is no evidence to support the logistics of proceeding with the project in its present form. A more targeted approach will be adopted to explore

specific areas identified as important in this study. In the event some tests will be excluded and the reasons for this will be discussed in context.

6.2 Dermatophytosis

Dermatophytes were only cultured from one patient (8%) in this study as compared with six (50%) from the control group. It is possible that the culture method was inadequate but when present, dermatophytes grew strongly and were very evident within several days. The collection method was vigorous and large quantities of skin scales were collected as demonstrated by microscopy and it seems unlikely that dermatophytes growing in the upper layers of the SC were missed on both occasions. However the study population consisted of more men than women and the group of matched controls happened to contain a high proportion of ex servicemen, a group known to produce an increased frequency of athlete's foot. These findings demonstrate differences from the studies presented in the literature. Dermatophytosis was found to be a strong risk factor in studies conducted by Dupuy et al (1999) but only assumed by the presence of toe web intertrigo. Roujeau et al (2004) in a study of 243 patients and 267 controls used culture and microscopy to demonstrate dermatophytes in 42% of patients as compared with 24% of controls. Björnsdóttir et al (2005) in a similar but smaller study (100 patients and 200 controls) cultured both fungi and bacteria from the toe webs but concluded that the presence of dermatophytes only became significant when bacteria were excluded from the analysis. From the study by Dupuy the frequency of dermatophytosis cannot be calculated and from the remaining studies differences exist because of the tests performed and the analytical techniques used. In all these studies the controls were recruited from amongst a hospitalised patient population, the only information given is that their medical conditions were acute. No details of medication are supplied so it is impossible to know if these conditions could somehow affect the numbers of dermatophytes confirmed by culture. Day (1996) has shown that the presence of bacteria on the skin can in some cases drive fungi deeper into the skin where they may be inaccessible to the methods used to obtain culture samples and this could have happened in the previous studies. It is an unlikely cause in this study because the condition is accompanied by maceration and malodour and neither this or toe web intertrigo was identified on either patients or controls.

There is a commonly held misconception that dermatophytes gain access to the feet via the toe web skin. However this is not the case, as the initial infestation invariably affects the footsole, possibly as a result of the lack of sweat glands on the medial plantar aspect (Miller 1959) thought to encourage a more alkaline environment. Invariably spread extends to toe web skin where ideal conditions of humidity assist proliferation. Cracks and fissures assist bacteria and fungi alike but both result from thickened skin, which ceases to be supple so although the mechanical implications are obvious the immune response may also be affected. If skin is thick it is more difficult for toxic agents to penetrate below the surface and consequently they may evade detection by the immune system, which requires to remain primed in order to provide the best defence. Hard skin was found on the heels of most patients and on some controls and the presence of fissures on heels and foot soles were found to be significantly higher in patients than in controls. Thickened skin may therefore be more the important finding than lack of fungi on the feet and it may be that overall better foot care would be a suitable method of prevention.

The presence of cultured Trichophyton Rubrum (TR) was confirmed by serum levels of IgE to Trichophyton Rubrum (TR) in all cases where TR was grown and additionally in two patients where it was not. The latter finding is proof of exposure to TR at some time in the past since this particular fungus was not cultured during the study. One of these negative results was patient (P2) with elevated cytokine results and the other (P5) who grew the superficial dermatomycosis Tinea Nigra, from the sole of his cellulitic foot on both occasions in great quantity. Increased levels of IgE to TR suggest an immunological mechanism for its control but previous studies have shown that individuals are in some cases able to produce varying immunological responses to the same fungal species after infection on subsequent occasions (Woodfolk 1998, Svejgaard 1985) and that such diverse responses could have implications for the immunological control of other infective organisms (Woodfolk 1998, Svejgaard 1985, Walters (1974). Therefore although dermatophytes per se may not be responsible for cellulitic events their infestation whether past or present may contribute to immune disruption such as decreased lymphocyte trafficking, lymphoproliferation and differentiation Maleszka (2001). Aside from this the results obtained from this study indicate that IgE detection is a reliable method of confirming exposure to fungal disease although as a stand

alone it would be unable to discriminate between past or present infective episodes. In order to produce a comprehensive assessment, IgE status would need to be determined for all of the various dermatophyte strains.

Some participants grew a large number of different types of microorganism from a single sample. It was interesting to note that penicillium was frequently included and in several cases colonies grew up beside dermatophytes. In this association there appeared to be a mutual inhibition of the growth patterns so that when the organisms touched both colonies ceased to expand (figure 17). Penicillium is a ubiquitous organism and this finding demonstrates that natural flora have a limiting effect upon other microorganisms including dermatophytes. The ongoing prescription of antibiotic therapy has the potential to negate this relationship and this may be important for the study where a significantly higher lifetime use of antibiotic therapy was shown in the patient group.

6.3 Prophylaxis

Healthy skin is colonised by an indigenous population of commensal bacteria who assist in control over transient pathological species by numerical dominance, secretion of bacteriocins and maintaining the immune system in a constantly primed state (Holland 1993, Scheffel 1979). Concomitant with this the intercellular epidermal matrix contains natural antimicrobial peptides known as cathelidicins and β-defensins which have been shown to confer a degree of natural prophylaxis against bacteria and fungi (Nizet 2007, Nizet et al 2001, Ricketts at al 1951) and an additional antibiotic peptide has also been identified in sweat (Schittek at al 2001). These combined effects help to decrease the risk of infection and maintain the immune system in a constantly primed state (Holland 1993, Scheffel 1979).

Preliminary results from the PATCH study (Dermatology Clinical Trials Network ONLINE) suggest that the prophylactic use of antibiotics reduces the number of recurrent cellulitic attacks but at a cost. Sprunt (1971) showed that high doses of antibiotic upset the balance of the natural flora present upon surface skin, facilitating the opportunistic overgrowth of pathogenic forms, and thereby offering the potential for dominance in one species such as SA which has a propensity for superantigen activity. Three patients in this study P2, P7 and P12 failed to produce any cultured organisms from either foot or at either visit; interestingly these participants also recorded a high lifetime exposure to prescription antibiotics.

However this study did not set out to culture bacteria and those that grew are only the small sample who managed to survive antibacterial agents present in the culture medium which was primarily developed for fungal identification. A useful addition to a future study would therefore be to produce cultures from foot scrapings for both bacteria and fungi. The protocol would only need slight adaptation and the data, particularly in respect of commensal bacterial forms and superantigen activity, would greatly advance the understanding.

The PATCH study has problems with recruitment. As patients become aware of the consequences of prolonged antibiotic usage most decide in favour of alternative treatments and try to implement their own selfcare. Many podiatrists assume the presence of athlete's foot and prescribe antifungal treatment without culture. Many patients assume the same and treat themselves with over the counter medication and this management has been reported to reduce the number of recurrences. There was one such patient in this study whose data was not paired because of difficulties with venesection at either visit. This patient had suffered several recurrent attacks and was displaying classic signs of chronic oedema. Trichophyton Interdigitale was cultured from the foot sole of the cellultic foot on both occasions; the patient was informed and the GP made aware. Consequently antifungal treatment was applied and the patient had remained episode free at last contact (twelve months after the second visit). This patient, like many others, expressed concern that no identifiable portal of entry could be identified in the area of the calf recurrently affected. In such cases health care workers naturally turn their attention to the feet but patients do not always reason in this way and it is often found that the feet have previously been ignored. Attention to such issues as hard skin and toe web intertrigo are provided in dedicated foot clinics available to those who suffer from diabetes where neuropathy and vascular perfusion issues make them high risk for foot disease and ulceration. Podiatrist care also offers such intervention but unless this is free few assess the benefit as worthy of the cost. Two diabetic patients took part in the study one (P12) previously mentioned, was receiving foot care and cultured nothing from the feet on either occasion. The other was not attending a foot clinic and cultured virulent pseudomonas from both feet which persisted at the second visit. This patient was prescribed two additional courses of antibiotic therapy by the GP because the skin condition continued to exhibit signs of cellulitis. At visit two

CPR levels were above normal at 14 and the neutrophil count remained high; following this the GP was contacted. It may be that the IV and oral antibiosis administered throughout this episode was inappropriate to deal with this infection or that immune dysfunction allowed it to rekindle when the systemic effects were withdrawn, in either case knowledge of the offending organism would have informed the intervention and influenced the further management.

There is no doubt that application of topical antifungal emollients appear to be of benefit to those who suffer from fungal foot disease but podiatrist opinion suggests that the benefits may be negated by continuous usage and current advice suggests intermittent breaks of several weeks between courses. The logic is that small amounts of fungi are necessary to keep the immune system primed. An alternative method of control may be to inhibit fungal growth by using a natural biological preparation the rationale for this has been derived from the literature and the methodology is being considered.

With regard to prophylaxis, it may be the awareness of feet and shoes, rather than medication, that serves to enhance foot hygiene and implement an element of control in cases of recurrent cellulitis. The mechanism concerning fungal foot disease and its association with cellulitis is still not understood but the prevention may be simpler. Foot care is cheaper than antibiotics and advice costs even less.

6.4 Skin physiology

Physiological skin testing produced some interesting results and showed significance in some areas. Because of the observed erythema and concomitant radiated heat it was expected that skin blood flow would be increased during the episode and that values of evaporative water loss would be high and these expectations were confirmed. All cases recorded a decline in resting microcirculatory blood flow by the second visit, which was normal by comparison with results from control subjects. It would appear that an adequate mechanism for the delivery of the immune response was demonstrated. Two patients P4 and P5 had gross oedema. Despite this P5 produced the expected responses at both visits. P4 displayed much lower perfusion levels overall and the response at the second visit was well below normal. This patient had oedema in both legs and was much older, the contralateral foot was dropped and the range of movement limited. The reason for the low results can only be speculated but the persistent low perfusion in this case is a likely sequel to oedema.

The microcirculatory blood measurements have produced the expected results and no additional information has been obtained therefore it would be unnecessary to include this test in any future study.

In his original thesis concerning evaporative water loss through the epidermis Nilsson (1971) showed a correlation between water loss and temperature. These findings were confirmed by this study but although the cellulitic skin produced higher values than contralateral legs or controls at visit one the results did not achieve the expected significance. However at visit two patient data for cellulitic legs produced a significantly higher value for TEWL when comparison was made to contralateral and control legs. This latter finding in P1, P2, P8 and P10, although within the normal range, might indicate altered physiology. P3 however, produced a considerable increase from 7.9 – 20 g/hr/cm^2 and this is interesting as this patient was clinically the least representative of cellulitis with respect to skin characteristics. In addition some readings that were well below the accepted normal were obtained in both patient and control groups and it may be that the lower end of normal needs to be revised. However the performance of the Tewameter was a cause for concern throughout the study as it was consistently difficult to obtain valid calibrations thus undermining the confidence that should be commanded by a reliable scientific test. The data obtained with respect to TEWL are unremarkable with respect to cellulitis although they may have relevance to oedema. For this reason and on the grounds of reliability it has been decided to exclude TEWL measurements from any further investigations.

Variations in skin pH values have been shown to have relevance for the balance of the microflora at the skin surface (McBride 1993) and elevated pH readings within the acidic range were expected in relation to fungal/bacterial cultures within the study population. The data obtained showed that pH readings for the lower leg reached levels of 6.5 and above in some patients and also some controls but there were no significant differences between the patient and control groups and no association was found within groups between pH values and numbers of cultured organisms. However pH values are difficult to interpret because even transient changes have dramatic effects on the results. Soaps, creams and emollients applied to the skin may have long lasting effects, however if such creams are constantly applied the readings obtained reflect the ongoing value for the individual and must be noted and this information may be a useful addition to the

patient history. Although pH values on calf skin do not appear to be increased, values recorded from the plantar aspect of the foot may be more important (see dermatophyte section) and these would be included as part of future studies.

6.5 Oedema

No significant differences were found for circumferential measurements of the cellulitic legs between visits. Significant differences were shown between cellulitic and contralateral legs at visit one but differences were less at visit two and significance was not found. Persistent swelling remains a feature in those who have suffered from cellulitis and this is confirmed by patients who report that legs never return to 'normal size'. The expected difference between legs at the return visit was not found and this is probably due to increased dependency after return to normal daily life.

The epidermal thickness did not appear to change between episodes although seven out of the twelve patients reported skin peeling following the cessation of antibiotic treatment and imaging appearances indicated that the skin was stretched. Peeling is considered to be part of the innate (inherited) mechanism for clearing pathogens including fungal organisms from the epidermis (Milstone 2004) and is an expected feature of infections such as scalded skin syndrome. Badour and Bisno (1984) comment on this in some cases of patients recovering from episodes of cellulitis and the mechanism may have implications for closure of the episode and/or future immunity. This finding deserves attention in a future study where the timing and nature of the event would need more careful investigation.

In the inflammatory state fluid content between skin layers is greatly increased due to an influx of water and plasma proteins, which exit the small blood vessels and pool in the extravascular space. In this study dermal thickness measurements were significantly greater in the cellulitic legs as compared to the cellulitic control legs at the first visit and the findings nearly reached significance at visit two. High levels of venous incompetence were also found in the patient group and the links between this and dependant leg swelling are well known (Ryan 2005). Ultrasound dermal thickness measurements confirm the theory that leg swelling is the consequence of increased depth of tissue in the dermis but characterisation of this tissue is important. Figure 30a shows that acute oedema manifests as fluid collection in the papillary dermis just beneath the SC. Post episode the appearances (30b) suggest that this case has almost completely resolved leaving

small and reduced pockets of fluid interspersed throughout this layer which would be consistent with the normal daily rise and fall in fluid levels resulting from circadian processes (Gniedieka 1994). Despite this, the patient who provided this image, reported swelling in excess of normal in this leg with subsequent increases after each three cellulitic attacks. This patient was middle aged, without comorbidities, and reasonably fit but there were no apparent structural changes in the dermis although incompetence was demonstrated in this leg. In contrast, P5 with gross oedema, demonstrated no incompetence but displayed a dermal image almost completely devoid of structure (figure 31). Although the latter patient was younger he had experienced many more attacks and the originating factor was almost certainly an injury sustained during a sporting event with subsequent oedema originating as a consequence of trauma. Previous injury upon the ipsilateral side was found be significantly higher in the patient population and appears to be the most likely initiating event, whether by tissue damage or residual infection retained at the site of insult. Cox (2006) maintains that oedema is a predisposing factor for cellulitis; this new evidence suggests that it is not an initial cause but a deleterious sequel of the initiating event, which deteriorates with time. Oedema may provide consequences that facilitate future episodes and create a self perpetuating series of events. Consistent oedema may have a role in persistent low level inflammation, the trapping of left over antigen within the tissue or masking the presence of infective organisms from immune system surveillance and the wide variation in time scales between attacks may be associated with the time taken for the oedema to manifest. It is known that with age oedema impedes the circulation and promotes the risk of ulceration and that such oedema displays fluid pockets interspersed with islands of tissue. The chronic oedema that has been demonstrated in this study appears to have a different form and it may be possible to characterise it more precisely than with the quantification of low echogenic pixels chosen for the image analysis in this study; the application of mathematical transforms may provide this information.

The ultrasound imaging techniques developed in this study have been shown to be feasible, valid and reliable but time and precision are required to obtain images of suitable quality. In this study imaging was the final test because application of contact jel was expected to affect results of the other physiological tests, it was difficult to obtain the perfect views required for confident analysis because patients

were beginning to tire; consequently the number of good quality images was small. Because of these limitations it seems appropriate that further studies should address oedema as a separate issue.

6.6 Immunology and stress factors

Patient history revealed significantly higher values for allergy including asthma, recurrent skin rashes and bacterial infections excluding cellulitis. This finding may be suggestive of immune problems and the finding of significantly higher neutrophil counts and elevated levels of IL-8 (chemotactic agent for neutrophils) in the patient group at visit one supports this theory although the numbers for the latter test are too small to make positive conclusions.

The construction of blood profiles (figure 38) was useful to plot the course of the various cell types during the episode and highlight individual differences between patients. In addition it has revealed a limitation for this study. When cellulitis is uncontrolled a maintained increase in CRP is observed (figure 38a), while falling levels signal that response is underway. Because patients were recruited at varying stages of the cellulitic episode/antibiotic treatment, levels of CRP were in some cases already falling and this affects the understanding of the cytokine analysis. In order to effect meaningful comparisons only patients displaying the initial high levels of CRP as well as subsequent results should be included so that levels of cytokines can be matched at the appropriate stages.

The cytokine analysis as a whole appears promising; detection levels were good and in accordance with those expected from the patient with the systemic fungal infection. The markers which support this diagnosis (IL-4 and IL-5) were elevated with respect to the other samples and although normal values of these cytokines are unknown there is some confidence that others displaying an IH reaction could be identified. In this case IgE was only of benefit to corroborate the evidence because levels in serum can be very high without any particular inference.

Evaluation of psychological stress using the stress questionnaire was the most problematic because of the difficulties with interpretation experienced by many participants. Patients reported higher levels of stress and the results just reached significance. This area of research is particularly important and many studies are investigating relationships between psychological stress, immune function and skin disease. The effects of stress hormones, cortisol in particular, are just beginning to be understood (Nakano 2004, Dhabhar 2003, Nakano 2002,

O'Sullivan 1998). The results obtained from this study suggest a possible association between psychological stress and cellulitis but the subject is too complex for assessment by questionnaire alone. This area requires the attention of an independent study combining qualitative methods with quantitative investigation through immunological pathways. Scientists at Southampton General Hospital have developed such quantitative methods, which are currently being used to examine brain function with respect to skin challenges, but presently only in animal models. A future objective will be to devise a methodology for use in collaboration at a later date.

6.7 Limitations/benefits

Lack of recruitment proved to be a major limitation in this study; even so the study aims have been met except in the comparison between acute and recurrent patients and in the cytokine analysis. The paucity of acute/recurrent data does not allow meaningful comparisons between the groups and no clear indications have emerged; this aim will therefore need to be carried forward into all aspects of the future work. The cytokine analysis was hampered by lack of funds, it is an important component of the study and requires to be completed in advance of further studies. However a limitation has been identified (see previous section) and this must be considered in the interpretation of results.

The following addition limitations have also been identified:

- The TEWL equipment was found to be unreliable with respect to calibration, this is a crucial aspect of the validation procedure and the results obtained have been reviewed with caution.
- The Dermascan protocol was exacting and arduous and imaging had to be abandoned in some cases, however recruitment into a standalone study should permit the time and attention required to obtain the information needed for additional analysis.
- The stress questionnaire proved less user friendly than anticipated although results suggest that this aspect of the study may have important consequences for cellulitic events. A purpose designed version will be devised for use in future investigations.

The large amount of data generated by this study has been reviewed in great detail and this may not have been the case if the expected recruitment numbers

had been realised. Aspects such as blood profiling and updates of patient history could not have been pursued in such depth. As a consequence additional information has been acquired which will assist in the planning of subsequent work.

6.8 Summary

The major difficulty was recruitment and this impacted on all parts of the study particularly the comparison between acute and recurrent cases. At the end of the study only twelve matched pairs of patients and controls had completed both visits. Sufficient numbers of patients fulfilling the entry criteria were identified but some patients refused because they were unwell, short of time or unwilling to attend for tests in the research facility situated some distance from the treatment clinic. Despite this the study aims have been met except in the comparison between acute and recurrent patients and in the cytokine analysis. Some logistical and technical difficulties were discovered and will be addressed.

From the tests conducted the major findings were:

- Fewer dermatophytes were grown from the feet of patients as compared with matched controls and this finding was also consistent for opportunistic dermatomycoses, bacteria and yeasts. Where TR was cultured the findings were confirmed by specific IgE to TR in all cases.
- Physiological tests for microcirculatory blood flow, transepidermal water loss and pH produced results expected from cellulitic skin.
- Differences in epidermal thickness did not reach significance between patients and controls but dermal thickness, venous incompetence and observed leg swelling were all found to be greater in the patient group and this is consistent with enduring oedema. Ultrasound images of the oedematous legs revealed dermal patterns whose appearance differs from the presentation of classic oedema.
- Ipsilateral injuries, allergies, history of other bacterial infections and excessive life prescription of antibiotics were found to be significant in the patient group as compared to matched controls.
- The cytokines tested were detected throughout a wide range. Levels of IL-12 and IL-8 almost reached significance in the patient group but the

numbers compared were low. Blood profiling showed evidence of increased neutrophil count post episode in the patient group.

- The stress questionnaire produced problems with interpretation. Despite this scores were significantly higher in the patient group.

6.9 Conclusion

In this preliminary investigation into the risk factors associated with cellulitis of the lower limb a large number and diversity of tests were required in order to evaluate the existing knowledge base. It was hoped to contribute clear indications for more focussed studies and, although numbers were small, this has been achieved. Fungi, bacteria, immunology, patient history and foot care together with oedema assessment and stress investigations have been identified as areas worthy of further investigation and powered studies are now required to increase our understanding of this debilitating condition.

CHAPTER 7
FUTURE WORK

7 FUTURE WORK

The pilot study has generated large quantities of data, which would be difficult to manage in a larger study. For this reason and also because of the difficulties experienced with recruitment it has been decided to divide the future work into the following three focus areas:

Fungi/bacteria/immunology/history and foot care

These studies will be conducted within the ACU in conjunction with treatment protocol and this approach is expected to increase recruitment rates. Tests will include fungal/bacterial cultures, blood profiles and cytokine analysis, investigation into the frequency and timing of episodes of skin peeling, pH of the feet, patient history and foot care issues.

Oedema assessment

These studies will be conducted within the Wellcome Clinical Research Facility and will target recurrent cases. Serial ultrasound skin images will be collected and investigated by image analytical transforms.

Stress investigations

These studies will be carried out in conjunction with other groups who are currently researching in this area. Tests will include evaluation of stress by biochemical methods and purpose designed questionnaire.

APPENDIX

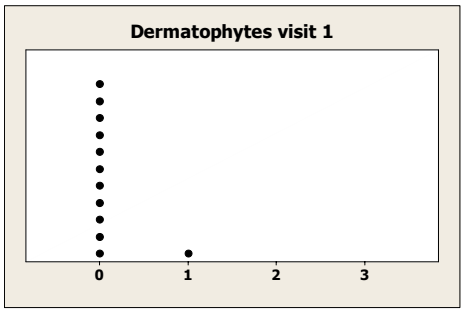
Appendix 1

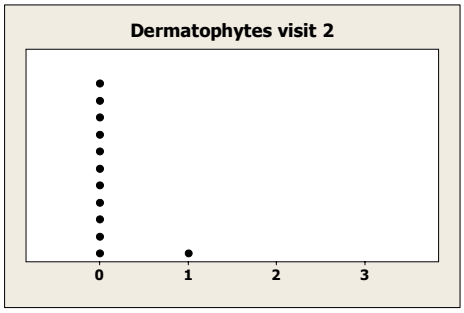
Questionnaire of Perceived Stress
Ref: Cohen , S., Kamarck, T., Mermelstein, R. (1983)
A global measure of perceived stress,
Journal of Health and Social Behavior pp 385-396.

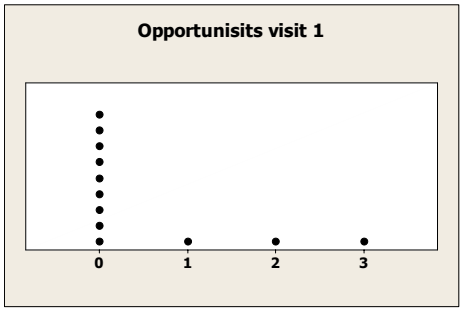
		never	almost never	sometimes	fairly often	very often
1	In the last month, how often have you been upset because of something that happened unexpectedly?	0	1	2	3	4
2	In the last month, how often have you felt that you were unable to control the important things in your life?	0	1	2	3	4
3	In the last month, how often have you felt nervous and 'stressed' ?	0	1	2	3	4
4	In the last month, how often have you dealt successfully with irritating life hassles?	4	3	2	1	0
5	In the last month, how often have you felt that you were effectively coping with the important changes that were occurring in your life?	4	3	2	1	0
6	In the last month, how often have you felt confident about your ability to handle your personal problems?	4	3	2	1	0
7	In the last month, how often have you felt that things were going your way?	4	3	2	1	0
8	In the last month, how often have you felt that you could not cope with all the things that you had to do?	0	1	2	3	4
9	In the last month, how often have you been able to control irritations in your life?	4	3	2	1	0
10	In the last month, how often have you felt that you were on top of things?	4	3	2	1	0
11	In the last month, how often have you been angered because of things that happened that were outside of your control?	0	1	2	3	4
12	In the last month, how often have you found yourself thinking about things that you have to accomplish?	0	1	2	3	4
13	In the last month, how often have you been able to control the way you spend your time?	4	3	2	1	0
14	In the last month, how often have you felt that difficulties were piling up so high that you could not overcome them?	0	1	2	3	4

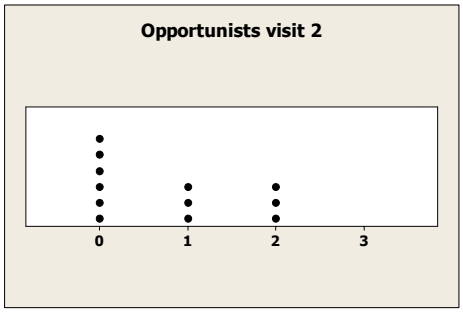
Appendix 2

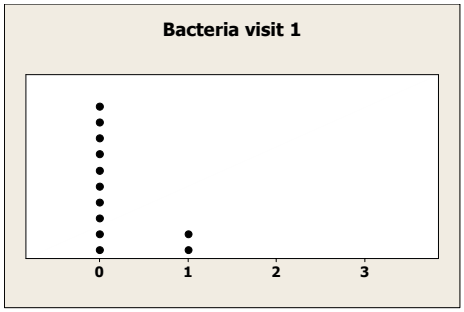
Dot plots to show distribution of dermatophytes, opportunists, bacteria and yeasts cultured from the foot sole of the **cellulitic foot** (n = 12)

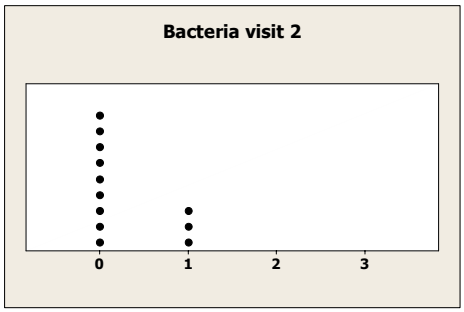


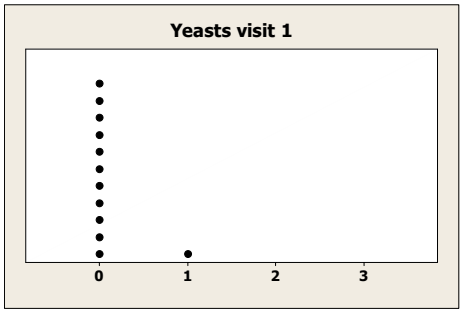


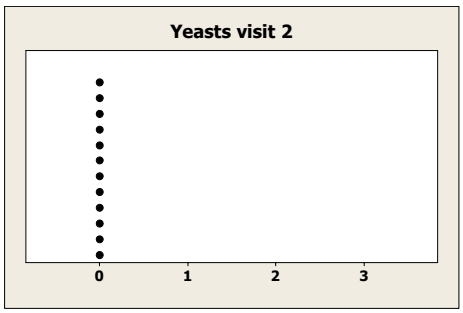






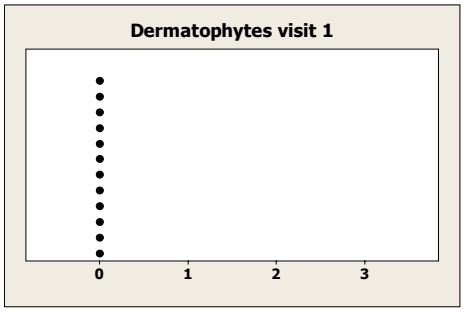


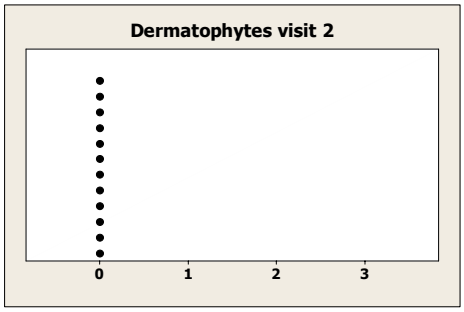


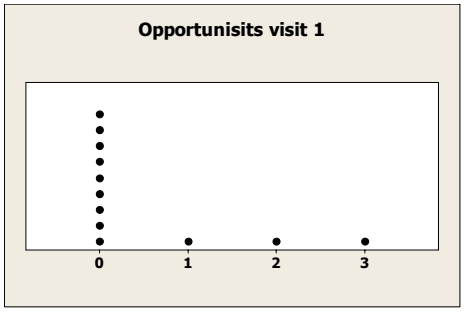


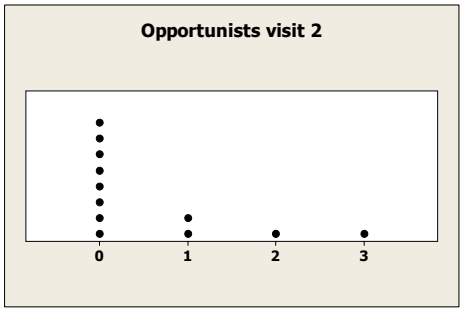
Appendix 3

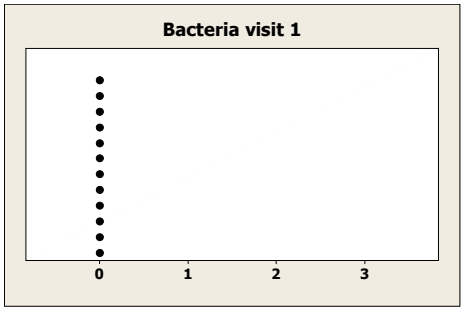
Dot plots to show distribution of dermatophytes, opportunists, bacteria and yeasts cultured from the interdigital spaces of the **cellulitic foot** (n = 12)

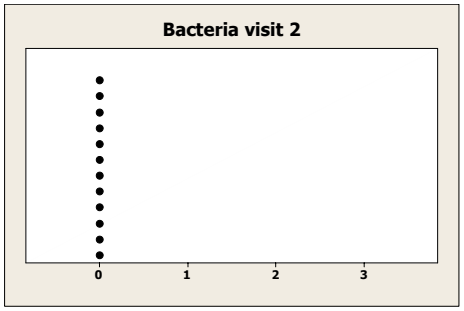


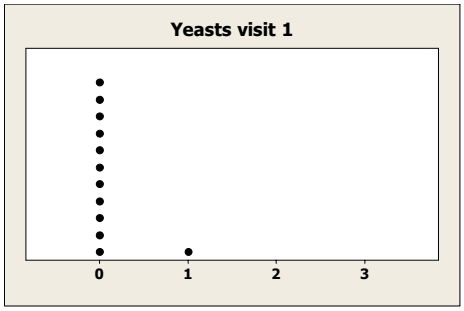


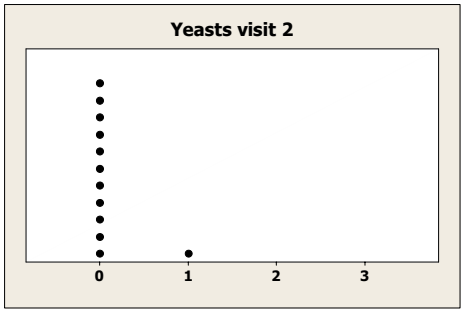






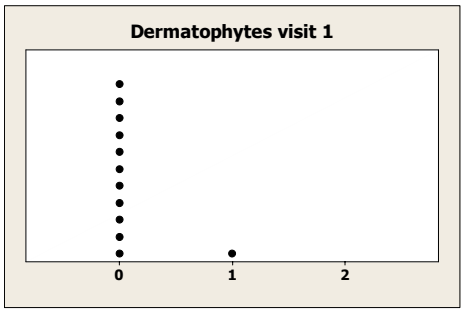


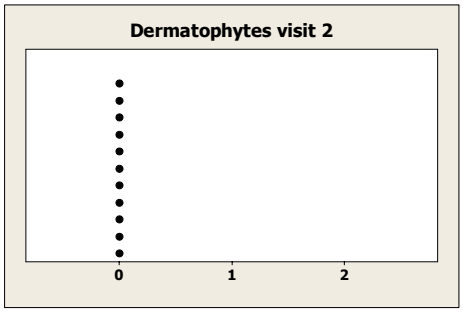


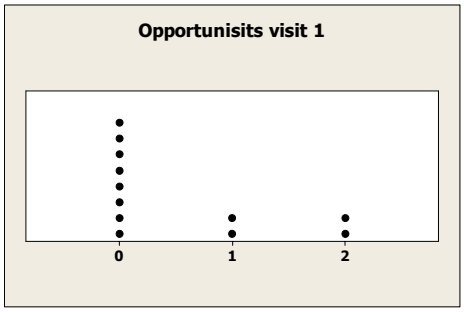


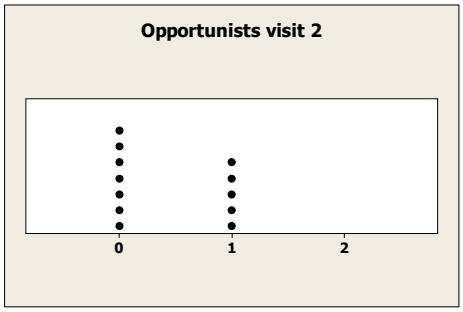
Appendix 4

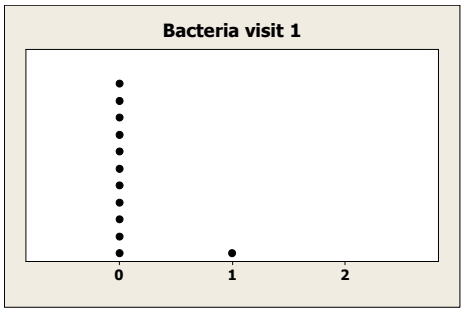
Dot plots to show distribution of dermatophytes, opportunists, bacteria and yeasts cultured from the foot sole of the **contralateral foot** (n = 12)

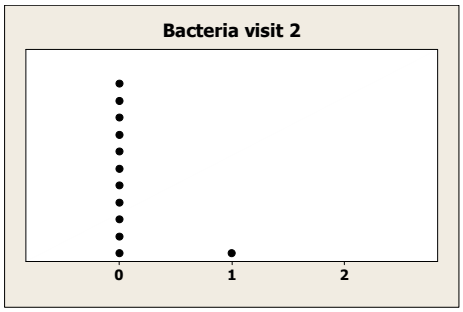


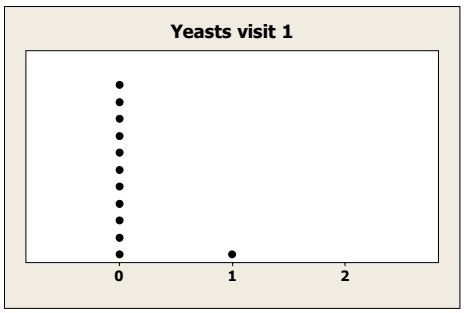


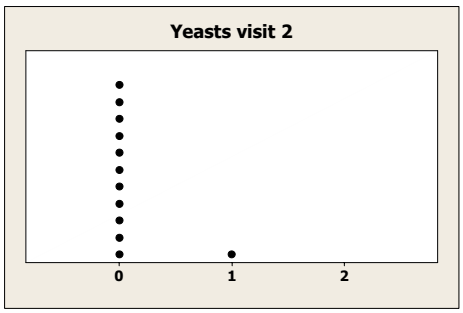






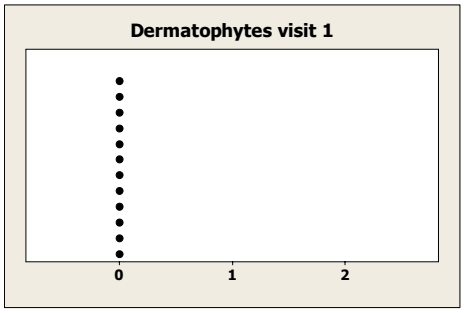


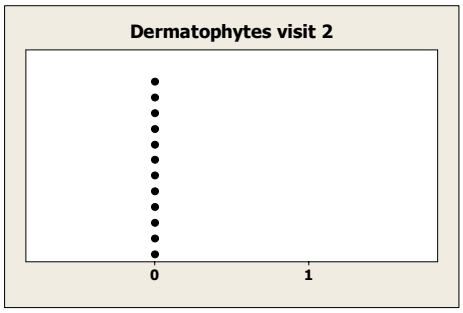


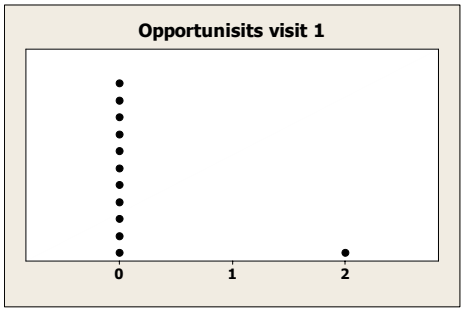


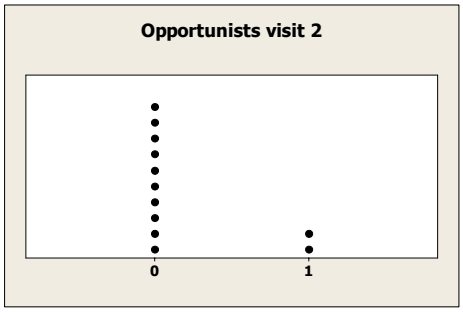
Appendix 5

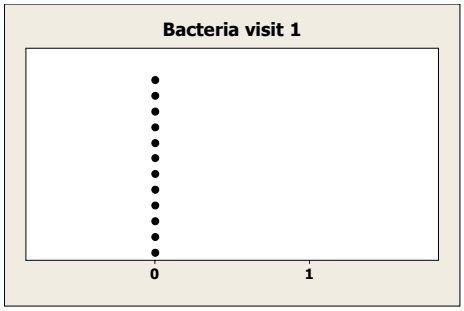
Dot plots to show distribution of dermatophytes, opportunists, bacteria and yeasts cultured from the interdigital spaces of the **contralateral foot** (n = 12)

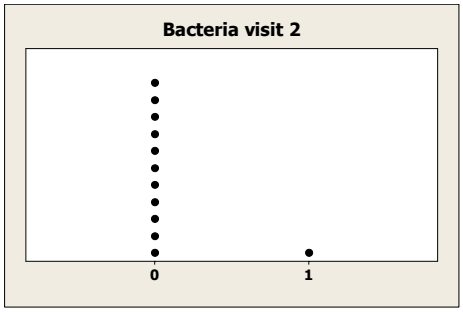


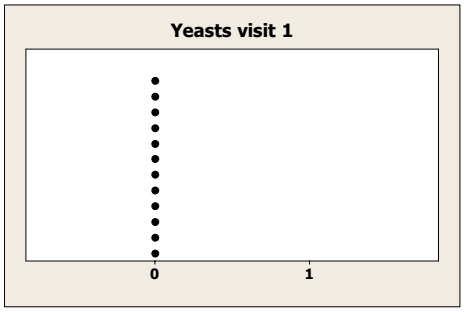


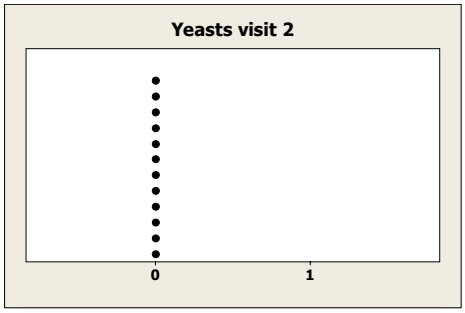






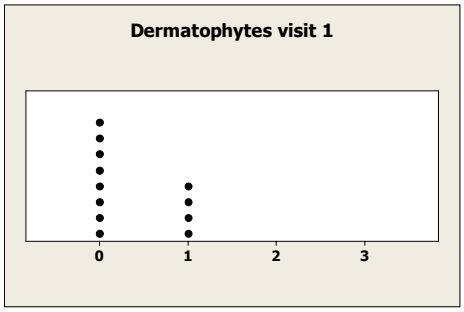


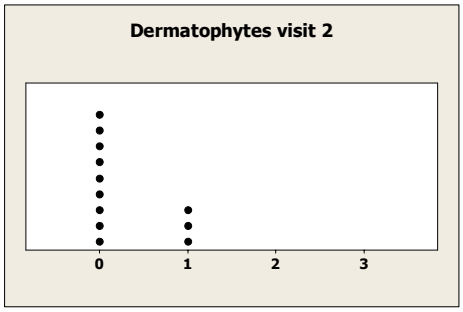


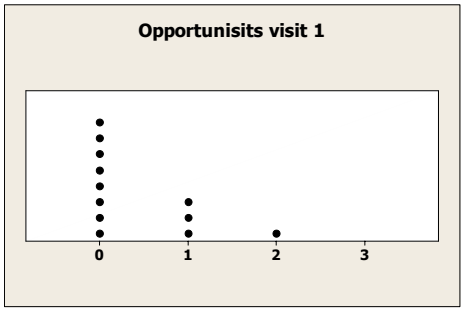


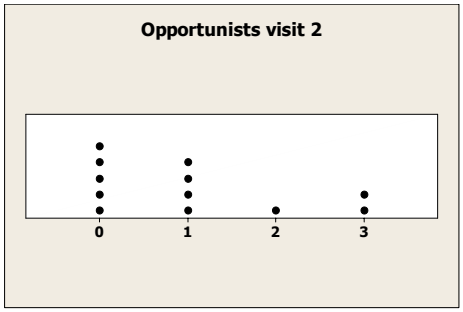
Appendix 6

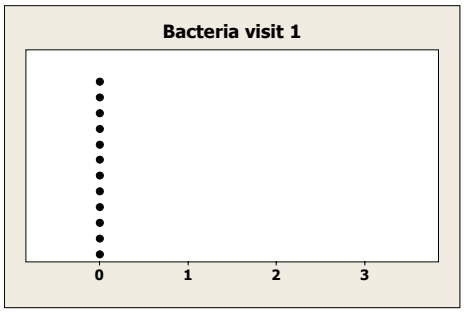
Dot plots to show distribution of dermatophytes, opportunists, bacteria and yeasts cultured from the foot sole of the **control foot** (n = 12)

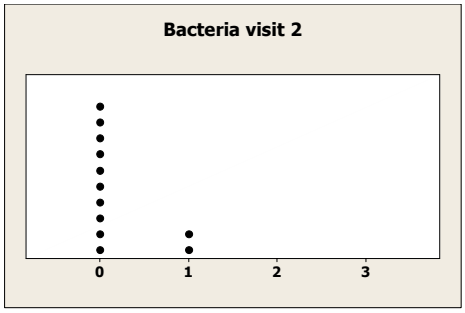


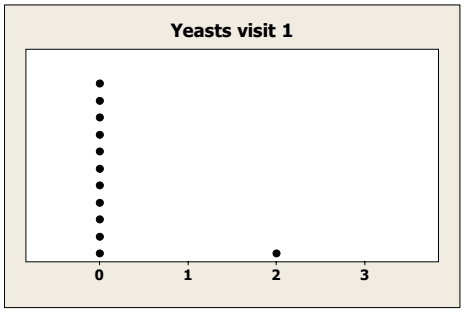


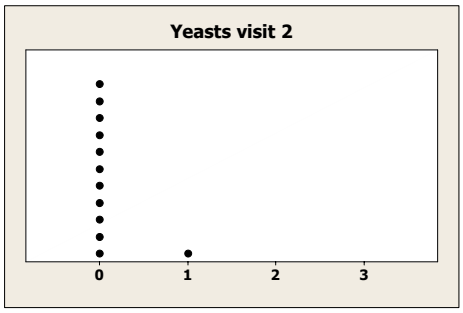






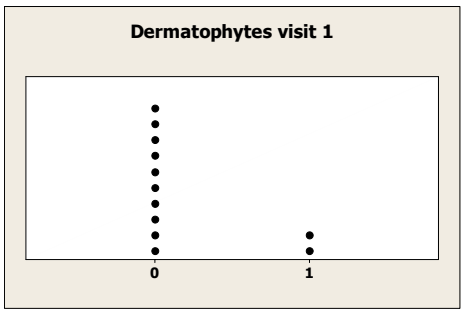


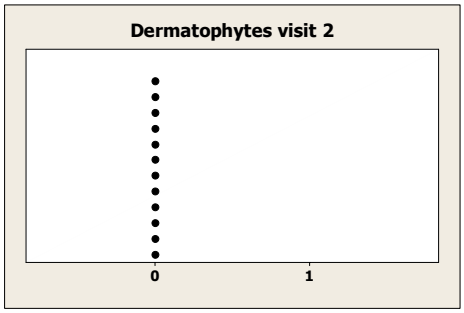


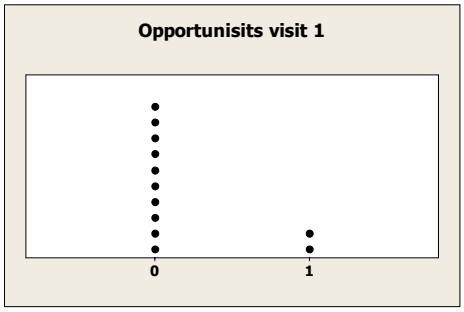


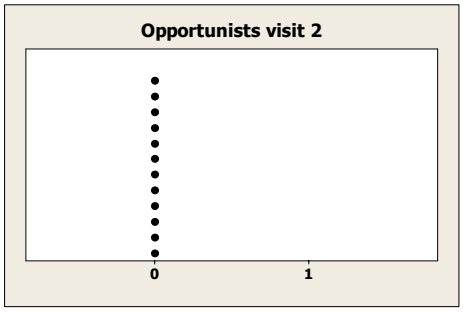
Appendix 7

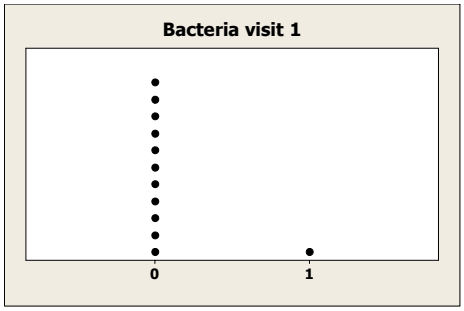
Dot plots to show distribution of dermatophytes, opportunists, bacteria and yeasts cultured from the interdigital spaces of the **control foot** (n = 12)

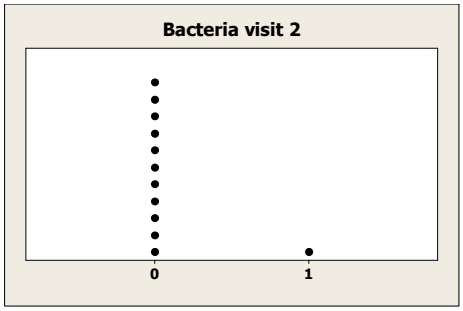


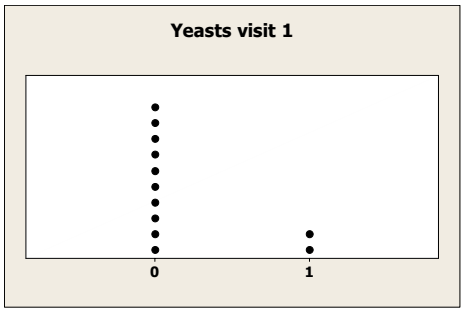


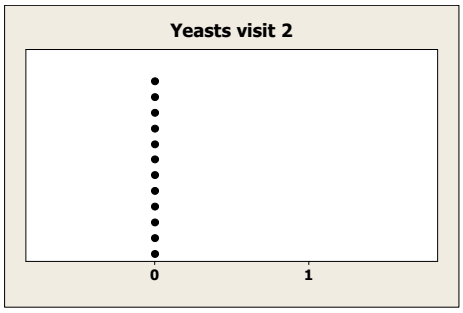






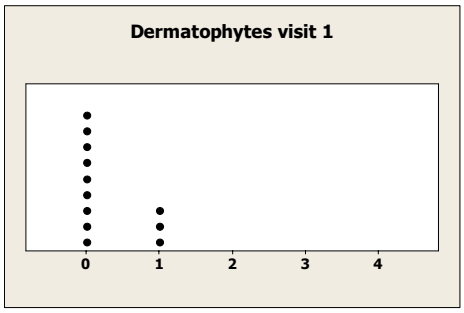


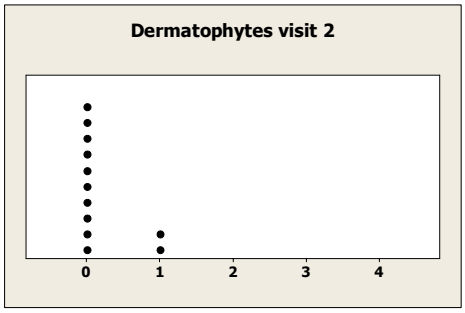


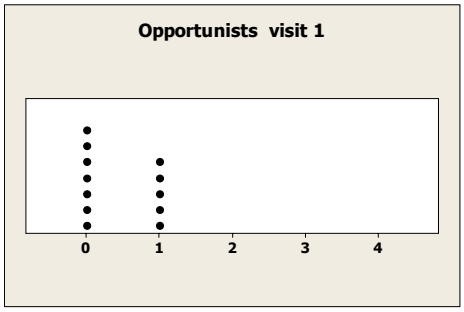


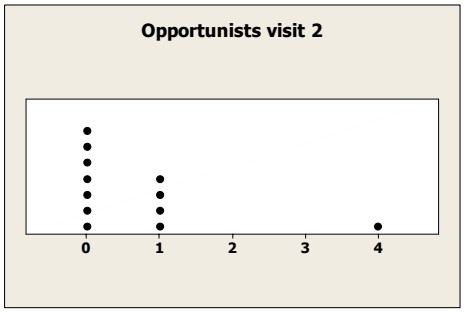
Appendix 8

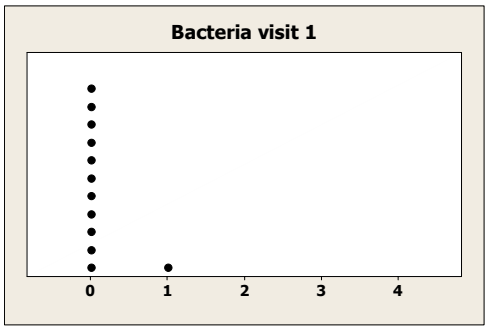
Dot plots to show distribution of dermatophytes, opportunists, bacteria and yeasts cultured from the foot sole of the **contralateral control foot** (n = 12)

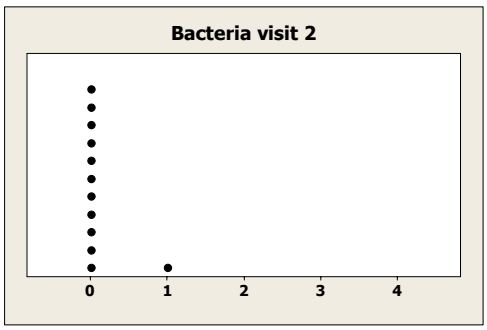


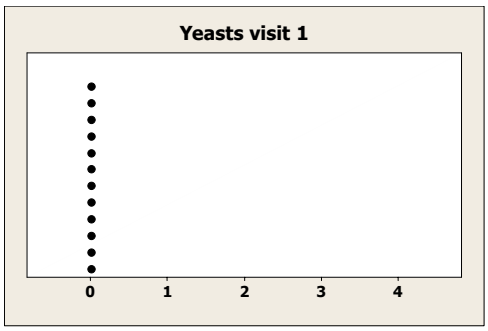


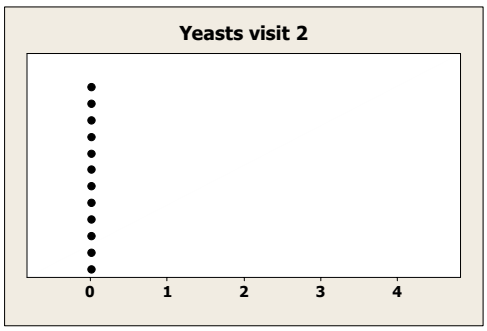






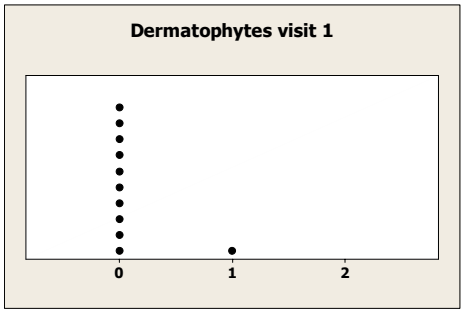


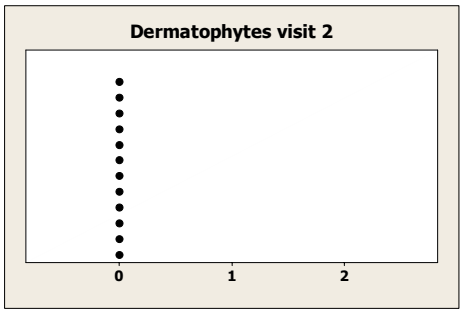


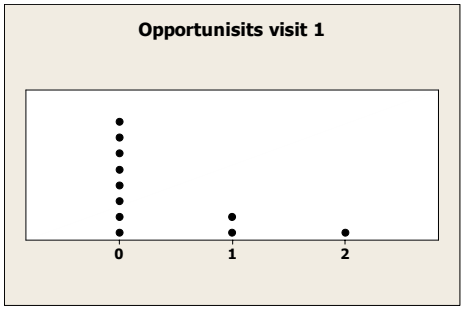


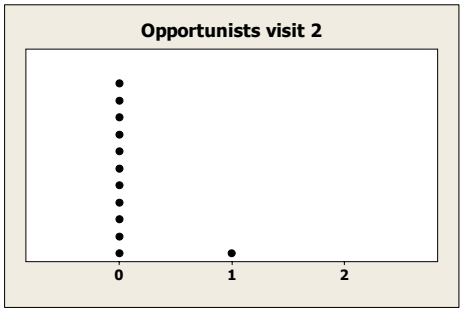
Appendix 9

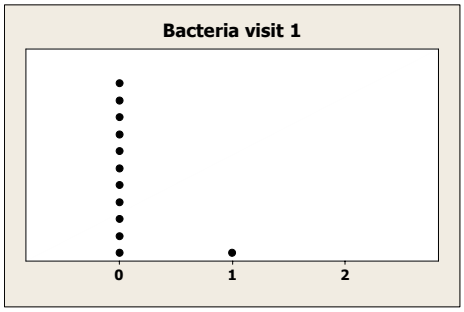
Dot plots to show distribution of dermatophytes, opportunists, bacteria and yeasts cultured from the interdigital spaces of the **contralateral control foot** (n = 12)

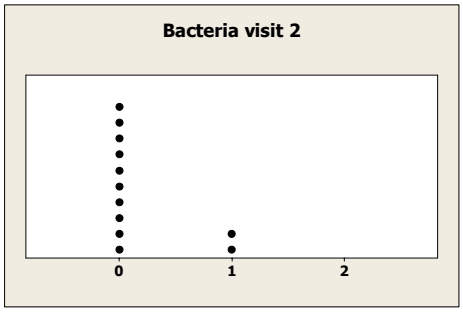


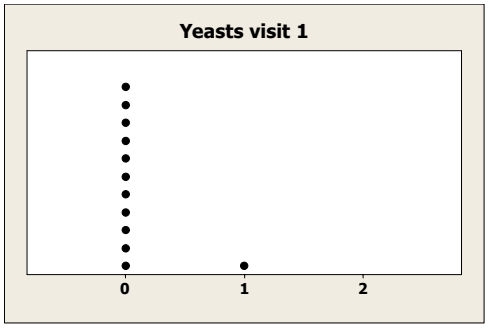


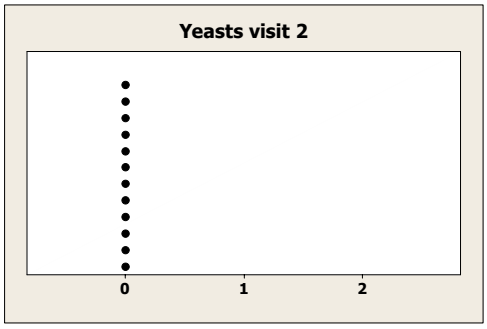












Appendix 10

Significance tests for results of Opportunist cultures

N = 12	Min - Max	N = 12	Min-max	Test	P value
Cell foot (1)	0 –6	Contra foot (1)	0 – 4	WSR	0.656
Cell foot (1)	0 –6	Control foot (1)	0 - 2	WSR	0.586
Cell foot (1)	0 –6	Cell foot (2)	0 – 5	WSR	0.250
Cell foot (2)	0 – 5	Contra foot (2)	0 - 1	WSR	0.754
Cell foot (2)	0 – 5	Control foot (2)	0 - 3	WSR	0.797
Contra foot (1)	0 – 4	Contra control foot (1)	0 - 3	WSR	1.0
Contra foot (1)	0 – 4	Contra foot (2)	0 - 1	WSR	1.0
Contra foot (2)	0 - 1	Contra control foot (2)	0 - 4	WSR	1.0
Control foot (1)	0 - 2	Control foot (2)	0 - 3	WSR	0.250

N = numbers compared (column 1 and column 3)
(1) = visit 1 (2) = visit 2

WSR = Wilcoxon Signed Ranks Test (exact 2 tailed test)

Appendix 11

Significance tests for results of Bacterial cultures

N = 12	Min-Max	N = 12	Min-Max	Test	P value
Cell foot (1)	0 – 1	Contra foot (1)	0 – 1	WSR	1.0
Cell foot (1)	0 – 1	Control foot (1)	0 - 1	WSR	1.0
Cell foot (1)	0 – 1	Cell foot (2)	0 – 1	WSR	1.0
Cell foot (2)	0 – 1	Contra foot (2)	0 - 2	WSR	1.0
Cell foot (2)	0 – 1	Control foot (2)	0 - 1	WSR	1.0
Contra foot (1)	0 – 1	Contra control foot (1)	0 - 1	WSR	1.0
Contra foot (1)	0 – 1	Contra foot (2)	0 - 2	WSR	1.0
Contra foot (2)	0 - 2	Contra control foot (2)	0 - 1	WSR	1.0
Control foot (1)	0 - 1	Control foot (2)	0 - 1	WSR	0.750

N = numbers compared (column 1 and column 3)
(1) = visit 1 (2) = visit 2

WSR = Wilcoxon Signed Ranks Test (exact 2 tailed test)

Appendix 12

Significance tests for results of Yeast cultures

N = 12	Min-Max	N = 12	Min-Max	Test	P value
Cell foot (1)	0 - 1	Contra foot (1)	0 - 1	WSR	1.0
Cell foot (1)	0 - 1	Control foot (1)	0 - 3	WSR	1.0
Cell foot (1)	0 - 1	Cell foot (2)	0 - 1	WSR	1.0
Cell foot (2)	0 - 1	Contra foot (2)	0 - 1	WSR	1.0
Cell foot (2)	0 - 1	Control foot (2)	0 - 1	WSR	1.0
Contra foot (1)	0 - 1	Contra control foot (1)	0 - 1	WSR	1.0
Contra foot (1)	0 - 1	Contra foot (2)	0 - 1	WSR	1.0
Contra foot (2)	0 - 1	Contra control foot (2)	0 - 0	WSR	1.0
Control foot (1)	0- 3	Control foot (2)	0 - 1	WSR	0.750

N = numbers compared (column 1 and column 3)
(1) = visit 1 (2) = visit 2

WSR = Wilcoxon Signed Ranks Test (exact 2 tailed test)

Appendix 13

Significance tests for IgE to Trichophyton Rubrum results

N = 12	Min-Max	N = 12	Min-Max	Test	P value
A = Patients (1)	0 – 1.46	B = Patients (2)	0 – 1.14	WSR	0.500
A = Controls (1)	0 – 17.30	B = Controls (2)	0 – 20.6	WSR	1.0
A = Patients (1)	0 – 1.46	B = Controls (1)	0 – 17.30	WSR	1.0
A = Patients (2)	0 – 1.14	B = Controls (2)	0 – 20.6	WSR	1.0

N = numbers compared (column 1 and column 3)
(1) = visit 1 (2) = visit 2

WSR = Wilcoxon Signed Ranks Test (exact 2 tailed test)

Appendix 14

Patient/Volunteer Serum levels of IgE to Trichophyton Rubrum (kUA/L)

	Patients	**Controls**
	P1	**C1**
Visit 1	0	0
Visit 2	0	0
	P2	**C2**
Visit 1	1.46	1.72
Visit 2	0.85	1.05
	P3	**C3**
Visit 1	0	0
Visit 2	0	0
	P4	**C4**
Visit 1	0	0
Visit 2	0	0
	P5	**C5**
Visit 1	0.81	0
Visit 2	0.44	0
	P6	**C6**
Visit 1	0	0.42
Visit 2	0	0.36
	P7	**C7**
Visit 1	0	0
Visit 2	0	0
	P8	**C8**
Visit 1	0	0
Visit 2	0	0
	P9	**C9**
Visit 1	0	17.3
Visit 2	0	20.6
	P10	**C10**
Visit 1	0	0
Visit 2	0	0
	P11	**C11**
Visit 1	1.0	0
Visit 2	1.14	0
	P12	**C12**
Visit 1	0	0
Visit 2	0	0

Clinical relevance of result
0 = undetectable, 0.4 - 0.7 = low level
0.7 – 3.5 = moderate, 3.7 – 17.5 = high
17.5 - > 100 = very high

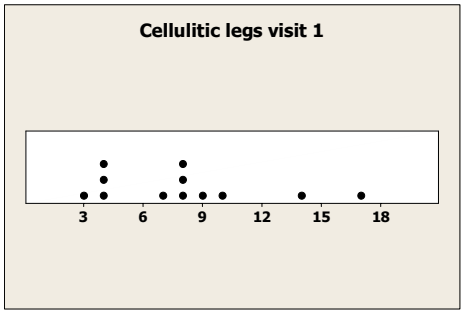
Appendix 15

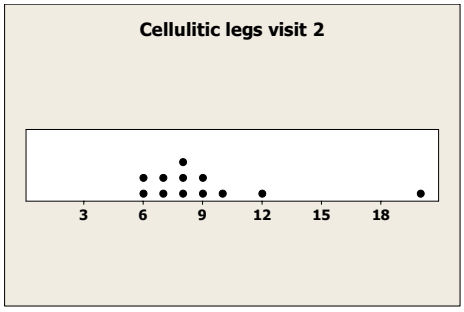
Patient/Volunteer TEWL Scores g/hm^2

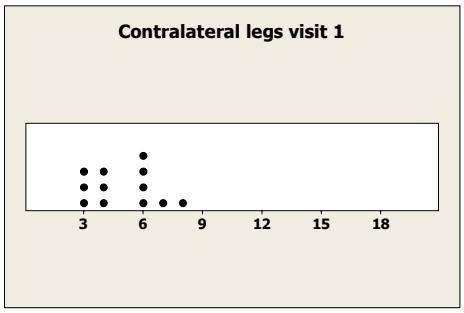
	Cellulitic Leg	Matched Control	Contralateral Leg	Matched Control
	P1	**C1**	**P1**	**C1**
Visit 1	2.5	7.1	2.8	6.6
Visit 2	6.4	7.6	4.6	5.9
	P2	**C2**	**P2**	**C2**
Visit 1	4.3	3.1	6.2	2.6
Visit 2	7.1	6.0	5.5	9.4
	P3	**C3**	**P3**	**C3**
Visit 1	7.9	4.9	3.5	6.0
Visit 2	20.0	3.8	4.8	3.5
	P4	**C4**	**P4**	**C4**
Visit 1	13.6	5.0	6.3	5.3
Visit 2	8.7	1.2	6.0	2.5
	P5	**C5**	**P5**	**C5**
Visit 1	17.2	8.5	8.1	6.8
Visit 2	12.3	6.2	7.0	7.2
	P6	**C6**	**P6**	**C6**
Visit 1	7.0	5.4	3.0	2.7
Visit 2	6.6	9.0	1.5	5.5
	P7	**C7**	**P7**	**C7**
Visit 1	8.3	1.4	5.5	1.4
Visit 2	8.0	2.6	4.8	3.1
	P8	**C8**	**P8**	**C8**
Visit 1	4.0	4.4	3.5	2.5
Visit 2	7.8	4.7	6.0	5.7
	P9	**C9**	**P9**	**C9**
Visit 1	8.9	4.0	3.3	3.6
Visit 2	10.0	5.9	6.0	3.5
	P10	**C10**	**P10**	**C10**
Visit 1	4.0	10.0	4.0	9.0
Visit 2	8.2	9.1	7.5	3.7
	P11	**C11**	**P11**	**C11**
Visit 1	10.0	7.4	6.4	6.6
Visit 2	6.1	6.5	4.5	7.1
	P12	**C12**	**P12**	**C12**
Visit 1	8.2	6.5	6.4	6.5
Visit 2	9.2	7.4	7.6	8.5

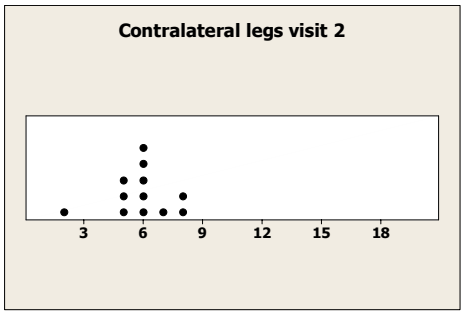
Appendix 16

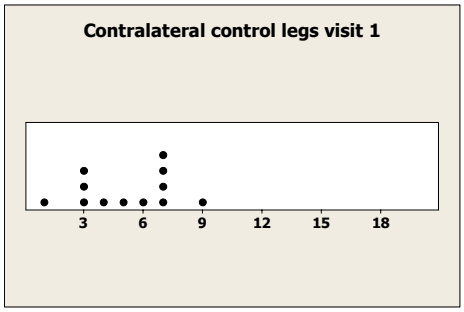
Dot plots to show distribution of data for Transepidermal water loss (TEWL) (n = 12)

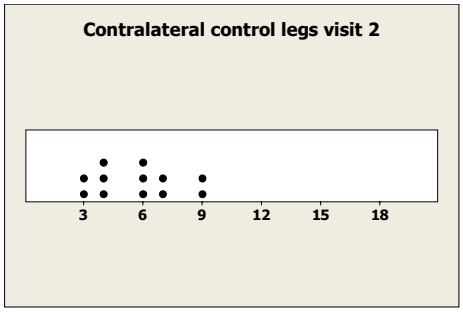












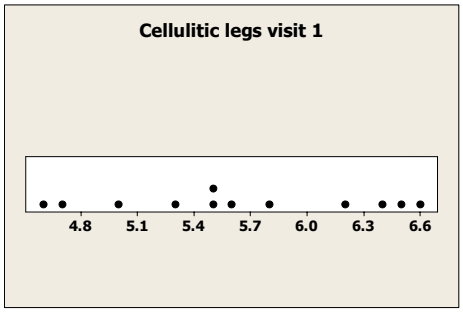
Appendix 17

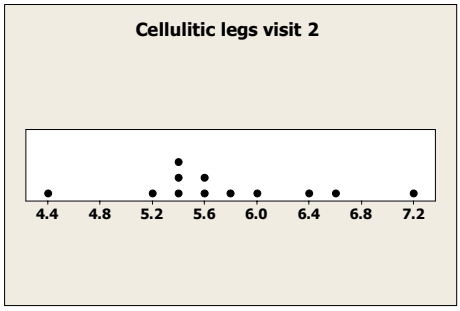
Patient /Volunteer pH scores

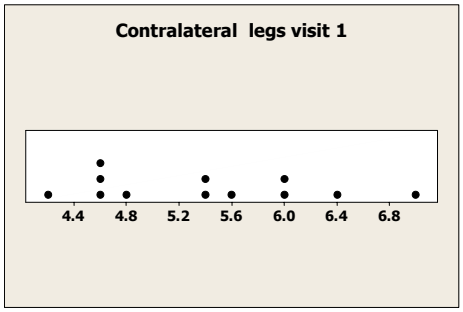
	Cellulitic Leg	Matched Control	Contralateral Leg	Matched Control
	P1	**C1**	**P1**	**C1**
Visit 1	5.8	6.0	5.5	6.5
Visit 2	5.2	6.4	5.1	6.7
	P2	**C2**	**P2**	**C2**
Visit 1	6.5	4.8	4.6	4.6
Visit 2	5.4	5.0	5.9	5.2
	P3	**C3**	**P3**	**C3**
Visit 1	6.6	6.5	6.9	6.6
Visit 2	5.6	7.0	5.8	6.7
	P4	**C4**	**P4**	**C4**
Visit 1	5.6	5.3	4.2	5.2
Visit 2	5.5	6.2	5.8	6.2
	P5	**C5**	**P5**	**C5**
Visit 1	6.2	6.2	5.9	6.2
Visit 2	6.3	6.7	5.6	6.8
	P6	**C6**	**P6**	**C6**
Visit 1	5.0	5.6	4.7	6.3
Visit 2	5.9	6.0	5.7	6.0
	P7	**C7**	**P7**	**C7**
Visit 1	5.5	6.2	5.4	6.2
Visit 2	6.5	6.6	6.8	6.6
	P8	**C8**	**P8**	**C8**
Visit 1	6.4	6.0	6.3	5.7
Visit 2	7.1	5.7	7.4	6.0
	P9	**C9**	**P9**	**C9**
Visit 1	4.6	5.3	4.5	5.2
Visit 2	4.4	5.3	5.6	5.2
	P10	**C10**	**P10**	**C10**
Visit 1	5.3	7.1	6.0	7.2
Visit 2	5.3	6.2	5.1	6.4
	P11	**C11**	**P11**	**C11**
Visit 1	4.7	5.7	4.5	6.4
Visit 2	5.7	6.7	5.4	6.6
	P12	**C12**	**P12**	**C12**
Visit 1	5.5	5.9	5.4	6.3
Visit 2	5.4	5.6	5.6	5.7

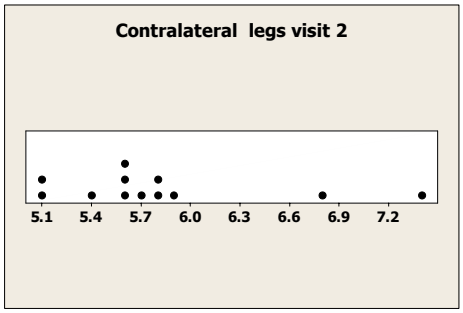
Appendix 18

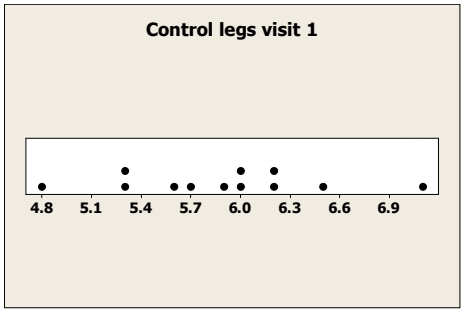
Dot plots to show distribution of pH data

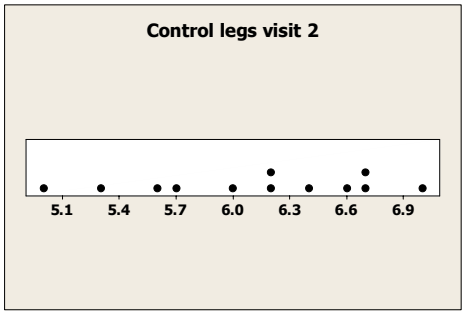


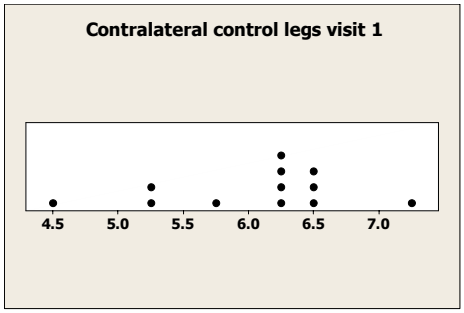


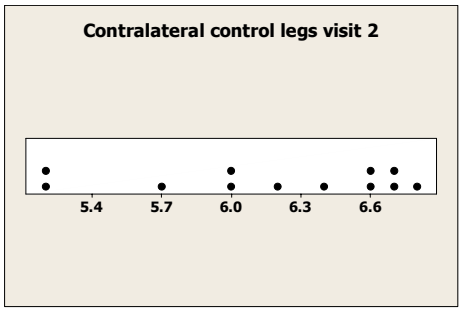






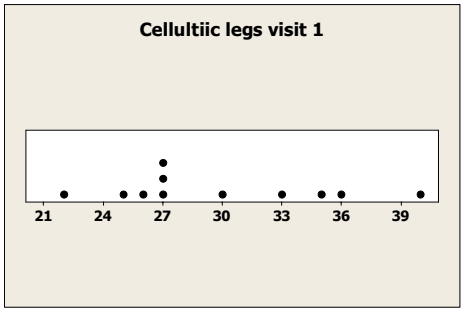


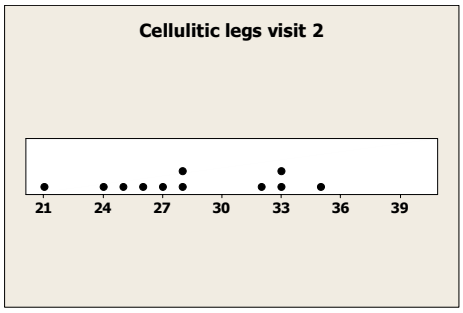


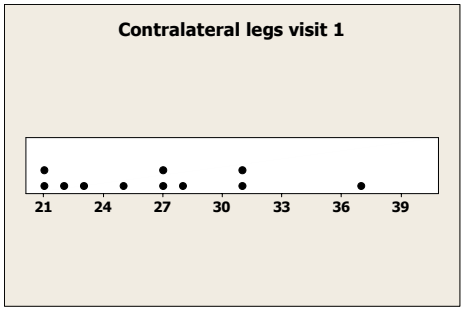


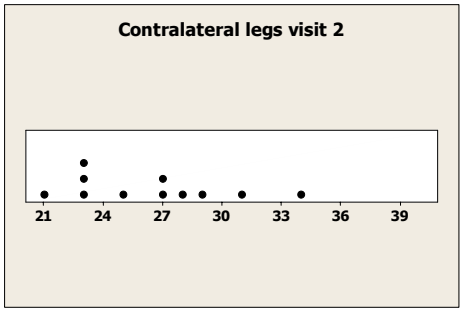
Appendix 19

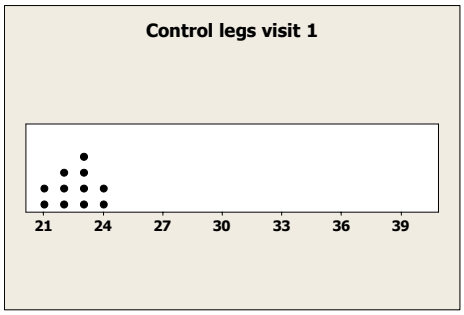
Dot plots to show distribution of ankle circumference measurements (cms**)** (n = 11)

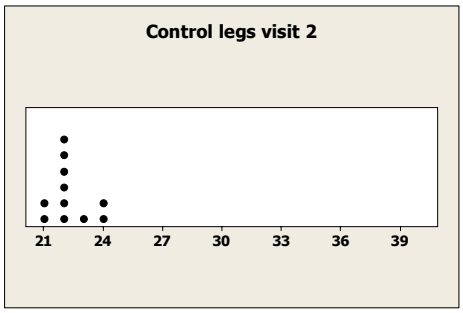


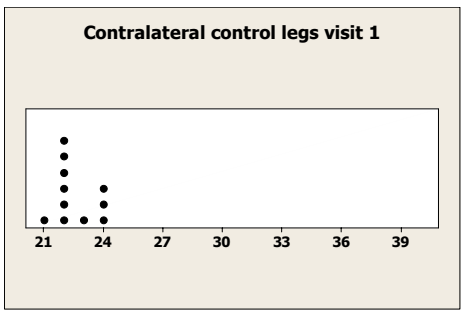


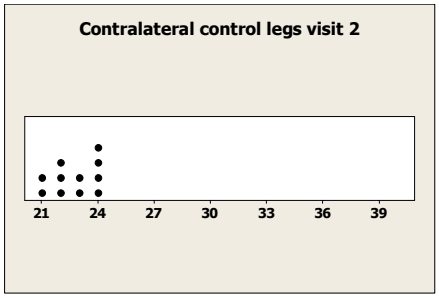






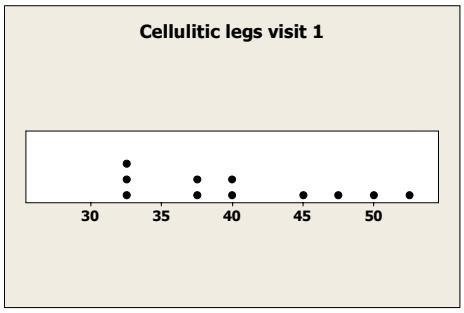


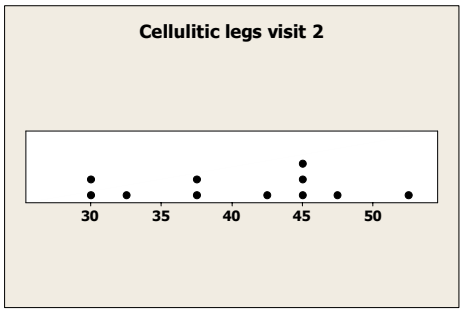


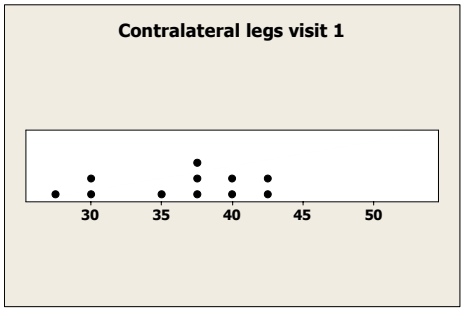


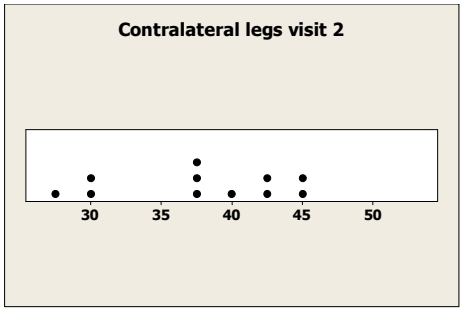
Appendix 20

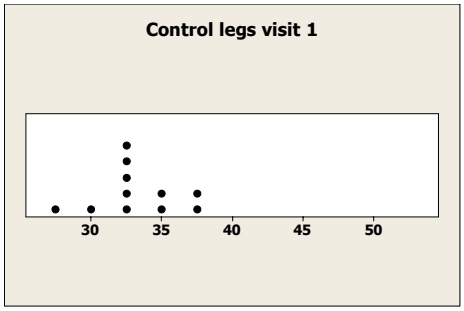
Dot plots to show distribution of mid calf circumference measurements (cms) (n = 11)

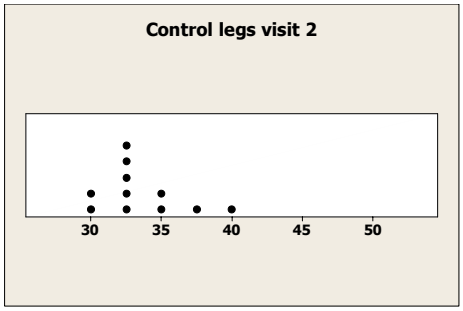


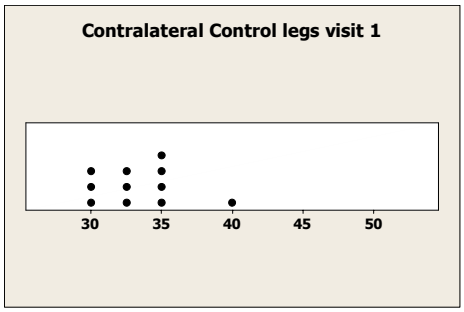


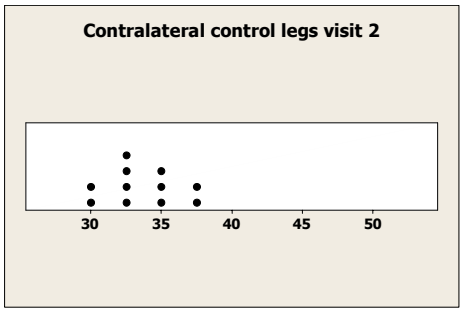






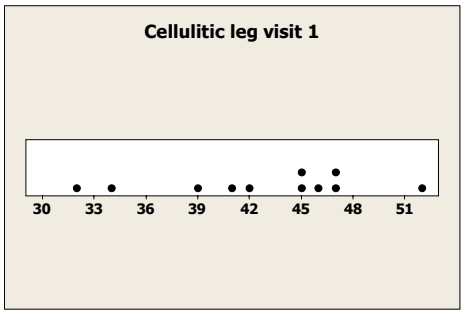


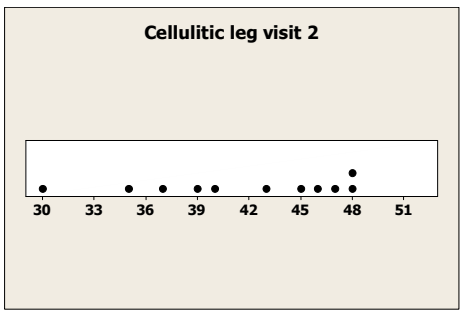


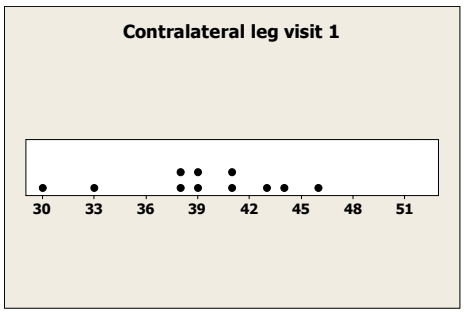


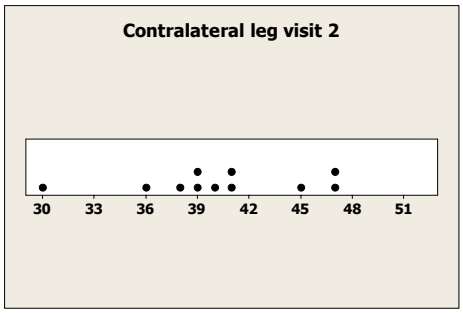
Appendix 21

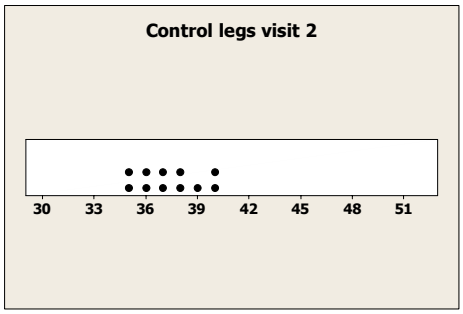
Dot plots to show distribution of upper calf circumference measurements (cms) (n = 11)

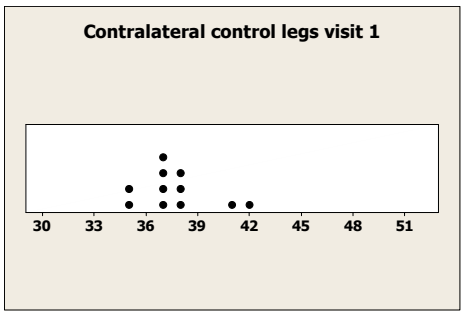


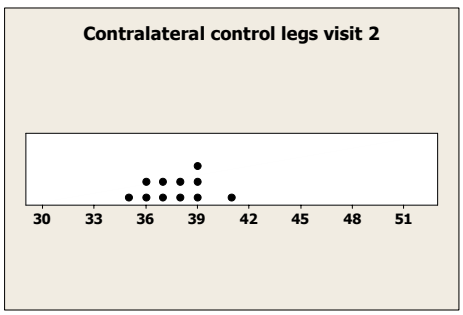












Appendix 22

Significance tests for leg measurements (cms) taken at the ankle

N = 11	Mean (SD) Min-Max	N = 11	Mean (SD) Min-Max	95% mean CI (A - B)	Test	DF	P value
A = Cell legs (1)	29.5 (5.45) 21.5 – 39	B = Contra legs (1)	26.4 (4.92) 21 – 36	-0.80 – 6.98	PT	10	0.11
A = Cell legs (1)	29.5 (5.45) 21.5 – 39	B = Control legs (1)	22.2 (0.96) 20 – 23.5	3.40 – 11.14	PT	10	0.002
A = Cell legs (1)	29.5 (5.45) 21.5 – 39	B = Cell legs (2)	28.2 (4.47) 21 - 35	-0.653 – 3.199	PT	10	0.172
A = Cell legs (2)	28.2 (4.47) 21 - 35	B = Contra legs (2)	26.1 (3.94) 20 – 33.5	-0.61 – 4.89	PT	10	0.114
A = Cell legs (2)	28.2 (4.47) 21 - 35	B = Control legs (2)	22.1 (0.83) 21 – 23.5	2.94 – 9.33	PT	10	0.002
A = Contra legs (1)	26.4 (4.92) 21 – 36	B = Contra control legs (1)	22.3 (0.88) 21 – 23.5	0.71 – 7.57	PT	10	0.023
A = Contra legs (1)	26.4 (4.92) 21 – 36	B = Contra legs (2)	26.1 (3.94) 20 – 33.5	-1.85 – 2.491	PT	10	0.751
A = Contra legs (2)	26.1 (3.94) 20 – 33.5	B = Contra control legs (2)	22.4 (1.1) 20.5 – 23.0	0.72 – 6.65	PT	10	0.020
A = Control legs (1)	22.2 (0.96) 20 – 23.5	B = Control legs (2)	22.1 (0.83) 21 – 23.5	-0.203 – 0.475	PT	10	0.391

N = numbers compared (column 1 and column 3)
(1) = visit 1 (2) = visit 2

CI = confidence interval (A = parameter column 1, B = compared parameter column 3)
PT = Paired T-test
DF = degrees of freedom

= significant (P = 0.05)

Appendix 23

Significance tests for leg measurements (cms) taken at mid calf level

N = 11	Mean (SD) Min-Max	N = 11	Mean (SD) Min-Max	95% mean CI (A - B	Test	DF	P value
A = Cell legs (1)	40.1 (7.2) 31.5 - 53	B = Contra legs (1)	36.6 (4.6) 37 – 42.5	-0.53 – 7.44	PT	10	0.082
A = Cell legs (1)	40.1 (7.2) 31.5 - 53	B = Control legs (1)	33.2 (2.75) 28 - 37	2.49 – 11.33	PT	10	0.006
A = Cell legs (1)	40.1 (7.2) 31.5 - 53	B = Cell legs (2)	40.3 (7.68) 29.5 - 53	-2.66 – 2.30	PT	10	0.873
A = Cell legs (2)	40.3 (7.68) 29.5 - 53	B = Contra legs (2)	37.5 (5.87) 28.5 - 46	-0.38 – 5.83	PT	10	0.079
A = Cell legs (2)	40.3 (7.68) 29.5 - 53	B = Control legs (2)	33.6 (0.91) 29 – 39.5	1.63 – 11.64	PT	10	0.014
A = Contra legs (1)	36.6 (4.6) 37 – 42.5	B = Contra control legs (1)	33.3 (2.7) 29 - 39	0.64 – 6.0	PT	10	0.020
A = Contra legs (1)	36.6 (4.6) 37 – 42.5	B = Contra legs (2)	37.5 (5.87) 28.5 - 46	-2.717 – 0.899	PT	10	0.289
A = Contra legs (2)	37.5 (5.87) 28.5 - 46	B = Contra control legs (2)	33.6 (2.4) 30.5 - 38	-0.03 – 7.94	PT	10	0.051
A = Control legs (1)	33.2 (2.75) 28 - 37	B = Control legs (2)	33.6 (0.91) 29 – 39.5	-1.398 – 0.489	PT	10	0.308

N = numbers compared (column 1 and column 3)
(1) = visit 1 (2) = visit 2

CI = confidence interval (A = parameter column 1, B = compared parameter column 3)
PT = Paired T-test
DF = degrees of freedom

= significant (P = 0.05)

Appendix 24

Patient leg measurements (mm)

	Cell Leg ankle	Cell leg mid calf	Cell leg upper calf	Contra leg ankle	Contra leg mid calf	Contra leg upper calf
P1						
Visit 1	25.5	32.5	34.0	21.0	28.5	29.5
Visit 2	21.0	30.0	30.0	22.5	29.5	29.5
P2						
Visit 1	27.0	43.0	41.5	25.0	42.5	39.0
Visit 2	25.0	37.0	39.5	25.0	39.0	41.0
P3						
Visit 1	26.5	36.5	45.5	36.5	37.5	43.5
Visit 2	27.0	45.0	45.0	27.0	41.5	44.5
P4						
Visit 1	35.0	47.0	52.0	28.0	40.5	41.0
Visit 2	33.0	47.0	46.0	28.5	41.5	41.0
P5						
Visit 1	35.5	53.0	46.5	22.5	38.5	38.0
Visit 2	35.0	53.0	48.0	22.5	38.5	38.5
P6						
Visit 1	32.5	44.0	47.0	30.5	39.5	43.0
Visit 2	33.0	44.5	47.5	33.5	44.0	46.5
P7						
Visit 1	25.0	31.5	32.0	21.0	31.0	33.0
Visit 2	27.5	29.5	36.5	22.5	30.5	35.5
P8						
Visit 1	27.0	39.0	39.0	26.5	35.5	39.0
Visit 2	25.5	37.5	39.0	27.0	37.5	38.0
P9						
Visit 1	29.5	36.5	40.5	27.0	31.0	38.0
Visit 2	28.0	32.0	35.0	27.5	28.5	38.5
P10						
Visit 1	Unable to measure because of bandages					
Visit 2	Not done					
P11						
Visit 1	39.5	49.0	45.0	31.0	41.5	45.5
Visit 2	32.0	46.0	47.0	30.5	46.0	47.0
P12						
Visit 1	21.5	39.0	44.5	21.5	37.0	41.0
Visit 2	23.5	41.5	42.5	20.5	36.5	39.5

Appendix 25

Control leg measurements (mm)

	Cont leg ankle	Cont leg mid calf	Cont leg upper calf	Contra cont leg ankle	Contra cont leg mid calf	Contra cont leg upper calf
C1						
Visit 1	22.0	33.0	36.5	22.0	33.0	38.0
Visit 2	22.5	35.0	36.5	23.5	36.5	38.0
C2						
Visit 1	23.5	34.5	37.0	23.5	33.0	40.5
Visit 2	23.5	33.0	38.0	23.0	34.5	38.0
C3						
Visit 1	21.5	30.0	37.5	21.5	32.0	37.5
Visit 2	22.0	29.0	36.0	21.0	31.5	37.0
C4						
Visit 1	23.5	36.5	39.5	23.5	39.0	37.0
Visit 2	23.5	39.5	39.5	23.5	38.0	36.5
C5						
Visit 1	20.5	33.0	33.0	21.0	34.0	35.0
Visit 2	21.0	33.5	34.5	20.5	33.5	35.5
C6						
Visit 1	22.5	36.0	36.5	21.5	36.0	36.5
Visit 2	22.0	35.0	37.0	22.0	34.5	38.5
C7						
Visit 1	22.5	28.0	38.0	22.0	31.0	37.0
Visit 2	22.0	29.5	37.5	23.5	32.0	38.5
C8						
Visit 1	22.5	32.5	36.0	23.5	29.0	36.5
Visit 2	22.0	32.5	35.0	22.5	30.5	34.5
C9						
Visit 1	21.0	31.5	35.5	22.0	31.0	35.0
Visit 2	21.0	33.0	36.0	22.0	30.5	36.0
C10						
Visit 1	21.5	38.5	37.5	22.0	34.5	34.5
Visit 2	21.5	37.5	38.0	22.0	36.0	37.0
C11						
Visit 1	22.0	33.0	38.0	22.0	34.0	38.0
Visit 2	21.5	33.0	39.0	21.5	33.0	39.0
C12						
Visit 1	23.0	37.0	40.0	22.5	34.5	41.5
Visit 2	22.0	37.0	40.0	23.5	35.0	41.0

Appendix 26

Patient/Volunteer Dermascan Results area A and area B. (Average of 3 measurements in mms)

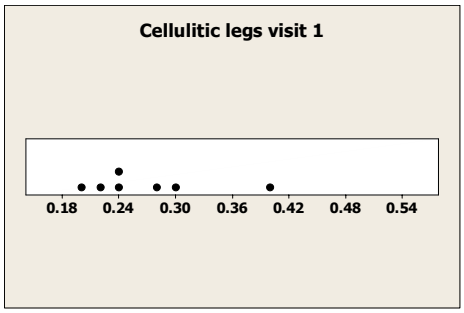
Cellulitic legs and matched controls

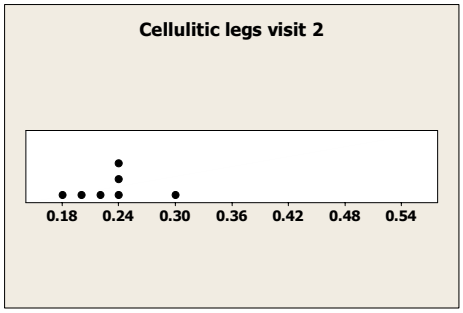
	Lt visit 1		Rt visit 1		Lt visit 2		Rt visit 2	
	Area A mms	Area B mms	Area A mms	Area B mms	Area A mms	Area B mms	Area A mms	Area B mms
P1	0.23	2.3	0.18	1.36	0.18	1.87	0.2	1.44
P2	0.3	1.8	0.2	2.0	0.3	1.9	0.2	2.0
P3	0.26	1.6	0.28	2	0.17	1.7	0.22	1.7
P4	0.25	1.8	0.3	1.6	0.23	1.5		
P5	0.27	1.3	0.39	1.36	0.23	1.33	0.29	1.29
P6	0.3	2.14	0.24	2.2				
P7								
P8	0.22	2.49	0.24	2.2	0.23	2.31	0.22	2.22
P9					0.26	2.01	0.23	2.17
P10	0.23	3.09	0.2	1.27	0.24	2.05	0.27	1.21
P11	0.18		0.27	1.87	0.31	1.9		
P12								
C1	0.16	1.51	0.17	1.75	0.16	1.7	0.19	1.67
C2	0.18	2.04	0.18	1.7	0.23	2.01	0.2	1.7
C3	0.26	1.66	0.24	1.56	0.23	1.76	0.22	1.76
C4			0.22	1.76			0.25	1.7
C5			0.24	1.39			0.2	1.26
C6	0.56	1.7	0.34	1.64	0.4	1.5	0.33	1.8
C7					0.2	2.26	0.24	1.77
C8					0.28	1.56	0.24	1.53
C9	0.26	1.4			0.21	1.5		
C10	0.23	1.39	0.29	1.62	0.3	1.43	0.25	1.43
C11	0.22	1.91	0.25	1.46	0.2	1.47	0.17	1.37
C12	0.20	1.45	0.35	1.22	0.28	1.47	0.26	1.41

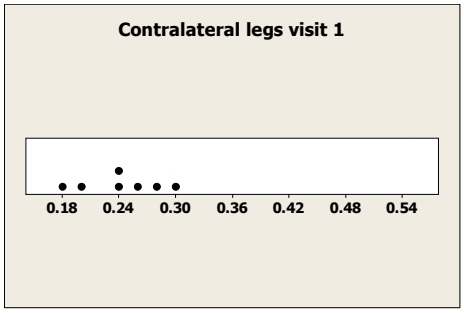
Measurements for cellulitic legs and matched controls are highlighted.
Soma data are missing – unable to perform tests or measure accurately or equipment was not functioning.
Numbers entered into the data analysis:
Area A = 7 pairs
Area B = 6 pairs

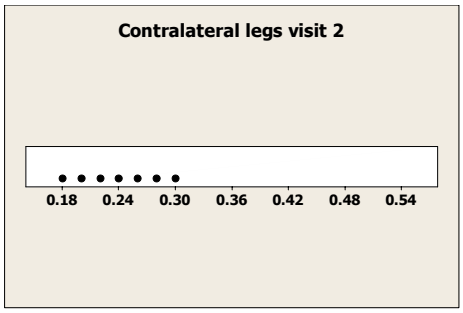
Appendix 27

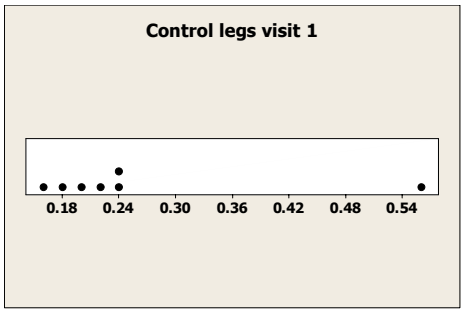
Dot plots to show distribution of ultrasound thickness measurements
Area a (mms)
(n = 7)

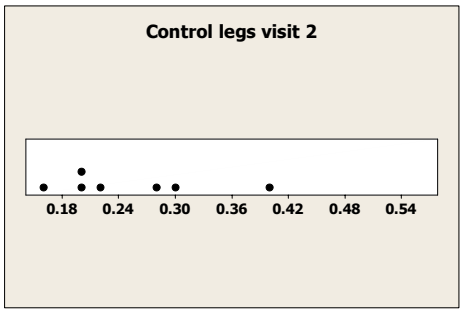


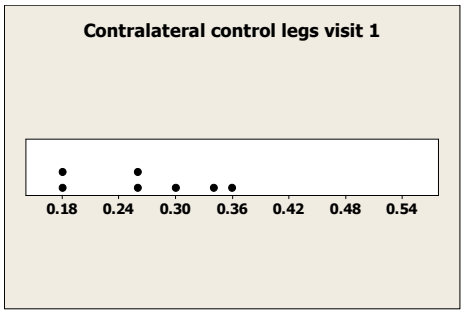


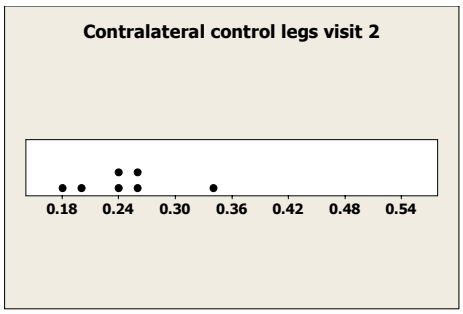












Appendix 28

Significance tests for ultrasound measurements (area a) mms

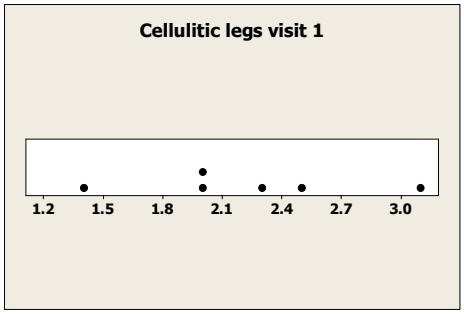
N = 7	Mean (SD) Min-Max	N = 7	Mean (SD) Min-Max	95% mean CI (A - B)	Test	DF	P value
A = Cell legs (1)	0.264 (0.07) 0.2 – 0.39	B = Contra legs (1)	0.241 (0.04) 0.18 – 0.30	-0.047 – 0.092	PT	6	0.452
Cell legs (1)	0.2 – 0.39	Control legs (1)	0.16 – 0.56		WSR		0.438
A = Cell legs (1)	0.264 (0.07) 0.2 – 0.39	B = Cell legs (2)	0.227 (0.03) 0.18 – 0.29	-0.019 – 0.094	PT	6	0.158
A = Cell legs (2)	0.227 (0.03) 0.18 – 0.29	B = Contra legs (2)	0.235 (0.04) 0.17 – 0.30	-0.059 – 0.042	PT	6	0.692
A = Cell legs (2)	0.227 (0.03) 0.18 – 0.29	B = Control legs (2)	0.251 (0.08) 0.16 – 0.40	-0.072 – 0.023	PT	6	0.257
A = Contra legs (1)	0.241 (0.04) 0.18 – 0.30	B = Contra control legs (1)	0.262 (0.07) 0.17 – 0.35	-0.098 – 0.553	PT	6	0.520
A = Contra legs (1)	0.241 (0.04) 0.18 – 0.30	B = Contra legs (2)	0.235 (0.04) 0.17 – 0.30	-0.041 – 0.053	PT	6	0.777
A = Contra legs (2)	0.235 (0.04) 0.17 – 0.30	B = Contra control legs (2)	0.24 (0.05) 0.17 – 0.33	-0.056 – 0.054	PT	6	0.951
Control legs (1)	0.16 – 0.56	Control legs (2)	0.16 – 0.40		WSR		1.0

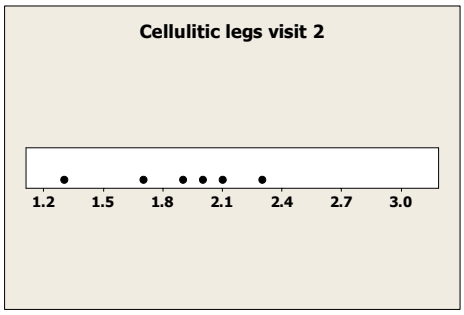
N = numbers compared (column 1 and column 3)
(1) = visit 1 (2) = visit 2

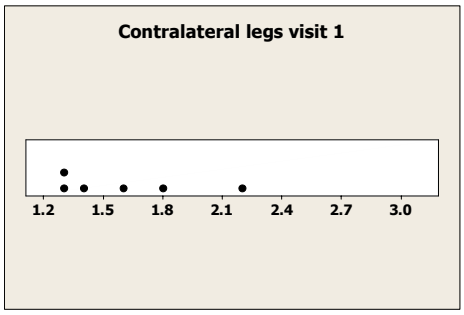
CI = confidence interval (A = parameter column 1, B = compared parameter column 3)
PT = Paired T-test
WSR = Wilcoxon Signed Ranks Test (exact 2 tailed test)
DF = degrees of freedom

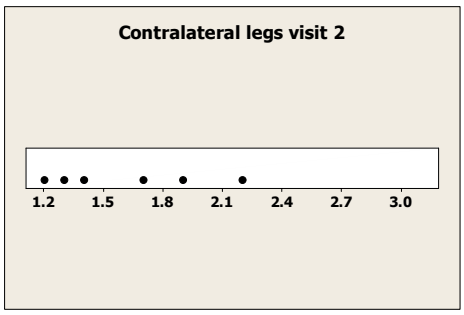
Appendix 29

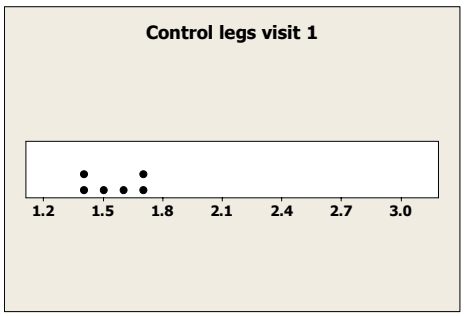
Dot plots to show distribution of ultrasound thickness measurements
Area b (mms)
(n = 6)

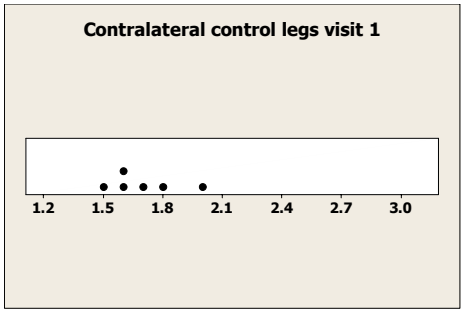


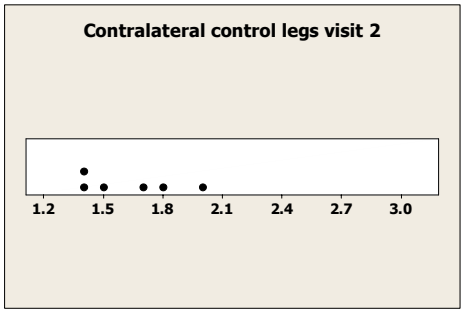












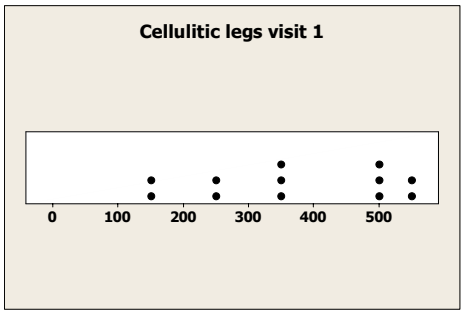
Appendix 30

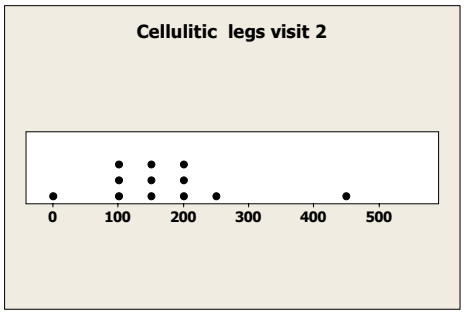
Patient/Volunteer LDI scores (Mean flux within AOI)

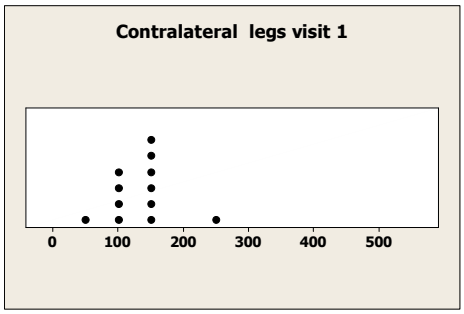
	Cellulitic Leg	**Matched Control**	**Contralateral Leg**	**Matched Control**
	P1	**C1**	**P1**	**C1**
Visit 1	338	132	101	113
Visit 2	149	113	92	135
	P2	**C2**	**P2**	**C2**
Visit 1	354	204	114	182
Visit 2	113	181	114	210
	P3	**C3**	**P3**	**C3**
Visit 1	533	187	154	224
Visit 2	207	154	118	246
	P4	**C4**	**P4**	**C4**
Visit 1	162	178	45	138
Visit 2	21	147	16	
	P5	**C5**	**P5**	**C5**
Visit 1	489	152	153	
Visit 2	89	230	176	
	P6	**C6**	**P6**	**C6**
Visit 1	519	132	97	113
Visit 2	168	113	96	135
	P7	**C7**	**P7**	**C7**
Visit 1	266	93	240	101
Visit 2	256	110	263	115
	P8	**C8**	**P8**	**C8**
Visit 1	138	147	107	132
Visit 2	112	213	102	180
	P9	**C9**	**P9**	**C9**
Visit 1	246	128	133	
Visit 2	164	167	228	
	P10	**C10**	**P10**	**C10**
Visit 1	500	135	153	131
Visit 2	436	109	280	126
	P11	**C11**	**P11**	**C11**
Visit 1	567	214	140	164
Visit 2	207	128	205	119
	P12	**C12**	**P12**	**C12**
Visit 1	340	124	174	140
Visit 2	195	128	155	147

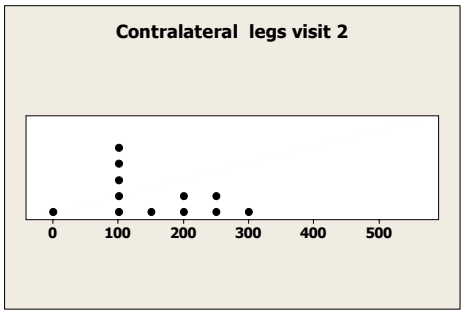
Appendix 31

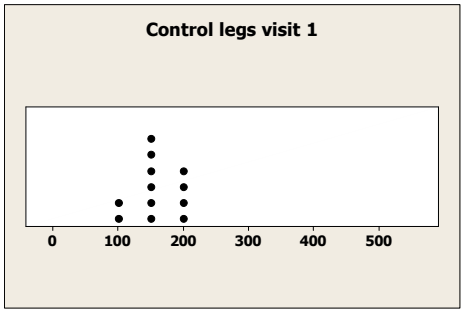
Dot plots to show distribution from Laser Doppler Imaging (mean flux values) (n = 12, *n = 10)

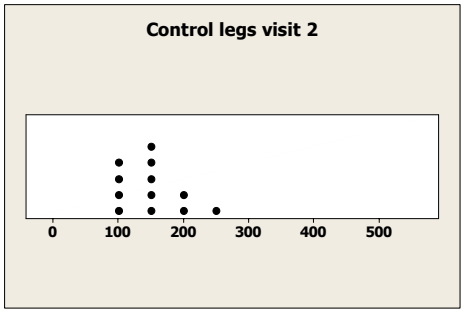


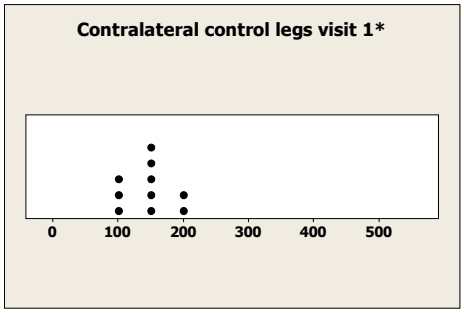


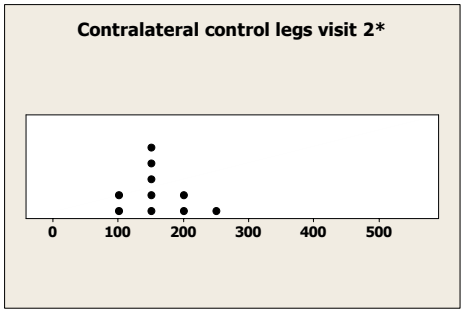












Appendix 32

Patient/Volunteer LDI Scores

	Cellulitic Leg	Matched Control	Contralateral Leg	Matched Control
	P1	**C1**	**P1**	**C1**
Visit 1	3	2	2	2
Visit 2	2	2	2	2
	P2	**C2**	**P2**	**C2**
Visit 1	3	2	2	2
Visit 2	2	2	2	2
	P3	**C3**	**P3**	**C3**
Visit 1	4	2	2	2
Visit 2	2	2	2	2
	P4	**C4**	**P4**	**C4**
Visit 1	2	2	1	2
Visit 2	1	2	1	
	P5	**C5**	**P5**	**C5**
Visit 1	4	2	2	
Visit 2	2	2	2	
	P6	**C6**	**P6**	**C6**
Visit 1	4	2	2	2
Visit 2	2	2	2	2
	P7	**C7**	**P7**	**C7**
Visit 1	3	2	3	2
Visit 2	3	2	3	2
	P8	**C8**	**P8**	**C8**
Visit 1	2	2	2	2
Visit 2	2	2	2	2
	P9	**C9**	**P9**	**C9**
Visit 1	3	2	2	
Visit 2	2	2	2	
	P10	**C10**	**P10**	**C10**
Visit 1	4	2	2	2
Visit 2	3	2	2	2
	P11	**C11**	**P11**	**C11**
Visit 1	4	2	2	2
Visit 2	2	2	2	2
	P12	**C12**	**P12**	**C12**
Visit 1	3	2	2	2
Visit 2	2	2	2	2

1 = Low relative flux
2 = Normal relative flux
3 = High relative flux
4 = Very high relative flux

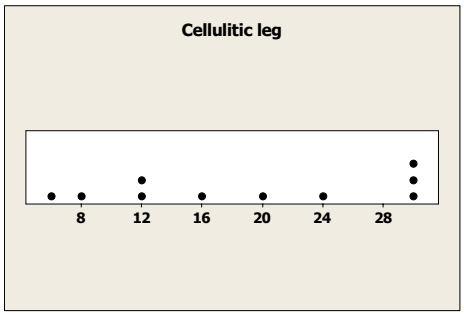
Appendix 33

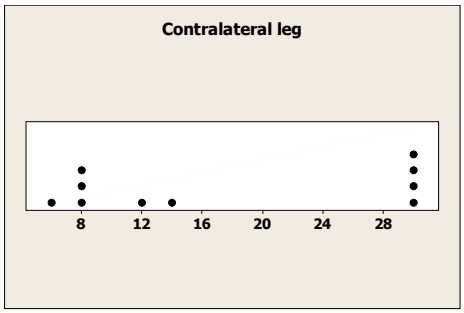
Patient/Volunteer Venous refilling times (secs)

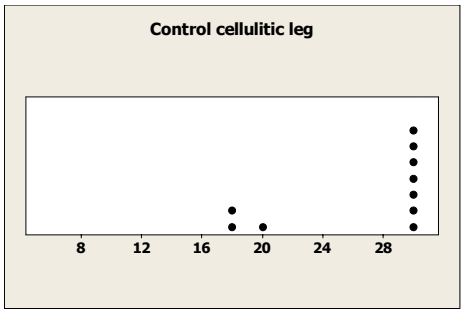
Cellulitic Leg	Matched Control	Contralateral Leg	Matched Control
P1	**C1**	**P1**	**C1**
> 25	> 25	> 25	> 25
P2	**C2**	**P2**	**C2**
> 25	> 25	> 25	> 25
P3	**C3**	**P3**	**C3**
7	18	7	18
P4	**C4**	**P4**	**C4**
Unable to measure			
P5	**C5**	**P5**	**C5**
> 25	23	> 25	23
P6	**C6**	**P6**	**C6**
Unable to measure			
P7	**C7**	**P7**	**C7**
5	18	7	> 25
P8	**C8**	**P8**	**C8**
23	> 25	> 25	> 25
P9	**C9**	**P9**	**C9**
16	> 25	14	> 25
P10	**C10**	**P10**	**C10**
11	> 25	5	> 25
P11	**C11**	**P11**	**C11**
20	> 25	11	> 25
P12	**C12**	**P12**	**C12**
11	20	7	12

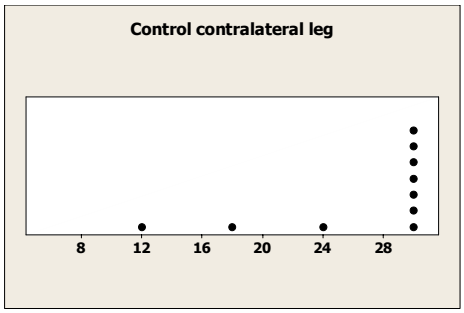
Appendix 34

Dot plots to show distribution of venous refilling times (VRT) in secs (n = 10)









Appendix 35

Patient/Volunteer LDF Scores

Cellulitic Leg	Matched Control	Contralateral Leg	Matched Control
P1	**C1**	**P1**	**C1**
1	1	1	1
P2	**C2**	**P2**	**C2**
1	1	1	1
P3	**C3**	**P3**	**C3**
2	2	2	2
P4	**C4**	**P4**	**C4**
Patient unable to perform test			
P5	**C5**	**P5**	**C5**
1	1	1	1
P6	**C6**	**P6**	**C6**
Patient unable to perform test			
P7	**C7**	**P7**	**C7**
2	2	2	1
P8	**C8**	**P8**	**C8**
2	1	1	1
P9	**C9**	**P9**	**C9**
2	1	2	1
P10	**C10**	**P10**	**C10**
2	1	2	1
P11	**C11**	**P11**	**C11**
2	1	2	1
P12	**C12**	**P12**	**C12**
2	2	2	2

1 = Competent, 2 = Incompetent

Appendix 36

Table of routine blood results (patients)

	CRP	WBC	Neutrophils	Lymphocytes
Normal range	0-6 mg/L	4 -11 (10^9/L)	2 -7.5 (10^9/L)	1.5 -4 (10^9/L)
P1 episode	490.0	13.7	12.0	0.9
FU visit	2.0	6.0	4.0	1.6
P2 episode	138.0	8.9	6.9	1.1
FU visit	5.0	6.6	3.7	1.7
P3 episode	3.0	4.2	2.2	1.4
FU visit	4.0	4.5	2.3	1.3
P4 episode	198.0	9.6	7.1	1.7
FU visit	4.0	6.3	3.8	1.8
P5 episode	171.0	10.2	7.7	1.4
FU visit	0.0	5.6	3.3	1.5
P6 episode	51.0	12.7	8.2	3.3
FU visit	14.0	10.9	6.9	3.0
P7 episode	10.0	5.6	4.2	0.6
FU visit	3.0	5.0	3.8	0.6
P8 episode	18.0	4.2	1.9	1.1
FU visit	1.0	4.4	1.9	1.5
P9 episode	56.2	7.7	5.8	1.4
FU visit	2.0	6.5	4.5	1.5
P10 episode	283.0	14.3	11.5	1.3
FU visit	3.0	9.7	6.0	2.6
P11 episode	98.0	10.1	6.8	2.0
FU visit	5.0	9.0	5.4	2.5
P12 episode	4.0	5.7	4.2	1.0
FU visit	3.0	7.0	5.1	1.2

	High result

	Low result

Appendix 37

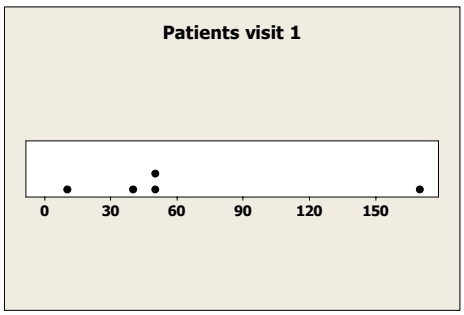
Table of blood results (controls)

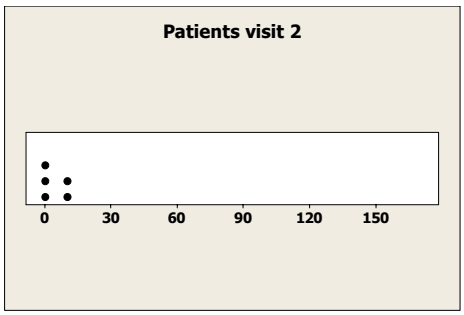
	CRP	WBC	Neutrophils	Lymphocytes
Normal range	0-6 mg/L	4-11 (10^9/L)	2-7.5 (10^9/L)	1.5-4 (10^9/L)
C1 first visit	1.0	6.0	4.0	1.6
FU visit	1.0	5.2	2.9	1.6
C2 first visit	0.0	5.9	2.5	2.5
FU visit	0.0	6.1	2.5	2.6
C3 first visit	0.0	5.8	3.3	1.8
FU visit	4.0	4.5	2.3	1.3
C4 first visit	4.0	7.2	4.4	2.0
FU visit	4.0	7.2	4.3	2.1
C5 first visit	0.0	4.8	3.2	1.1
FU visit	0.0	6.4	4.2	1.4
C6 first visit	2.0	5.3	3.2	1.6
FU visit	2.0	6.2	3.6	2.0
C7 first visit	0.0	6.9	4.0	2.2
FU visit	0.0	7.2	3.9	2.4
C8 first visit	0.0	5.3	3.0	1.5
FU visit	0.0	4.3	2.5	1.2
C9 first visit	0.0	5.5	3.4	1.4
FU visit	0.0	5.6	3.5	1.5
C10 first visit	2.0	4.3	2.4	1.5
FU visit	3.0	4.4	2.5	1.5
C11 first visit	0.0	4.6	2.8	1.6
FU visit	2.0	5.2	3.1	1.6
C12 first visit	6.0	4.5	2.7	0.9
FU visit	5.0	4.4	2.4	1.0

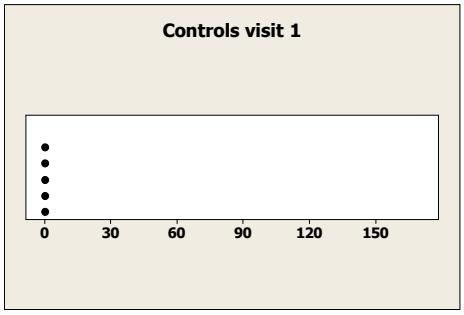
	High result		Low result

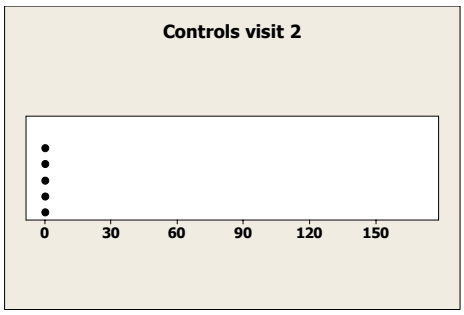
Appendix 38

Dot plots to show distribution of CRP (mg/mL)

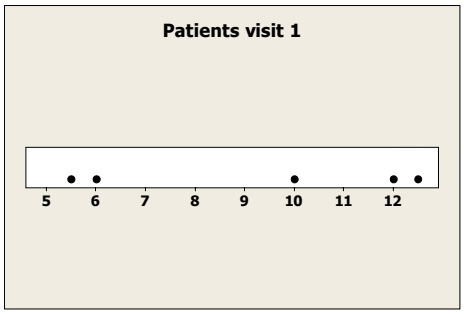


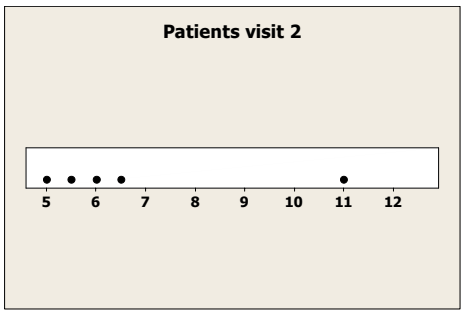


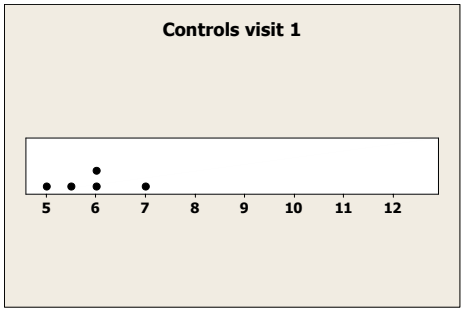


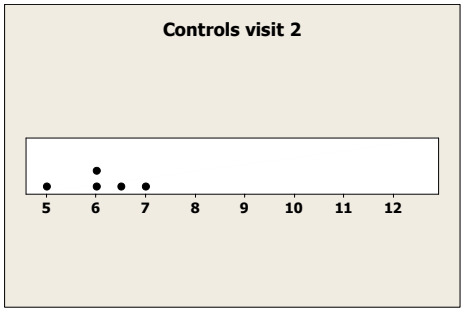


Dot plots to show distribution of total white cell count (10^9/L)



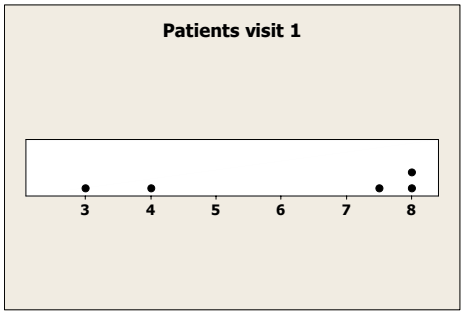


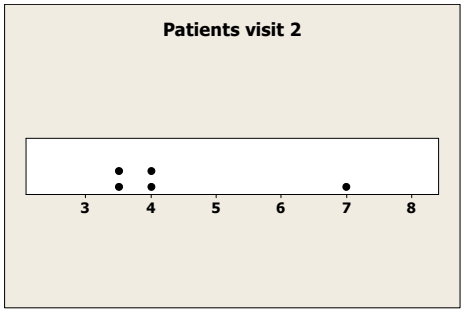


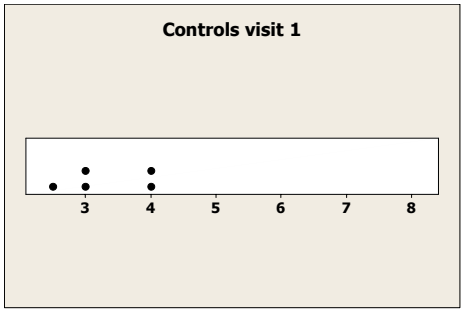


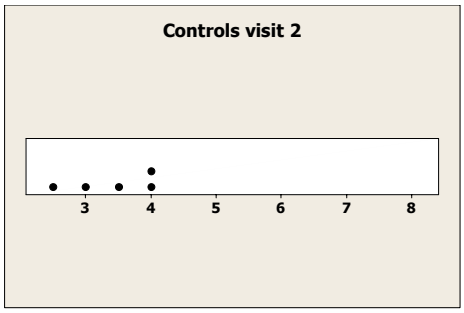
Appendix 39

Dot plots to show distribution of neutrophils (10^9/L)

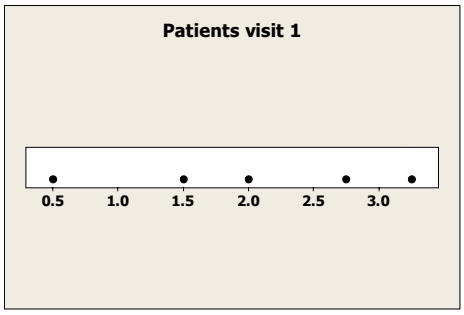


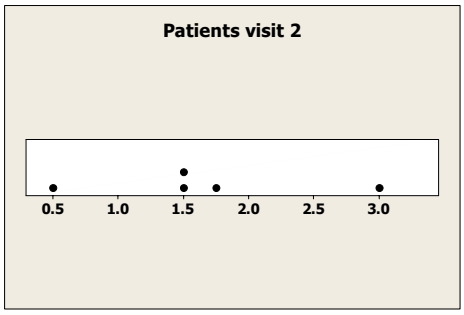


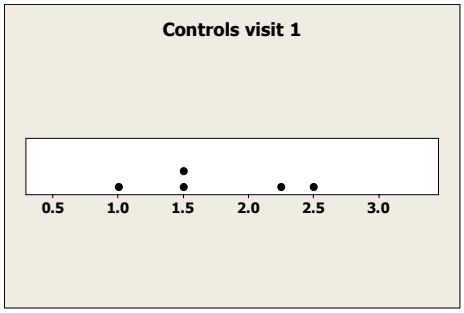


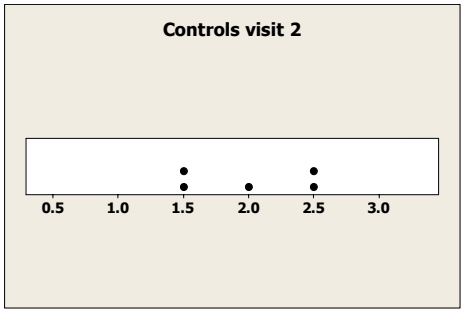


Dot plots to show distribution of lymphocytes(10^9/L)









Appendix 40

Significance tests for CRP (mg/L)

N = 12	Mean (SD) Min-Max	N = 12	Mean (SD) Min-Max	95% mean CI (A - B)	Test	DF	P value
A = Patients (1)	122.5 (140.6) 3 - 490	B = Patients (2)	3.83 (3.54) 0 - 14		WSR		0.001
A = Controls (1)	1.25 (1.96) 0 - 6	B = Controls (2)	1.75 (1.87) 0 – 5.0	-1.335 – 0.335	PT	11	0.214
A = Patients (1)	122.5 (140.6) 3 - 490	B = Controls (1)	1.25 (1.96) 0 - 6		WSR		0.001
A = Patients (2)	3.83 (3.54) 0 - 14	B = Controls (2)	1.75 (1.87) 0 – 5.0	-0.22 – 4.39	PT	11	0.072

Significance tests for WBC (10^9/L)

N = 12	Mean (SD) Min-Max	N = 12	Mean (SD) Min-Max	95% mean CI (A - B)	Test	DF	P value
A = Patients (1)	8.91 (3.53) 4.2 - 14	B = Patients (2)	6.79 (2.06) 4.4 – 10.9	0.490 – 3.743	PT	11	0.015
A = Controls (1)	5.64 (0.993) 4.3 – 7.2	B = Controls (2)	5.633 (1.15) 4.3 – 7.2	-0.480 – 0.496	PT	11	0.971
A = Patients (1)	8.91 (3.53) 4.2 - 14	B = Controls (1)	5.64 (0.993) 4.3 – 7.2	0.91 – 5.62	PT	11	0.011
A = Patients (2)	6.79 (2.06) 4.4 – 10.9	B = Controls (2)	5.633 (1.15) 4.3 – 7.2	-0.263 – 2.580	PT	11	0.100

N = numbers compared (column 1 and column 3)
(1) = visit 1 (2) = visit 2

CI = confidence interval (A = parameter column 1, B = compared parameter column 3)
PT = Paired T-test
WSR = Wilcoxon Signed Ranks Test (exact 2 tailed test)
DF = degrees of freedom

= significant (P = 0.05)

Appendix 41

Patient/Volunteer cytokine values (pg/ml)

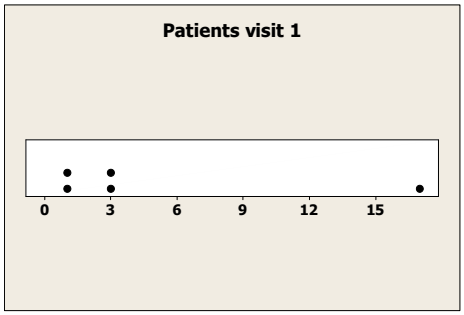
	IFN-λ		IL-1β		IL-2		IL-4		IL-5	
	P1	**C1**	**P1**	**C1**	**P1**	**C1**	**P1**	**C1**	**P1**	**C1**
Visit 1	2.79	0.68	0.08	0.15	0.34	0.10	0.43	0.10	0.08	0.21
Visit 2	0.54	0.89	0.17	0.15	0.31	0.15	0.37	0.09	0.03	0.31
	P2	**C2**	**P2**	**C2**	**P2**	**C2**	**P2**	**C2**	**P2**	**C2**
Visit 1	17.4	6.1	0.53	0.18	2.43	0.54	4.62	2.12	369.3	0.14
Visit 2	16.4	5.49	0.69	0.35	3.02	0.62	5.69	2.04	448.7	0.14
	P5	**C5**	**P5**	**C5**	**P5**	**C5**	**P5**	**C5**	**P5**	**C5**
Visit 1	0.76	1.0	0.19	0.02	0.10	0.11	0.38	0.50	0.08	0.09
Visit 2	0.12	1.70	0.07	0.15	0.10	0.26	0.62	0.47	0.13	0.16
	P6	**C6**	**P6**	**C6**	**P6**	**C6**	**P6**	**C6**	**P6**	**C6**
Visit 1	1.32	1.30	0.00	0.10	1.82	0.22	0.32	0.25	0.16	0.30
Visit 2	1.62	0.30	0.01	0.03	2.77	0.21	0.16	0.15	0.27	0.27
	P7	**C7**	**P7**	**C7**	**P7**	**C7**	**P7**	**C7**	**P7**	**C7**
Visit 1	2.51	0.34	0.19	0.00	0.33	0.08	0.15	0.14	0.09	0.01
Visit 2	0.74	0.77	0.28	0.05	0.43	0.13	0.22	0.03	0.16	0.16

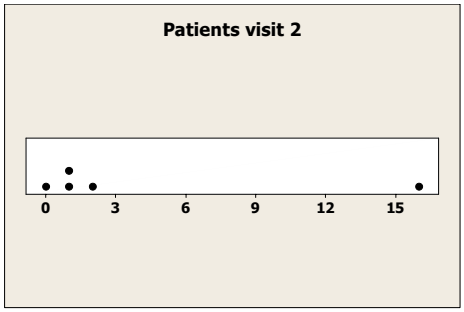
	IL-8		IL-10		IL-12p70		IL-13		TNF-α	
	P1	**C1**	**P1**	**C1**	**P1**	**C1**	**P1**	**C1**	**P1**	**C1**
Visit 1	1.05	0.35	0.54	0.37	0.20	0	1.63	1.39	2.09	0.93
Visit 2	1.12	0.73	0.26	0.47	0	0.09	0.03	1.65	1.70	1.50
	P2	**C2**	**P2**	**C2**	**P2**	**C2**	**P2**	**C2**	**P2**	**C2**
Visit 1	1.66	0.75	1441.0	0.65	3511.0	1.16	972.0	2.12	7.09	0.89
Visit 2	2.19	0.17	1559.0	0.74	4624.0	1.38	1213.0	1.85	5.46	1.08
	P5	**C5**	**P5**	**C5**	**P5**	**C5**	**P5**	**C5**	**P5**	**C5**
Visit 1	1.75	0.44	0.34	0.14	0.95	0.05	0.98	0.28	2.42	0.73
Visit 2	0.61	0.46	0.25	0.67	1.22	0.31	0.31	0.65	1.72	1.26
	P6	**C6**	**P6**	**C6**	**P6**	**C6**	**P6**	**C6**	**P6**	**C6**
Visit 1	1.70	0.61	0.19	0.67	0.93	0.23	0.66	1.21	2.07	5.43
Visit 2	0.43	0.31	0.95	0.45	3.85	0.06	3.99	0.54	3.47	4.58
	P7	**C7**	**P7**	**C7**	**P7**	**C7**	**P7**	**C7**	**P7**	**C7**
Visit 1	3.90	0.81	0.63	0.21	0.10	0	0.34	0.47	3.13	2.35
Visit 2	0.48	3.27	0.83	0.47	0.26	0.02	0.89	0	3.50	5.05

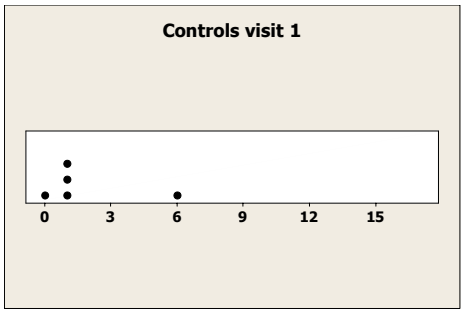
Below the detection limit of the assay

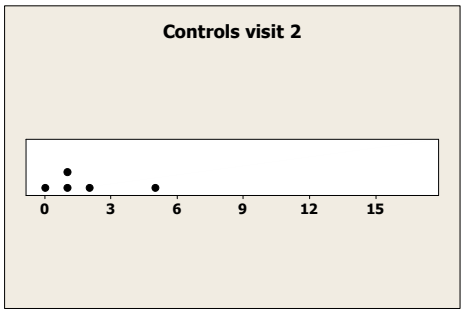
Appendix 42

Dot plots to show distribution of IFN-γ (pg/ml)
(n = 5)

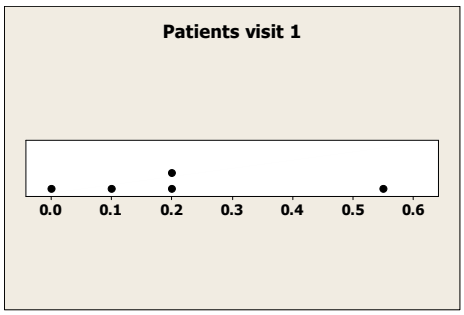


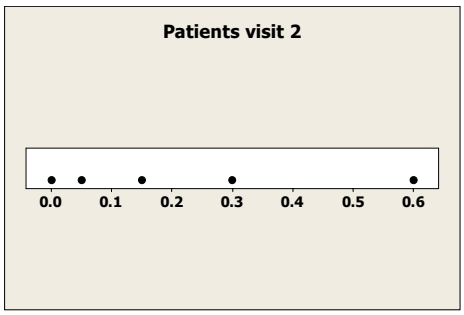


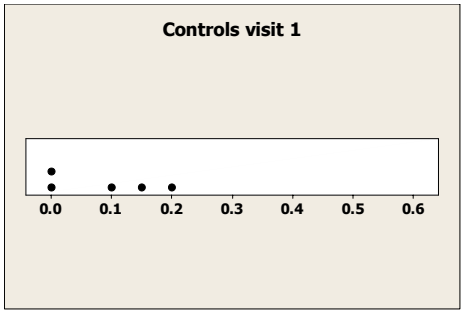


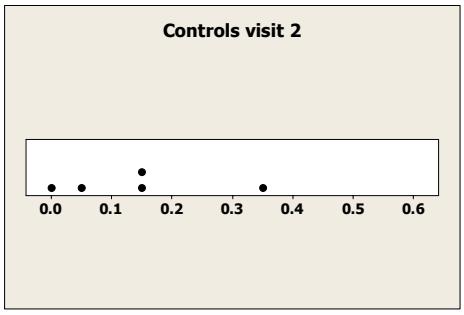


Dot plots to show distribution of IL-1β (pg/ml)
(n = 5)



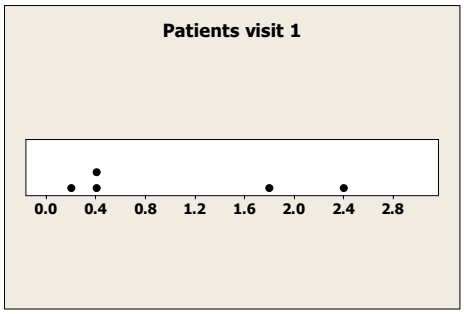


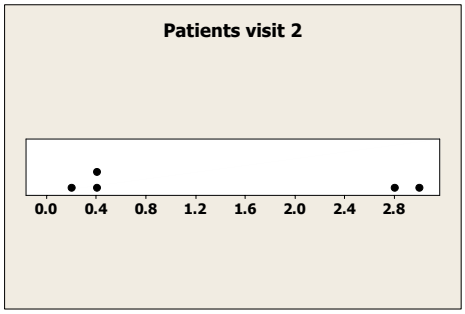


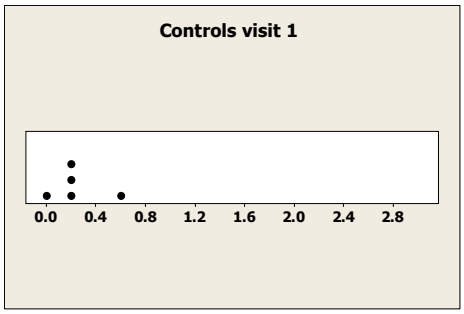


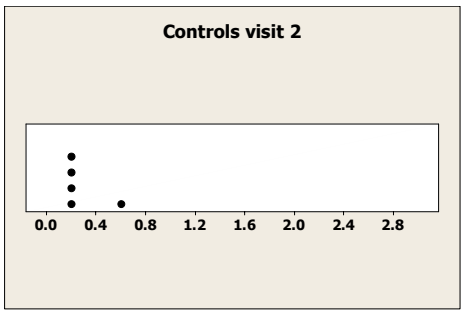
Appendix 43

Dot plots to show distribution of IL-2 (pg/ml)
(n = 5)

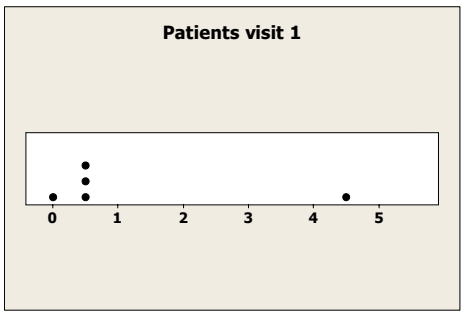


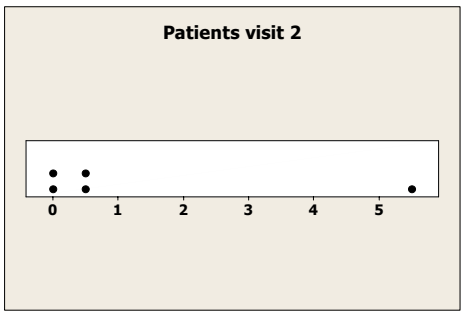


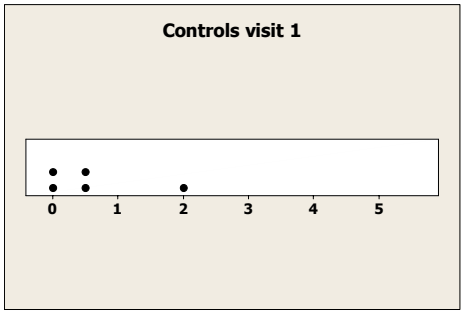


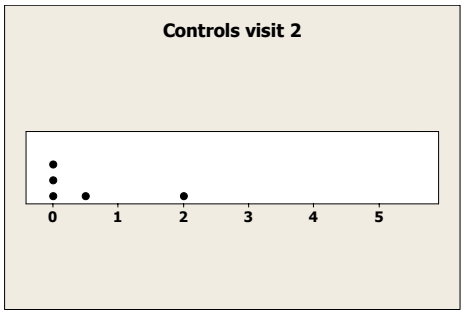


Dot plots to show distribution of IL-4 (pg/ml)
(n = 5)



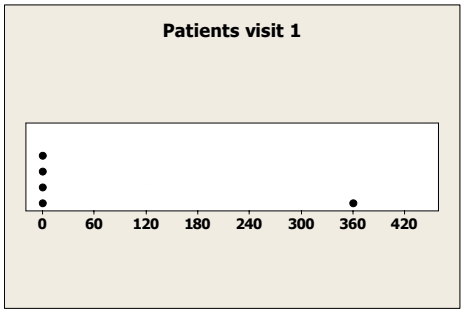


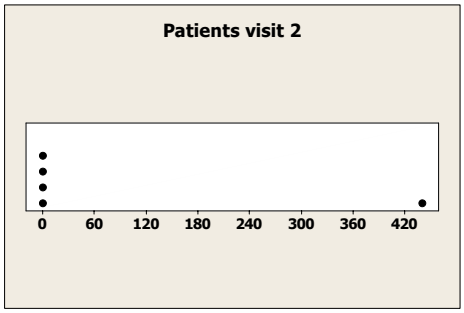


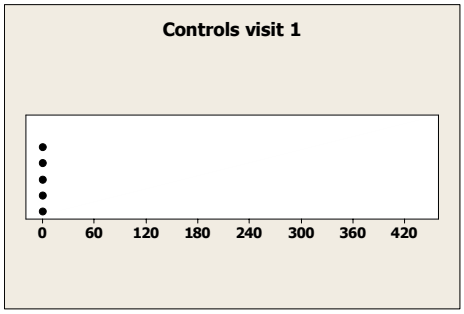


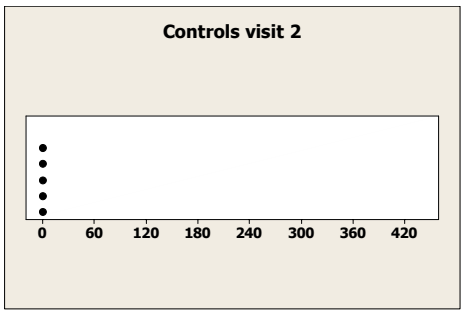
Appendix 44

Dot plots to show distribution of IL-5 (pg/ml)
(n = 5)

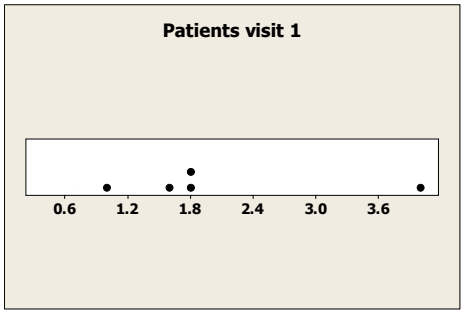


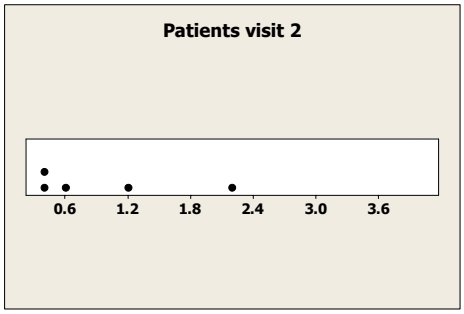


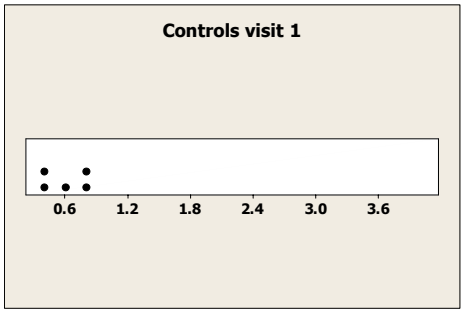


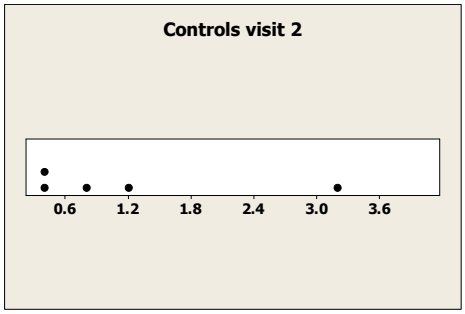


Dot plots to show distribution of IL-8 (pg/ml)
(n = 5)



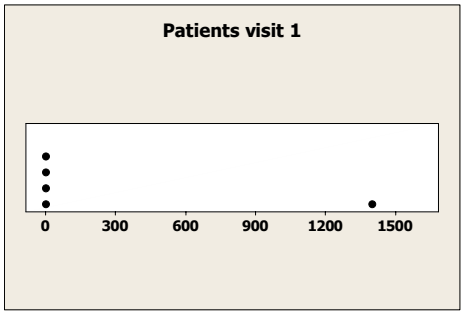


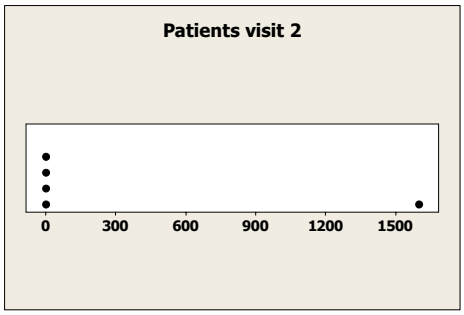


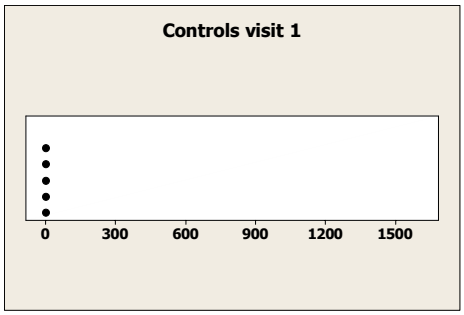


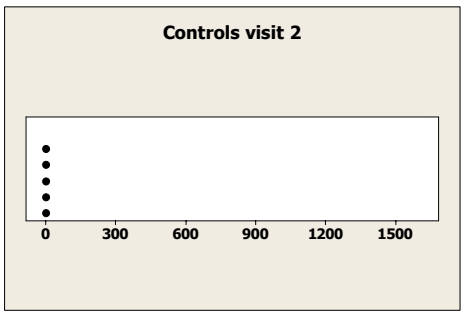
Appendix 45

Dot plots to show distribution of IL-10 (pg/ml)
(n = 5)

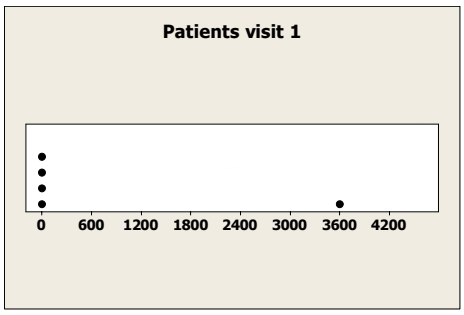


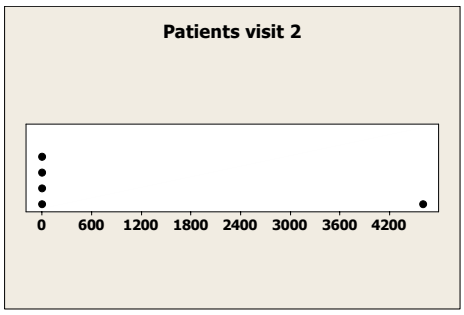


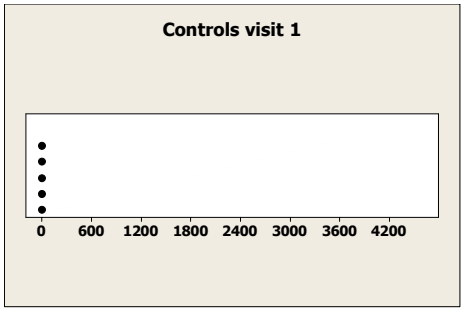


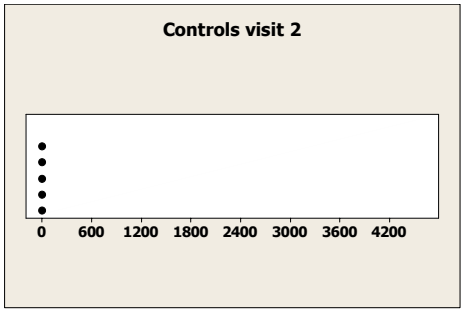


Dot plots to show distribution of IL-12p70 (pg/ml)
(n = 5)



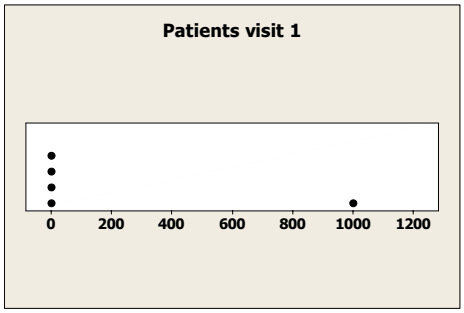


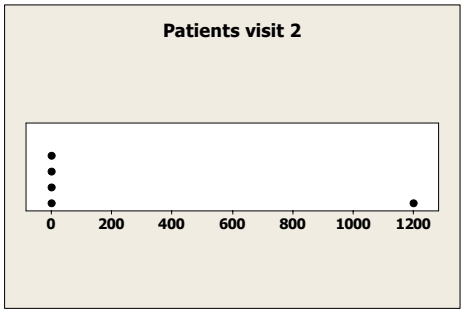


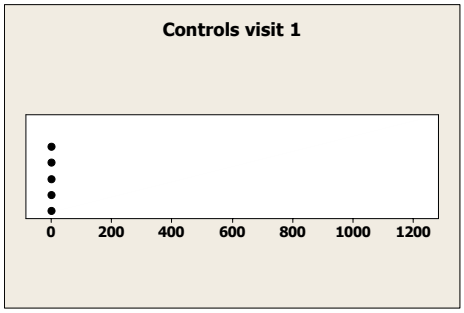


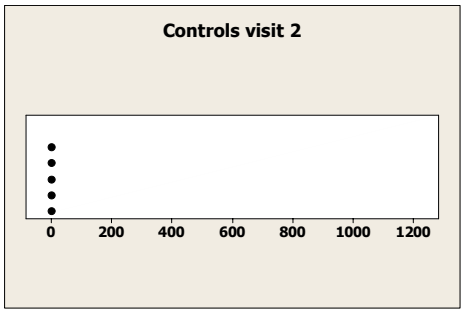
Appendix 46

Dot plots to show distribution of IL-13 (pg/ml)
(n = 5)

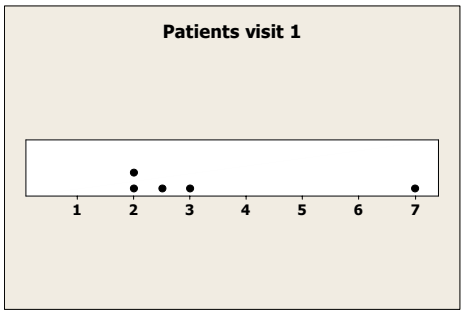


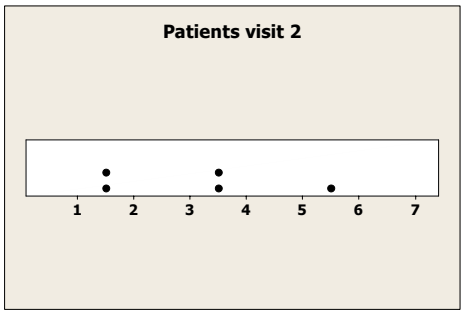


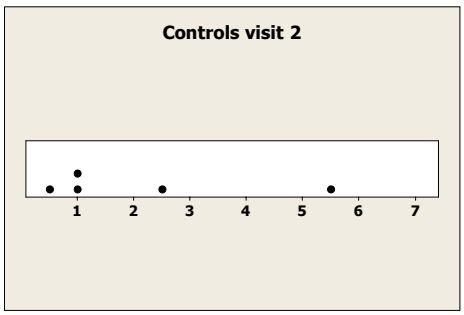


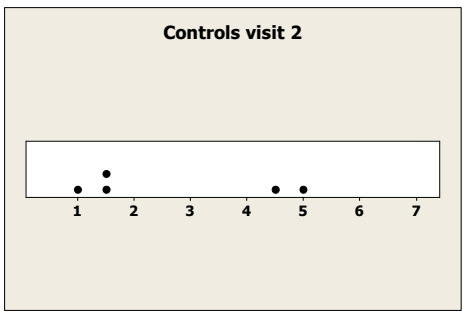


Dot plots to show distribution of TNF-α (pg/ml)
(n = 5)









Appendix 47

Significance tests for serum IFN-λ results (pg/ml)

N = 5	Min-Max	N = 5	Min-Max	Test	P value
Patients (1)	0.76 – 17.4	Patients (2)	0.12 – 16.4	WSR	0.125
Controls (1)	0 – 6.1	Controls (2)	0 – 5.49	WSR	1.0
Patients (1)	0.76 – 17.4	Controls (1)	0 – 6.1	WSR	0.188
Patients (2)	0.12 – 16.4	Controls (2)	0 – 5.49	WSR	0.813

Significance tests for serum IL-1β results (pg/ml)

N = 5	Mean (SD) Min-Max	N = 5	Mean (SD) Min-Max	95% mean CI (A - B)	Test	DF	P value
A = Patients (1)	0.198 (0.20) 0 – 0.53	B = Patients (2)	0.224 (0.23) 0 – 0.6	-0.137 – 0.085	PT	4	0.552
A = Controls (1)	0.086 (0.08) 0. – 0.18	B = Controls (2)	0.14 (0.13) 0 – 0.35	0.191 – 0.839	PT	4	0.338
A = Patients (1)	0.198 (0.20) 0 – 0.53	B = Controls (1)	0.086 (0.08) 0. – 0.18	-0.125 – 0.349	PT	4	0.261
A = Patients (2)	0.224 (0.23) 0 – 0.6	B = Controls (2)	0.14 (0.13) 0 – 0.35	-0.099 – 0.267	PT	4	0.272

N = numbers compared (column 1 and column 3)
(1) = visit 1 (2) = visit 2

CI = confidence interval (A = parameter column 1, B = compared parameter column 3)
PT = Paired T-test
WSR = Wilcoxon Signed Ranks Test (exact 2 tailed test)
DF = degrees of freedom

Appendix 48

Significance tests for serum IL-2 results (pg/ml)

N = 5	Mean (SD) Min-Max	N = 5	Mean (SD) Min-Max	95% mean CI (A - B)	Test	DF	P value
A = Patients (1)	1.005 (1.05) 0.10 – 2.43	B = Patients (2)	1.32 (1.44) 0.10 – 3.02	-0.857 – 0.213	PT	4	0.170
A = Controls (1)	0.21 (0.19) 0.08 – 0.54	B = Controls (2)	0.27 (0.20) 0.13 – 0.62	-0.136 – 0.008	PT	4	0.070
A = Patients (1)	1.005 (1.05) 0.10 – 2.43	B = Controls (1)	0.21 (0.19) 0.08 – 0.54	-0.299 – 1.887	PT	4	0.114
A = Patients (2)	1.32 (1.44) 0.10 – 3.02	B = Controls (2)	0.27 (0.20) 0.13 – 0.62	-0.581 – 2.685	PT	4	0.148

Significance tests for serum IL-4 results (pg/ml)

N = 5	Min-Max	N = 5	Min-Max	Test	P value
Patients (1)	0 – 4.62	Patients (2)	0 – 5.69	WSR	0.625
Controls (1)	0 – 2.12	Controls (2)	0 – 2.04	WSR	0.250
Patients (1)	0 – 4.62	Controls (1)	0 – 2.12	WSR	0.375
Patients (2)	0 – 5.69	Controls (2)	0 – 2.04	WSR	0.125

N = numbers compared (column 1 and column 3)
(1) = visit 1 (2) = visit 2

CI = confidence interval (A = parameter column 1, B = compared parameter column 3)
PT = Paired T-test
WSR = Wilcoxon Signed Ranks Test (exact 2 tailed test)
DF = degrees of freedom

= P value close to 0.05

Appendix 49

Significance tests for serum IL-5 results (pg/ml)

N = 5	Mean (SD) Min-Max	N = 5	Mean (SD) Min-Max	95% mean CI (A - B)	Test	DF	P value
Patients (1)	0.1 – 369.3	Patients (2)	0 – 448.7		WSR		0.313
A = Controls (1)	0.148 (0.114) 0 – 0.3	B = Controls (2)	0.208 (0.076) 0.14 – 0.31	-0.155 – 0.035	PT	4	0.154
Patients (1)	0.1 – 369.3	Controls (1)	0 – 0.3		WSR		1.0
Patients (2)	0 – 448.7	Controls (2)	0.14 – 0.31		WSR		1.0

Significance tests for serum IL-10 results (pg/ml)

N = 5	Mean (SD) Min-Max	N = 5	Mean (SD) Min-Max	95% mean CI (A - B)	Test	DF	P value
Patients (1)	0 - 1441	Patients (2)	5 - 1559		WSR		0.438
A = Controls (1)	0.408 (0.245) 0.140– 0.67	B = Controls (2)	0.560 (0.135) 0.45 – 0.74	-0.492 – 0.188	PT	4	0.282
Patients (1)	0 - 1441	Controls (1)	0.140– 0.67		WSR		0.438
Patients (2)	5 - 1559	Controls (2)	0.45 – 0.74		WSR		0.438

N = numbers compared (column 1 and column 3)
(1) = visit 1 (2) = visit 2

CI = confidence interval (A = parameter column 1, B = compared parameter column 3)
PT = Paired T-test
WSR = Wilcoxon Signed Ranks Test (exact 2 tailed test)
DF = degrees of freedom

Appendix 50

Significance tests for serum IL-13 results (pg/ml)

N = 5	Mean (SD) Min-Max	N = 5	Mean (SD) Min-Max	95% mean CI (A - B)	Test	DF	P value
Patients (1)	0 - 972	Patients (2)	0 – 1213		WSR		0.625
A = Controls (1)	1.09 (0.743) 0.28 – 2.12	B = Controls (2)	0.938 (0.784) 0 – 1.85	-0.408 – 0.720	PT	4	0.485
Patients (1)	0 - 972	Controls (1)	0.28 – 2.12		WSR		0.438
Patients (2)	0 – 1213	Controls (2)	0 – 1.85		WSR		0.438

Significance tests for serum TNFα results (pg/ml)

N = 5	Min-Max	N = 5	Min-Max	Test	P value
Patients (1)	2.07 – 7.09	Patients (2)	1.7 – 5.46	WSR	0.625
Controls (1)	0.73 – 5.43	Controls (2)	1.08 – 5.05	WSR	0.438
Patients (1)	2.07 – 7.09	Controls (1)	0.73 – 5.43	WSR	0.438
Patients (2)	1.7 – 5.46	Controls (2)	1.08 – 5.05	WSR	1.0

N = numbers compared (column 1 and column 3)
(1) = visit 1 (2) = visit 2

CI = confidence interval (A = parameter column 1, B = compared parameter column 3)
PT = Paired T-test
WSR = Wilcoxon Signed Ranks Test (exact 2 tailed test)
DF = degrees of freedom

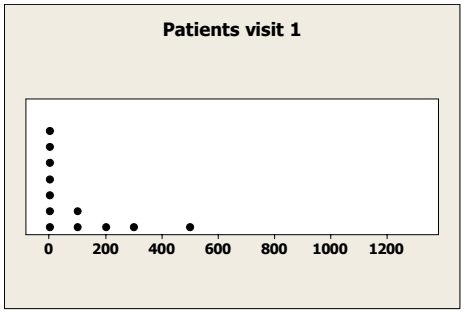
Appendix 51

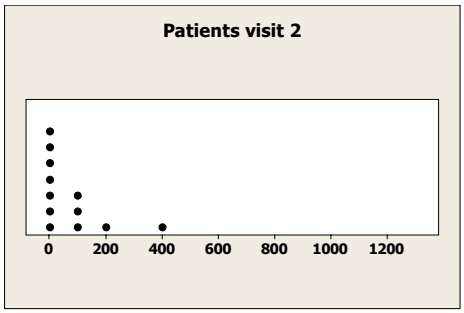
Patient/Volunteer IgE Scores (iu/mL)

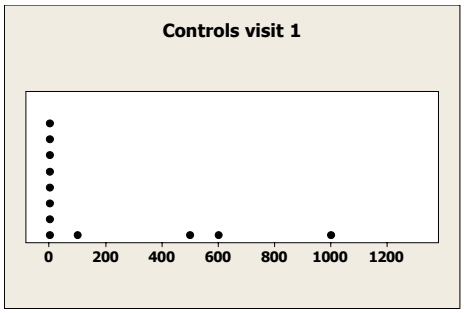
	Patients	**Controls**
	P1	**C1**
Visit 1	8.4	9.3
Visit 2	11.2	15.2
	P2	**C2**
Visit 1	474	46.1
Visit 2	373	33.5
	P3	**C3**
Visit 1	7.3	22.1
Visit 2	6.3	13.1
	P4	**C4**
Visit 1	0	111
Visit 2	21.3	107
	P5	**C5**
Visit 1	86	476
Visit 2	59	677
	P6	**C6**
Visit 1	256	574
Visit 2	208	641
	P7	**C7**
Visit 1	0	26.1
Visit 2	113	18.7
	P8	**C8**
Visit 1	10.4	0
Visit 2	8.3	13.2
	P9	**C9**
Visit 1	0	950
Visit 2	38.1	1260
	P10	**C10**
Visit 1	68.7	10.8
Visit 2	20.0	6.8
	P11	**C11**
Visit 1	189.0	31.2
Visit 2	114.0	30.7
	P12	**C12**
Visit 1	7.4	14.0
Visit 2	10.2	13.0

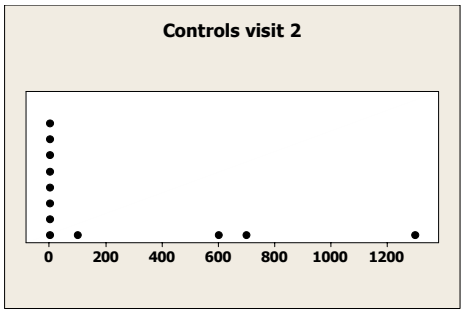
Appendix 52

Dot plots to show distribution of serum IgE iu/mL
(n = 12)









Appendix 53

Significance tests for IgE results

N = 12	Min-Max	N = 12	Min-Max	Test	P value
Patients (1)	0 - 474	Patients (2)	6.3 - 373	WSR	0.556
Controls (1)	0 - 950	Controls (2)	7 - 1260	WSR	0.424
Patients (1)	0 - 474	Controls (1)	0 - 950	WSR	0.556
Patients (2)	6.3 - 373	Controls (2)	7 - 1260	WSR	0.424

N = numbers compared (column 1 and column 3)
(1) = visit 1 (2) = visit 2

WSR = Wilcoxon Signed Ranks Test (exact 2 tailed test)

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