

Factors Predicting Patient Outcomes in a UK Burn's Unit; The influence of Acinetobacter Baumannii and the antimicrobial peptide LL-37 in burn wounds.

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**Thesis submitted to the University of London for the Degree of
Doctor of Philosophy (PhD)**

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London 2011

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Abstract

Sepsis and multi-organ failure are the most frequently reported causes of death in burn injuries. Their early identification allows therapies and resources to be targeted in a more effective and efficient way. Due to its frequent antibiotic drug resistance *Acinetobacter baumannii* (MRAB) is increasingly causing a problem in burns units. New strategies need to be found to combat infection and sepsis in the burn ICU.

This study examines the potential of the Albumin Creatinine Ratio, a marker of systemic endothelial dysfunction in predicting outcomes, sepsis and multi-organ failure; the role of *Acinetobacter* in causing organ failure; and explores for the presence of the cathelicidin, LL-37 in the burn wound and examines its potential utility for treating infection and sepsis.

It was found that ACR on admission and at 48 hours is predictive of patient outcomes and the development of sepsis, and may be of use predicting multi-organ failure.

Multi-organ failure occurs more frequently in MRAB patients compared to those patients with drug sensitive *Acinetobacter baumannii*. The number of agency nursing staff and work intensity are possible contributing factors in MRAB acquisition. LL-37 has been found in both acute burn wounds as well as in the grafted healing burn wound and is active against drug resistant *Acinetobacter baumannii*.

ACR can therefore identify those patients at risk of sepsis and may have a role in predicting multi-organ failure.

MRAB acquisition in the burns intensive care unit is a significant cause for concern as patients are more likely to suffer from multi-organ failure as well as prolonging their hospital stay and resulting in poorer outcomes.

LL-37 has many functions and importantly plays a role in the body's innate immune system. In the era of increasing antibiotic resistance it may provide a novel therapeutic role in treating MRAB infection.

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Abbreviations

ACCP	American College of Chest Physicians
ACR	Albumin creatinine ratio
AMP	Antimicrobial peptide
APACHE	Acute Physiology and Chronic Health Evaluation
ARDS	Adult respiratory distress syndrome
AUC	Area under curve
CAMP	Cathelicidin anti-microbial peptide
CEA	Cultured epithelial autographs
CLP	Cecum ligation and puncture
Cp	Crossing point
Ct	Crossing threshold
EC	Effective concentration
EGF	Epidermal growth factor
EPOR	Erythropoietin receptor
ESBLs	Extended spectrum β -lactamases
Fio ₂	Fraction of inspired oxygen
FTB	Full thickness burn
GGT	Gamma glutamyl transpeptidase
G-CSF	Granulocyte colony- stimulating factor
hCAP18	Human cathelicidin
HDU	High Dependency Unit
ICAM	Intercellular adhesion molecule
IL-1 beta	Interleukin-1 beta
IL	Interleukin
IGF-1	Insulin-like growth factor 1
IFN- γ	Gamma interferon
ICU	Intensive Care Unit
LC50	Lethal concentration
LL-37	Cathelicidin (human)
LTB ₄ /C ₄	Leukotriene B ₄ /C ₄

MARC	Microalbuminuria/urinary creatinine ratio
MBC	Minimum bacterial concentration
MCP-1	Monocyte chemoattractant protein-1
MMP	Matrix Metalloproteinase
MIC	Minimum inhibitory concentration
MIP-1	Macrophage inflammatory protein-1
MOF	Multiorgan Failure
MRAB	Multi-resistant <i>Acinetobacter baumannii</i>
NK cells	Natural killer cells
NPV	Negative Predictive Value
OR	Odds Ratio
PAF	Platelet Activating Factor
PAI	Plasminogen Activator Inhibitors
Pao2	Partial pressure of arterial oxygen
PCR	Polymerase chain reaction
PDECGF	Platelet derived endothelial cell growth factor
PDGF	Platelet Derived Growth Factor
PGD2/E2	Prostaglandin D2/E2
PPIA	Peptidylprolyl isomerase A
PPV	Positive Predictive Value
qPCR	Quantitative real time Polymerase Chain Reaction
ROC	Receiver Operating Characteristic
SAPS	Simplified Acute Physiology Score
SCCM	Society of Critical Care Medicine
SCLP	Sham cecum ligation and puncture
sFlt-1	Soluble fms-like tyrosine kinase-1
SIRS	Systemic Inflammatory Response Syndrome
SOFA score	Sequential Organ Failure Assessment score
TBSA	Total Body Surface Area
TGF- β	Transforming growth factor beta
Th-cells	T- helper cells
TNF	Tumour necrosis factor
VCAM	Vascular cell adhesion molecule

VEGF

Vascular endothelial growth factor

Acknowledgements

I would like to thank my supervisors Professor F Fortune and Dr E. Hagi-Pavli for giving me the opportunity to conduct this work and for their support, encouragement and guidance through out the process.

I would like to thank Professor P Dziewulski for allowing me access to the St Andrew's burns unit for collection of my data and also thank and acknowledge the patients who have made this study possible.

Many people have helped me in the laboratory, many thanks to all of them, Dr Alan Cruchley, Dr Simon Rawlinson, Steve Cannon, Dr Rob Whiley, Dr Rob Allaker, Dr Melanie Lodrick, Tanya Novak and Anand Lalli. Statistical help was gratefully received from Mike Parker.

Lastly, and most importantly I would like to thank my wife and children who have offered constant support and love throughout my work.

Chapter 1 Burn injuries and treatment- an overview

1.1 Introduction

Until a few hundred years ago fire was considered one of the four main ingredients from which all other substances could be formed. This hypothesis has long since been debunked but it is without doubt that fire and the ability to tame it has been key to the evolution and development of man.

Man has used fire for around a million years, from the time of *Homo erectus*, man's predecessor, and it is safe to assume that burns have been sustained from this time.

Until relatively recently, few statistics were gathered about the nature or extent of burns sustained and most reports of fires, such as the Great Fire of London, centred their attention on the number of buildings destroyed rather than the number of deaths and certainly little if any mention was made of non lethal injury.

In recent years the information regarding burns and scalds has been collected in a more systematic and thorough way. The government publish yearly figures for the number of scalds and burns in the UK (Mortality Statistics, Accidents and Violence <http://www.statistics.gov.uk/hub/population/deaths/mortality-rates>) and the general trend has been a reduction in the number of deaths over the last 40 years. Deaths attributable to fires (even if the death occurred weeks or months later) number between 400-500 per annum (United Kingdom Fire Statistics 2008, www.communities.gov.uk) with the majority being house fires.

Most burns units collect their own data and the minimal information would include age, sex, cause and extent of injury, type of burn, time of accident and time between injury and hospital assessment or admission, 1st aid and treatment prior to formal assessment, family size, socio/marital status. An appreciation of these factors is essential to directing burn care and especially burn prevention programmes.

A variety of burns units have looked at their data in more detail and the types of burns and ages of patients affected. Despite local differences the results are broadly similar when comparing units in different parts of the world. For instance, looking at a specific group, scald burns accounted for 65.7% of the total

number of children treated for burns in a study in the USA (Drago 2005). A 10-year retrospective study in India showed similar results with 63.1% of burns in the paediatric population being due to scalds (Kumar et al., 2001) and in the UK figures for paediatric scalds attending an emergency department were 51% (Rawlins et al., 2007). In general children are more likely to suffer scalds and adults, flame related injuries.

The treatment and management of burns has certainly been advanced and driven by a variety of factors. These include increasing industrialisation which increased the number of work related incidents, including flame, scald and chemical; and secondly, within the last century, major world wars which meant large numbers of casualties attending hospitals at one time. In combination with advances in other fields' i.e. microbiology, anaesthesia and surgery, post war burn care has made great leaps in understanding the nature of the injuries, the healing process and the ability to return function and cosmetic appearance to the patients.

The last 30 years have seen a new age in burns care and research as the scientific advances are being translated to the clinical setting. There is still much to be learnt however, in the prevention, treatment and rehabilitation of burn injuries.

1.1.2 Skin Pathophysiology

Normal skin Anatomy

The skin is one of the largest organs in the body ranging from 0.025 m² in the newborn to 1.8 m² in the adult (Arturson 1996 B) it comprises 16% of body weight (Gawkrodger 2002). The skin consists of two layers; the epidermis and dermis. The epidermis ranges in depth from 0.05 mm thick (eyelids) to over 1mm thick on the soles of the feet; and the dermis is usually 10 times thicker than the associated epidermis. An average total skin depth is 1-2mm (Williams 2001).

Skin is thin in infants and increases in adults to the ages of between 30-40 years old whereupon it tends to decrease with age.

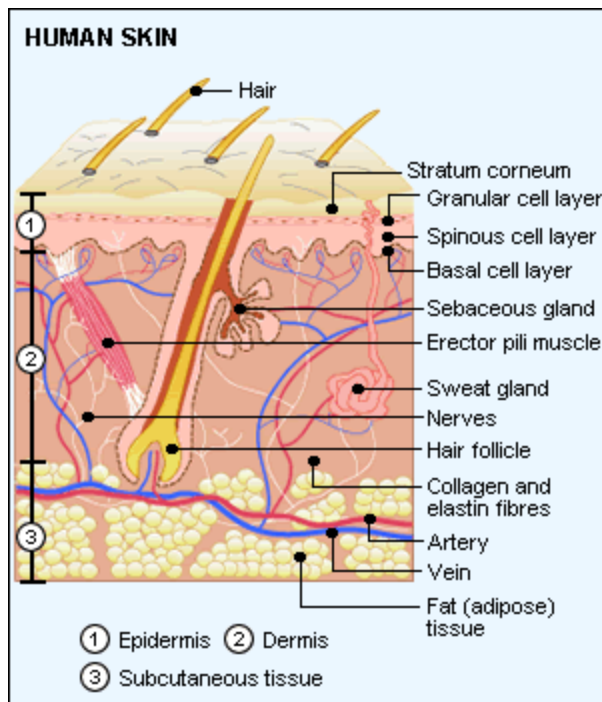


Figure 1.0 A schematic cross-section of the skin showing the layers of the skin and structures found within the skin (<http://www.mydr.com>).

Skin Development

Epidermis

The epidermis originating from ectoderm is formed of stratified squamous epithelium; it is mainly composed of keratinocytes and is arranged in four layers:

stratum corneum (or five layers if the stratum lucidum, found only in the palms and soles is counted), stratum granulosum, stratum spinosum and stratum basale. The basal layer is the deepest layer and rests on the basement membrane, which attaches it to the dermis. It consists mostly of young keratinocytes that are mitotically active, as well as melanocytes (5-10% of the basal cell population) and infrequently, merkel cells. It is a single layer of columnar cells, the basal surfaces of which sprout fine processes and hemi desmosomes, anchoring them to the *lamina densa* of the basement membrane. From the basal layer, cells migrate up and outwards maturing to become the stratum spinosum; a layer where mitosis no longer occurs but protein synthesis is prominent (Williams 2001). *Langerhans* cells are mostly found in this layer. The next layer of maturation is the stratum granulosum in which keratin production is prominent. It can be seen that cells become flattened and lose their nuclei. The last layer of keratinocyte maturation is the stratum corneum, a dead layer of cells composed of keratin and cellular debris. This is a relatively impervious layer that eventually desquamates.

Dermis

The dermis is derived from the mesoderm and is comprised of fibrous connective tissue containing specialized structures. The main cell type is the fibroblast, which produces extracellular protein, primarily collagen and elastin. Collagen makes up 70% of the dermis. It is secreted into the extracellular matrix where it undergoes maturation (cross-linking and coiling) into strong fibres oriented so as to allow stretchability whilst providing tensile strength (Herndon 2002).

Elastin fibres are loosely arranged in all directions of the skin and provide elasticity and an element of resting tension in the skin.

There is a constant turnover (degradation, re-modelling and production of collagen) of these elements. This occurs at a lower rate in unstressed skin but at a higher rate when healing is occurring or chronic mechanical stresses are applied.

The extracellular matrix, or ground substance, of the skin is made up of glycoaminoglycans, proteoglycans and other macromolecules; it provides a semi fluid matrix that lubricates the cellular and fibrillar components. It is through the ground substance that cells including inflammatory cells migrate, and nutrients diffuse.

The dermis is divided into 2 main layers, the superficial papillary dermis which interdigitates with the epidermal rete ridges and the thicker reticular dermis. A large plexus of vessels beneath the dermis, known as the sub-dermal plexus sends vessels upward to form a plexus between the reticular and papillary dermis. More superficially, there lies a network of small vessels, the papillary plexus. The dermis also contains hair follicles, sweat glands, lymphatics and open-ended nerve fibres (Arturson 1996 B). It is from some of the adnexal structures that re-epithelialisation occurs in superficial burns.

At the dermo-epidermal junction exists the basement membrane, a layer composed of mucopolysaccharides, which is also rich in fibronectin. Cells of the basal epidermal layer are joined to the basement membrane by hemidesmosomes. The basement membrane is anchored to the dermis with the help of special fibrils. (Herndon 2002)

Skin Function

Protective-barrier.

The stratum corneum is an effective barrier to the penetration of external agents, its structural integrity also protects against invasion by microorganisms. Barrier function is compromised when the stratum corneum is removed i.e. skin disease or injury. Penetration of a substance through the epidermis is indirectly proportional to the thickness of the stratum corneum and directly proportional to its concentration difference across the barrier layer (Hunter 2002). A rise in skin temperature aids penetration (Hunter 2002).

As previously mentioned, melanocytes are found interspersed between the keratinocytes in the basal layer. Melanocytes are dendritic cells, derived from the neural crest, which are responsible for melanin pigment production. The main stimulus to melanin production is ultraviolet radiation. Melanin protects the cell nuclei in the epidermis from the harmful effects of ultraviolet (UV) radiation.

The Protective/Immunological Role of Skin

The skin may be defined as an immunological organ as it contains nearly all the elements of cellular immunity (with the exception of B cells which are not found in normal skin but are in some disease states). The epidermal barrier can be

considered an example of innate immunity since the majority of microorganisms that have contact with the skin do not penetrate it (Lawrence 1996). The profuse blood supply and lymphatics to the dermis provide conduits through which immune cells can pass to or from their site of action.

The next immunological aspects of the skin to consider are the cells. Keratinocytes function as the first line of defence; as well as providing a physical barrier they are immune cells in their own right. As such, they produce a number of cytokines and on their surface express intracellular adhesion molecules (ie ICAM-1) and immune reactive molecules (ie MHC class II antigens) as well as preparing external antigens for presentation to T lymphocytes which then mount an immune response.

Keratinocytes also store the antimicrobial peptide cathelicidin, (a peptide involved in immune defense that is part of the innate immune system) in lamellar bodies (Braff et al., 2005) as well as the defensins and a range of other antimicrobial peptides that help protect the skin from bacterial invasion. Langerhans cells found in skin are dendritic cells derived from the bone marrow; they play a role in antigen presentation.

T lymphocytes can circulate through normal skin. Different T lymphocytes perform different functions i.e. helper cells, cytotoxic T cells and suppressor cells. Mast cells, like macrophages, are resident in the dermis and are recruited in inflammatory reactions.

Fluid and electrolyte homeostasis.

The stratum corneum prevents the loss of interstitial fluid from within.

Thermoregulation.

Thermoregulation of the body is achieved by a variety of systems in the body of which the skin plays an important role.

Blood flow is controlled by the sympathetic nervous system at the arteriovenous anastomoses at the superficial venous plexus. Local factors, physical and chemical i.e. H^+ , CO_2 also have an effect. The blood flow can be varied from 1-100 ml/min per 100g of skin (skin of fingers and forearm) (Gawkrodger 2002) depending on environmental and physiological influences.

The production of sweat also cools the body through evaporation. Sweat levels can vary between 0.5-10 litres in a day (Gawkrödger 2002).

Neurosensory

The skin is well supplied by the nervous system. Different areas of the body have different amounts of nerve endings, the skin of the hands, face and genitalia being particularly well supplied. The nerves supplying the skin have their cell bodies in the dorsal root ganglia. The nerve supply is by dermatome with varying degrees of overlap. Free sensory nerve endings are found in the dermis, extending to the epidermis where they are often associated with *Merkel* cells (Gawkrödger 2002). These nerve endings can detect heat, cold, pain and itch/tickle. There are also specialised corpuscular receptors in the dermis, the *Pancinian corpuscle*, which detects pressure and vibration, and *Meissner's corpuscle*, which is sensitive to touch (Gawkrödger 2002).

1.2 Burn Wounds

Thermal Energy

Thermal injury occurs as a result of an energy transfer from a heat source to the body. This may be by three main methods:

Conduction; whereby the energy is transferred from high-energy molecules to lower energy by direct contact.

Convection; the transfer of heat, in a gas or liquid, by the circulation of currents, from one region to another.

Radiation; where a molecule may give up kinetic energy through conversion to electromagnetic energy.

The energy transfer may depend on a variety of factors (Arturson, 1996 B.)

1. The conductivity, which varies in different kind of tissues.
2. The rate of absorption or dissipation of heat, which is dependent initially upon the function of the peripheral circulation.
3. The presence or absence of insulation such as hair; cornified layers of surface epithelium, natural skin oils etc.
4. The total water content of the skin.
5. The amount of surface pigmentation.
6. The presence or absence of clothing of different kinds.

As these factors are impossible to measure directly in an injured patient it is therefore better to classify burn injury by factors such as the intensity of heat exposure, duration of exposure and anatomically by depth of injury relative to the thickness of the epidermis and dermis in different areas of the body. These details frequently cannot be obtained from the patient (if confused or unconscious) and may have to be deciphered anecdotally from emergency personnel or witnesses, making their accuracy difficult to verify.

Duration of heat exposure

The body can resist heat damage of even a high temperature if it is for a short duration. Prolonged heat exposure of a moderate temperature can likewise not cause significant damage. If the rate of heat dissipation is less than heat

absorption the tissue temperature will gradually rise to a point at which cellular disintegration will occur. The heat and time for which the temperature is sustained determine the level of damage incurred by the tissues.

Using a porcine model Moritz and Henriquez (1947) established the time-temperature relationship in thermal injury to skin. Using temperatures ranging from 44° to 100° C and between 1 second and seven hours they studied the relationship to damage incurred to the skin. From their findings they concluded that the rate at which irreversible cellular injury was sustained increased rapidly as the surface temperature was raised and that for each degree rise in surface temperature between 44° and 51° C the time required to produce such injury was reduced by approximately one-half. Above 51° C the relationship was no longer linear but followed the previously established trend.

At sustained temperatures enzyme systems begin to malfunction, early denaturation of proteins occurs and cellular functions are impaired, one of which is the Na⁺ pump. This failure results in high Na⁺ intracellular concentrations and concomitant swelling. At 44° C and above, damage to the cells exceeds the speed of repair, which eventually leads to cellular necrosis (again depending on the length of exposure to the heat source) (Williams 2001).

If the heat source is withdrawn, damage will continue until the temperature of the tissues is reduced to a range that the tissues can tolerate normally. This is the basis of emergency management of thermal burns; quickly reducing the temperature of the cells (usually by cold water in the emergency setting) may reduce cell damage.

Tissue Destruction

As has been mentioned, fairly low temperatures for a sustained period can cause enzyme systems to begin to malfunction. Increased temperatures can cause severe changes to proteins, known as coagulation, which involves destruction of all levels of protein architecture. At this stage cell necrosis is complete starting from the area where the heat energy was most focused (usually the surface of the skin) extending downwards. This is called the zone of coagulation. This area corresponds to the first of three areas of burn injury described by Jackson (1953). This area of coagulation is the burn eschar.

The next area of injury is the zone of stasis, so called because the damage leads to a slowing of the circulation, which eventually becomes static. A number of factors contribute to circulatory stasis such as formation of obstructing platelet microthrombi and microemboli (Boykin et al., 1980).

If this compromised area sustains further insults i.e. an ischaemic episode, dehydration, hypovolaemia or infection then the entire zone may become necrotic and be converted to dead eschar. Stasis may be reversed with cell recovery occurring within a week if the wound is treated optimally. Zawacki (1974) found in a study on burns induced on guinea pigs that in undressed wounds with the blisters removed reversal of capillary stasis was least and the necrosis full thickness in depth, the worst possible outcome. However reversal of capillary stasis was complete and necrosis absent when the blister was removed and the underlying tissue covered with a split thickness skin (porcine) graft, giving the best result.

Beyond the zone of stasis is the zone of hyperaemia. This area has minimal cellular injury but exhibits vasodilation as part of the inflammatory response. Provided this area does not undergo any further physiological insults complete recovery of the cells usually occurs in this area.

The role of wound oedema in the burn wound.

Severe burns cause a systemic inflammatory response and a microvascular leak of protein and fluid into the interstitium termed oedema.

Oedema formation occurs in a variety of disease processes in any part of the body and is due to a number of physiological causes. The oedema has a detrimental effect on nutrient and oxygen delivery. Oedema formation has positive and negative contributions to the burn wound. Firstly it can beneficially deliver substances into the interstitium to help fight bacteria. In addition, with the lymphatic system it aids bacterial and debris removal (Williams 2001). Its negative effects are the reduction of oxygen and nutrients delivered to the tissues and the increased risk of infection.

In animal models systemic capillary leak occurs during the first 24 hours, when thermal injury exceeds 30 TBSA% (Arturson 1961, Carvajal et al., 1979).

Oedema formation is quick with an immediate transit phase lasting 10-15 minutes followed by a sustained phase of oedema formation. The speed and extent of oedema formation is dependent upon the severity of the burn injury. The oedema starts to be reabsorbed immediately and continues as long as the oedema is present depending on the welfare of the patient. Typically oedema is maximal at 12-24 hours and is maintained at a high level for 48-72 hours (Williams 2001).

Why does oedema form?

Arturson (1967) established that oedema formation occurred rapidly after burn injury by observing a dog paw before and after scalding inside a plethysmograph. It was found that lymph flow increased after injury. In addition to increased lymph flow after injury, protein levels in the lymph were also increased, suggesting that the microvasculature had become more permeable. The two possible reasons for this are:

1. Direct damage to the microvasculature (capillary and venular endothelial cells) by the heat. This causes cells to swell disrupting intracellular connections, creating pathways through which fluid can be lost (Cotran 1965)
2. A variety of chemical mediators are released (histamine, bradykinins, oxygen free radicals, sensory neuropeptides) which increase vascular permeability (Arturson 2000).

Arturson (1967, 2000) also established that very large pressures (200-300mmHg) would be needed to drive fluid to create the large amounts of fluid seen in the oedematous burn. This was subsequently endorsed by Pitt et al., (1987) who demonstrated that capillary pressures almost double early after burn injury, and who proposed histamine as one of the agents responsible due to its dilatory properties on arteries. It was also established that interstitial hydrostatic pressures are dramatically reduced in the post-burn period, dependant on the severity of the burn (Lund et al., 1988). The reason for this is thought to be that the collagen fibres and their connections to cells are damaged, resulting in a mechanical expansion of the interstitial space creating a vacuum. This, in effect, sucks fluid into the interstitial space.

Finally, in addition to the changes in hydrostatic pressure there is an increase in interstitial osmotic pressure. This can occur because of a greater number of osmotically active particles being present due to the breakdown of collagen and other interstitial proteins.

Endothelial dysfunction

An important aspect of the systemic inflammatory response is the endothelial dysfunction that contributes to the leak of protein and oedema formation. Methods of assessing this leak have traditionally involved radioisotope or dye labelling of albumin and artificial lymphatic fistulas in animal models. Immunoassays have subsequently been developed that can detect low concentrations of albumin in the urine in a range of 30-200 mg/L which is termed microalbuminuria (Vlachou et al., 2006). An increase of radiolabelled albumin escape rate has been demonstrated during surgery, sepsis and malignancy with a time frame similar to microalbuminuria (Fleck et al., 1972 Fleck et al., 1985). In humans, acute inflammatory insults such as surgery have also resulted in microalbuminuria (Gosling et al., 1988). These results have led to the hypothesis that microalbuminuria is a reflection of systemic vascular dysfunction (Gosling 1995) which has subsequently been proved by Jensen et al., (1995).

Microalbuminuria has since been used in a variety of settings to predict outcome as it is considered a surrogate marker of systemic inflammation. It has been used in acute medical illnesses such as pancreatitis (Shearman et al., 1989) and

meningitis (Roine 1993); surgery (Tsang et al., 1998); trauma (De Gaudio et al., 1999, Gosling et al., 1986, Gosling et al., 1994) and to predict outcome in patients admitted to intensive care (Gosling et al., 2003, 2006). Currently there have been limited studies in burn patients (Vlachou et al., 2006, 2008, Cochran et al., 2008) and it is not clear if microalbuminuria can predict outcomes in the ICU burn population.

1.3 Principles and Practice of Burn Management

Burn Wound Depth

The depth of a burn wound determines the treatment and is classified in relation to the anatomical level of injury. Burns traditionally used to be divided into first, second, third and fourth degree, they are now generally divided into the following categories:

- i. Superficial burns (erythema),
- ii. Partial thickness which can be superficial dermal or deep dermal, and
- iii. Full thickness burns.

Superficial burns

Superficial burns affect only the outer epidermis and are characterised by mild pain or discomfort and erythema due to vasodilation (Benson et al., 2006). The pain usually lasts for two to three days. Eventually desquamation of the damaged superficial epithelium occurs and is followed by scar less healing in 7-10 days. These burns were formally known as first degree burns.

Partial thickness burns

Partial thickness burns are divided into two categories, again dependent on the depth of the burn. In a partial thickness burn the entire epidermis and a varying amount of the dermis is destroyed. Because of survival of nerve endings in the superficial to mid part of the dermis these burns can be acutely painful. There is often a protein rich oedema resulting in separation of the damaged epidermis and blister formation.

Superficial partial thickness: describes destruction involving the epidermis and upper third of the dermis. (See table 1). A burn of this depth will normally heal well within ten days to two weeks with minimal scarring. This is due to rapid re-epithelialisation of the surface due to migration of epithelial cells that have survived in deeper parts of the hair follicles and other adnexal structures such as the sweat and sebaceous glands.

One caveat is that these wounds may have a significant zone of stasis underneath the superficial damage and are therefore at risk of conversion to a deeper wound if factors such as infection, ischaemia or inadequate rehydration intervene.

The treatment for a superficial dermal burn consists of simple dressings and wound checks.

ii Deep partial thickness: describes injuries involving the epidermis along with most of the underlying dermis. At this level, only a few of the remaining epithelial cells that are located in the deepest parts of the epithelial appendages are viable. As a result, it would take months for re-epithelialisation to complete if this type of wound were left to heal by itself. In this instance, blisters do not occur because of the thick burn eschar which is adherent to the underlying dermal collagen (Arturson 1996 B). In addition pain sensation may be limited because the nerve endings have been destroyed, although some deep pressure sensors may still function.

If the wound is allowed to heal without intervention a dense scar forms with poorly functioning skin and a thin epidermis. The prolonged time for re-epithelialisation results in a long inflammatory phase allowing a large amount of collagen to be deposited, thus resulting in the dense scar. For this reason deep dermal burns are treated as full thickness burns (Arturson 1996 B)

Full thickness burns

Full thickness burns involves necrosis of the whole thickness of the skin and may involve underlying structures such as muscle, nerve, tendon cartilage or bone. As the whole thickness of the skin is involved there is limited opportunity for the wound to heal without extensive scarring which inevitably takes a long time as the epithelium has to migrate from the wound edges. The wound may also be reduced to some degree by contraction of the wound as it heals.

The wound is not painful, as all the nerve endings have been destroyed. It can have a characteristic appearance due to the heat coagulating the dermal blood vessels (rendering the damaged tissue avascular) which is a white waxy colour although depending on the injury the skin can appear leathery brown as well or even charred and black if prolonged heat exposure has occurred. To avoid the extensive scarring and contractures the recommended treatment for these burns is

excision of the burnt tissue and subsequent skin grafts. These wounds were formally called third degree burns.

If structures deeper than the skin were involved (bone, tendon or muscle) then these burns could frequently require more extensive reconstruction than just skin grafts such as local or distant tissue transfer or even amputation if reasonable function could not be restored.

Table 1.0 Classification and clinical appearance of thermal injury of different depth.

Based on ABC of wound healing-Burns (Benson et al., 2006).

Burn Type	Usual History	Skin colour	Blisters	Capillary refill	Sensation	Pinprick	Healing
Superficial	Sunburn	Red	Absent	Brisk	Painful	Bleeds	Heals in 7 days
Superficial dermal	Scalds of limited duration	Pale pink Dark pink	Present (small)	Brisk	Painful	Bleeds	Heals in 14 days
Deep dermal	Scalds of long duration Contact with high temperature	Dry blotchy red	May be present	Absent	No pain	No bleeding	Heals in months
Full thickness	Contact with high temperature Chemicals Electrical injury	Dry white/black	Absent	Absent	No pain	No bleeding	Granulates

Burn Eschar

In burn eschar all the protective functions of the skin (immunological, homeostasis, thermoregulation etc) have been lost and the burn is considered an open wound and therefore liable to infection. Pruitt et al., (1967) state that the eschar actually provides a medium for bacterial growth. The eschar has a high degree of permeability (Jelenko et al., 1971) and as a result the ability to retain fluid and electrolytes and plasma components is diminished.

Assessment of the burn wound

The ability to accurately assess the depth of a burn is notoriously difficult, and inaccurate analysis could easily lead to unnecessary or more extensive surgical procedures that may otherwise have been avoided.

Factors that are used in the assessment include;

1. History of the injury i.e. causative agent (scald, flame, chemical, cold etc), duration of exposure, first aid and care given prior to assessment etc.
2. Appearance of the wound (see table 1)
3. Assessment of pain. A gauge of the depth of the wound can be made by assessing how sensate it is to soft touch, and more accurately by pinprick.
4. Capillary return. The return of capillary filling after blanching caused by pressure on the wound (by pressing a finger onto the burnt tissue) gives an estimate of wound depth. Superficial burns blanch and capillary refill is rapid. Deep burns may be pale and show no capillary return due to the fact that the skin is avascular because the heat has coagulated the dermal vessels.
5. Surface temperature of the burn. It has been found that the temperature difference between burned and unburned skin correlates with the depth of injury (Wyllie and Sutherland 1991). In reality this is seldom used clinically as first aid (usually cold water) applied to the burn gives artificial results making for inaccurate assessment.
6. Laser Doppler. This is a method of determining blood flow in the burn wound and thus getting an idea of depth of damage to the tissues. The basic principle is that light from a helium-neon laser is shone over the skin where it 'interacts with

both stationary structures and moving blood cells. Back-scattered light from the moving cells is shifted in velocity using the Doppler principle, while back scattered light from stationary light from stationary objects remains at its original frequency. The mixing of these light waves is translated to an electrical signal, and mathematical estimation of blood flow can be made in normal versus study areas of skin (Herndon 2002).

When the type of wound has been determined the extent of injury is established by working out the percentage of body surface area affected. In children burns of >10% total body surface area must be admitted to hospital for treatment. In adults the cut off point is 15%. These are not hard and fast rules and obviously depend on the type of burn, age of patient and other co-morbid factors. Even if the injury does not require surgery burns of this size will require fluid replacement (due to the large losses through the damaged skin), which will have to be monitored.

Surgical treatment of a burn

Superficial burns and partial thickness burns of the superficial dermal variety of almost any size can be treated conservatively with appropriate dressings and fluid replacement. The required fluid replacement will be determined by the percentage of total body area affected.

Very small deep dermal and full thickness burns can be fully excised and closed directly by suturing the wound edges together. Only a few burns however fall into this category, the majority are larger and cannot be closed directly. In these circumstances the wound are typically tangential excised (Janzekovic 1970). This technique removes the necrotic tissue whilst preserving as much of the underlying tissue as possible. Partial thickness burns are debrided to a white, shiny dermal surface until punctate bleeding is apparent. In deep dermal burns this bleeding will be less frequent as the excision will uncover bigger vessels. Sometimes the tangential excision has to be continued to normal healthy fat (seen as yellow and glistening) is seen.

In small to medium sized injuries the wound can be closed by a skin graft, typically a split thickness skin graft (obtained by shaving a normal area of skin with a specialised knife or a dermatome and taking a layer of 8-10/1000th inch in

depth). This can be applied as a 'sheet' of unmeshed skin and secured in place and dressed appropriately.

If the burns are more extensive or donor sites are limited then the skin may be meshed at a variety of ratios, 1.5:1, 4:1 or even 6:1 to enable the skin to be stretched out to cover the resulting defect.

In the smaller burns and depending on the affected site of the body the skin graft may be a full thickness skin graft, as the cosmetic appearance is generally better.

In extensive or deep burns the tangential excision method may be redundant and instead the treatment consists of surgical excision down to the level of the underlying fascia. This includes the full depth of the skin as well as underlying subcutaneous fat down to the layer of investing fascia.

Burns affecting tendon, muscle and bone will require excision of all necrotic tissue and reconstruction using local or distant flaps as appropriate.

Recent advances in wound closure

Cultured Epithelial Autografts (CEA) and skin substitutes

Extensive burns (50-70%) result in a massive physiological stress on the patient and unique challenges as the donor sites are possibly going to be limited. One solution is the use of Cell cultured epithelial autograft (CEA) procedures that utilise skin cells from the patient usually taken at the time of primary burn excision. These are used to grow 'sheets' of new skin cells in a laboratory. These new sheets are used as grafts. Because these grafts are very thin (only a few cell layers thick) they are fragile and have a variable success rate. Because of this, and the time taken to produce the new skin the uptake of this method is limited. However, newer grafting procedures combine CEA with a dermal matrix for more support.

One of several relatively new materials being used is IntegraTM, which is a synthesized dermal replacement. It is a 'bilaminar composite that has a neodermis of bovine collagen held in a matrix pattern with shark cartilage chondroitin-6-sulphate. A layer of rubberised silicone is pressed sealed onto this and acts as a neoepidermis' (Herndon 2002). Over the next 2-3 weeks the neodermis becomes vascularised from the underlying wound and once this has

occurred the silicon sheet can be carefully removed and the underlying wound bed grafted with 6-8/1000th inch autograft or even CEA.

1.4 The Inflammatory Response to Burn Wounds

Inflammation is an essential component of normal wound healing when the wound is small and localised. In large burn injuries multiple biological cascades are initiated in a temporal fashion. There is initially an acute hyper-reactive immune response followed later by a hyporeactive phase. In critical burn injuries that inflammatory response can become deranged due to the global involvement of multiple tissue beds and recruitment of immune and non immune cells which puts a significant metabolic and strain on the repair process and the patient.

The inflammatory response is complex and many of the biological pathways overlap, influence each other and sometimes work in opposition. The balance of these factors determines the healing of the burn wound and is examined below.

When an organ or tissue is damaged in general it is unable to regenerate but instead forms a fibrotic scar. In the wound healing process the organ/tissue may not be fully restored to its previous state but repaired sufficiently to return to or maintain function. If the normal wound healing process is altered in any way it can result in greater tissue or organ damage and sub-optimal functional results.

Wound healing is complex with multiple processes interacting. It cannot be seen as a linear process but rather as a series of overlapping processes that have variable degrees of influence on each other.

Conventionally cutaneous wound repair has been divided into three parts as shown in the diagram below.

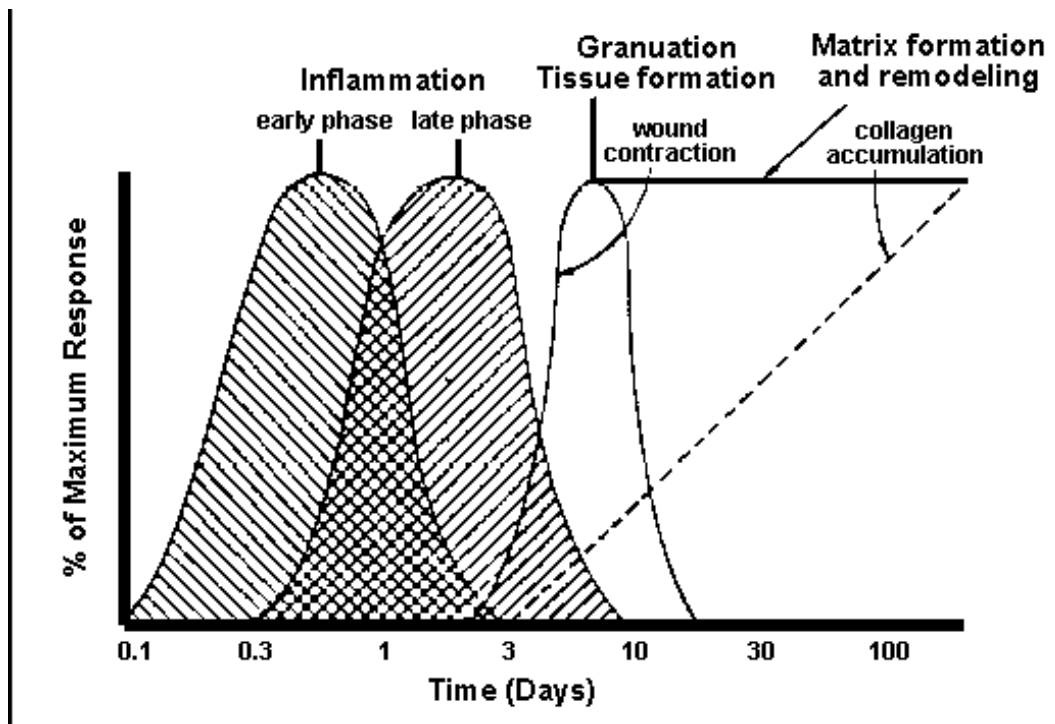


Figure 1.1 Phases of wound healing

Firstly the inflammatory stage which can be sub-divided into early and late stages. The second phase is re-epithelialisation and granulation tissue formation and then lastly matrix formation and re-modelling.

Collagen accumulation starts shortly after the onset of granulation tissue formation. (Clark 1996)

The following section gives an overview of cutaneous wound healing with specific reference to the process that occurs in a burns wound. Like any inflammatory reaction, burn wound inflammation comprises a whole cascade of interacting and overlapping mechanisms, which serve to respond to and treat the severe physiological challenges that a burn constitutes. The ‘insult’ of a major burn will result in both local effects and generalised responses involving the whole body. Because the effects of a large thermal injury are so complex with multiple interactions between various systems in the body some authors suggest looking upon a major burn wound as “a specialised organ with its own blood supply and unique metabolic characteristics” (Arturson 1996 A).

During the inflammatory phase of wound healing many of the inflammatory cascades overlap and occur concurrently; however for the sake of clarity they will be mentioned in the order that they tend to occur.

Inflammatory stage

Coagulation-fibrinolytic cascade

The first process that occurs during a major injury is damage to the blood vessels with the blood constituents being released into the wound, in the case of a burn wound this may occur with increased blood flow to the injured area due to vasodilatation and as was described in the chapter on burn wounds, oedema formation also occurs due to increased extravascular osmotic activity. Exposure of the blood to injured tissue results in the blood clotting. The blood clots as a result of 1) Surface activation of Hageman factor, 2) tissue pro-coagulant factor released from damaged cells, 3) surface membrane coagulation factors and phospholipids expressed on activated platelets and endothelial cells (Furie & Furie 1988). By a similar process Hageman factor activates the coagulation-fibrinolytic system in a burn. 2-3 hours after scalding the lymph and plasma in thermally damaged tissue displays hypercoagulability (Edery 1963).

With blood coagulation, platelet aggregation also occurs. This results in the formation of a fibrin rich clot that helps stop bleeding by plugging the damaged vessels and to some extent fills in wound space resulting from the injury. The fibrin matrix (a co-polymer of fibrin and fibronectin) can trap cellular debris and bacteria. The blood clot in the wound space will provide a provisional matrix for cell migration and act as a provisional matrix for the influx of monocytes.

Blood coagulation and platelet aggregation will cease when the stimuli to form the clot disappear. A number of factors are responsible for this including prostacyclin, antithrombin III, protein C, and plasminogen activator. (Clark 1996) Platelets also release chemotactic factors for blood leukocytes and growth factors such as platelet-derived growth factor (PDGF), transforming growth factor - α (TGF) and TGF- β which in turn activate macrophages and fibroblasts (Arturson 1996 A).

Complement

Concurrently with activation of the coagulation cascade the complement cascade is activated. The complement system is a group of ~20 different proteins found in the serum, which control inflammation. The complement cascade has two methods of activation, the classical or alternative pathways.

The classical pathway is activated by immune complexes containing antibodies of the IgG and IgM types as well as a number of other substances such as heparin, bacterial lipopolysaccharides and polynucleotides. The alternative pathway is activated by less specific mechanism in response to substances such as foreign substances (Arturson 1996 A). Activation of complement results in vasodilatation and increased microvascular permeability.

There are three major biological activities of the complement system (Roitt 1993)

1. The activation of phagocytes, including macrophages and neutrophils.
2. Opsonization (coating) of microorganisms and immune complexes so they can be recognised by cells expressing complement receptors.
3. Lysis of target cells.

Activation of either pathway results in the formation of fragments of complement factor C3. These fragments are activated by receptors that promote chemotaxis and phagocytosis of foreign particles and bacteria, in addition to facilitating cytotoxic reactions and the release of enzymes from the cells of the immune system. (Arturson1996 A).

At the same time the complement system is activated a whole host of other inflammatory mediators and pathways are activated including the kinin system, the arachadonic acid cascade, platelet activating factor and a variety of cytokines and chemokines.

Cytokines and Chemokines

The initial inflammatory response to trauma (whether it is a burn, infection or traumatic episode) is designed to maintain cellular function and restore homeostasis whilst responding to microbial challenges. The following anti-inflammatory phase helps restore function to normal. One group of molecules that modulates all of these aspects of inflammatory and anti-inflammatory response is the cytokine family.

The term cytokine includes interleukins, lymphokines and some other signalling molecules such as interferon and tumour necrosis factor- α (TNF- α). They are produced by a number of cells but are derived mainly from cells of the reticuloendothelial system (e.g. monocytes, macrophages and Kupffer cells)

(Gosain and Gamelli 2005). Cytokines work by their ability to bind to specific cellular receptors and influence immune cell differentiation and proliferation via intracellular pathways and gene transcription. Cytokines also influence their own and other cytokine production and suppression by positive and negative feedback loops.

Cytokines, when released work both locally and systemically where they exert their effects through endocrine, paracrine, autocrine and intracrine mechanisms (Clark 1991). An appropriate response results in inflammation at the injury site removal of any infection and then progress to normal wound healing. An inadequate or reduced cytokine response may result in delayed wound healing whilst an exaggerated response can result in haemodynamic instability, multi-organ failure and death (Gosain and Gamelli 2005).

A number of studies have looked at cytokine profiles post burn injury or wound manipulation to elucidate the relationship. Many of the studies have used animal models and in burns particularly the murine or rat model. Extrapolating animal model results to humans is not always accurate but Finnerty et al., (2009) assessed the similarities between humans and murine models in cytokine expression after burns. They found the post burn cytokine expression of 11 cytokines was similar in mice and children that confirmed the utility of the burned mouse model for studying human cytokine profiles.

Interleukins form a large group of cytokines. The majority are produced by T-cells, but they are also formed from mononuclear phagocytes. Whilst many interleukins have been studied in relation to thermal injury this study will focus on some of the more important proinflammatory molecules, in particular IL-6 and IL-8. Furthermore IFN- γ another pro-inflammatory cytokine will also be examined in more detail.

Cytokines in burns

It has long been established that cytokines levels are altered in response to a thermal injury. Early work by Drost et al. (1993 A) showed that plasma samples with detectable amounts of IL-1 beta and IL-6 were significantly more frequent in burned patients than in controls. Further work by the same team also showed that IL6 and TNF alpha levels were increased in severely infected burn patients as compared to patients who remained free of infection, and the IL6 level was

higher in infected patients who died than those who survived (Drost et al., 1993 B). Subsequent work has demonstrated that the relationship between cytokines and the factors that stimulate or suppress them are complicated and although many advances have been made in the last decade it will take many years before the complex response in burns is understood.

In general, however, the cytokine expression over time in burn patients follows a similar course if not complicated by organ failure or sepsis. Finnerty et al., (2006) demonstrated the normal trend by comparing cytokine expression profiles from severely burned children without signs of infections or inhalation injury to the cytokine profiles from normal, non-infected, non-burned children. The expression of pro-inflammatory and anti-inflammatory cytokines was maximal during the first week after thermal injury. Significant increases were measured for a large number of mediators during the first week after thermal injury: interleukin (IL) 1 β , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 p70, IL-13, IL-17 and interferon gamma (IFN- γ). Within five weeks the serum concentrations of most cytokines started decreasing towards normal levels. This data was correlated by a larger study (of 242 paediatric patients). Of 17 cytokines studied, 16 were drastically increased, most significantly IL-6, IL-8, monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1 (MIP-1), and granulocyte colony-stimulating factor (G-CSF). Even so called anti-inflammatory cytokines increased significantly post-burn. Only IL-5 was in the normal range post-burn and decreased over time (Jeschke et al., 2008).

At this stage it was not appreciated how prolonged the cytokines response could be. It was only after a further study by Jeschke et al., (2011); the largest series to date (977 paediatric patients) that again confirmed the previous findings but also demonstrated how long the inflammatory and metabolic response continues for. They followed up their patients for up to three years. Whilst most cytokines again showed a response to the burn injury dramatic changes were observed for serum IL-6, IL-8, G-CSF and MCP-1. These cytokines demonstrated an up to 2,000-fold increase immediately upon burn trauma and remained significantly elevated throughout the time period studied when compared with the concentrations detected in non-burned controls. INF- γ , TNF- α , IL-1 β , IL-2, IL-5, IL-7, IL-10, and IL-17 significantly increased by 2- to 20-fold in response to

burn injury and revealed relatively constant, but significantly increased levels for most of the three- year period post-burn compared to non-burned patients. IL-12p70 was not significantly altered compared to controls.

Whilst this cytokine response is generally consistent for most major burns there are significant differences between the adult and paediatric population. In a study comparing adults and children who had received burns greater than 20% TBSA it was found that during the first week following burn injury, IFN-gamma, IL-10, IL-17, IL-4, IL-6, and IL-8 were detected at significantly higher levels in adults compared with children. There were no differences in IL-12, IL-2, IL-7, or TNF levels in adult compared with pediatric burn patients at any time point. The authors concluded that this might provide an insight to the higher morbidity rate in adults. Also, the dramatic discrepancies observed in plasma cytokine detection between children and adults may mean that these two patient populations may benefit from different therapeutic interventions to attenuate the post-burn inflammatory response (Finnerty et al., 2008).

The next question is whether there is a relationship between the size of the burn and the cytokine response. Several small studies looking at only one or two cytokines have found conflicting results but the largest study by Jeschke et al., (2007) looked at 189 paediatric patients divided into different groups according to burn size. They found firstly that the burn size determines the inflammatory and hypermetabolic response. In terms of cytokines only six were significantly affected. Serum IL-8, TNF, IL-6, IL-12p70, monocyte chemoattractant protein-1 and granulocyte-macrophage colony-stimulating factor were significantly increased in the large burns. In general, it was found that the smaller the burn size, the lower the cytokine concentration, perhaps indicating a relationship between burn size and cytokine expression. Other cytokines, such as IL-1 β , macrophage inflammatory protein-1 β , IFN γ , IL-10, granulocyte colony-stimulating factor, IL-17, IL-13, IL-7, IL-5, IL-4, and IL-2, were affected by the burn but not by the size of the burn. These cytokines showed no significant difference between the burn size groups, but did present with significant changes over time.

Whilst these are significant findings the clinically relevance of the response of each cytokine is yet to be determined, it is not known which mediators are

induced directly by the burn injury and which are secondary mediators indicative only of systemic inflammation. One way of doing this has been to look at the cytokine response in specific circumstances. Certain cytokines seem to play a more prominent role in the post burn response especially if there is a concomitant injury or a complication and many studies have therefore looked at the cytokine profile of patients under these conditions to investigate this.

Gauglitz et al., (2008) compared severely burned children suffering from inhalation injury who did not survive with those who survived. They found significant differences in the serum levels of IL-4, IL-6, IL-7, IL-10 and IL-13. Of note IL-6 and IL-10 were significantly elevated in non-survivors at admission compared with survivors. Non-survivors had significantly lower IL-7 serum levels 5-7 days after hospital admission compared with survivors. A number of other cytokines were not significantly different between the groups (IL-2, IL-4, IL-5, IL-6, IL-8). By using logistical regression they found that the risk for mortality increased as the levels of IL-6 and IL-10 increased and that the risk of mortality increased as the levels of IL-7 decreased. A further study by the same group, again looking at paediatric patients and comparing a group with inhalation injury with a group without inhalation injury found that an inhalation injury causes alterations in IL-7 and IL-12p70. However there were no increased levels of pro-inflammatory cytokines, indicating that an inhalation injury in addition to a burn injury does not augment the systemic inflammatory response early after burn (Finnerty et al., 2007 A).

In an animal model (rats) Orman et al., (2012) looked at serial values of cytokines in response to a burn and septic shock (so called double-hit). The rats received a burn injury followed two days later by cecum ligation and puncture (CLP) to induce sepsis. Following this the rats were sacrificed at different time points and their cytokine profiles analysed. They found IL-6 (exhibiting both pro and anti inflammatory properties) was up regulated on the 7th and 10th days and postulated it might be to protect the host system from the subsequent complications caused by the burn and sepsis. In a further study by the same group (Orman et al., 2011), the response following the cecum ligation and puncture (CLP) or its corresponding control treatment (sham-CLP or SCLP) in male rats was analyzed by measuring different cytokines and chemokines. Whilst a number of cytokines were significantly altered in both burn and CLP (B+CLP)

and B+SCLP groups, IL-10 and IL-6 were significantly up regulated in the B+CLP group when compared to the B+SCLP group.

In humans a number of studies have found an increased risk of sepsis linked to certain cytokines. A recent study by Pileri et al., (2008) looked at the plasma levels of IL-6 and IL-10 in burn patients and correlated the levels with the severity of sepsis. They looked at 60 burn patients (adults), 34 of which developed sepsis (the control group was healthy volunteers). They found that both IL-6 and IL-10 levels, were significantly higher in septic patients than in non-septic patients. In the case of IL-6 this was after three days and for IL-10 throughout all points of the study. In addition Finnerty et al., (2007 B) found serum IL-6, IL-12 p70, and TNF can be used to identify paediatric burned patients who are at high risk of death from sepsis. Earlier work by Yeh et al., (2002) had suggested this by noting that a marked increase in serum levels of IL-6 before death by sepsis in their study of burned patients.

From the studies above it appears that the cytokine profile can be heavily influenced by either a concurrent injury (i.e. an inhalation injury) or a double hit such as sepsis. However, it must be noted that there is also evidence that the cytokine response can be altered significantly by interventions that occur during the care of the burn patients. Papini et al., (1997) examined the response of tumour necrosis factor (TNF) and IL-6 after burn wound dressing changes and débridement. They found that concentrations of tumour necrosis factor changed little during surgery or dressing changes, whilst there were dramatic increases (X3) in plasma concentrations of IL-6 during surgery or dressing change. A further part of the study found that bacteraemia can further increase the IL-6 response but preventing Gram- positive bacteraemia (by using teicoplanin) did not significantly affect IL-6 concentrations which may mean release of IL-6 is not the direct result of infection.

The above studies demonstrate that although significant insights have been gained into the inflammatory response and cytokine release over the last twenty years further studies are required to determine the clinical relevance of these findings as it is not clear which mediators are induced directly by the burn injury and which are secondary mediators indicative only of systemic inflammation.

Interleukin-6

IL-6 is produced by, T and B cells, endothelial cells, macrophages and fibroblasts. Its expression can be induced by other proinflammatory mediators such as IL-1, TNF and LPS (Cicco et al., 1990). It has multiple actions. It can induce B cells to differentiate into antibody forming cells, the proliferation of T cells and stimulates the production of a range of acute phase proteins (Gosain and Gamelli 2005). In patients with multiple trauma or sepsis, the degree of IL-6 elevation correlates with the risk of morbidity and mortality (Calandra et al., 1991) although a causative role for IL-6 has not been found, suggesting that it may be a marker for disease severity. Increased levels of IL-6 have been seen after major burns (Yeh et al., 1999, Jeschke et al., 2008) and most recently Jeschke et al., (2011) have demonstrated an up to 2,000-fold increase immediately upon burn trauma and found that levels remained significantly elevated up to sixty days post injury when compared with the concentrations detected in non-burned controls.

Abdel-Hafez et al., 2007 demonstrated a significantly higher level of IL-6 in a paediatric burn population that developed sepsis, those that died and also found a correlation of IL-6 with the TBSA% burned.

Interleukin-8

Interleukin-8 (IL-8, CXCL8) is a chemokine associated with acute inflammation and is found elevated in human wounds (Garner et al., 1994). IL-8 was discovered in 1987 (Yoshimura et al., 1987) It is a 8-10 kDa protein found in the form of two active isomers, a 77 amino acid form and the more active 72 amino acid form. It modulates the function of a number of inflammatory cells involved in the healing process.

Since its discovery its structure, gene location, and receptor structure and properties have all been established (Iocono et al., 2000).

IL-8 is secreted constitutively or in response to proinflammatory stimuli from a wide variety of cells including monocytes/macrophages, neutrophils, eosinophils, T-cells, keratinocytes, fibroblasts, endothelial cells and chondrocytes (Iocono et al 2000).

IL-8 production in macrophages is stimulated by a number of factors including TNF- α , IL-1 α and IL-1 β .

IL-8 is resistant to temperature and proteolysis and relatively resistant to an acidic environment (Remick 2005). These characteristics help it to survive in the suboptimal conditions that occur in sites of acute inflammation. Again, in burns patients, levels have been found to be raised very soon after injury and like IL-6 can be elevated for prolonged periods (Jeschke et al., 2008, Jeschke et al., 2011). Whilst the main studies that have looked at IL-8 in burns have been mentioned above, IL-8 has also been examined in regards to its role in lung injury. Rodriguez et al., (1993) looked at 88 patients with acute thermal injuries. Forty-eight hours after injury IL-8 (as well as TNF and IL-6) was significantly present in the systemic circulation, lung, normal skin, and thermally injured skin.

The presence of IL-8 (and TNF and IL-6) proteins in the lung, normal skin, and thermally injured skin were associated with TNF, IL-6, and IL-8 mRNA up-regulation. Using logistic regression analysis this team demonstrated that the presence of IL-8 in the lung was associated with early pulmonary physiologic dysfunction and nosocomial pulmonary infection. They concluded that acute thermal injury initiates an early systemic, lung, and skin response involving IL-8 TNF and IL-6 and that the TNF, IL-6, and IL-8 protein present in the lung and skin in response to acute thermal injury are generated locally and do not originate from the systemic cytokine pool. Furthermore they postulated that the lung cytokine response to acute thermal injury might initiate local organ failure.

Just a year later Donnelly et al., (1994) examined the cytokine profile associated with adult respiratory distress syndrome (ARDS) a complication seen after severe accidental trauma, including burns. Plasma samples were examined post injury and at 4 hourly intervals. Plasma IL-8 (and IL-6) concentrations were markedly increased starting in the immediate post injury period in both ARDS and non-ARDS patients, but no significant differences were found between the two groups until 16 hours after injury when plasma IL-8 concentrations became significantly higher in the ARDS group. From their results they concluded that measurements of plasma concentrations of proinflammatory cytokines, were not helpful in predicting the development of ARDS after severe accidental injury.

Kurzius-Spencer et al., (2008) also examined inflammatory markers in the lungs to assess the value of initial concentrations as predictors of subsequent lung

injury. Using a population of intubated, inhalation smoke victims they assayed the tracheobronchial fluid collected every two hours to determine which cytokines were present. In addition the partial pressure of arterial oxygen (Pao₂) and the fraction of inspired oxygen (Fio₂) were recorded approximately every 6 hours. The Pao₂/Fio₂ ratio is used to determine hypoxemia and is one of the criteria used to diagnose ARDS. Of the cytokines examined only initial IL-8 was associated with increased Pao₂/Fio₂ and with a minimum Pao₂/Fio₂ during the first 72 hours. In smoke inhalation victims, tracheobronchial IL-8 increases rapidly and the authors thought that high initial IL-8 may predict improved oxygenation. So it appears that IL-8 levels alone may not predict subsequent lung injury but used in conjunction with measures of lung function there may yet be a clinical use for determining IL-8 in lung injury.

Interferon (IFN)

There are three groups, IFN- α made by leukocytes, IFN- β made by fibroblasts and IFN- γ made by predominantly T and NK lymphocytes.

IFN- γ , which has been studied most, has been found to have antiviral activity, broad proinflammatory activity inducing the expression of TNF and IL-1 and be involved in the priming of circulating and tissue macrophages (Nathan et al., 1983). IFN- γ is produced by type 1 Th (T Helper) cells (CD4⁺ and CD8⁺) and natural killer cells. IFN- γ also promotes the differentiation of CD4⁺ T cells to the T_H1 phenotype, producing a positive-feedback loop and augmenting its own production (Gosain and Gamelli, 2005). Because of its role in priming macrophages as well as the anti-infective effects of IFN- γ observed in animal models and other clinical studies IFN- γ was used in a Phase III multicentre European trial to see if giving IFN- γ subcutaneously reduced infection rates or mortality in burn victims. After comparing the treatment group with the control group (who received a placebo) no difference was found in the mortality at 90 days, incidence of infectious complications and duration of intensive care unit or hospital stay (Wasserman et al., 1998).

IFN- γ production is reduced in mice from 2-7 days post burn (Suzuki and Pollard 1982). In human patients however, there are significant time-dependent changes in plasma concentrations of IFN- γ . Initially levels were found to be low but

began to rise from day 5 to 10 and decreased subsequently (Vindenes et al., 1995).

In the study by Jeschke et al., (2011) $\text{INF-}\gamma$, significantly increased by 2- to 20-fold in response to burn injury and revealed relatively constant, but most interestingly displayed significantly increased levels for most of the three-year period post-burn compared to non-burned patients. Again like other cytokines there are variations between the paediatric and adult population with IFN-gamma being detected at significantly higher levels in adults compared with children in the first week post burn (Finnerty et al., 2008).

Platelets

Platelets originate from megakaryocytes in the bone marrow. Their main role has long been considered to be in blood clotting but increasingly their role in inflammation is being recognised. When the endothelial cells are injured platelets stick to the exposed cell surface. Platelets release into the wound a number of cytokines including insulin like growth factor 1 (IGF-1), platelet factor IV, transforming growth factor beta ($\text{TGF-}\beta$), platelet derived growth factor (PDGF) and epidermal growth factor (EGF). All of these factors in turn attract and activate macrophages and fibroblasts (Arturson 1996 B).

Mast Cells

Mast cells can be activated by immunological stimuli, toxins, and some drugs. All of these stimuli cause an influx of Ca^{2+} ions, which causes degranulation of the mast cell. There is an exocytosis of the granule mast cell contents with the release of a number of preformed mediators.

Histamine is one of the main mediators released. These mediators have three main areas of physiological effects. There are chemoattractants (Platelet activating factor (PAF), Leukotiene B4 (LTB_4), IL-8, and $\text{TNF-}\alpha$), vasoactivators (histamine, PAF, kininogenase) and spasmogens (histamine, prostaglandin D2 (PGD_2), LTC_4 , LTD_4) (Arturson 1996 B).

The release of chemoattractants from the mast cells contributes to the recruitment and interstitial migration of leukocytes. Also due to the release of mediators from mast cells there is arteriolar constriction this will result in a reduced blood flow.

This will create the right haemodynamic conditions for blood clotting and the migration of leucocytes (Arturson 1996 A)

Neutrophils

As haemostasis is achieved neutrophils are attracted to the wound site (monocytes are also attracted but neutrophils initially arise in greater numbers). They migrate to a site of injury via the blood stream; they then attach to the endothelium and pass through to the surrounding tissue.

The role of neutrophils is to initially bind to the invading organisms, ingest and kill them. Arturson (1996 B) reports that a number of studies have shown that after thermal injury there may be defects in neutrophil chemotaxis, phagocytosis and killing but other functions may be enhanced.

Neutrophil activity is determined by the amount of wound contamination. Through phagocytosis and subsequent enzyme and oxygen free radical release, contaminating bacteria will be destroyed. When the wound is cleared of debris and bacteria neutrophil infiltration will cease in a few days. Most of the neutrophils will become enmeshed in the wound clot and desiccated tissue. This eschar will slough in time as the tissue regenerates.

In a heavily contaminated wound where there are excessive micro-organisms or indigestible particles neutrophil infiltration will be prolonged and may be damaging due to the enzyme and oxygen free radical release.

Those neutrophils which are in viable tissue, become senescent after a few days, and will be phagocytosed by macrophages (Newman et al., 1982).

Monocytes

Regardless of whether the neutrophil infiltration stops or continues monocyte will migrate into the tissue and bind to specific extracellular matrix proteins. Once activated, (by cytokines such as TNF- α and IFN- γ) the monocytes will become macrophages. They will then phagocytose and digest pathogenic organisms, redundant neutrophils and tissue debris resulting in wound débridement.

In addition to the production of cytokines, wound macrophages also produce a range of extracellular matrix molecules. These molecules with the addition of fibrin and fibrinogen from the blood clot, fibroblasts, endothelial cells and matrix

molecules from degranulating platelets form the granulation tissue or provisional matrix.

Re-epithelialisation and granulation tissue formation

After the epithelial barrier has been breached many of the protective functions of the skin are lost resulting in an increased morbidity and mortality unless the deficit can be restored. This process actually starts within hours after an injury as epithelial cells from residual epithelial structures (either the wound edges or adnexal structures) start to move across the wound deficit.

There are ranges of phenotypic changes that occur in the epithelial cells that allow lateral movement (i.e. decreased binding between the epidermis and the dermis and between the epidermis and the basement membrane) and enable the cell to be mobile (through formation of peripheral cytoplasmic actin filaments) (Singer and Clark 1999). The epithelial cells at the wound edge lose their apical-basal polarity and external pseudopodia from their free basolateral sides into the wound. (Clark 1996)

Krawczyk (1971) found that the epithelial cells at the wound edge started to proliferate one to two days after injury. There are a variety of postulated reasons for this such as absence of neighbouring cells, or increase in local growth factors. Growth factors are released from macrophages and the keratinocytes themselves. Many of these growth factors have stimulated re-epithelialisation in animal models (Brown et al., 1989) or have been absent in models of deficient re-epithelialisation (Werner et al., 1994). It is likely that they are present during the repair that occurs in a normal wound and the most likely substances include epidermal growth factor, transforming factor- α (TGF- α) and keratinocyte growth factor.

A few days after injury, macrophages, wound fibroblasts or migrating epidermal cells deposit fibronectin. Wound keratinocytes not only cover the wound with a provisional matrix but also express cell surface receptors that facilitate their migration across the matrix.

If the basement membrane has been destroyed by the injury, the epidermal cells migrate over a provisional matrix of fibrin, fibronectin, type I and type V collagen, tenascin and vitronectin. If the basement membrane is intact fibronectin

infiltrates the basement membrane. Fibronectin and fibrin originally comes from the circulation but after a few days is produced from a variety of cells, such as wound fibroblasts, macrophages and migrating epidermal cells. (Clark 1996)

Sometimes the epidermis dissects through a wound through the production of collagenase separating non-viable tissue from viable tissue. The epidermal movement through tissue relies on the production of collagenase produced by the epidermal cells.

As re-epithelialisation continues the basement membrane reappears sequentially from the wound margins inwards. The epidermal cells revert to their normal phenotype and attach to the basement membrane through hemidesmosomes and the underlying neoderms through type VII collagen fibrils.

Granulation tissue

Granulation tissue is formed about four days post injury. It consists of new blood vessels, macrophages, fibroblasts and loose connective tissue. The granulation tissue is stimulated by the range of cytokines and growth factors released from the platelets, monocytes and macrophages. The macrophages supply a continuing source of growth factors necessary to stimulate fibroplasia and angiogenesis. The fibroblasts are stimulated to construct the new extracellular matrix by a range of cytokines with chemotactic, mitogenic and modulatory activities.

The structural molecules of newly formed extracellular matrix, called the provisional matrix contribute to formation of granulation tissue by providing a scaffold for cell migration. These molecules include fibrin, fibronectin and hyaluronic acid (Singer and Clark 1999)

The provisional extracellular matrix is gradually replaced with a collagenous matrix, initially types I and III.

Once enough collagen has been deposited in the wound, fibroblasts cease collagen production. Factors that may down-regulate fibroblast proliferation and matrix synthesis are thought to include gamma interferon and the collagen matrix. (Clark 1996) Gradually a relatively acellular scar replaces the fibroblast rich, granulation tissue.

Concurrently with the newly formed extracellular matrix and fibroblast complex there must also be angiogenesis. As early as the second day endothelial cells migrate through the basement membrane in response. A whole variety of

substances have been shown to have angiogenic properties including TGF- α , TGF- β , TNF- α , PDECGF (platelet derived endothelial cell growth factor), vascular endothelial growth factor (VEGF), and IL-8 (Clark 1996).

The migrating endothelial cells form capillary sprouts that lengthen and branch at their tips and join forming capillary loops through which blood flows. New sprouts can then extend from these loops to form a capillary plexus.

Tissue Remodelling

The third phase of wound repair is the tissue remodelling which is a process of cell maturation, cell apoptosis and extracellular remodelling. This starts at the wound margin as granulation tissue is still invading the wound space. When the granulation has filled the wound space and has been covered with a neodermis the fibroblasts transform into myofibroblasts, which start to contract the wound.

The collagen remodelling and that occurs during the transformation from granulation tissue to scar tissue relies on a fine balance between continued breakdown of collagen and continued synthesis of collagen. The breakdown of collagen is controlled by molecules, called matrix metalloproteinases (MMPs). These are proteolytic enzymes produced by macrophages, epidermal cells, fibroblasts and endothelial cells. (Singer and Clark 1999) The eventual wound repair depends on a balance between MMPs and their respective tissue inhibitors. As healing proceeds, the number of inflammatory cells and fibroblasts decreases and the increasing collagen matrix becomes organised into thicker, more heavily cross-linked bundles. During this time the ratio of collagen types during wound healing varies with type III collagen predominating in the early wound but type I collagen takes over later in the healing process.

Scars continue to remodel for a considerable length of time and are generally considered to be in a steady state two years post the initial injury.

Abnormal wound healing

As might be imagined from such a complex process with so many interacting processes and molecules with multiple functions there are many physiological and biochemical changes and defects that can lead to impaired healing. It is too big a subject to examine these multiple factors in detail but sufficient for the moment to give some examples of the potential causes of impaired healing. An

impaired supply of oxygen or nutrients to the wound site, prolonged inflammation (possibly due to infection), impaired neovascularization or decreased synthesis of growth factors or collagen will all impede the formation of a strong healthy scar.

Further complications of scar formation are the fibroproliferative disorders that result in the excessive accumulation of collagen resulting in hypertrophic or keloid scars. These conditions have been linked to a range of problems including prolonged inflammation and disorders of the synthesis and secretion of extracellular proteins and cytokines as well as disordered remodelling (Singer and Clark 1999).

Infection in burns wounds-The clinical consequences of burn wounds.

Burn mortality has decreased significantly in recent years (Lionelli et al., 2005, Xiao et al., 1992) and this has been due to advances in many aspects of burn care, including nutritional support, pulmonary care, changes in surgical practice and the use and development of antibiotics.

Burn mortality has been linked to a number of factors including age, inhalation injury and total body surface area (TBSA) percentage greater than 40 (Ryan et al., 1998). With the skin playing such a vital role as a defensive barrier it is not surprising that the greater the TBSA % burned the greater the mortality. It has been shown that a greater number of deaths are due to sepsis in those patients with a greater percentage TBSA burned, (Bang et al., 2002) and following on from this the greater the TBSA % burned, the greater the risk of infection (Kagan et al., 1985). As well as an increased mortality, infected burn wounds also result in a greater morbidity with prolonged hospital stays, greater scarring and a greater need for revision surgery.

Due to changes in management of the burns patients' mortality due to sepsis has also dropped over the years, thus echoing the advances in other aspects of burn care. Before early surgical excision of the burn was advocated, management was far more conservative. The natural colonisation of the wound was allowed to occur as it allowed the burn eschar to separate spontaneously. The resulting wound would re-epithelialise leaving pronounced scarring and contractures. Subsequently treatment used to consist of immersion hydrotherapy and cleaning of the wound until granulation tissue appeared, after which the site could be skin

grafted (Church et al., 2006). At times other methods gained popularity such as the open or exposure method in which its advocates noted the improved comfort of the patients (Sneve 1905). This technique was revived briefly in the 1940s in both the US and UK and it was seen not as a form of abandonment but a way of actively managing the wound.

Exposure meant the wound rapidly became colonized with bacteria, the only benefit being that the relative dryness discouraged growth of some bacteria such as *Pseudomonas*.

Current treatment consists of early excision (for full thickness and deep partial thickness burns) and grafting to achieve wound closure whether it is by autograft, allograft or occasionally skin substitutes. This more aggressive approach has not only reduced the mortality rate (in patients without inhalation injuries) (Ong et al., 2006) and length of the hospital stay (Heimbach 1987, Ong et al., 2006) but has also lowered hospital costs (Munster et al., 1994). By shortening the hospital stay, early closure of the burn wound also reduces infective complications (Atiyeh et al., 2005, Kagan et al., 1985). Despite these advances, sepsis is still responsible for a large proportion of the deaths of burns victims (Bang et al., 1998) and infection still contributes significantly to the patient morbidity.

The first studies into burns and bacteria came shortly after the discovery of bacteria and the theory of germs. Lustgarten (1891) implicated *Proteus* in sepsis in 1891 but it was not until many years later that other bacterial studies made an impact. Pack (1926) and Aldrich (1933) reported a high incidence of *Strep. pyogenes* and other bacteria in burns (Lawrence 1992). Further studies endorsed this work, Cruickshank (1935) noted that haemolytic streptococci rates rose six-fold from admission to 3-6 days later. He also noted that haemolytic streptococci were found in higher numbers in the air of the burn ward compared to the general surgical ward. In 1940 other bacteria were implicated in burn sepsis such as *Pseud. aeruginosa*, *Staph. aureus* and *Proteus* (Heggie and Heggie 1940). In 1943 penicillin became available and topical applications with antimicrobial substances such as silver nitrate became part of mainstream care in the 1960s and 1970s.

It has taken many years however for the factors that influence burn wound colonisation and the consequences to become clearer.

Immediately following a burn, the wound surface is sterile, however microorganisms rapidly colonize it. Factors that may influence this include the depth and extent of the injury, the causative factor (i.e. flame burns are more susceptible to sepsis (Bang et al., 1998)) and history of the injury. For instance contamination is more likely in explosive injuries and some industrial injuries than a scald by a kettle at home. The site of the burn may increase the risk of colonization and type of organism isolated. A perineal burn is difficult to keep clean and is more likely to become contaminated by gram-negative gastrointestinal flora than a burn of similar size and depth on the forearm.

Some bacteria can survive the initial insult (particularly in deep partial thickness burns) as they maintain a presence in the sweat glands and hair follicles. These are typically *staphylococci*, which colonise the wound within 48 hours unless a topical antimicrobial agent is used (Church et al., 2006, Erol et al., 2004).

Subsequently (around 5-7 days) other microbes colonise the wounds, often from the patients' endogenous microflora from the respiratory tract, gastrointestinal tract or skin. These may be gram positive or gram-negative bacteria. Colonisation by yeasts or fungi tends to occur later due to the frequent use of empirical broad-spectrum antibiotics. The type of organism changes during the history of the burn wound and as a result drug sensitivities and resistance also changes. These factors should be taken into consideration when starting empirical antibiotic therapies (Altöparlak et al., 2004). Erol et al., (2004) took periodic swabs from patients on admission, on the 7th, 14th and 21st days of hospitalization. The swabs were from the wound site as well as other areas on the body (nasal, axillary, inguinal and umbilical). On admission coagulase negative *staphylococci* and *staphylococcus aureus* were most prevalent. Over time there was a decrease in the number of coagulase negative *staphylococcus* isolated and an increase in *staphylococcus aureus* and *pseudomonas*. These findings endorse a previous study by Vindenes et al., (1995). Of note Erol et al., (2004) found 35.3 % of wounds were sterile on admission but 86.3 % were colonised by the end of the first week and that methicillin resistance of *staphylococcal* strains increased during the study period. At any one of these stages described above microorganisms can also be transferred to the patient's skin or wound by contact with the hospital environment (contaminated surfaces, equipment, air, water or

fomites) in addition to contamination transferred from the hands of healthcare personnel working with and in close proximity to the patient.

In recent years gram negative organisms have played a more important role in burn wound infections partially due to their large number of virulence factors and also increasing strains with antimicrobial resistance (Geyik et al., 2003, Singh et al., 2003). One of the key gram-negative organisms that have gained prominence in recent years is *Acinetobacter baumannii*. It is an aerobic, gram negative, oxidase negative non-fermenting bacterium (Appleman et al., 2000) that is normally a commensal organism but due to growing antibiotic resistance is now a key opportunistic pathogen in burns and intensive care units. Nosocomial outbreaks of *A. baumannii* have previously been described in the literature and many of the outbreaks have been associated with multiresistant strains (Lyytikainen et al., 1995, Mulin et al., 1995 and Simor et al., 2002). Multiresistant strains of these species causing bacteraemia, pneumonia, meningitis, urinary tract infections and surgical wound infections have been isolated from hospitalised patients worldwide (Van Looveren et al., 2004). The overall incidence of *A. baumannii* as a nosocomial infection was 9% in Europe in 1995 (Vincent et al., 1995) but a higher reported incidence has been found in burn units, 13% in a Canadian burns unit (Simor et al., 2002) and 14% in a Brazilian unit (Santucci 2003). A number of factors have been identified for the risk of acquisition and subsequent mortality of multi resistant *Acinetobacter baumannii* (MRAB). (*Acinetobacter baumannii* that is resistant to carbapenem has been termed MRAB-C). The factors for acquisition of MRAB include receipt of blood products and duration of mechanical ventilation (Simor et al., 2002), Acute Physiology and Chronic Health Evaluation (APACHE II) score on admission (a measure of physiological variables to determine severity of disease) and number of intravascular lines placed (Wong et al., 2002). Previous broad-spectrum antibiotics have been found to play an important role in MRAB acquisition (Mulin et al., 1995, Villiers et al., 1998, Ayan et al., 2003). Factors associated with mortality include immunosuppression, shock, recent surgery, invasive procedures such as central venous or pulmonary catheterization and mechanical ventilation (Chen et al., 2005). In the same study mortality was significantly associated with lower platelet count and lower serum albumin concentration.

In addition many outbreaks have been linked to equipment or the inanimate hospital environment. This is partly due to the ability of *Acinetobacter* to survive for long periods on dry surfaces (Wendt et al., 1997). Catalano et al., (1999) describe the survival of *Acinetobacter* on hospital bed rails causing sporadic outbreaks whilst Neely et al., (1999) report the presence of *Acinetobacter* on computer keyboards in the burns unit. Beck-Sague et al., (1990) again found higher rates of *Acinetobacter* in ventilated patients although found no link to the equipment.

One important aspect not to be forgotten is that transmission via the hands of hospital staff can be a frequent cause of spread of infection. *Acinetobacter* is considered a low-grade pathogen and can remain on or in the human body without causing illness, and for this reason dissemination via the hands of hospital staff may remain undetected (Joly-Guillou 2005). Close adherence to standard levels of hand hygiene can have an important impact on reducing or eliminating outbreaks of colonization or infection (Chan et al., 2007). Other factors that have been implicated in the transmission and spread of infection within a hospital or unit include bed occupancy and therefore the turnover of patients. The number of procedures or points of contact between the patient and members of staff as well as the level of training of the staff involved may also be important.

For those patients with infections possibly caused by *Acinetobacter*, a β -lactam alone or in combination with an aminoglycoside or fluoroquinolone was the initial empirical therapy (Meyer 2005). This regimen has become less effective due to the appearance of β -lactamases (Rahal and Urban 2000), extended spectrum β -lactamases (ESBLs) and enzymes that inactivate aminoglycosides (Bergogne-Bérézin and Towner 1996).

The speed in which *Acinetobacter baumannii* has become a significant problem in hospitals is illustrated by the rise in cases resistant to carbapenem in one unit increasing from 0% to 55% between 1998 and 2006 This overall increase coincides with the emergence of the MRAB-C OXA-23 clone-1 as the most prevalent strain in London and South East England (Wareham et al., 2008). There appears to be little clinical difference in clinical outcome in patients with bacteraemia from *Acinetobacter baumannii* and other *Acinetobacter* species

(Choi et al., 2006) but it may be that different clones of MRAB result in different patient morbidity or mortality rates.

Innate and adaptive immune responses

Host defences against infection can be divided into innate and adaptive immune responses. The innate response is initiated rapidly after a thermal injury by stimulating local and systemic inflammatory reactions, which allows time for the adaptive immune response to become active. The innate immune system is composed of natural barriers to microbial invasion as well as cellular (neutrophils, macrophages, NK-cells, and monocytes) and humeral (complement) elements (Church et al., 2006). In contrast the adaptive immune system is related to T and B cell activation in response to antigens.

Part of the innate immune system that is being extensively investigated is a group of compounds that make up a family of proteins called antimicrobial peptides. They are thought to play a key role in skin defence by being able to directly kill or inhibit a large variety of bacteria and fungi (Pazgier et al., 2006, Durr et al 2006). In addition to this they can also influence other cells in both the innate and adaptive immune system. In mammalian skin two groups of antimicrobial peptide have been discovered, cathelicidins, of which the sole human cathelicidin is LL-37, and the defensins of which there are three sub-families.

LL-37 can promote the recruitment of inflammatory cells directly by stimulating leukocyte chemotaxis (Yang et al., 2001) and LL-37 may also have a key role in wound healing as it has been shown to induced angiogenesis (Koczulla et al., 2003). Whilst both defensins and LL-37 have been found in burn wounds studies have been limited. Milner and Ortega (1999) studied defensins and showed reduced expression in human burn wounds and staining in a number of subcutaneous structures and reticular dermis, most notably in eccrine and apocrine gland acini (Milner et al., 2004).

Kaus et al., (2007) examined LL-37 in addition to defensins in burn wounds. They found no differences in hCAP-18/LL-37 expression levels when comparing burned skin with healthy controls. Further experiments showed *In situ* hybridisation revealed expression of hCAP-18/LL-37, hBD2 and hBD3 at the surface of burns was independent of burn depth.

The study of LL-37 is not only confined to cutaneous wounds, it has also been isolated from serum. Lippross et al., (2011) examined the serum profiles of defensins, cathelicidins and pro-inflammatory cytokines post trauma and found that hBD-2, hBD-3 and LL-37 concentrations were significantly elevated after

trauma and followed different characteristic concentration curves. In addition, similar patterns of concentration profiles were recorded for hBD-2/IL-6 and hBD-3/IFN-gamma.

The anti microbial properties of LL-37 and other AMPs have led researchers to speculate that it may offer an alternative to conventional antibiotics especially in the era of growing antibiotic resistance.

As yet the role of LL-37 in burns wounds is yet to be fully defined, especially what happens post the acute injury. In addition no studies have looked at systemic levels of LL-37 in burn patients to determine if there is any relationship between the magnitude of the injury and it's subsequent expression.

1.5 Hypothesis

Great advances have been made in burn care in the last forty years with a resultant decrease in morbidity and mortality.

Despite these advances, infection and organ dysfunction are still the most frequently reported causes of death. Multi-organ failure (MOF) is responsible for 25-65% of all burn deaths (in Europe) and sepsis for 2%-14% of all burn deaths. The increasing presence of multi-resistant organisms makes treating these complications even more difficult. Instead of continued reliance on conventional antibiotic therapies new treatments should be sought for the treatment of infections.

One problem in burn care is the early recognition of the patients who go on to develop sepsis and organ failure. Early identification allows therapies and resources to be targeted in a more effective and efficient way.

This study hypothesizes that the Albumin-Creatinine Ratio (ACR), a marker of endothelial dysfunction can be used as a diagnostic tool to predict outcome (mortality) and can identify those patients that may develop sepsis and organ failure.

Despite the improvements in burn care and management we hypothesize that the reason sepsis and organ failure still make such a contribution to the mortality rate is due to the increased prevalence of multi-drug resistant organisms.

The impact of acquisition of a multi-drug resistant organism (*Acinetobacter baumannii*) is studied particularly looking at patient outcomes and multi-organ failure.

Because of the growing drug resistance to many antibiotics alternative treatments for infections are being sought. One such candidate, an antimicrobial peptide, the cathelicidin, LL-37 serves in a protective role in the skin. Its role in the burn patient is still unknown. This study hypothesizes that LL-37 is present in the acute and healing burn wound and may have activity against multi drug resistant organisms.

It is hoped the results of this study will be clinically relevant and continue to improve burn care.

1.6 Aims

By a retrospective analysis of admissions to a burn ICU the clinical utility of urinary ACR sampling at admission and post resuscitation in determining outcome will be examined. Secondary measures are to investigate the prevalence of microalbuminuria in burns patients and to determine any relationships between ACR and mechanism of injury, inhalation injury, body surface area burnt and length of stay.

In addition by a retrospective analysis of adult burn patients we aim to determine whether ACR can predict the development of sepsis and multi-organ failure in the burn ICU setting.

The frequent cause of sepsis and organ failure is infection. We aim to determine the impact *Acinetobacter baumannii*, a multi-drug resistant organism on a UK burn unit and its relationship to patient outcomes.

Because of the growing drug resistance to many antibiotics alternative treatments for infections are being sought one potential candidate is the peptide LL-37. It's presence and role in acute burn injuries is yet to be defined. We aim to determine the presence of LL-37 in skin after a burn injury and subsequently in the healing phase compared with normal, healthy controls by immunohistochemistry and rt PCR and to examine serum levels of LL-37 and pro-inflammatory cytokines post burn injury to investigate any relationships with injury or outcome.

Finally we plan to examine whether LL-37 has antimicrobial properties against *Acinetobacter baumannii* and therefore potential use in the clinical burn setting.

Chapter 2 Albumin creatinine ratio in the burn ICU

2.1.1 Introduction

Definition of Albumin Creatinine Ratio

Inflammation is an essential part of normal healing. In a small burn this may manifest itself as a mild rise in cytokines with no clinical signs. In a large burn a major systemic inflammatory response may be seen resulting in endothelial dysfunction and a microvascular leak of fluid and protein into the interstitium. Almost 40 years ago Parving et al., (1974) showed that there was a correlation between microalbuminuria and the increased rate of transcapillary escape of radiolabelled albumin in hypertensive and diabetic patients. A link between increased systemic vascular permeability to albumin and microalbuminuria was therefore made.

Microalbuminuria is thought to reflect the glomerular component of the systemic capillary leak (Gosling 1995). As a result of the increased glomerular permeability renal resorption cannot cope with an increase in filtered albumin and urinary albumin increases. Small increases in glomerular permeability are amplified by the renal concentrating mechanism to produce large changes in albumin excretion. This is because normally >99% of filtered albumin is reabsorbed by the kidney by mechanisms close to saturation (Sarti et al., 2001). Due to the fact the kidneys receive around a quarter of the cardiac output the kidneys can convert a 1% increase in vascular permeability to a nine-fold increase in urinary albumin (Gosling et al., 1994).

Since Parving's findings microalbuminuria has been used as a surrogate marker for systemic endothelial dysfunction and a predictor of outcome in a variety of surgical and intensive care settings (Vlachou et al., 2006).

Clinically proteinuria is defined as a urine protein concentration above 0.2g/L (Vlachou et al., 2006) whilst microalbuminuria is defined as a urinary albumin concentration of 30-200 mg/litre⁻¹ (Yew and Pal 2006). To correct for variations of urinary flow it is normally expressed as the urinary albumin to creatinine ratio (ACR). This is variably referred to as ACR or microalbuminuria/urinary

creatinine ratio (MACR). For clarity, in this text it will be referred to as ACR even if MACR was used in the original reference. Normally to calculate the albumin excretion rate an overnight or twenty-four hour collection of urine is used, however assuming the urinary creatinine ratio remains constant (Gosling 1995) the ACR can be calculated from a bedside spot urine test.

In many of the earlier references when an overnight or twenty-four hour collection of urine was used to determine the albumin excretion the results are expressed in mg/litre⁻¹. In later references the ACR is expressed in mg mmol⁻¹ or mg/g. In this work the ACR is expressed in mg mmol⁻¹ however when quoting other results the units used in the original reference will be used.

The normal value of ACR is defined as less than 2.3 mg mmol⁻¹. The presence of microalbuminuria in normal healthy adults is 3.3% but may be up to 29% in adults with diabetes or 18.8% in those with hypertension (Jones et al., 2002).

Patients sustaining burns injuries are at a high risk of systemic inflammatory response syndrome (SIRS). When severe, SIRS leads onto organ failure. The inflammatory response to the injury, trauma or infection is wide spread and may damage organs distant from the initial insult. Although the sequence of inflammation, organ failure and sometimes ultimately death is well recognised the exact mechanisms are still poorly understood. One difficulty encountered when caring for the patients is identifying at an early enough stage those that may develop SIRS and multi-organ failure in order that the clinician may try and modulate the inflammatory and immune response.

One of the first features of inflammation is an increased capillary permeability to plasma proteins. This can occur within a few minutes after injury and can take 12 hours to return to normal (Vlachou et al., 2006).

Albumin, globulin and fibrinogen are the major proteins present in the plasma. In health albumin, which is formed by the liver, plays a number of roles in the human body. Albumin helps in the transport of bilirubin and free fatty acids and well as playing a lesser role in the transport of cortisol and some of the thyroid hormones. The major function of albumin however, is to provide colloid osmotic pressure in the plasma. The colloid osmotic pressure tends to cause fluid movement by osmosis from the interstitial spaces into the blood. This osmotic pressure exerted by the plasma proteins normally prevents significant loss of

fluid volume from the blood into the interstitial spaces. Due to its charge and weight in the healthy patient almost all the albumin is reabsorbed and not filtered by the kidneys.

Using radiolabelled albumin it has been found that the transcapillary escape of albumin increases significantly in patients with malignancies and infection, septic shock and cardiac surgery (Fleck et al., 1985). Gosling et al., (1988) found urinary total protein and albumin levels were raised after a variety of general surgical procedures on humans and postulated that measurements of protein excretion may be used to predict postoperative complications. Subsequent work by Shearman et al (1989) found low proteinuria is a very early response in acute pancreatitis, and thought that it may reflect the severity of inflammation.

This combined evidence resulted in a hypothesis that following acute inflammatory insults urine albumin reflects systemic endothelial dysfunction as part of the early inflammatory response (Gosling et al., 1988).

2.1.2 ACR in non-burn patients

Medical patients

In both diabetic and non-diabetic patients microalbuminuria has been recognised as an important risk factor for renal and cardiovascular disease (Ritz et al., 2010.) Further studies have demonstrated an association between urine albumin and albumin transcapillary escape rate and evidence of systemic endothelial dysfunction in normal subjects and those with diabetes (Feldt-Rasmussen 2000, Pedrinelli et al., 2001).

Microalbuminuria has been found to be predictive of outcome or has shown an association between the urinary albumin and the severity of the inflammatory insult in a number of acute medical conditions such as ischaemia reperfusion injuries (Matsushita et al., 1996), meningitis in children (Roine 1993), acute pancreatitis (Shearman et al., 1989) and anaphylactic shock (Wood et al., 2000). Disease activity (Mahmud 1994) and tumour necrosis levels in patients with inflammatory bowel disease (Mahmud 1995) have been correlated with urinary albumin. Furthermore ACR after an acute myocardial infarction has been found

to be a good predictor of short-term mortality and long-term mortality (after a 10 year follow-up) (Berton et al., 2010).

Trauma, Surgery and Urinary Albumin

Urinary albumin has been shown to be raised post surgical procedures (Gosling et al., 1988) and after trauma. In trauma patients the urinary excretion of total protein, albumin and gamma glutamyl transpeptidase (GGT) activity was assessed over the 6 days post trauma. The excretion of total protein and albumin was positively correlated with injury severity (Gosling and Sutcliffe 1986). Microalbuminuria seems not only to be raised according to the severity of the injury but also seems to be useful for predicting postoperative complications. Gosling (1995) has reported that in both surgical and trauma patients whose microalbuminuria is maintained and does not return to normal within 4 and 8 hours (respectively) post insult it has been possible to predict subsequent organ failure. Patients who sustained blunt trauma have shown that the urinary albumin is proportional to the severity of the injury (Gosling et al., 1994, Gosling and Sutcliffe 1986) and is predictive of acute lung injury and acute respiratory distress syndrome (Pallister et al., 1997). The urinary albumin levels taken within 4 hours of the start of aortic surgery have been found to be able to predict those patients who would later develop pulmonary complications that were non cardiogenic in origin (Smith et al., 1994). Honarmand et al., (2009) demonstrated that microalbuminuria (determined by a sample taken on the first day of admission) in critically ill trauma patients also estimates the risk of prolonged mechanical ventilation. Not all studies have found that ACR predicts postoperative complications. Norwood and Sayers (2005) studied forty-four patients undergoing elective infrarenal abdominal aortic aneurysm repair and 10 control patients undergoing major abdominal surgery. Urine samples were taken before, immediately after and 24 h after the procedure. Whilst the urine samples of the control patients were not raised post operatively compared with the study group, the changes in ACR in the study group had no correlation with the development of postoperative complications.

Sarti et al., (2001) studied forty children undergoing elective surgery (all patients with diabetes, nephropathies, recent infections and malignancies were excluded) and found that the ACR showed a progressive increase during surgery and

subsequent decrease after surgery. In most cases the pre-operative value was achieved within 24 hours after the end of surgery. There was a significant correlation between the increase in ACR and the severity of the surgical trauma as measured by the Oxford Surgical Stress Score (This is a scoring system based on the main factors that contribute to surgical stress: volume of blood loss, the degree of superficial dissection, the extent of visceral trauma and the duration of the operation). This study is important because it shows that the paediatric population responds in the same way as adults.

In addition two patients showed a rise in ACR after the initial postoperative value returned to normal but before the clinical appearance of a surgical complication (peritonitis and a large subcutaneous haematoma). A further patient showed a persistent rise in ACR before the appearance of a septic complication. This useful finding, of ACR rising prior to a septic episode has been endorsed by De Gaudio et al., (2000). They examined 55 patients post major abdominal or vascular surgery. Again any patient with any pathological condition (such as hypertension, diabetes or kidney failure) that may be associated with a chronic baseline presence of microalbuminuria was excluded. They found that the postoperative patients that developed sepsis showed an increase in glomerular permeability as shown by the ACR. The increase of the ACR was also positively correlated to the increase in the sepsis related organ failure assessment (SOFA) score (This is a scoring system based on physiological parameters that determines the degree of organ failure a patient may be suffering from).

A similar pilot study by MacKinnon et al., (2000) looking at a similar number but broader range of patients demonstrated that microalbuminuria 6 hours after admission to a general ICU could identify patients at risk of developing multi-organ failure and those at a greater risk of death.

2.1.3 Microalbuminuria as a predictive tool for outcomes in the Intensive Care Unit (ICU).

Further studies have examined the use of the urine albumin-creatinine ratio in the ICU setting (Abid et al., 2001, Gosling et al., 2003, Gosling et al., 2006, Thorevska et al., 2003). The general conclusion from these studies is that ACR may have a predictive role in determining organ failure and /or mortality. One of the problems is that the studies tend to have wide patient demographics with some not differentiating between medical, surgical, or trauma patients, there are also different cut-off values for ACR, and variability in the timing of the tests and methods of quantification. This was confirmed in a systematic review by Gopal et al., (2006) who looked at all studies between 1951 and 2004 that provided data on the diagnostic accuracy of microalbuminuria on the intensive care unit. From 35 potentially relevant abstracts only 9 were included in their final review. Their conclusion was that microalbuminuria may hold promise as a predictor of illness severity and mortality on the ICU but further studies should be carried out.

2.1.4 ACR and physiology scoring systems

Although ACR seems to have a useful potential for predicting outcomes, it is necessary to see how well it fairs against the conventionally used outcome measures.

Gosling et al., (2003) looked at a mixed cohort of 140 patients (including 11 burns patients) by measuring ACR within 15 minutes of admission to the intensive care unit and comparing it to APACHE II and SAP II scores (The APACHE and SAP scores are two scoring systems looking at physiological parameters that are frequently used in the ICU setting to predict outcome and severity of illness). They found that for surgical, trauma and burns patients but not medical patients microalbuminuria predicted death as well as APACHE II and SAP II scores calculated after 24 hours.

Of note they found that in the burns cases ACR was positively associated with ICU stay (as well as highest serum CRP and highest bilirubin).

A further larger study by Gosling et al., (2006) examined a mixed cohort of medical (205 patients) and surgical (225) patients. In this study ACR was

measured on admission and after 4-6 hours. It was found that the urinary albumin changes rapidly within the first six hours post ICU admission and that ACR predicted ICU mortality and inotrope requirements as well or better than APACHE II and SOFA scores. Again this study had a small number of burns patients (n=21).

Although both of these studies include burns patients the numbers examined are too small to be able to extrapolate the results to all burns patients. Neither study comments on the size of the burn. Previous work, mentioned above, has shown that the rise in urinary albumin is related to the severity of the trauma and one might hypothesize that the greater the total body surface area involved in the injury may be reflected in the level of the rise of the microalbuminuria.

Secondly although the presence of inhalation injuries was noted in the first study (Gosling et al., 2003) no mention was made of whether it affected microalbuminuria. Again it might be hypothesized that as urinary albumin has predicted pulmonary complications post surgery and blunt trauma it may also do so where there has been a direct insult to the respiratory system.

2.1.5 Burn wounds and ACR

There have been only a limited number of studies looking directly burns and microalbuminuria. The first by Vlachou et al., (2006) looked at 43 adult burns patients. Serial urine samples were taken between 2-36 hours after injury (every hour for the first 12 hours and then 4 hourly to 36 hours. In addition urine samples to determine the ACR were taken during 44 episodes of wound manipulation during the same period. Their results showed that median ACR was highest 2 hours after injury returning to normal within 6 hours. Furthermore the ACR remained high for up to 8 hours in the presence of inhalation injury, accelerant use or alcohol intoxication. The ACR showed a positive correlation with TBSA at 3 and 7 hours post injury ($p<0.05$). However the percentage of full thickness burn (%FTB) was significantly correlated with ACR at 3-7 hours post injury.

One of the most important results to come out of the study was the strong association between ACR and wound manipulation (escharotomy or wound scrubbing). During wound manipulation the urinary specimens were collected at

more frequent intervals (half hourly during the procedure) with a baseline level prior to the manipulation.

An increase in ACR within 30 minutes of a surgical insult has been noted previously when it was recorded to rise 20 minutes after the start of cardiopulmonary bypass (Tsang et al., 1998) but most importantly in Vlachou's study (2006) was the demonstration of how sensitive the microvascular response is to pro-inflammatory stimuli as even simple wound scrubbing provoked a measurable response. This obviously has a profound impact on using ACR as a predictive tool.

One fault common to many of the studies previously listed is that no mention is made of the timing of the urinary sampling and any prior procedures. Patients, especially those admitted to ICU may undergo a variety of invasive tests or monitoring modalities that could subsequently, albeit transiently affect the ACR. The first study to look at outcomes was by Yew and Pal (2006) and was a retrospective analysis of burns of at least 40% TBSA or significant inhalation injury in 21 patients. Non-thermal burns and paediatric patients were excluded from this study.

They found that a mean ACR of 20 mg mmol⁻¹ or more was associated with a poorer outcome in their cohort. They also noted a biphasic variation of mean ACR in those patients that died with either an early peak in the first week or a later peak a month later, which they ascribe to the "two cascades of organ failure". The early peak occurring during the first week is caused by failure of reversal of burn shock. The later cascade is typified by an infectious process and is usually associated with pneumonia (Yew and Pal 2006). Unlike other studies they did not find a correlation between ACR on admission and outcome. As the authors themselves note, this is most likely due to the fact that most patients in their cohort are stabilised in a peripheral hospital prior to transfer therefore the admission ACR in ICU may not reflect early ACR elevation due to the burn injury.

A further study by Vlachou et al., (2008) looked specifically at ACR during burn excision. Serial half hourly ACR levels were measured in 25 adult patients during 44 burn excision procedures. They found that ACR increases during surgery (maximally a three-fold rise 1.5 hours from the start of surgery); there was no significant difference between the pre-operative ACR for those that

received blood transfusions and those that did not. Most importantly was the finding that in 15 patients that suffered post operative complications the ACR was significantly higher both pre and peri-operatively. Lastly, they demonstrated that there was an increased ACR response to surgery if the surgery was carried out seven days or more after the injury.

In the same year, Cochran et al., (2008) published their results investigating ACR compared with injury severity and resuscitation demands. They examined 30 patients and looked at time to resuscitation (defined as the time of conversion from lactated Ringers solution to maintenance fluids or when the calculated maintenance rate for fluids was maintained for two hours). They hypothesized that an ideal marker of inflammation and associated resuscitation demands would help optimise burn shock resuscitation and also minimise secondary complications and therefore that ACR would correlate with injury severity or burn shock parameters. Their study found no correlation with TBSA, volume of resuscitation fluid used and in contradiction to other studies the presence of inhalation injury.

There may be several reasons for their results; firstly the sample size may be too small. Secondly they make no mention of the precise timings of the ACR sampling. Each of the three measurements could have been taken over a wide range of times. As previous studies have demonstrated the sensitivity of the ACR may mean the timing or the limited number of samples may not have reflected the severity or duration of the inflammatory response.

2.2 Aims

To examine the clinical utility of urinary ACR sampling at admission and post resuscitation in determining outcome by a retrospective analysis of burns admissions to a burn ICU between November 2003-September 2009. Secondary measures were to investigate the prevalence of microalbuminuria in burns patients admitted to ICU and to determine any relationships between ACR and mechanism of injury, inhalation injury, body surface area burnt and length of stay.

2.3 Methods

2.3.1 Clinical Setting

The Burns Service comprises twenty beds including four intensive care and four high dependency beds. There are twelve low dependency / rehabilitation beds. The unit is a tertiary referral centre and accepts patients from a population of over 6 million people. Patients are also taken from out of traditional catchment areas and occasionally internationally.

A multidisciplinary team including microbiologists, pharmacists, anaesthetists and burn surgeons review all major burns daily. The Parkland formula is used in the initial resuscitation to maintain a urine output of 0.5 ml/kg/h in adults and 1 ml/kg/h in children. Nutritional supplementation is via nasoduodenal enteral nutrition with high calorie, low fat formulas. As part of the burn treatment protocol, gastric ulcer prophylaxis is performed with H₂ antagonists. Low molecular weight heparin (40mgs/day) is prescribed daily. Early excision and grafting is routinely performed for deep burns that would take more than two weeks to heal without surgical intervention using allografting, auto-grafting or dermal substitutes depending on the individual characteristics of burn injuries.

Retrospective collection of data regarding demographic details, mode of injury, examination findings was from clinical records. All other results were obtained from the Burns ITU computer system MetaVision (*iMDsoft*, Massachusetts, USA).

All patients admitted to the burns unit between November 2003 and September 2009 that fulfilled the unit's admission criteria were included in the study.

2.3.2 Exclusions

Exclusions were those patients with non-thermal processes including desquamating skin disorders, meningococcal septicaemia, necrotising fasciitis and purpura fulminans; patients who stayed in the ITU for less than 24 hours were excluded; patients who were transferred from other hospitals more than 5 days post injury; patients with multiple injuries (where the burn was clinically judged not to be the main injury); patients for compassionate care only; patients with no or inadequately recorded ACR results.

Patients with a diagnosis of Type I or Type II diabetes mellitus were included.

Parameters recorded included patient characteristic data such as age, gender, co-morbidities, injury data including time of injury, mechanism and TBSA. ACR was routinely measured on admission and recorded daily.

Inhalation injuries were determined by appearance on bronchoscopy. Smoke inhalation injury is restricted to injury below the glottis caused by products of combustion. The diagnosis requires both of the following: History of exposure to products of combustion and bronchoscopy relevant signs below the glottis. The signs observed below the glottis that indicate smoke inhalation include carbonaceous material, oedema, or ulceration. Erythema or carbon staining in the absence of evidence of other signs of injury was not considered an indicator of smoke inhalation injury.

2.3.3 Analysis of urine

On admission a sample of urine was collected within the first six hours, subsequently a fresh sample of urine was collected daily via an in-dwelling urinary catheter at 06.00 daily for the duration of the admission.

ACR was measured using the DCA 2000 Analyser manufactured by Bayer (Parsons et al 1999). It runs a quantitative assay for microalbumin and creatinine in the urine sample. Only 0.4mls of urine is required to perform the analysis. The principle of measurement of microalbumin is based on immunoturbidmetry, and that of creatinine is based on colourimetry. The albumin to creatinine ratio is calculated by the machine and expressed in mg mmol⁻¹ after 7 minutes. (Yew and Pal 2006)

2.3.4 Statistics

The analysis performed was a bootstrap analysis and regression analysis. Regression analysis has been performed using the computer program R (R development Core Team 2011).

Bootstrap analysis was chosen because a paper submitted to the journal Burns as part of this work had been rejected as the statistical analysis (ANOVA) was thought by the reviewers to be too simple. Advice was then sought from a medical statistician (Dr Mike Parker, Anglia Ruskin University, Chelmsford) who advised the bootstrap analysis. All bootstrap and regression analysis in this thesis was performed by Dr Mike Parker.

Bootstrap analysis is a computer based method for assigning measures of accuracy to sample estimates. For this analysis it is assumed the observations form an independent and identically distributed population. The computer programme can implement bootstrapping by constructing a number of re-samples of the dataset and of equal size to the observed dataset, which is obtained by random sampling with replacement from the original dataset. The idea of bootstrapping is that the sample collected is the best guess of the shape of the population it was taken from, for instance two peaks in distribution in the sample would not be well approximated by a Gaussian curve, which has only one peak.

Bootstrapping is used when parametric inference is in doubt or requires very complicated formulas for the calculation of standard errors. Bootstrapping also checks the reliability of the dataset.

When bootstrapping is performed, repeated sampling is used (in this case 9999 times) which gives a histogram of bootstrap means i.e. it produces a simulated distribution of the statistic we are interested in. This gives an estimate of the distribution of the mean from which one can answer how much the mean varies.

Bootstrapping of the mean allows one to construct a confidence interval for the mean. It has been found that the simulated distribution has a shape that distorts the confidence limits and the distribution tails off more slowly than the Normal distribution (so called bias, although it is not bias in the conventional sense) so a correction is applied to make it tail off quicker (the accelerated bootstrap). This is remedied by using the "accelerated and bias-corrected" approach. When an

accelerated bootstrap was performed it is noted in the main text. This is to improve the accuracy of the confidence limits (rather than correcting for bias in the conventional sense of the word) and is performed within the computer package.

A 95% confidence limit (interval) has been used throughout this study when looking at the bootstrapping results. The confidence interval describes a range of values within which we can be reasonably sure that the true effect actually lies. (The true effect being the experimental intervention's effect compared with the control intervention). The confidence interval is based on the hypothetical notion of considering the results that would be obtained if the study were repeated many times. So if a study were repeated infinitely often, and on each occasion a 95% confidence interval calculated, then 95% of these intervals would contain the true effect. A narrow confidence interval gives us greater certainty in this, but if the interval is wider the uncertainty is greater. If the confidence interval includes 0 we can say that there is no significant difference between the means of the two populations, at a given level of confidence (in this study 95%). In addition whilst a result may show statistical significance it should always be examined in the clinical context to determine the relevance of the result to clinical practice.

(http://handbook.cochrane.org/chapter_12/12_4_1_confidence_intervals.htm).

2.4 Results

2.4.1 Demographics

A total of 547 patients were eligible for the study from which 202 were excluded. The reasons for exclusion are summarised in the flow chart Figure 1.

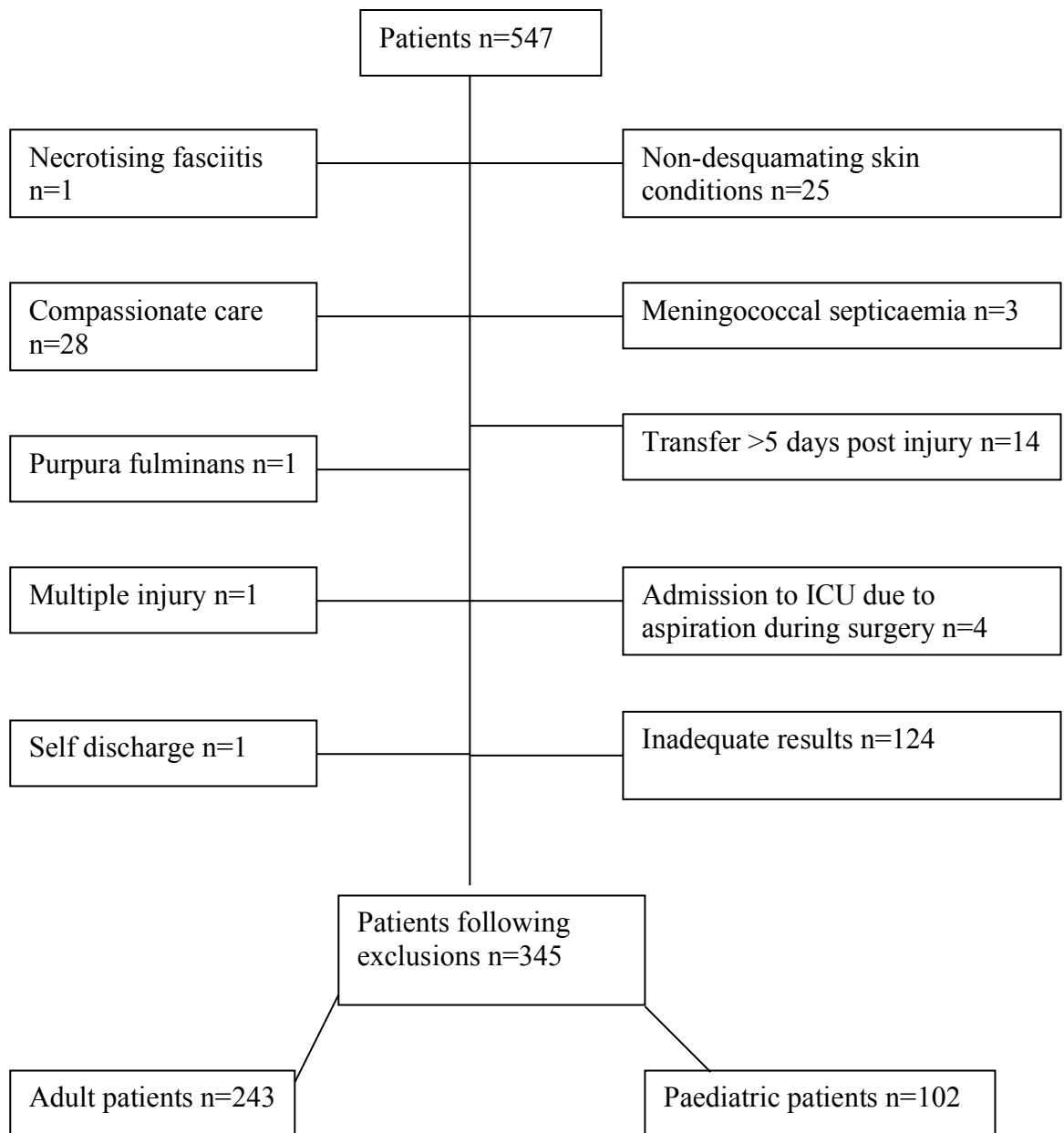


Figure 2.1. Flow chart showing the number of burns patients recruited and reasons for exclusions. Data shows 547 patients admitted to the burn ICU between November 2003-September 2009 were eligible for the study. After 202 exclusions, 345 adult and paediatric patients were studied of which 243 were adults and 102 children.

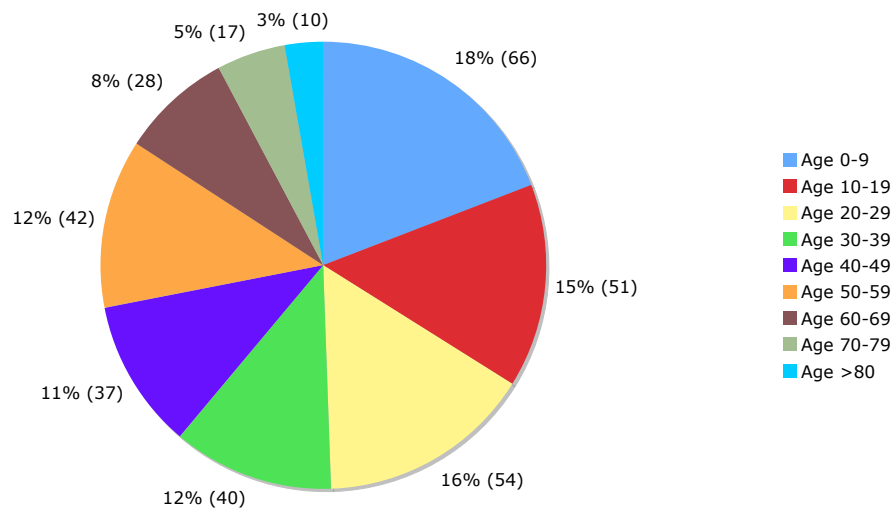


Figure 2.2. The number of patients in each age group admitted to the burns ITU. The data demonstrates the age in years, of the patients admitted to the burn unit in the study period, November 2003-September 2009. The data shows the percentage of the total each group contributes with the total number in the group in brackets. Each age range is a decade except for the patients aged 80 years or greater which form one group.

The demographics of the population studied showed that out of the 345 patients studied the mean age (range) is 33.2 years (1 month-95 years old). Figure 2.2 demonstrates the number of patients in each age group and shows that almost half the patients (49%) were in the three youngest age groups (ages 0-9, 10-19 and 20-29). This is in keeping with similar studies of westernised urban populations. Of note the majority of injuries in the 0-9-age range tended to be scalds (water or oil), whilst flame burns predominated in the older age ranges (data is not shown) again this is in keeping with similar studies of westernised urban populations.

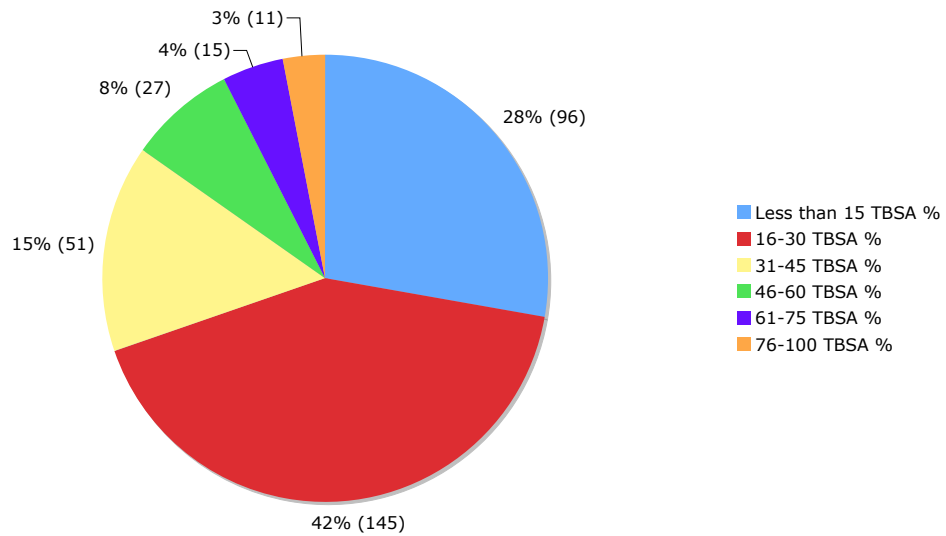


Figure 2.3. The number of patients within each range of TBSA% burned. The data shows the percentage and number (in brackets) of patients within each range of TBSA% burned admitted to the burn unit in the study period, November 2003-September 2009. Each group is a range of 15% except for the group representing burns of 75-100%.

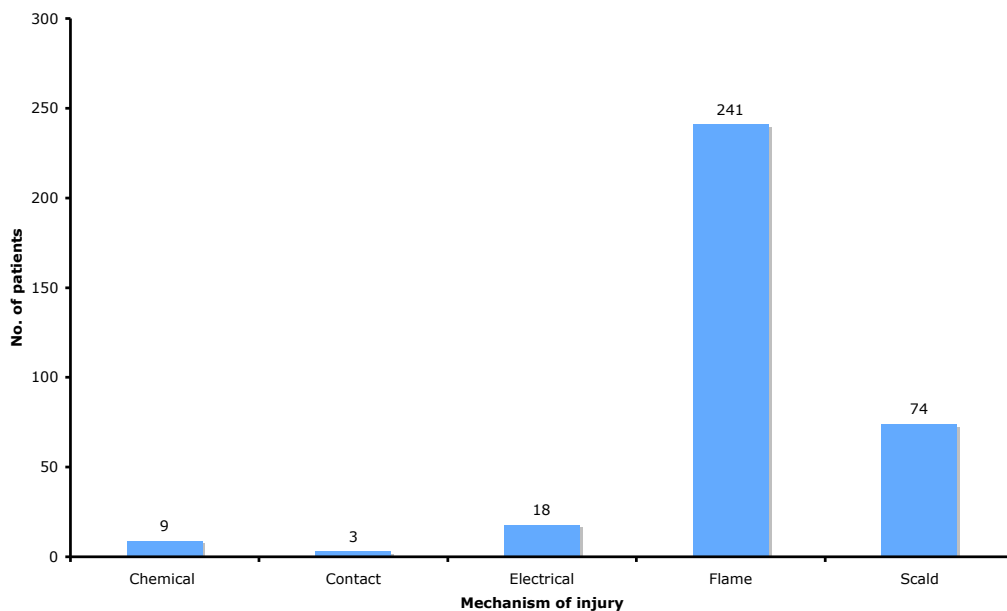


Figure 2.4. The mechanism of injury resulting in the patients being admitted to the burns ITU. The data shows the cause of the injury leading to admission for both the adult and paediatric population.

Within the 345 patients, 90 patients sustained inhalation injuries (26.1%) (Table 2.3) and 44 patients died (12.75%). There were 220 male patients and 125 female patients (table 2.2). The mean ACR on admission was 7.21 mg mmol⁻¹ (range 0.09-192 mg mmol⁻¹) from the 301 patients that had a value recorded on admission (table 2.1).

The mean length of stay was 15.3% (1-175 days) with the mean stay per percentage burn being 0.6-days/%burn.

Normally, a burn ITU admits patients with significant injuries only and does not typically admit patients that do not need formal resuscitation. The cut off figure is 10% for children and 15% for adults. Burns small than this can be treated in the standard burn ward. There are exceptions however, which include chemical burns and small body surface area burns in high-risk populations (paediatric or elderly) or particular areas of the body such as the face (particularly eyes and mouth) or the perineum. Figure 2.3 summarises the number of patients and the percentage of body surface area burn sustained. It can be seen that 28% of burns are in the group 0-15 TBSA%. This large number of smaller burns tended to be in the younger age groups and the elderly where even a small burn is potentially life threatening. The mean TBSA% sustained (range) was 28.2% (2-90%) demonstrated in table 2.1.

The mechanism of injury contributing to admissions to the burn unit is summarised in figure 2.4. In keeping with other units that serve a generally urban population the majority of injuries are flame burns and scalds. The lack of major industry locally means there are relatively few chemical burns, of those admitted about half were accidents in or around the home compared with in factories or at work. The three contact burns were due to falls against radiators or other heating equipment. The electrical burns were mainly high voltage burns associated with industrial cables being severed or touched rather than domestic supply accidents.

2.4.2 ACR on admission

On admission 301 patients had a urinary sample taken and ACR recorded on admission (defined as being taken within 6 hours of coming to the unit). One hundred and thirty seven of these patients (45.5%) had an elevated ACR (>2.3 mg mmol⁻¹). Forty-four patients did not have an ACR recorded within 6 hours of admission. The mean ACR on admission (range) was 7.2 mg mmol⁻¹ (0.3-120). Post resuscitation, (defined as 48 hours post admission) mean ACR was 4.38 mg mmol⁻¹ (0.34-77.1).

Variable	Mean	Standard deviation	Percentiles					Number of values
			Smallest 0%	25%	Median 50%	75%	Largest 100%	
ACR at admission	7.21	19.45	0.09	0.98	2.12	4.55	192.00	301
Age	33.25	23.78	0.10	14.00	31.00	51.00	95.00	345
% TBSA	28.23	19.02	2.00	15.00	25.00	35.00	90.00	345

Table 2.1: Summary of statistics of variables used in analysis of ACR on admission

Sex	Count	Percentage
Male	220	63.8
Female	125	36.2
Overall	345	100.0

Table 2.2: Sex of patients-number and percentage of total

Inhalation	Count	Percentage
No	255	73.9
Yes	90	26.1
Overall	345	100.0

Table 2.3: Number of inhalation injuries

Tables 2.1-2.3 are summaries of the statistics used in the regression model. They are included to demonstrate that the population studied is typical of a large burn ICU in Western Europe and are comparable with data produced by similar units.

When looking at the data for the mechanism of injury it became apparent that the numbers of patients suffering from some of the mechanisms was very small. The first three categories in Table 2.4 below contain small numbers and would mean that if the variable were included in a regression model some regression coefficient estimates would be unreliable.

	Count	Percentage
Chemical	9	2.6
Contact	3	0.9
Electrical	18	5.2
Flame	241	69.9
Scald	74	21.4
Overall	345	100.0

Table 2.4: Mechanism of injury resulting in admission for all patients eligible for inclusion in the study. Data shows the number and percentage of patients being admitted for each mechanism of injury.

To avoid this, these three categories (contact, chemical and electrical) have been combined in a derived variable- Mechanism 3 (Table 2.5). One problem with grouping mechanisms of injury together is that different mechanisms can affect the patient in radically different ways and thus the results can also be skewed. Electrical burns, for example may have occult injuries that the total body surface area percentage may not reflect. For instance a high voltage electrical injury may only ‘burn’ a few percent of TBSA (at the entry and exit points or where clothing has caught fire) but also cause extensive internal damage, such as muscle damage. It is unknown what effect this may have on the ACR compared with a straightforward thermal injury. Of the few other studies looking at burns ACR in the ITU setting Vlachou et al., (2008) looked at only flame burns making direct comparison to our study group difficult because of the mixed aetiology of this cohort of patients. Gosling et al., (2003) examined 11 burn patients but did not specify mechanism of injury.

After statistical advice (Dr Mike Parker, Anglia Ruskin University, Chelmsford) the three mechanisms, chemical, contact and electrical injury were grouped together to ensure the regression model was as accurate as possible accepting

that larger data sets for the burns caused by chemical, contact or electrical injuries may change the results.

Mechanism	Count	Percentage
Flame	241	69.9
Scald	74	21.4
Mechanism 3	30	8.7
Overall	345	100.0

Table 2.5: The mechanism of injury sustained-revised table. The revised data from table 2.4 for mechanism of injury having combined chemical, contact and electrical as one mechanism (mechanism 3).

A multiple regression model was performed looking at the log ACR at admission versus a number of variables that might influence ACR. Only 301 patients had their admission ACR values recorded so all statistics discussed in relationship to admission ACR refer to these patients only. The studied variables were: Age, TBSA%, sex, inhalation and mechanism. Please see Appendix I for full details and statistical analysis performed (Appendix I, section 2, figure 1). Further analysis with different regression models is also included in Appendix I (Section 3).

In summary the sex of the patient, inhalation injury and mechanism had no influence on the ACR value on admission. The two other factors that consisted of continuous variables, TBSA% and age are looked at in more detail.

The fit of simple regression lines with continuous variables are shown below. The standard errors and 95% confidence limits for the fitted regression line have been obtained using a bootstrap. This involves taking repeated random samples of the data and performing calculations of the resulting distribution of the estimate of interest. In the bootstrap analyses here 9999 samples have been selected. The bootstrap analyses were performed using R package boot (Canty and Ripley 2010).

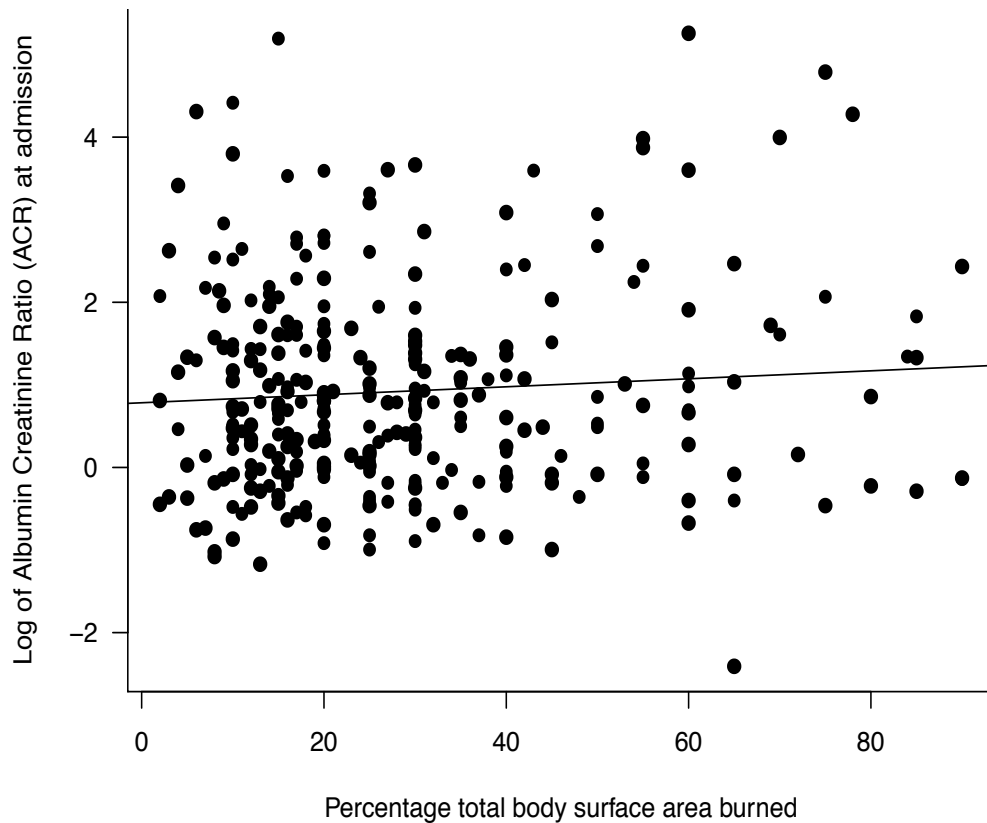


Figure 2.5. Log ACR at admission against TBSA%. Data shows the correlation between the log ACR at admission against the percentage of total body surface area burned. Regression analysis was carried out using R package boot (Canty and Ripley 2010). The regression slope obtained was 0.00484; Bootstrap CI (95%) (-0.00359, 0.01426) shows there was no statistical significance.

Looking at the regression line in Figure 2.5 demonstrates that in the 301 patients who had ACR taken at admission (within 6 hours of arrival) there was no statistically significant link between the ACR on admission to the size of the burn sustained. Although the slope of the regression line is positive the breadth of the confidence interval shows that any effect of TBSA% on admission ACR would be zero or very small. This corresponds with what can be found clinically. The TBSA%, whilst giving an indication of the scale of the injury does not always reflect the severity of the burn i.e. a 10% burns that extends to muscle of bone may be more severe than a 25% partial thickness burn.

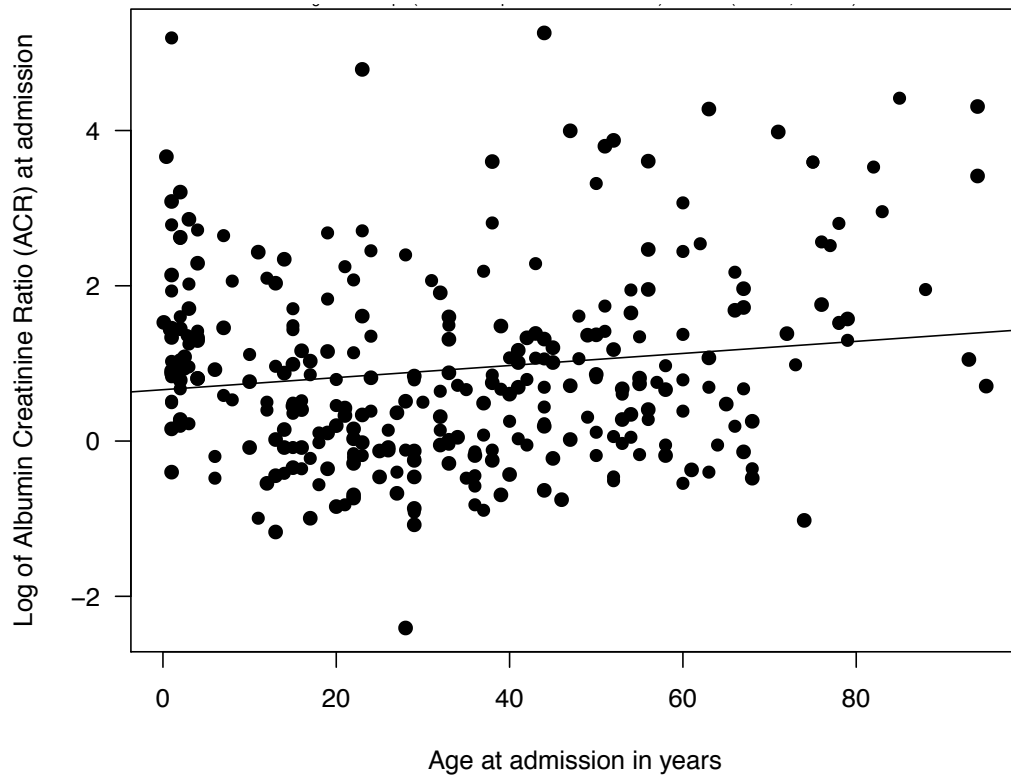


Figure 2.6. Log ACR at admission against age. Data shows a scatter plot of the correlation between Log ACR at admission against age. Regression analysis was carried out using R package boot (Canty and Ripley 2010). The regression slope obtained was 0.00779; Bootstrap 95% confidence interval (0.00097, 0.01455) shows there was statistical significance.

Figure 2.6 demonstrates that as the age of the patients increased their admission ACR was also higher. This was statistically significant. There are several ways to interpret this result. Firstly it is known that the ability to respond to injury and illness tends to decrease with age, as organ function tends to deteriorate with time. It may be possible that with age the physiological response to burn injury is altered and this is reflected in an increased capillary permeability and therefore higher ACR. However it must be noted that diabetic patients and a number of patients with cardiovascular disease also have raised ACR (Ritz et al., 2010 and Feldt-Rasmussen 2000, Pedrinelli et al., 2001) and diabetes and cardiovascular disease are more likely to be seen in an older population. It is therefore unclear whether the higher ACR seen in older patients is due to increased vascular permeability that occurs with age or with other medical illness that are more frequently associated with age, or elements of both. It would require further

analysis of the data to determine the influence of associated medical illnesses on the ACR the patients.

2.4.3 Length of stay in the ICU related to ACR

The use of log length of stay and log ACR produce straight-line relationships. However, for the data considered here this has not produced model residuals that are normally distributed as required for statistical inference. A residual is the difference between the actual value of a variable and the value of variable that was predicted by a statistical model. Bootstrapping is called "model-based" where the model residuals are repeatedly sampled, and each sample of residuals provides a new set of estimates of the model's fitted terms. By producing a large number of such estimates one can use the observed statistical distribution of the estimates to obtain confidence limits. The confidence limits for the regression coefficients are therefore biased-corrected and accelerated bootstrap estimates obtained using R functions `boot` and `boot.ci` from the R package `boot` (Canty and Ripley 2011, Davison and Hinkley 1997). Further explanation of confidence intervals, bias correcting and the accelerated bootstrap is given on pages 80-81.

Peak ACR

To determine if any relationship existed between the length of stay in the ICU and the ACR the first aspect to be examined was the peak ACR during the in-patient stay or until time of death. A summary of the statistics used are in the analysis of peak ACR are detailed in Appendix I Table 1.

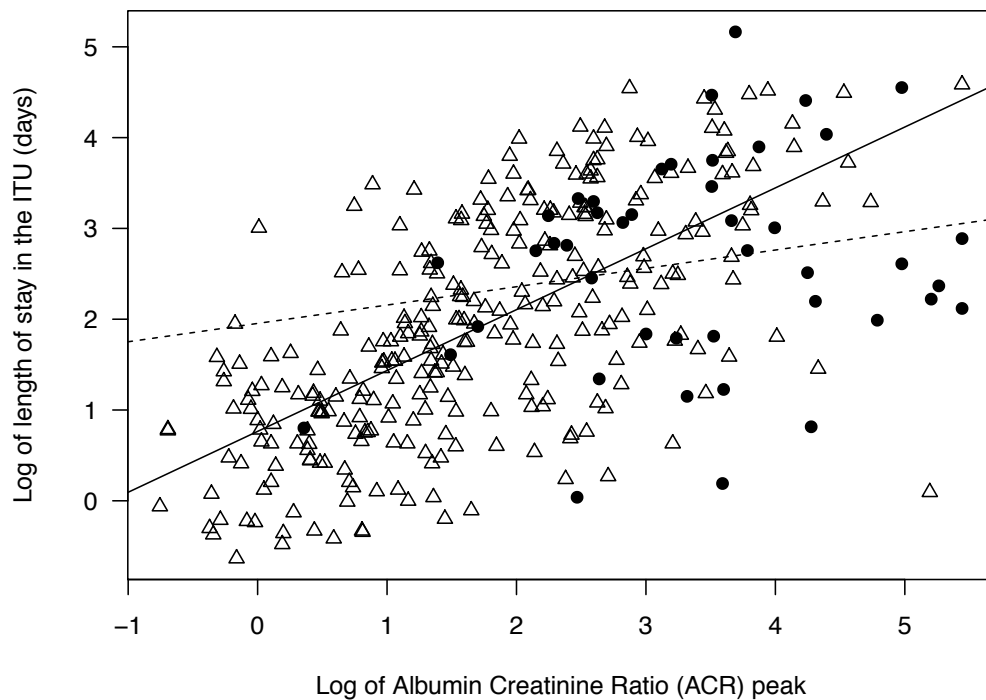


Figure 2.7. Log of length of stay against log of peak ACR. Data shows the correlation between log of length of stay in the ITU (days) against log peak ACR. Regression analysis was carried out using R package boot (Canty and Ripley 2010). The scatter plot has regression lines fitted separately to the groups. The alive group (open triangle and solid line) regression slope obtained was 0.67; Boot strap confidence levels of 95% (0.581, 0.761) showed statistical significance. Died group (closed circles and dashed regression line) regression slope was 0.202; Boot strap 95% confidence levels (-0.091, 0.482) showed no statistical significance.

There is an increasing trend in length of stay with increasing peak ACR for the patients that survived which is demonstrated in figure 2.7, this is statistically significant. If it is accepted that ACR reflects the degree of inflammation and therefore injury, then it may be expected that those patients with worse injuries have a larger ACR. Those patients with more significant injuries (if they survive) tend to need more surgical interventions and normally have a longer course of treatment than those with small injuries. This association of length of stay with peak ACR is not statistically significant for the patients that died. The trend fails to be established for the patients that died because there are few cases with the

lower values of peak ACR, and hence the range of the values is too limited to make it clear that a relationship exists.

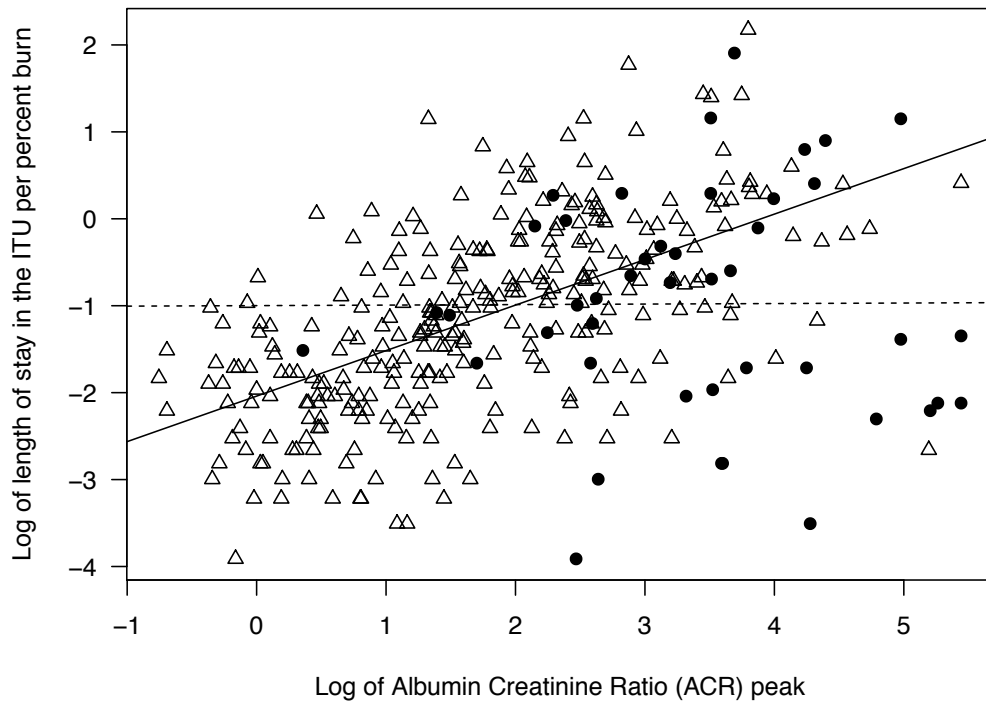


Figure 2.8. Log of length of stay per percent TBSA against log of peak ACR

Data shows the correlation between log of length of stay per percent TBS against log peak ACR. Regression analysis was carried out using R package boot (Canty and Ripley 2010). The scatter plot has regression lines fitted separately to the groups. The alive group (open triangle and solid line) regression slope obtained was 0.523; Boot strap confidence levels of 95% (0.441, 0.605) showed statistical significance. Died group (closed circles and dashed regression line) regression slope was 0.006; Boot strap 95% confidence levels (-0.325, 0.336) showed no statistical significance.

Traditionally the length of stay has been estimated by the TBSA% with every percentage of the body affected equalling one day in the hospital so a 30% burns might be expected to stay 30 days. Now the average length of hospital stay for most serious burn injuries can be expected to be only 0.5 days per percent of TBSA that is burned. However as noted before a burn of 10% full thickness may be a more significant injury that a 20% superficial burn. For this reason the length of stay per percent TBSA is often used as it gives an idea of the severity

of the injury and allows a clearer comparison when looking at different units data. We therefore looked at the length of stay per TBSA% against peak ACR. Like figure 2.7, figure 2.8 shows that there is an increasing trend of length of stay per percent burn with increasing peak ACR for the patients that survived. This is statistically significant. Although the TBSA% does not always correlate with the extent of the injury in general the larger the TBSA% burned the greater the injury and therefore the greater the endothelial dysfunction and ACR. This trend is not statistically significant for the patients that died. The trend fails to be established for the patients that died because again there are few cases with the lower values of peak ACR, and hence the range of the values is too limited to make it clear that a relationship exists.

From the above data it is clear in the patients that survived there was a clear relationship between the peak ACR and the length of stay. Whilst this may have no clinical use, (as one can never predict when the peak ACR value will be either in the patients that survive or die) from those results one might hypothesize that those patients with greater injuries or inpatient complications (displayed by a higher ACR peak) have more systemic endothelial dysfunction and therefore require more intensive therapy intervention (requiring a longer in-patient stay). Clinically it would be far more useful to see if the ACR at admission could predict length of stay. Establishing which patients may stay a prolonged time in ICU could help target therapies to individual patients or aid decision-making in resource allocation.

A regression analysis was performed looking at this using R package boot (Canty and Ripley 2010).

A summary of the statistics of ACR at admission is shown in table 2.1.

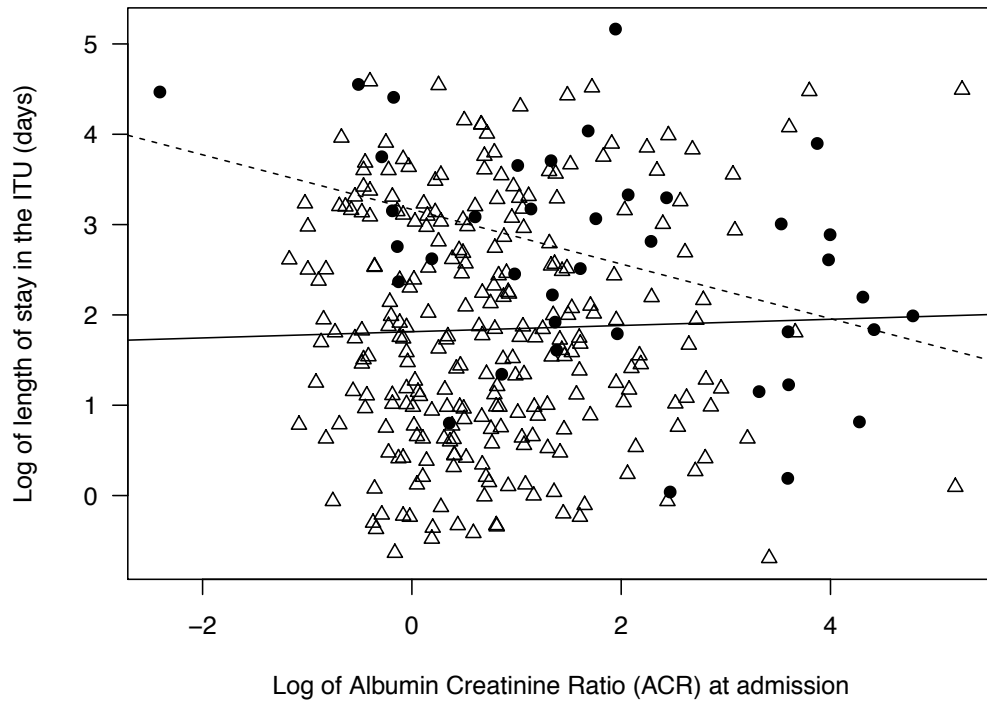


Figure 2.9. Log of length of stay against log of ACR at admission.

Data shows the correlation between log of length of stay per in the ITU (days) against log of ACR at admission. Regression analysis was carried out using R package boot (Canty and Ripley 2010). The scatter plot has regression lines fitted separately to the groups. The alive group (open triangle and solid line) regression slope obtained was 0.034; Bootstrap confidence levels of 95% (-0.108, 0.177) did not show statistical significance. For the Died group (closed circles and dashed regression line) the regression slope was -0.302; Boot strap 95% confidence levels (-0.508, -0.102) showed statistical significance.

Figure 2.9 demonstrates that there is a decreasing trend in length of stay with increasing ACR at admission for the patients that died. This is statistically significant. If higher ACR values indicate increasing ill health this result would be expected because the patients who die early have a reduced length of stay. For the patients that survived there was no statistically significant link between admission ACR and length of stay. For those patients that survived, the ACR at admission was unable to distinguish those patients that might have prolonged in-patient stays compared to the peak ACR that could (figure 2.7). This is probably

because the peak ACR whilst an in-patient will reflect events such as sepsis or organ failure that will generally cause a prolonged stay in ITU.

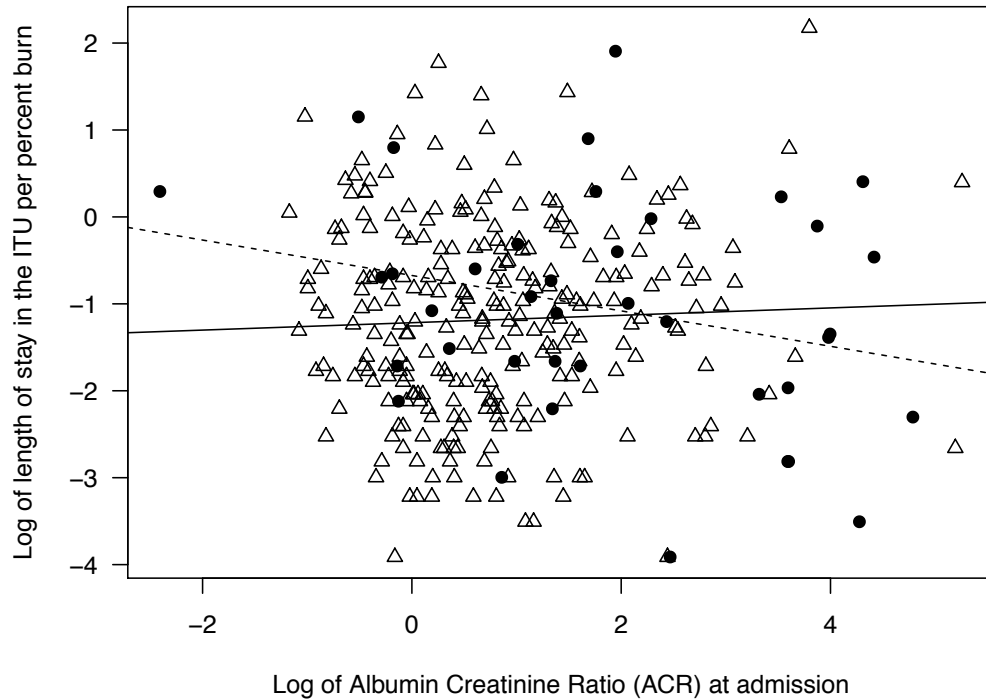


Figure 2.10. Log of length of stay per percent TBSA against log of ACR at admission. Data shows the correlation between log of length of stay per percent TBSA against log of ACR at admission. Regression analysis was carried out using R package boot (Canty and Ripley 2010). The scatter plot has regression lines fitted separately to the groups. The alive group (open triangle and solid line) regression slope obtained was 0.043; Boot strap confidence levels of 95% (-0.83, 0.164) did not show statistical significance. For the Died group (closed circles and dashed regression line) the regression slope was -0.204; Boot strap 95% confidence levels (-0.439, 0.027) showed statistical significance.

In figure 2.10 both for the patients that survived and died there was no trend between log ACR at admission and Log of length of stay per percent TBSA. The apparent trends in the graph are not statistically significant. A summary table of statistics for length of stay per percent burn and ACR is found in Appendix 1, Table 2. As previously described the reason for these results may be related to the fact that the TBSA% does not always correlate with the extent of the injury.

Because large burns can cause such a rapid and profound systematic response it is argued that the physiological response seen in the first twenty-four hours may not be representative of the true nature of the injury. With adequate resuscitation many of the early physiological derangements can be correctable presenting a very different picture of the patients' physiological state and potential prognosis. To determine if the ACR echoed this picture and could be radically altered post resuscitation (and therefore be a more useful prognostic indicator of length of stay) a regression analysis was performed looking at ACR at 48 hours and length of ICU stay.

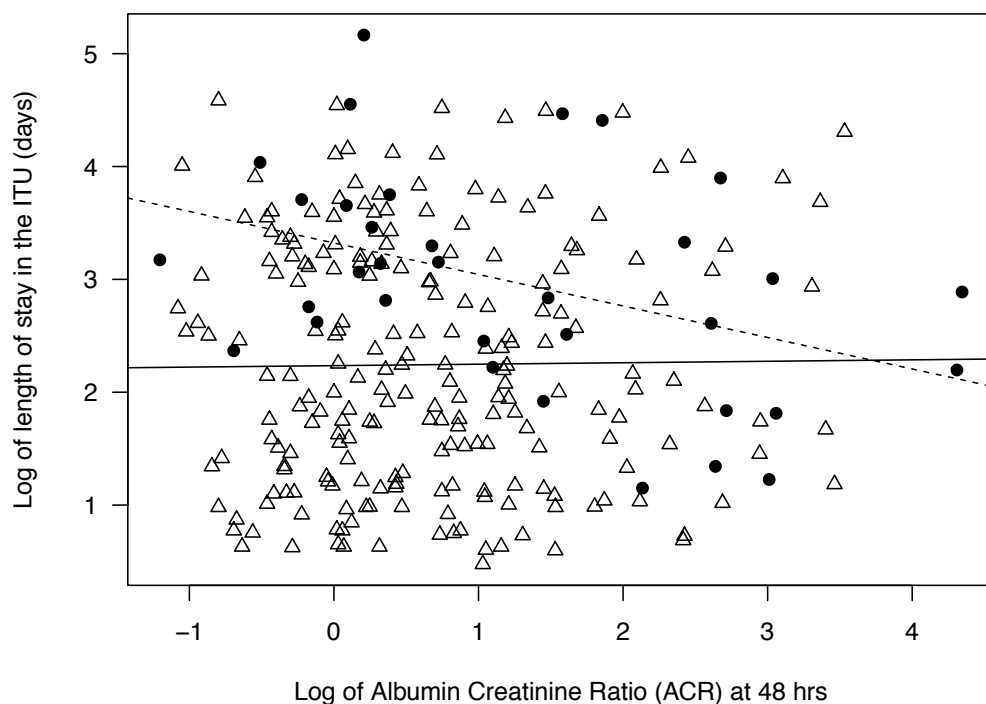


Figure 2.11. Log of length of stay (days) against log of ACR at 48 hours. Data shows the correlation between log of length of stay (days) against log of ACR at 48 hours. Regression analysis was carried out using R package boot (Canty and Ripley 2010). The scatter plot has regression lines fitted separately to the groups. The alive group (open triangle and solid line) regression slope obtained was 0.013; Bootstrap confidence levels of 95% (-0.132, 0.158) did not show statistical significance. For the Died group (closed circles and dashed regression line) the regression slope was -0.279; Boot strap 95% confidence levels (-0.483, -0.069) showed statistical significance.

Similar to figure 2.9 the results in Figure 2.11 shows there is a decreasing trend in length of stay with increasing log ACR at 48 hours for the patients that died. If higher ACR values do indicate increasing ill health then as the patients who die early have a reduced length of stay the trend is to be expected. For the patients that survive there is no trend between log ACR at 48 hours and length of stay.

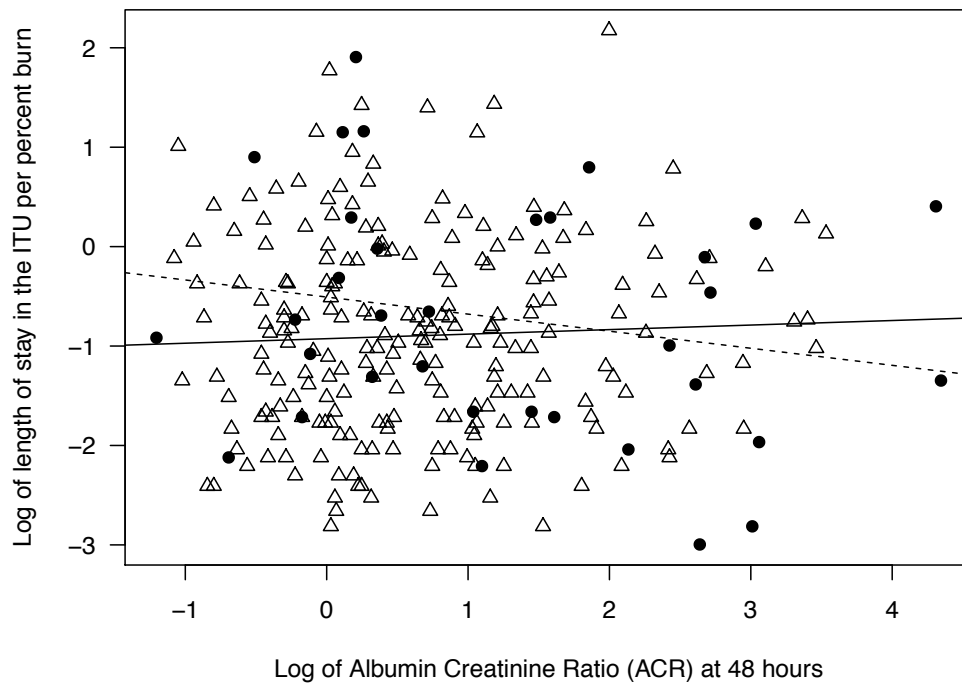


Figure 2.12 Log of length of stay per percent TBSA against log of ACR at 48 hours. Data shows the correlation between log of length of stay per percent TBSA against log of ACR at 48 hours. Regression analysis was carried out using R package boot (Canty and Ripley 2010). The scatter plot has regression lines fitted separately to the groups. The alive group (open triangle and solid line) regression slope obtained was 0.046; Boot strap confidence levels of 95% (-0.89, 0.177) did not show statistical significance. For the Died group (closed circles and dashed regression line) the regression slope was -0.172; Boot strap 95% confidence levels (-0.461, 0.093) showed no statistical significance.

As can be seen from the confidence intervals the apparent trends in figure 2.12 are not statistically significant. Normally one expects that the greater the injury the longer the stay in hospital. At 48 hours the patient has normally been resuscitated and their acute response to the injury has been treated (although may be ongoing). The measures required to resuscitate the patient will also have an effect on their endothelial dysfunction and figure 2.12 shows that an ACR value taken at this time has no prognostic value on the duration of their ITU stay.

2.4.4 ACR and outcome prediction

For all ROC curve statistics the analysis has been performed with computer programme R (R Development Core Team, 2011).

Diagnostic predictor cut-off value

This section examines the use of a simple cut-off value for diagnostic prediction of Outcome. For each set up considered below the receiver operating characteristic (ROC) curve shows the optimum cut-off value according to the maximum Youden Index. The analysis has been performed using the function ROC from the R package DiagnosisMed (Brasil P 2010). The bootstrap confidence limits have been obtained using 9999 bootstrap replicates with the function roc from the R package pROC (Robin et al 2011).

Receiver Operating Characteristics (ROC) Curve and The Youden index

The ROC curve is a graphical method of displaying the discriminatory accuracy of a marker (diagnostic test) for distinguishing two populations (Fluss et al., 2005) i.e. diseased or not diseased (or in this case dead or alive). The ROC curve is the true positive rate (sensitivity) versus the false positive rate (1-specificity) over all the threshold values of the marker. It gives the ability to choose an optimal cut-off value for an index given the investigators criteria. It is common to summarize the information in a ROC curve in a single value (or index). The area under the ROC curve (AUC) is a commonly used index of diagnostic accuracy as is the Youden Index (Youden 1950). The AUC may be computed and its difference relative to 0.5 statistically assessed. If the AUC is not different from 0.5 it means the considered index is as useful as tossing a coin. A ROC curve that operates no better than chance for detecting disease will lie along the 45 degree line that runs from the intersection of the x and y axis to the upper right hand corner of the graph. Points along this line would indicate that this test provides an equal number of true and false positives and that it does not discriminate between healthy and ill patients hence being as useful as a coin toss.

The Youden index is the difference between the true positive rate and the false positive rate. One advantage the Youden index has over the AUC is that it provides a criterion for choosing the optimal value or cut off point, so called

maximising the index. This index ranges between 0 and 1, with values close to 1 indicating that the biomarker's effectiveness is relatively large and values close to 0 indicating limited effectiveness. By identifying the optimal cut-point for calling a patient diseased, the numbers of correctly classified individuals are maximized.

The data used to help determine both the ROC curve and Youden index (in figure 2.13) is shown below in table 2.6. This contingency table allows the sensitivity, specificity and positive predictive value (PPV) and negative predictive value (NPV) to be worked out. The sensitivity is the probability that a test result will be positive when the disease is present (i.e. the true positive rate). The specificity is the probability that a test result will be negative when the disease is not present (i.e. the true negative rate). The PPV is the probability that the disease is present when the test is positive and the NPV is the probability that the disease is not present when the test is negative.

Using Peak ACR in outcome prediction in burns

		Predicted outcome		Total	Percentage correctly classified
		Alive	Died		
Observed outcome	Alive	186	105	291	63.7
	Died	4	38	42	90.9
Total		190	143	333	67.3

Table 2.6. Diagnostic classification table for using a cut-off value 8.58 mg mmol-1 for the peak ACR. Shows the data used to derive the contingency table from which the sensitivity, specificity, PPV and NPV were derived. Diagnostic performance values obtained from the above table and their 95% confidence intervals are: sensitivity 90.9% (CI: 77.9%, 96.2%), specificity 63.7% (CI: 58.3%, 69.2%), Positive predictive value (PPV) 26.6% (CI: 20.0%, 34.4%), and Negative predictive value (NPV) 97.9% (CI: 94.7%, 99.2%). The area under the curve with bootstrap 95% confidence interval is 0.8367 (CI: 0.7751, 0.8927).

The data in table 2.6 is used to help construct the ROC curve and Youden index in figure 2.13. It shows that by maximizing the Youden index a cut-off Peak ACR value of 8.58 mg mmol-1 predicts the death of a patient with a sensitivity of 90.9% and specificity of 63.7% (see also figure 2.13).

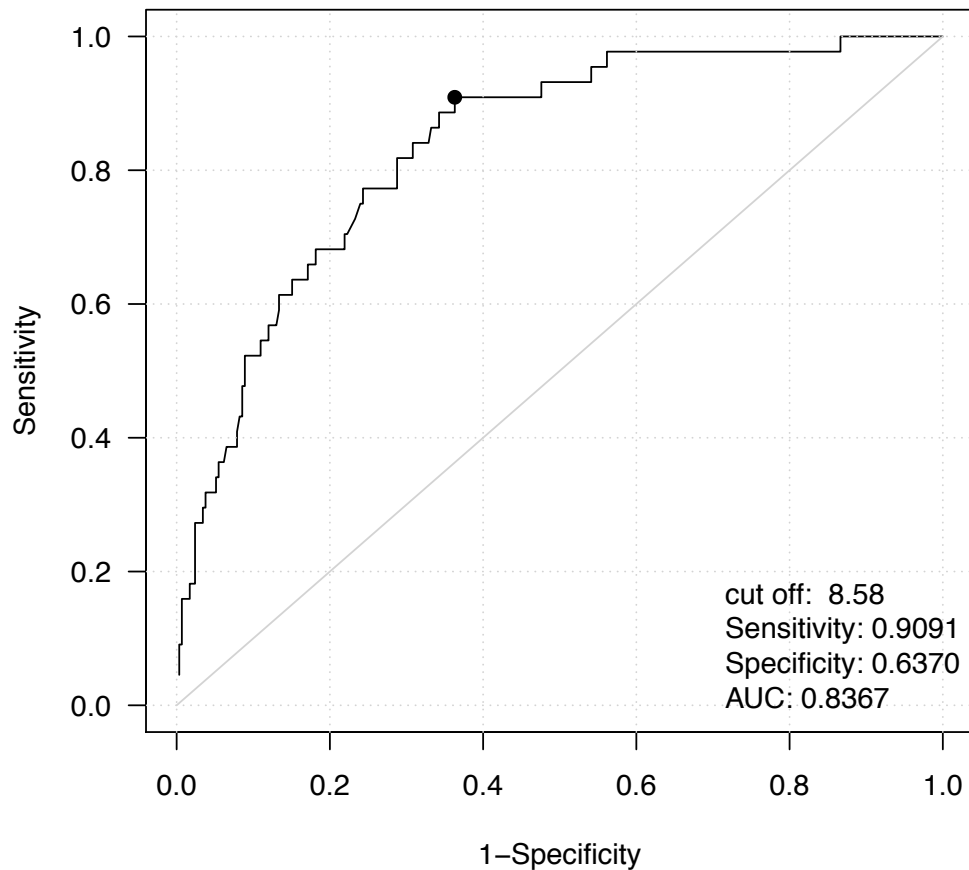


Figure 2.13. ROC curve for outcome versus peak ACR. Data shows a graphical plot of sensitivity against 1-specificity, (true positive against false positive) the ROC curve for outcome (death) against peak ACR. The cut off ACR value of 8.58 is estimated by maximizing the Youden Index.

Figure 2.13 demonstrates that using the Youden index a cut-off Peak ACR value of 8.58 mg mmol⁻¹ predicts the death of a patient with a sensitivity of 90.9% and specificity of 63.7%. The AUC gives the overall performance of a diagnostic test and an AUC value of .83 shows this is a good test in determining patient death when using the peak ACR. One problem is that it is impossible to know when the peak ACR may occur so its clinical relevance is limited. It does however strengthen the argument that ACR values are related to outcome. Clinically it would be more useful to see if the admission ACR has a role in predicting outcome.

ACR at admission to predict outcome in burns

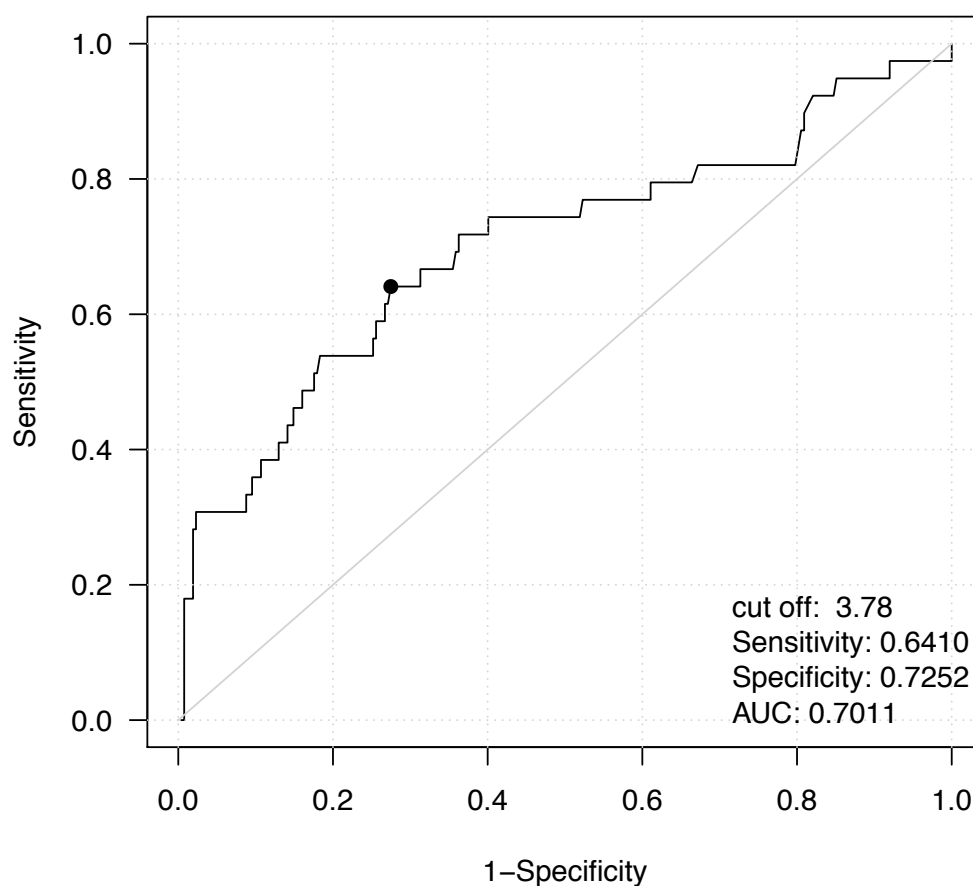


Figure 2.14 ROC curve for outcome versus ACR at admission. Data shows a graphical plot of sensitivity against 1-specificity, (true positive against false positive) the ROC curve for outcome (death) against admission ACR. The cut off ACR value of 3.78 is estimated by maximizing the Youden Index. The sensitivity for this test is 64.1 % and the specificity 72%. The AUC is .70, which shows this to be a fair test for determining outcome (death or survival) with a cut off value of 3.78 mg mmol-1.

		Predicted outcome		Total	Percentage correctly classified
		Alive	Died		
Observed outcome	Alive	190	72	262	72.5
	Died	14	25	39	64.1
Total		204	97	301	71.4

Table 2.7 Diagnostic classification table for using a cut-off value for ACR at admission. Shows the data used to derive the contingency table from which the sensitivity, specificity, PPV and NPV were derived. Diagnostic performance values obtained from the above table (table 2.7) and their 95% confidence intervals are: sensitivity 64.1% (CI: 48.4%, 77.3%), specificity 72.5% (CI: 66.8%, 77.6%), PPV 25.8% (CI: 18.1%, 35.3%), and NPV 93.1% (CI: 88.8%, 95.9%). The area under the curve with bootstrap 95% confidence interval is 0.7011 (CI: 0.5956, 0.7994).

The data in table 2.7 is used to help construct the ROC curve and Youden index in figure 2.14. It shows that by maximizing the Youden index an ACR at admission of greater than or equal to 3.78 mg mmol-1 it predicts the death correctly 64.1% of the time. Whilst this is better than tossing a coin it may not be of sufficient value to be used clinically as there are many other pieces of information that would be used in conjunction with the clinical findings that would be used to assess the chances of a patients survival or not. More useful is the negative predictive value of 93.1% which shows the probability that the disease is not present when the test is negative. Therefore we can say that a patient with an ACR less than 3.78 mg mmol-1 has a 93.1% chance of survival. Again because many of the physiological disturbances seen in burns are rapidly treated in the first 24-36 hours it was decided to see if the ACR at 48 hours would be better at predicting outcome. It may be that that if an ACR is still elevated at this time despite resuscitation then it is a more accurate reflection of the underlying inflammatory response and subsequently outcome.

ACR at 48 hours

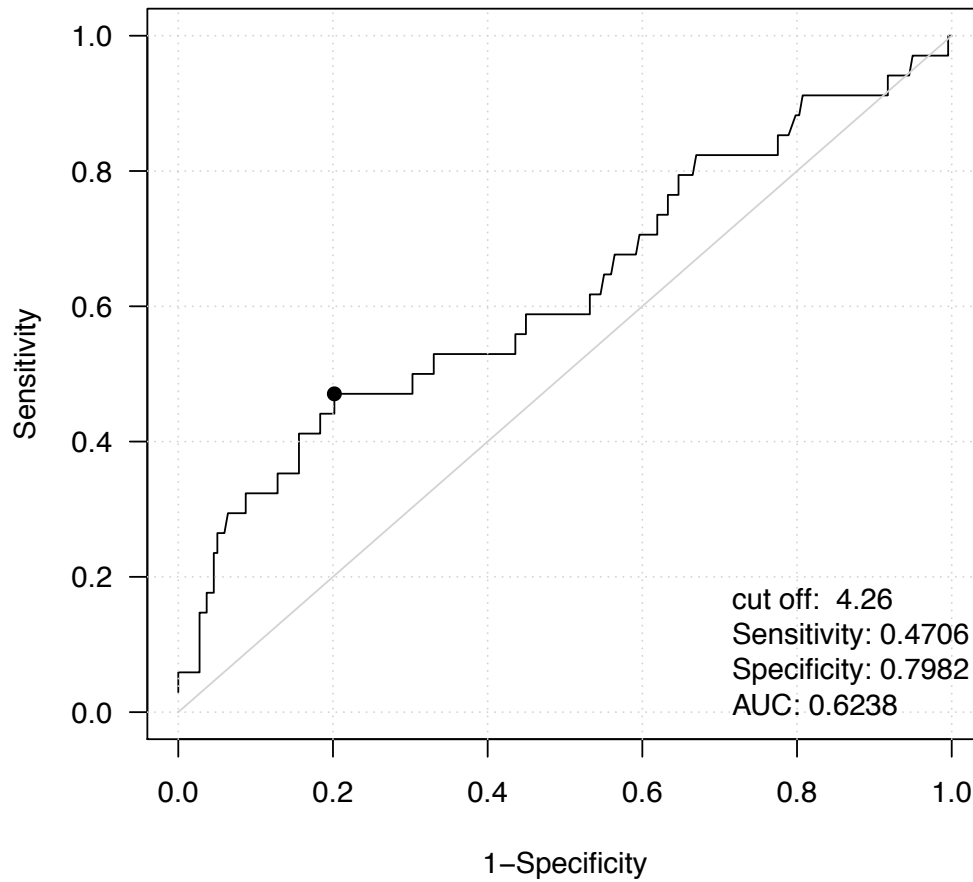


Figure 2.15 ROC curve for outcome versus ACR at 48 hours. Data shows a graphical plot of sensitivity against 1-specificity, (true positive against false positive) the ROC curve for outcome (death) against ACR at 48 hours. The cut off ACR value of 4.26 is estimated by maximizing the Youden Index. The sensitivity for this test is 47 % and the specificity 79%. The AUC is .62, which shows this to be a poor test for determining outcome (death or survival) with a cut off value of 4.26 mg mmol-1.

		Predicted outcome		Total	Percentage correctly classified
		Alive	Died		
Observed outcome	Alive	174	44	218	79.8
	Died	18	16	34	47.1
Total		192	60	252	75.4

Table 2.8 Diagnostic classification table for using a cutoff value for ACR at 48 hours

Shows the data used to derive the contingency table from which the sensitivity, specificity, PPV and NPV were derived. Diagnostic performance values obtained from the above table (table 2.8) and their 95% confidence intervals are: sensitivity 47.1% (CI: 31.5%, 63.3%), specificity 79.8% (CI: 74.0%, 84.6%), PPV 26.7% (CI: 17.1%, 39.0%), and NPV 90.6% (CI: 85.7%, 94.0%). The area under the curve with bootstrap 95% confidence interval is 0.6238 (CI: 0.5098, 0.7340).

The data in table 2.8 is used in conjunction with figure 2.15 to examine whether the ACR readings at 48 hours are of any use in outcome prediction. It is clear that the sensitivity of 47.1% (table 2.8) is poor and indeed the AUC is .62 (figure 2.15) showing this to be a poor test of outcome. There are a number of reasons why this maybe. The first is that the act of resuscitation itself may affect the body's response to inflammation and therefore the endothelial dysfunction. As the inflammatory process is ongoing, a snap shot picture when treatment has commenced may not reflect the dynamic situation that is evolving. Although there are protocols for resuscitation it does vary according to the patient's response to treatment and it may be that whilst many physiological parameters are normalized in the first 48 hours it does not reflect the inflammatory response at a molecular level and the endothelial dysfunction as a result. Another problem is the data collection and that as time progresses there is missing data possibly making the results less accurate.

It was decided to see if any trends in the first four days of admission would be a more accurate way of predicting outcome.

2.4.5 Area under the curve of initial ACR values

There are some problems with the recording of the ACR. To be able to use ACR for the prediction of patient outcomes requires the diligent recording of readings at the initial stages of the patients' stay in the ITU.

Out of the 345 cases, for "ACR at admission" there are 301 (87.2%) readings recorded, leaving 44 (12.8%) missing. Out of the 301 cases where there is a reading for "ACR at admission" there are 22 (7.3%) cases where the reading for "Day 1" is missing.

Out of the 345 cases, for "ACR at 48hrs" there are 252 (73.0%) readings recorded, leaving 93 (27.0%) missing. Out of the 252 cases where there is a reading for "ACR at 48hrs" there are 2 (0.8%) cases where the reading for "Day 2" is missing.

Out of the 345 cases, there are 65 (18.8%) of cases for which there is no reading for "Day 1", and there are 24 (7.0%) of cases for which there are no readings for either "Day 1" or "Day 2".

Out of the 345 cases, there are 280 (81.2%) of cases for which there is a reading for "Day 1", 209 (60.6%) of cases for which there are readings for both "Day 1" and "Day 2", 162 (47.0%) of cases for which there are readings for all the first three days, and 134 (38.8%) of cases for which there are readings for all the first four days.

To check whether the initial ACR readings could predict the final outcome the area under the curve of the ACR values for days 1 to 4 was calculated. To increase the number of cases where the area could be calculated in the presence of missing values, if the value for day 3 was missing it was assigned the value for day 2 (which might also be missing), and if the value for day 4 was missing it was assigned the value for day 3 (which might also be missing). The algorithm for calculating the area would only produce a result if there were an ACR reading for all four days. This left 140 cases out of 345 that could be used in the analysis.

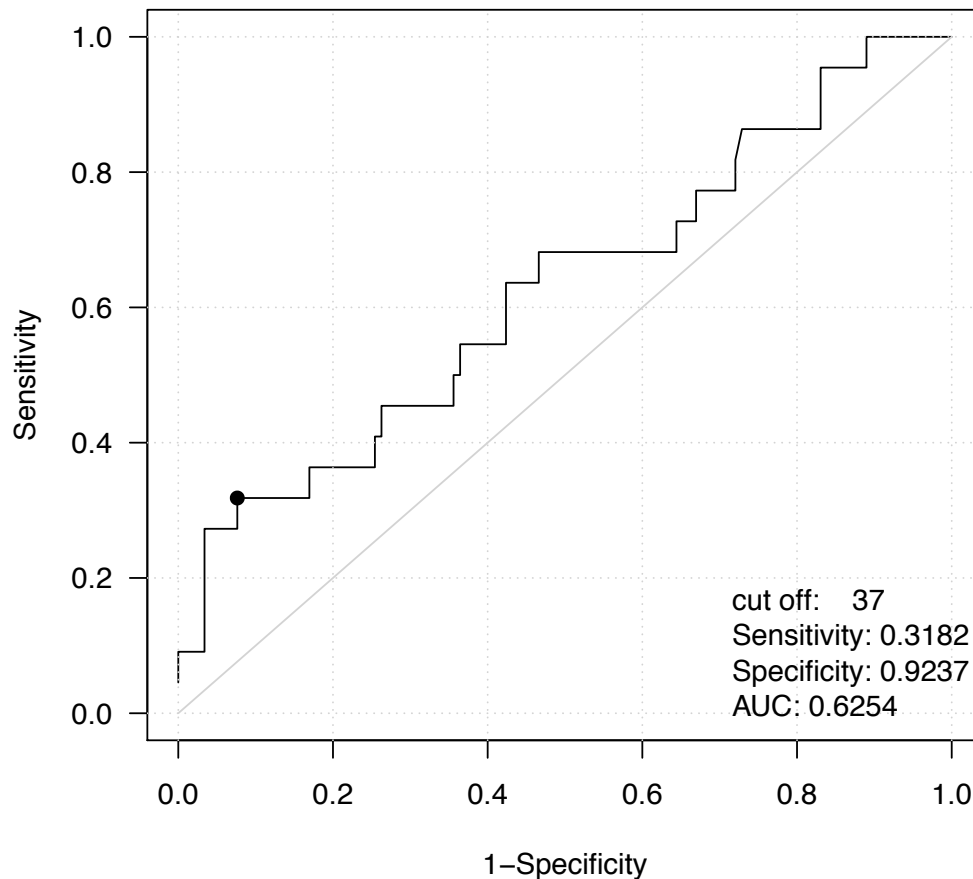


Figure 2.16 ROC curve for outcome versus area under the curve (AUC) of ACR for up to 4 days. A cut off of 37 mg mmol⁻¹ gives a sensitivity of 31.8% and specificity of 92.3%. Data shows a graphical plot of sensitivity against 1-specificity, (true positive against false positive) the ROC curve for outcome (death) against AUC of ACR for up to 4 days. The cut off ACR value of 37 is estimated by maximizing the Youden Index. The sensitivity for this test is 31 % and the specificity 92%. The AUC is .62, which shows this to be a poor test for determining outcome (death or survival).

The ROC curve in figure 2.16 shows that the use of the area under the curve of the initial ACR values in the first 4 days is not a successful diagnostic measure. Firstly the AUC of .62 suggests this is a poor test for separating survivors from those that die and secondly the cut off of 37 mg mmol⁻¹ is too high to be used in a clinical setting as the number of patients achieving this ACR is limited and the sensitivity is too low to be a useful clinical tool.

2.5 Discussion

This study of adults and children admitted to a UK burns ICU and requiring resuscitation shows a high prevalence rate (45.5%) for microalbuminuria on admission.

The prevalence of microalbuminuria in normal healthy adults is 3.3% (Jones et al., 2002). A number of studies have demonstrated an increased ACR in critically ill patients and after surgery and trauma (Matsushita et al., 1996, Shearman et al., 1989, Wood et al., 2000, Mahmud 1994, Gosling et al., 1988, Gosling and Sutcliffe 1996).

This study shows a lower prevalence than other critical care populations. Thorevska et al., (2003) found 69% of their patients' demonstrated microalbuminuria and clinical proteinuria on the first day of admission. This higher rate may be explained by a number of factors. Firstly their study examined medical patients, in which a higher proportion had diabetes or hypertension (co-morbidities that in isolation may increase ACR) than this study of burns patients. The patients admitted suffered from a variety of medical problems that may have a greater effect on endothelial dysfunction. It is not clear when their samples were taken, and from Vlachou's work (2008) it has been shown that the ACR can be very sensitive to surgical procedures or wound manipulations. It is possible that the timing of the samples may have influenced their results.

A further reason may be that the average age of their population was much higher (64.5 years) and as figure 2.6 demonstrates ACR values increase as age increases. Although the regression of log ACR on age is statistically significant it only explains about 2% of the variance of log ACR. And so is unlikely to be clinically useful.

This study demonstrates the usefulness of urinary ACR at admission to the burns unit in predicting outcome.

Looking firstly at the peak ACR and outcome, a cut-off ACR value of 8.58 mg mmol⁻¹ seems at first to be accurate at predicting those patients who may die from their injuries sensitivity 90.5% (CI: 77.9%, 96.2%) (Table 2.6 and Figure 2.13) although the positive predictive value, PPV 26.6% (CI: 20.0%, 34.4%) is

less impressive. These results, however are no use in the clinical scenario, as the clinician will never know when the peak ACR results may be, but do demonstrate the relationship between ACR and outcome. Whilst peak ACR has no clinical application in individual patient's treatment it may have a research role in comparing response to treatment between patients or even between burn centres.

Using a cut off value of 3.78 mg mmol⁻¹ the ACR at admission (Table 2.7) demonstrates a sensitivity of 64% with a specificity of 72%. The PPV was 25.8% and NPV 91.1% in determining outcome. Previously Yew and Pal (2006) had determined that a mean ACR of 20 mg mmol⁻¹ was associated with poorer outcomes in patients with extensive burns.

It is difficult to compare this data with other studies due to uncertainty of timings of ACR recordings and differences in the demographics of the patients studied.

However looking at diagnostic accuracy (to determine the development of ARDS, Acute Respiratory Distress Syndrome) rather than outcome, Pallister's study (2001) shows similar sensitivity and specificity rates (Sensitivity 63.4%, specificity 73.3%, PPV 54%, NPV 80%). The investigation by Gosling et al., (2003) provides the nearest demographic match to this study into burn patients. It examines the admission ACR in surgical and trauma patients admitted to intensive care and includes 11 burn patients. Their higher cut off ACR value of >5.9 mg mmol⁻¹ gave sensitivity for death of 100% and specificity of 59% (PPV, 25% and NPV, 100%). This was found to predict death as well as other commonly used measures, in this case APACHE II and SAP scores.

Whilst the sensitivity of ACR in determining outcome in burns patients may be improved by using a higher ACR value its clinical usefulness as a clinical tool will be reduced.

Vlachou et al (2006) showed a positive correlation between urinary ACR and TBSA at 3 and 7 hours post injury and with the percentage of full thickness burn between 3 and 7 hours. Vlachou's later work (2008) showed per-operative ACR was associated with % TBSA excised.

In contradiction this study found no correlation between admission ACR and TBSA. One reason for this is that TBSA may not accurately reflect the degree of

injury that the patient suffers from. For example a 10% full thickness burn, which has burnt tissue down to bone, may elicit a greater systemic response than a larger but more superficial burn. Further studies would have to include the %FTB as well as the TBSA% to clarify the relationship between ACR and burn size.

All the patients in Vlachou's study (2008) were flame related which may have an impact on interpretation of their results as the mechanism of burn injury has a significant influence on outcome. Flame burns result in a higher proportion of cases of multi-organ failure, and have a higher mortality rate (Kraft et al., 2011). In our study due to the low number of contact, chemical and electrical burns these mechanisms were grouped together and compared with scalds and flame injuries (Table 2.5). There was no association between ACR and the mechanism of injury found in our study which suggests Vlachou's results can be extrapolated to burns of all mechanisms.

Previously Gosling et al., (2003) have demonstrated that ACR on admission was related to intensive care length of stay in surgical, trauma and burn patients although not for medical patients. Our study found no link between admission ACR and length of stay in those patients that survived but as may be expected a decreasing trend of length of stay with increasing ACR at admission in those patients that died (Table 2.7, Figure 2.10). Because of the rapid and profound systemic changes that occur in a major burn it may be argued that the admission ACR may be misleading because adequate resuscitation will correct many of the initial physiological abnormalities and thus stabilise the patient. ACR measurements after the period of resuscitation may therefore be a more accurate determinant of systemic vascular dysfunction. ACR at 48 hours echoed the results found on admission and therefore in patients that survived there is no correlation between ACR and length of stay. The variance with Gosling's (2003) results may be the result of the same sample size of burns in that study.

Vlachou et al., (2006) also found that the ACR was higher for up to 8 h in the presence of inhalation injury. Our study looked at inhalation injuries and performed multiple regression models (summary data shown in Appendix 1, table 3 and table 4) and found that the standard errors were so large that no association between ACR (at admission) and inhalation injury could be made.

It has been made clear that the ACR is very sensitive to surgery and wound manipulations and therefore an inhalation injury could easily cause a rise in ACR shortly after injury. It must be noted that in Vlachou's study (2006) all patients who presented to the unit after 6 hours were excluded. In our unit, many burns do not reach the unit within 6 hours and excluding these patients would have resulted in a far smaller study group. The majority of patients transferred from other hospitals will also have had some form of resuscitation started or even a surgical procedure such as wound débridement or cleaning. It may be that the first few hours after a burn are the most crucial when gathering ACR samples. After 6 hours the ACR may have reduced either because of the natural response of the patient's body to the burn or fluid resuscitation has been started or been influenced by interventions in the referring hospital.

Studies have found that a number of other inflammatory mediators are affected by wound manipulation. Commonly analysed markers include IL-6, CRP and procalcitonin.

As little as one hour after the start of elective surgery rises in serum levels of IL-1 inhibitors, IL-6 and cortisol have been noted (Di Padova et al., 1991). However the rise of IL-6 can be even more sensitive. Papini et al., 1997 found a three-fold increase in IL-6 levels between pre-operative and recovery samples after dressing changes as well as débridement of both small and large burns. IL-6 levels are related to the degree of surgical stress and trauma, and there is an association between IL-6 levels and adverse outcome (Jawa et al., 2011). It is less clear that the CRP level is affected in the same way as studies have shown conflicting results. CRP has also commonly been used as a measure of inflammatory response (and as a measure of adverse outcome (Kushner et al., 2006)) and is sensitive to minor environmental irritants such as secondhand smoke exposure and air pollution (Peters et al., 2001), causing CRP elevation. However its significance when related to surgery is less clear, as no change (Cruickshank et al., 1990) or minor elevation (Nichol et al., 1998) has been found to occur to CRP levels after minor surgery when compared to more major surgical interventions. Procalcitonin has also been found to be elevated in a third of patients after minor surgical procedures (Meisner et al., 1998) however this like many of these studies fail to mention the precise timing of the blood sample

so it is difficult to compare to the sensitivity of ACR as demonstrated by Vlachou et al., (2006).

The initial analysis had looked at the ACR at specific time points (admission, 48 hours, peak ACR) and given only a 'snap-shot' of what may be going on. Therefore the trends in ACR were examined to see if they were useful in predicting outcome. One difficulty encountered was that there were days when the ACR had not been recorded.

To check whether the initial ACR readings could predict the final outcome the area under the curve of the ACR values for days 1 to 4 was calculated. To increase the number of cases where the area could be calculated in the presence of missing values, if the value for day 3 was missing it was assigned the value for day 2 (which might also be missing), and if the value for day 4 was missing it was assigned the value for day 3 (which might also be missing). The algorithm for calculating the area would only produce a result if there were an ACR reading for all four days. The consequence of this was that only 140 cases out of 345 could be used in the analysis.

The results showed a sensitivity of 31.8% and a specificity of 92.3%. The ACR cut-off value was 37 however, which would limit its clinical application. Trends in ACR do not seem to have a role in predicting outcome in burns cases.

	ACR cut off value mg mmol-1	Sensitivity %	Specificity %	PPV %	NPV %	AUC
Peak	8.58	90.5% CI: 77.9%, 96.2%	63.9% CI: 58.3%, 69.2%	26.6% CI: 20.0%, 34.4%	97.9% CI: 94.7%, 99.2%	0.83
Admission	3.78	64.1% CI: 48.4%, 77.3%	72.5% CI: 66.8%, 77.6%	25.8% CI: 18.1%, 35.3%	93.1% CI: 88.8%, 95.9%	0.70
48 hours	4.26	47.1% CI: 31.5%, 63.3%	79.8% CI: 74.0%, 84.6%	26.7% CI: 17.1%, 39.0%	90.6% CI: 85.7%, 94.0%	0.63
4 day trend	37	31.8%	92.3%			0.62

Table 2.9. A summary of analysis of ACR readings at different time points with outcome. Data show the ACR cut off points as maximized by the Youden index to determine outcome at the various time points when ACR was measured with the sensitivity, specificity, PPV and NPV for each category.

Table 2.9 summarizes the analysis of ACR readings at different time points with outcome. Peak ACR as previously discussed has no clinical application in individual patient's treatment but may have a research role in comparing response to treatment between patients or even between burn centres.

Clinically a test that can be performed easily and swiftly (as soon as the patient is admitted to the unit) and can accurately predict outcome would be most useful. This study has demonstrated that an admission ACR with a cut-off value of 3.78

mg mmol-1 with a sensitivity and specificity similar to other studies could be used as part of the assessment of the patient in determining outcome.

In conclusion, there have been limited studies determining the clinical utility of ACR in predicting burn patient outcome. This study suggests that ACR could be used as part of the assessment of the patient in determining outcome but the sensitivity and specificity are not high enough to use it as a clinical tool. It may be that its diagnostic accuracy could be improved by using it in conjunction with another marker of inflammatory response (such as C-reactive protein or procalcitonin). Another point to note, made clear by Vlachou's work is the importance of timing of the ACR sampling and that due to the nature of the patient referrals to our centre very early results post injury are rarely obtained. This could mean the key period where ACR is most useful is missed by the time the patients attend our centre.

ACR has been found to be of use not only in predicting outcome, but also in a number of other studies in ICU patients, can predict organ failure or sepsis.

ACR may therefore have a role in predicting complications, sepsis or multi-organ failure in burn patients.

Chapter 3 Sepsis in burns

3.1.1 Introduction

In addition to SIRS and multi-organ failure burns patients are also prone to the development of sepsis. Although defined criteria exist, sepsis can most simply be thought of as a change in the burn patient that triggers the concern for infection. Sepsis is a presumptive diagnosis when there is a concern of infection usually resulting in antibiotics being started whilst the source of the sepsis is being sought. Worldwide sepsis is a major cause of death and in the UK alone accounts for around 30,000 admissions to critical care units (Harrison et al., 2006). In Harrison's study, 27.0% of patients were identified as having severe sepsis in the first 24 hours following admission.

Sepsis in burn patients is not only a problem of increased morbidity, hospital stay and costs but also has a profound impact on mortality. The most recent review of severe burns injuries in Europe presented figures of between 2 % and 14% for the number of burn patients dying from sepsis (Brusselaers et al., 2010).

Like MOF the criteria to define and categorise sepsis has been the subject of much debate and also like SIRS and MOF, burn patients have often been excluded from studies into sepsis. There may be a number of reasons for this, including the systemic response seen in burn patients making them difficult to fit into some of the normal patterns of sepsis seen in other critical care populations. Secondly burn patients treated in most critical care units (rather than specific burn units) usually present in such small numbers that it is difficult to study such small populations and to show meaningful results.

Definition of sepsis

The concept of sepsis has been used for many years. Attempts to refine the definition have occurred with a number of consensus conferences culminating in the definition that is used most frequently worldwide. This was produced by the American College of Chest Physicians, (ACCP) and the Society of Critical Care Medicine (SCCM) and is shown in table 3.1 (Bone et al., 1992).

Condition	Definition
SIRS	2 or more of the following criteria without another identified cause Temperature > 38°C or < 36°C Heart rate > 90 beats/min Hyperventilation with respiratory rate >20/min or Paco2 <32 mm Hg White blood cell count >12,000 cells/L or <4000 cells/L
Sepsis	SIRS in the presence of documented infection
Severe sepsis	Sepsis with associated organ dysfunction, hypoperfusion, or hypotension
Septic shock	Sepsis with arterial hypotension despite adequate fluid resuscitation

Table 3.1. ACCP/SCCM Definitions for Sepsis and Organ Failure

In this definition sepsis can be categorised in three ways, reflecting the severity of the condition. The ACCP/SCCM definitions suffer a fault when applied to burns patients because of their reliance on the SIRS criteria. The systemic physiological changes in burn patients results in profound changes in many of the parameters used to define SIRS, such as a increased heart rate, temperature and respiratory rate as part of the hypermetabolic state seen in burn. Using these criteria, the majority of patients would be defined as having sepsis.

The American Burns Association Consensus Conference therefore produced their criteria for a definition of sepsis (Greenhalgh et al., 2007). There are six

categories looking at physiological parameters or clinical signs. Some categories have subgroups. It must be noted that the criteria for the definition are age-dependant with adjustments being made for children.

For sepsis a patient needs three of the triggers listed in table 3.2 and it is required that a documented infection is identified.

Physiological parameters	Criteria for definition of sepsis
I. Temperature	>39° or <36.5°C
II. Progressive tachycardia	A. Adults 110 bpm B. Children >2 SD above age-specific norms (85% age-adjusted max heart rate)
III. Progressive tachypnea	A. Adults >25 bpm not ventilated i. Minute ventilation >12 l/min ventilated B. Children >2 SD above age-specific norms (85% age-adjusted max respiratory rate)
IV. Thrombocytopenia (will not apply until 3 days after initial resuscitation)	A. Adults <100,000/mcl B. Children <2 SD below age-specific norms
V. Hyperglycemia (in the absence of pre-existing diabetes mellitus)	A. Untreated plasma glucose >200 mg/dl or equivalent mM/L B. Insulin resistance—examples include i. >7 units of insulin/hr intravenous drip (adults) ii. Significant resistance to insulin (>25% increase in insulin requirements over 24 hours)
VI. Inability to continue enteral feedings >24 hours	A. Abdominal distension B. Enteral feeding intolerance (residual >150 ml/hr in children or two times feeding rate in adults) C. Uncontrollable diarrhoea (>2500 ml/d for adults or >400 ml/d in children)
In addition, it is required that a documented infection is identified by A. Culture positive infection, or B. Pathologic tissue source identified, or C. Clinical response to antimicrobials	

Table 3.2. Criteria required for a diagnosis of sepsis (Greenhalgh et al., 2007).

As noted by Greenhalgh et al., (2007), sepsis is a presumptive diagnosis when there is a concern of infection usually resulting in antibiotics being started whilst the source of the sepsis is being sought.

Whilst the definition of septic shock was kept for burn patients (as haemodynamic instability is similar irrespective of cause) the consensus dropped the term severe sepsis. This was because it was felt there was little clinical difference between sepsis and severe sepsis in burns.

Whilst the treatment of sepsis has improved the focus has shifted to try and identify as early as possible those patients that may develop sepsis. In this way therapies and resources can be targeted in a more effective and efficient way.

3.1.2 Prevention of sepsis

As sepsis relies on the presence of infection it is logical to believe that reducing the chance of infection will reduce sepsis. From the moment the patient enters the burns unit steps can be taken to reduce the potential for infection.

Antibiotic prophylaxis

One possibility is whether this may be achieved by the prescription of prophylactic antibiotics?

The potential risks of antibiotic prophylaxis in burns patients are great. Antibiotic resistance is a growing problem throughout the medical world. In a population who frequently harbour multiple pathogens and that are at high risk of infection this is magnified, and the blind use of antibiotics is considered to be counterproductive. In addition there is the obvious risk of side effects of the individual antibiotics used and polypharmacy.

As noted by Chipp et al., (2010) the situation is less clear in paediatric patients due to the risk of toxic shock syndrome. This toxin-mediated febrile illness historically has a high death rate (McAllister et al., 1993) and the introduction of routine antibiotic prophylaxis in one unit (Rashid et al., 2005) was found to reduce its incidence. Toxic shock seems a particularly UK based phenomenon (Burd 2005) with three Dutch burn centres never having seen a case (all three centres used a different antibiotic and dressing protocol (Vloemans and Gorissen

2005). The result is that a third of hospitals in the UK give prophylactic antibiotics to children (Al-Benna et al., 2007).

Burn wound excision

Cope published evidence in over half a century ago that patients had a better outcome with early excision and grafting (Cope et al., 1947). This approach was popularised by Zora Janzekovic (1970) almost twenty years later who changed burn care by her method of tangential excision and grafting. By shaving sequential thin layers of burn eschar down to a layer of viable tissue, a clear visualisation of burn depth could be determined intra-operatively and definitive wound closure could be achieved at the same time. Although this is approach now forms the basis of burns management around the world it took time for this method to be accepted and the exact timing of burn wound excision can still be debated. Barret and Herndon (2002) hypothesized that acute burn wound excision (defined as being ‘in the first 24 hours after burning’) would be superior to conservative treatment and delayed excision in preventing bacterial colonization and invasion. They found that patients admitted and treated later had higher bacterial counts (more than 10^5 bacteria) and were more likely to suffer from infections and graft loss. Ong et al., (2006) performed a meta analysis of early excision of burns and only found six papers (that fulfilled the criteria for their study) that clearly demonstrated a benefit to early excision. Of these six studies only two looked at sepsis as an outcome measure.

It might seem logical to suppose that early burn excision by reducing bacterial counts and infection would also reduce sepsis rates. The little evidence however, is contradictory.

Herndon et al., (1989) showed that early excision in adults (age range 17-30 years and without inhalation injury) reduced mortality from 49% to 9% when compared with the conservative management group. No difference was seen in the groups however, when looking at length of stay, number of procedures or days septic. It must be noted that the criteria for sepsis were similar to the consensus definition in that they were based on physiological variables but were not as wide ranging.

Conversely Subrahmanyam (1999) found that early excision reduced the chance of sepsis. In this study all the three deaths from sepsis were in the conservatively

managed group. The problem with this study is firstly that the conservative management was the use of honey. Although used widely for the dressings of wounds in general, honey is not the first line treatment in most countries. Secondly the criteria used to define sepsis were not given. It therefore remains unclear whether early excision of burn wounds influences the development of sepsis.

Wound cover and grafting

After excising the burn tissue, the underlying exposed tissue bed is grafted, usually with a split thickness skin graft, either as a sheet or meshed. In larger burns there may not be enough donor skin available and the remaining exposed areas need suitable dressings to reduce the risk of infection.

In the majority of UK units the exposed areas are covered with a temporary biological dressing or skin substitute until the donor areas have healed sufficiently for re-harvesting more skin. The most frequently used dressings include cadaver allograft, xenograft (pig skin) as well as dermal scaffolds such as integra (Atiyeh et al., 2005) or matriderm (Ryssel et al., 2008).

Although there are no recent studies looking at skin substitutes and sepsis it seems logical that coverage of large exposed areas will reduce the possibility of infection and therefore sepsis (personal opinion).

Prevention of sepsis by controlling blood glucose

Critically ill patients frequently suffer from hyperglycaemia and increased insulin resistance and this is also true of burns patients. Stress hyperglycaemia may increase the risk of infection and polyneuropathy in the critical care population (Van den Burghe et al., 2001). This study showed that attempting to maintain normoglycaemia could reduce the morbidity and mortality in critically ill patients. (This is generally through intravenous insulin therapy.) The effect on sepsis was also marked. Intensive insulin treatment reduced episodes of septicemia by 46 percent compared to the control group. Furthermore, markers of inflammation were less frequently abnormal in the intensive-treatment group than in the conventional-treatment group. In addition the patients who received intensive insulin therapy were less likely to require prolonged use of antibiotics than were the patients who received conventional treatment, an effect that was

largely attributable to the lower rate of bacteremia in the intensive-treatment group.

It can be seen therefore that maintenance of normoglycaemia may be particularly important in burns. Hyperglycaemia results in increased rates of skin graft loss (Mowlavi et al., 2000), infections, (Pham et al., 2005) sepsis and mortality (Pidcoke et al., 2009).

The role of the gastrointestinal tract (GI) in burn sepsis

The gastrointestinal tract plays an important role in the immune response to injury. In the 1980s and 1990s there were a number of studies that looked at the mechanisms of gut barrier failure and bacterial translocation (Gosain and Gamelli 2007). After burn injury there is a loss of gut barrier function. The GI tract normally keeps bacteria confined within its lumen. Burn injury may allow increased bacterial infiltration into mesenteric lymph nodes and other extraintestinal sites (such as the liver or blood). This results in a process called translocation (Deitch and Berg 1987). Using an animal model (rats) Horton (1994) demonstrated that bacterial translocation was much higher in the burn rats compared to the control group and that intestinal blood flow was much reduced 5 hours after injury in the burn rats.

The problem of bacterial translocation forms the basis for the therapeutic strategy of selective decontamination of the digestive tract (SDD) as well as interventions aimed at improving gut barrier function such as early enteral feeding.

SDD is a prophylactic strategy consisting of treatment using enteral and parenteral antimicrobials that are used to decontaminate the oropharynx and intestine thus reducing the source of endogenous pathogenic organisms. A randomised, placebo controlled, double blind trial showed a mortality of 27.8% in the placebo group and 9.4 % in the SDD group in patients with severe burns (de la Cal et al., 2005). The use of SSD has been contentious however and Eiggiman et al., (2006) disputed the findings, believing the placebo group to be older and more severely injured and questioning the antibiotic regime of intravenous third-generation cephalosporin for initial prophylaxis due to its potential negative impact on the ecology of microorganisms. Since then there have been a number of studies in both the burn and critical care populations and more recently a systematic review of all the previous studies has concluded that

the protocol of SDD reduces mortality in critically ill patients, in particular when successful decontamination is obtained. However eighteen patients need to be treated with SDD to prevent one death (Silvestri et al., 2009).

3.1.3 Diagnosis and prediction of sepsis

A study by Poeze et al., (2004) found only a fifth of intensivists knew the ACCP/SCCM definition of sepsis and that the majority (83%) were concerned that sepsis was frequently missed. The Surviving Sepsis Campaign tackled this in 2008 and highlighted early recognition and treatment of sepsis (Dellinger et al 2008).

One method of detection of sepsis is an 'early warning system' as used on many medical and surgical wards where physiological parameters are routinely measured. The deviation of the parameters beyond pre-determined set levels should initiate further investigation or intervention. Whilst use of this system on admission has successfully predicted admission to critical care units and mortality in acute medical admissions (Groarke et al., 2008) they have not been used specifically in burns. Again because of the altered physiology of severe burns the scoring system would have to reflect the hypermetabolic state of these patients.

3.1.4 Treatment of sepsis

In terms of treatment, the sepsis that burns patients suffer from is no different from any other critical care population. There are international guidelines published as part of the Surviving Sepsis Campaign for managing severe sepsis and septic shock.

The use of prophylactic antibiotics in adults and children has already been discussed. However once a burn patient has developed sepsis the rapid delivery of the correct antibiotic targeted to the causative organism/s is paramount. This is obviously guided by the culture results or if these are not available broad spectrum antibiotics determined by local policy depending on the most likely commonly found organism. An important point to remember is that bacteria are not the causative agent in all cases of sepsis. Fungal samples are frequently isolated from burn patients (although usually found as a result of colonization

rather than infection) and can cause invasive fungal infections. The increasing threat of multi-drug resistant organisms can make treatment and eradication of the infective agent difficult or prolonged.

Attention has moved from treatment of the physiological consequences of sepsis to monitoring and altering the inflammatory response to sepsis.

3.1.5 Inflammatory markers in sepsis

Burn injuries even without the presence of sepsis or multiorgan failure result in the activation of inflammatory cytokines, interleukins and growth factors. Several studies have shown a variety of pro-inflammatory cytokines have a predictive role in outcomes in patients with severe sepsis (Oberholzer et al., 2001) whilst other studies have not confirmed this. Oberholzer et al., (2005) found that baseline total protein C, IL-8, IL-10, TNF-a, and procalcitonin concentrations, and the change in plasma cytokine concentrations from baseline over the initial 4 days were not useful in predicting outcome in patients with severe sepsis. However when used in conjunction with the APACHE score and MOF scores IL-6 was useful in predicting outcome in severe sepsis.

Despite the varying results, some pro-inflammatory cytokines can be used in outcome prediction. However this has not led to routine assays to monitor pro-inflammatory cytokines as part of standard clinical care. One reason is that the induction of several of these cytokines is a universal response to activation of the innate immune response, and elevated concentrations of some of these cytokines occurs in a variety of acute and chronic inflammatory diseases, including cancer and autoimmune diseases. Raised cytokine levels may not be sensitive or specific enough to monitor the presence of a systemic inflammatory response.

Instead of monitoring pro-inflammatory molecules the most frequently used biochemical markers to determine infection and inflammation clinically are the C-reactive protein (CRP), white cell count (WCC) and Erythrocyte Sedimentation Rate (ESR). One marker used less commonly is procalcitonin (PCT). Uzzan et al., (2006) showed in a meta-analysis that Procalcitonin represents a good biological diagnostic marker for sepsis, severe sepsis, or septic shock in critically ill patients. They concluded that Procalcitonin is superior to C-reactive protein in this respect and suggested that Procalcitonin should be

included in diagnostic guidelines for sepsis and in clinical practice in intensive care units. This work was further endorsed by Meynaar et al., (2011) who showed that PCT is more useful than LBP, CRP and IL-6 in differentiating sepsis from SIRS.

There have been limited studies in burns with conflicting results. Neely et al., (2004) studied PCT in 20 paediatric patients and found that PCT was inferior to CRP in the diagnosis of sepsis and lagged behind the clinical diagnosis by 0.8 days. In adult patients with burns, however, PCT has been found to be a useful diagnostic marker for sepsis (Lavrentieva et al., 2007), which has been confirmed in a meta-analysis by Mann et al., (2011). They concluded that PCT assay can be a helpful adjunct to clinical diagnosis of sepsis but is expensive and the ability to analyse it is not available in most hospitals limiting its usefulness. As yet limited availability of an inexpensive and rapid assay remains the obstacle to routine use of this test in mainstream burns care.

3.1.6. Inflammatory response in burns

With larger burns, the acute inflammatory response may not be contained to the burn site and the local surrounding area but may develop into the uncontrolled inflammatory response found in SIRS.

SIRS is associated with increased production of pro-inflammatory chemo- and cytokines, increased free radical production and activation of complement and coagulation cascades. The subsequent pathophysiological impact of this is demonstrated by increasing vascular permeability, leukocytosis, tachypnea, temperature, peripheral vascular resistance, increased leukocyte mobilization and recruitment (Shankar et al., 2007).

The innate immune response to inflammation and sepsis is a rapid response that is not pathogen specific (Oberholzer et al., 2001). After the discovery of inflammation associated cytokines many immunotherapies directed at neutralizing these bioactive compounds have been tried (Abraham 1999, Dubois and Vincent 2000). Using animal models many of these therapies have produced promising results, which unfortunately have not been reproduced in larger clinical trials (Abraham et al., 1998, Suntharalingam et al., 2006). One of the reasons for sepsis immunotherapies not to work is that they are monotherapies

directed against a single component of the inflammatory cascade. The complexity of the inflammatory immune cascade means that the pro- and anti-inflammatory molecules released changes with the duration and severity of the injury (Shankar et al., 2007). Therefore treating just one part of this cascade is an imprecise and blunt tool. Any treatment of this type needs to be modulated over time to reflect the changes going on.

Another factor that makes therapies targeting only one part of the immune cascade ineffective is multiple signalling mechanisms in inflammation and therefore the ability to evade pharmacological efforts to block certain key pathways such as IL-1 or TNF- α messaging. (Chipp et al., 2010)

Drug treatments may have to target the inflammatory process at a different level. A potential target is the vascular endothelium as there is increasing evidence that the endothelium plays a central and pathogenic role in sepsis. The endothelium is composed of a single layer of cells that lines the interior surface of all blood vessels. It is estimated to comprise around 10^{13} cells, representing a weight of 1.5 kg and covering 4,000–7,000 m² (Ait-Oufella et al., 2010) Endothelial cells are diverse in their roles and are involved in a number of physiological functions, These include prevention of coagulation, orchestration of the migration of blood cells into the tissues by expression of adhesion molecules, production of chemoattractant compounds, regulation of the microcirculation by dictating the tonus of the arterioles, regulation of blood pressure (via their effects on arterioles), and regulation of vasopermeability (Hack and Zeerleder 2001) They provide the interface between a liquid phase, blood plasma, and a solid structure, basal membrane, and as such are very responsive to their extracellular environment. As a result of chemical or physical stimuli they can rapidly modulate both their structure and function.

When exposed to certain agonists, such as lipopolysaccharide, cytokines, chemokines or growth factors, endothelial cells become activated. The activation state is manifested by enhanced permeability, increased leukocyte adhesion, a shift in the haemostatic balance towards a procoagulant phenotype, and altered regulation of vasomotor tone (Shapiro et al., 2010). This results in an increase of blood flow to the area of insult, local efflux of plasma proteins and leukocytes, and sequestering of the infection. The activated state may be considered

dysfunctional when instead of restoring it an overactive endothelium disturbs the homeostatic state.

The end-result of diffuse endothelial activation and dysfunction may be the loss of microvascular barrier integrity, leading to tissue edema, shock and multiple organ failure. Endothelial activation also leads to an increase in angiopoietin-2, which is known to destabilize barrier function and promote inflammation (Lee and Lilies 2011).

In sepsis, endothelial dysfunction typically involves some combination of increased leukocyte adhesion and transmigration, increased permeability, a shift in the hemostatic balance towards the pro-coagulant side and an alteration in vasomotor tone (Shapiro et al., 2010).

It can be seen therefore that in sepsis, endothelial activation and dysfunction are critical determinants of the host response and being able to measure the degree of endothelial dysfunction may be of benefit in early diagnosis or treatment of sepsis.

Shapiro et al., (2010) looked at a broad range of biomarkers of endothelial dysfunction in 221 patients and used the ACCP/SCCM guidelines to define sepsis. They measured circulating levels of soluble leukocyte adhesion molecules (soluble vascular cell adhesion molecule (VCAM)-1, soluble inter-cellular adhesion molecule (ICAM-1) and sE-selectin (the soluble form of the cell adhesion molecule E-selectin); procoagulant/antifibrinolytic mediators (plasminogen activator inhibitors (PAI)-1); and a marker of vascular endothelial growth factor (VEGF) signaling (sFlt-1). An association was found with severity of sepsis and biomarker levels for sFlt-1, PAI-1, sE-selectin, sICAM-1, and sVCAM-1. The level of a number of these markers also correlated with the SOFA score (multi-organ failure score) and APACHE score. One fault of the study that the authors' note is that the results do not test whether these changes were specific to sepsis, or whether endothelial cell activation occurs in critically ill patients with other insults such as trauma related inflammation. Whilst this study is very useful in identifying possible markers of endothelial dysfunction the molecules measured require expensive and time consuming tests.

An alternative measure of endothelial dysfunction that can be used cheaply and at the bedside may also provide sufficient information to prognosticate and

initiate the treatment of sepsis. One potential test that may fulfill this role is the albumin creatinine ratio.

Microalbuminuria has been found to be predictive of outcome or has shown an association between the urinary albumin and the severity of the inflammatory insult in a number of acute medical conditions such as ischaemia reperfusion injuries (Matsushita et al., 1996), meningitis in children (Roine 1993), acute pancreatitis (Shearman et al., 1989) and anaphylactic shock (Wood et al., 2000).

A recent study by Basu et al., (2010) suggests that ACR has a role in predicting sepsis with the median ACR on admission being significantly higher in the sepsis group. Of note the study population was a mixed medical and surgical population that did not include burn patients.

A study by Sarti et al., (2001) on a paediatric population noted a patient showed a persistent rise in ACR before the appearance of a septic complication. This useful finding of ACR rising prior to a septic episode has been endorsed by De Gaudio et al., (2000) who found that the postoperative patients that developed sepsis showed an increase in glomerular permeability as shown by the ACR.

There has been no study looking at ACR and the development of sepsis in the burn population.

3.1.7 Aim

Through a retrospective analysis of burn patient admissions to ICU to determine whether ACR can predict the development of sepsis and outcome in the burn ITU setting.

3.2 Methods

3.2.1 Clinical Setting

The Burns Service comprises twenty beds including four intensive care and four high dependency beds. There are twelve low dependency / rehabilitation beds. The unit is a tertiary referral centre and accepts patients from a population of over 6 million people. Patients are also taken from out of traditional catchment areas and occasionally internationally.

A multidisciplinary team including microbiologists, pharmacists, anaesthetists and burn surgeons review all major burns daily. The Parkland formula is used in the initial resuscitation to maintain a urine output of 0.5 ml/kg/h in adults and 1 ml/kg/h in children. Nutritional supplementation is via nasoduodenal enteral nutrition with high calorie, low fat formulas. As part of the burn treatment protocol, gastric ulcer prophylaxis is performed with H₂ antagonists. Low molecular weight heparin (40mgs/day) is prescribed daily. Early excision and grafting is routinely performed for deep burns that would take more than two weeks to heal without surgical intervention using allografting, auto-grafting or dermal substitutes depending on the individual characteristics of burn injuries.

Retrospective collection of data regarding demographic details, mode of injury, examination findings was from clinical records. All other results were obtained from the Burns ITU computer system MetaVision (*iMDsoft*, Massachusetts, USA).

All patients admitted to the burns unit between November 2003 and September 2009 that fulfilled the unit's admission criteria were included in the study.

3.2.2 Exclusions

Exclusions were those patients with non-thermal processes including desquamating skin disorders, meningococcal septicaemia, necrotising fasciitis and purpura fulminans; patients who stayed in the ITU for less than 24 hours were excluded; patients who were transferred from other hospitals more than 5 days post injury; patients with multiple injuries (where the burn was clinically judged not to be the main injury); patients for compassionate care only; patients with no or inadequately recorded ACR results.

Patients with a diagnosis of Type I or Type II diabetes mellitus were included.

Parameters recorded included patient characteristic data such as age, gender, co-morbidities, injury data including time of injury, mechanism and TBSA. ACR was routinely measured on admission and recorded daily.

Inhalation injuries were determined by appearance on bronchoscopy. Smoke inhalation injury is restricted to injury below the glottis caused by products of combustion. The diagnosis requires both of the following: History of exposure to products of combustion and bronchoscopy relevant signs below the glottis. The signs observed below the glottis that indicate smoke inhalation include carbonaceous material, oedema, or ulceration. Erythema or carbon staining in the absence of evidence of other signs of injury was not considered an indicator of smoke inhalation injury.

3.2.3 Analysis of urine

On admission a sample of urine was collected within the first six hours, subsequently a fresh sample of urine was collected daily via an in-dwelling urinary catheter at 06.00 daily for the duration of the admission.

ACR was measured using the DCA 2000 Analyser manufactured by Bayer (Parsons et al., 1999). It runs a quantitative assay for microalbumin and creatinine in the urine sample. Only 0.4mls of urine is required to perform the analysis. The principle of measurement of microalbumin is based on immunoturbidimetry, and that of creatinine is based on colourimetry. The albumin to creatinine ratio is calculated by the machine and expressed in mg mmol⁻¹ after 7 minutes (Yew and Pal 2006).

3.2.4 Statistics

The analysis performed was a bootstrap analysis and regression analysis.

Regression analyses have been performed using the computer program R (R development Core Team 2011). Both the bootstrap and regression analysis were performed by Dr Mike Parker.

Bootstrap analysis is a computer based method for assigning measures of accuracy to sample estimates. For this analysis it is assumed the observations form an independent and identically distributed population. The computer programme can implement bootstrapping by constructing a number of re-samples of the dataset and of equal size to the observed dataset, which is obtained by random sampling with replacement from the original dataset.

The re-sampling (in this case 9999 times) gives a histogram of bootstrap means i.e. it produces a simulated distribution of the statistic we are interested in. This gives an estimate of the distribution of the mean from which one can answer how much the mean varies.

Bootstrapping of the mean allows one to construct a confidence interval for the mean.

3.3 Results

Demographics

A total of 547 patients were eligible for the study from which 308 were excluded. The reasons for exclusion are summarised in Figure 3.1.

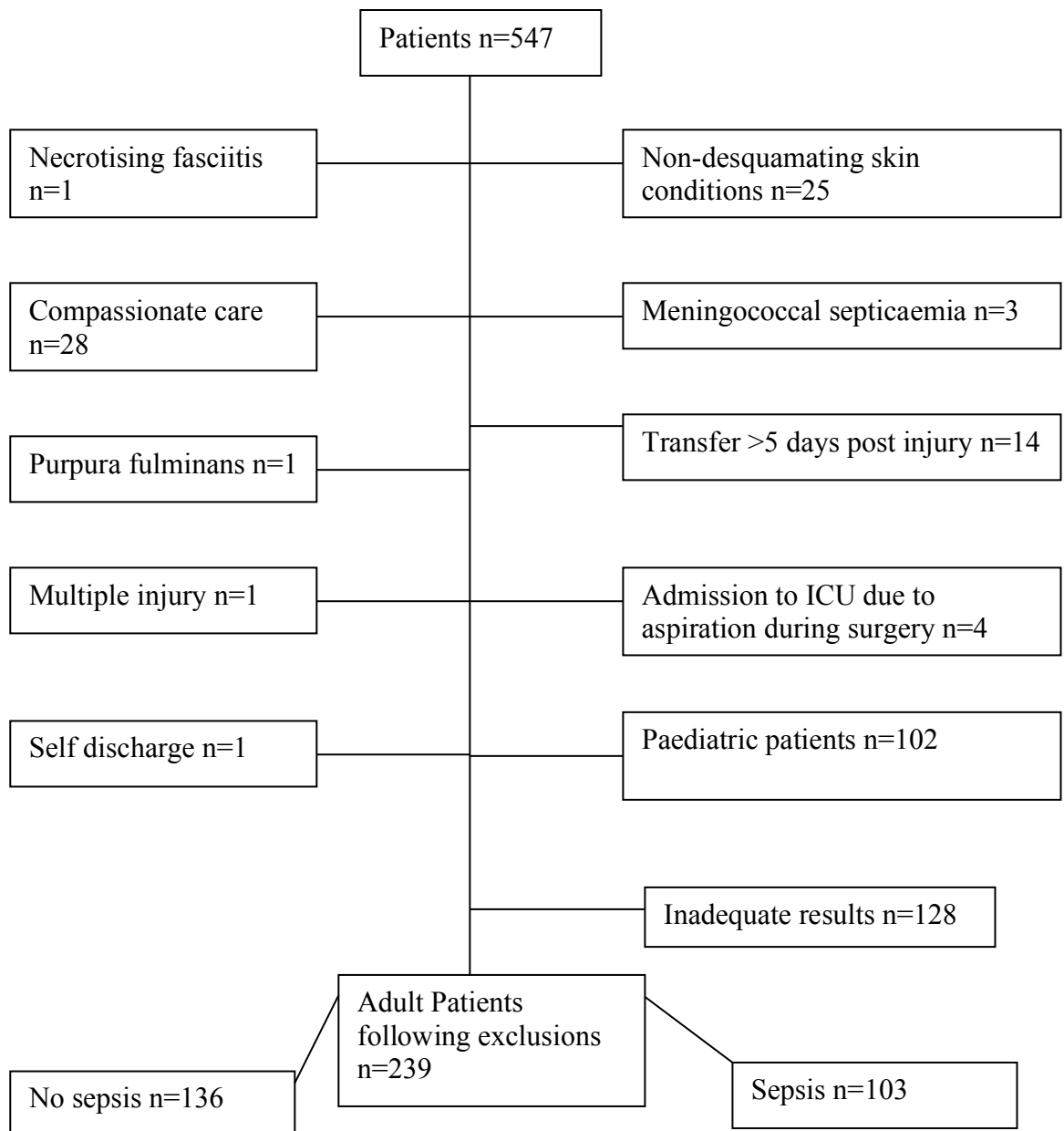


Figure 3.1. A flow chart showing the number of burns patients recruited and reasons for exclusions. 547 patients admitted to the burn ICU between November 2003-September 2009 were eligible for the study. After exclusions (308) 239 adult patients were studied of which 103 developed sepsis during their in-patient stay and 136 did not develop sepsis.

	No sepsis	Sepsis
No. Of patients	136	103
Age in years, mean. (Range)	42.4 (16-95)	44.97 (16-85)
No. Of patients with inhalation injury	20	49
No. Of deaths	6	32
Male: female	93: 43	63: 40
TBSA% (mean)	20.86	39.25
Length of ITU stay/%burn	0.46	1.15
Length of stay in days, mean	5.48	32.65
ACR admission (mean)	4.55	10.43
Peak ACR (mean)	7.58	33.19
ACR at 48 hours	3.74	4.31

Table 3.3. A summary of the septic and non-septic patients admitted to the burns ICU. This table summarises the main data examined in the study and allows a comparison of the two groups studied, septic vs. non septic.

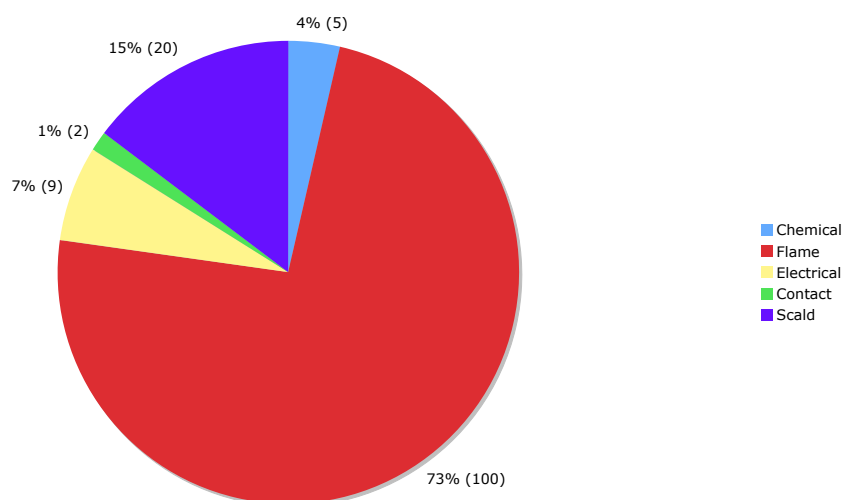


Figure 3.2 Mechanism of injury in non-septic adult patients. Data shows the mechanism of injury in the patients who did not develop sepsis as a percentage of the total number of non septic patients with the total number of patients in brackets.

Figure 3.2 shows the mechanism of injury in the non-septic patients. The mechanisms and proportions are typical of a tertiary referral unit in Western Europe accepting patients from a mainly urbanised population with limited large-scale industry nearby. The majority of burns are scalds and flame injuries (from household fires). In the adult population it can be seen that there are less scalds than when children are also included in the data (see figure 2.4 in which scalds make up 21.4% of the injuries).

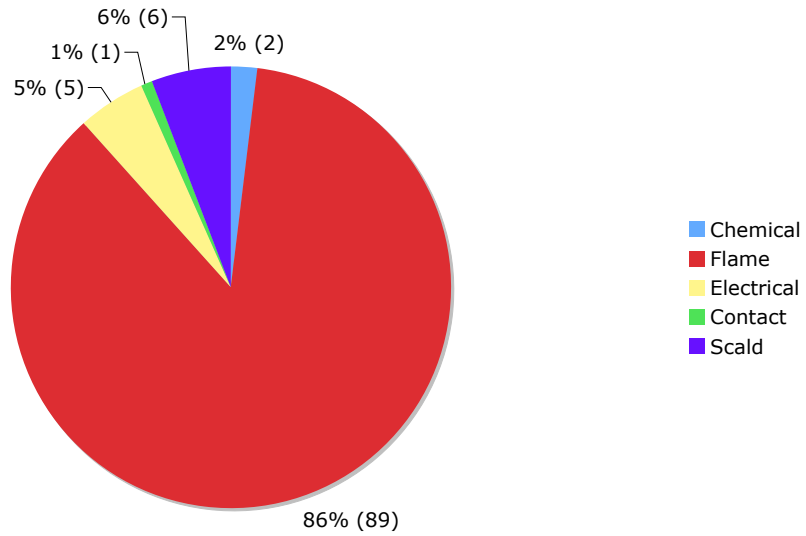


Figure 3.3 Mechanism of injury in adult patients who developed sepsis. Data shows the mechanism of injury in the patients who developed sepsis as a percentage of the total with the total number of patients in brackets.

Of note in figure 3.3 when compared to figure 3.2 there are even more flame related injuries. This is of importance, as there is evidence that flame burns result in a higher proportion of cases of multi-organ failure and has a higher mortality rate (Kraft et al 2011). It may also be the case that flame burns are more likely to cause sepsis.

Analysis

This analysis considers a comparison of patients who had episodes of sepsis with those that did not. The factor “Condition” is used to categorise patients as having had episodes of sepsis and those that had no sepsis.

Analyses have been performed using the computer program R (R development Core Team 2011).

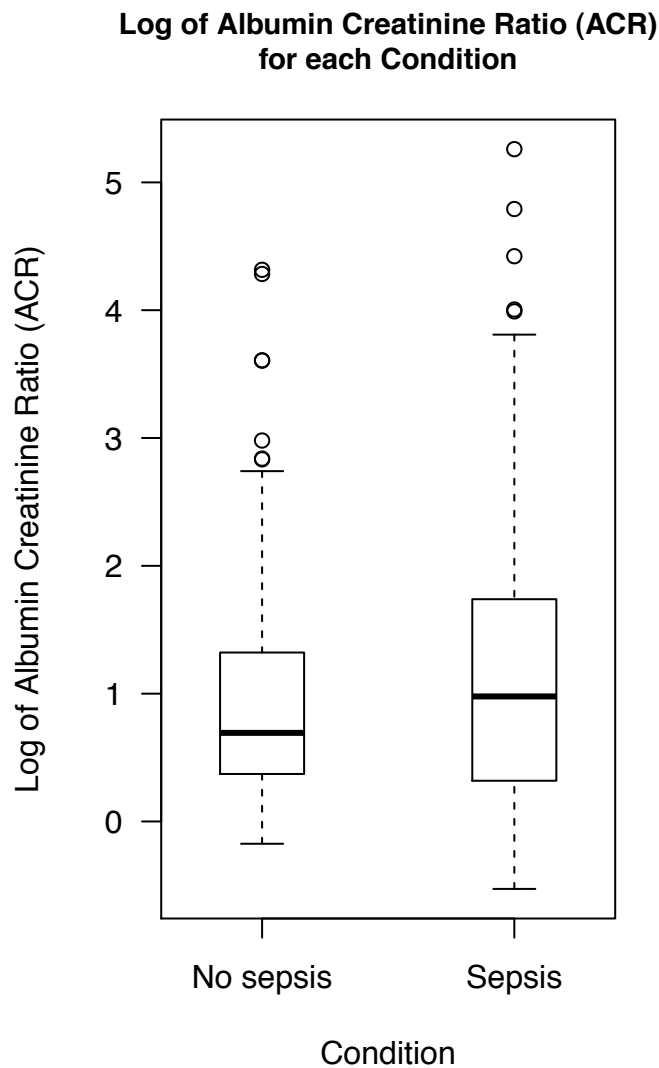


Figure 3.4. A boxplot graph of the log ACR at admission for the septic and non-septic patients. The length of the box covers the interquartile range, the lower edge of the box being the lower quartile, and the upper edge of the box being the upper quartile. The line across the box represents the median. The dotted lines represent the range and the circles as outlying results. The difference between the means of log ACR for the two groups is 0.298 with bootstrap 95% confidence limits (0.018, 0.604). This is statistically significant. (Data used to derive this graph is shown in Appendix II, Section I, Table I). N.B. The typical way to show a box plot or whisker plot graph is with the length of the box showing the interquartile range with a horizontal line representing the median. For statistical analysis the mean is used and referred to in the text (Dr Mike Parker).

Figure 3.4 illustrates that there is a statistically significant difference in the admission mean ACR values between the septic and non-septic patients. To see how useful this may clinically a ROC curve analysis was performed.

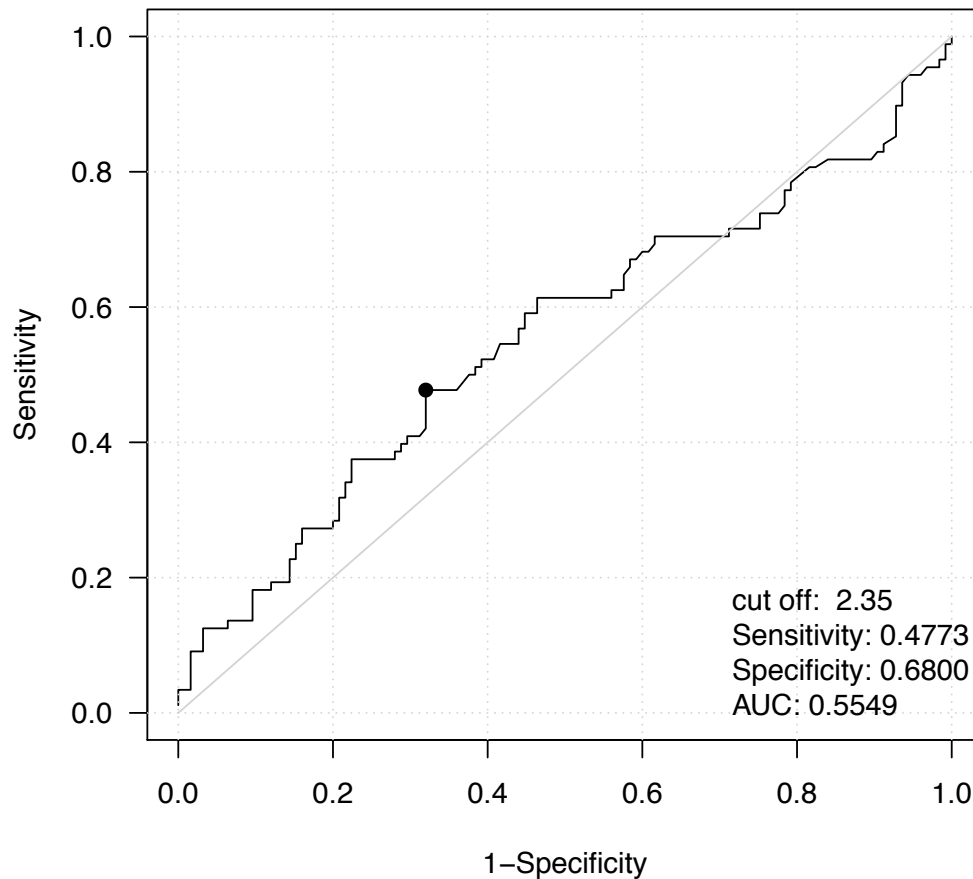


Figure 3.5 ROC curve for condition (sepsis/no sepsis) versus urinary ACR taken on admission to the burn unit.

Data shows a ROC curve, a graphical plot of sensitivity against 1-specificity, (true positive against false positive) for condition (of having sepsis) against admission ACR. The cut off ACR value of 2.35 mg mmol-1 is estimated by maximizing the Youden Index. Using a cut-off ACR of 2.35 mg mmol-1 sepsis can be predicted with a sensitivity of 47.7% and a specificity of 68%.

		Predicted outcome		Total	Percentage correctly classified
		No sepsis	Sepsis		
Observed outcome	No sepsis	85	40	125	68.0
	Sepsis	46	42	88	47.7
Total		131	82	213	59.6

Table 3.4 Diagnostic classification table for using a cut-off value for ACR at admission. The data used to derive the contingency table from which the sensitivity, specificity, PPV and NPV were determined. Diagnostic performance values obtained from the above table and their 95% confidence intervals are: sensitivity 47.7% (CI: 37.6%, 58.0%), specificity 68.0% (CI: 59.4%, 75.5%), PPV 51.2% (CI: 40.6%, 61.7%), and NPV 64.9% (CI: 56.4%, 72.5%). The area under the curve with bootstrap 95% confidence interval is 0.5549 (CI: 0.4747, 0.6352). The AUC show this is a poor test for determining sepsis.

Figure 3.5 gives the ROC curve for ‘condition’ (of having sepsis) against admission ACR. By maximizing the Youden index a cut off ACR value of equal or greater than 2.35 mg mmol-1 can determine whether a patient develops sepsis. The AUC is .55 however suggesting this is a poor test for determining sepsis. This is backed up by the data in table 3.4 that allows the PPV and NPV to be established. This test has a poor PPV and NPV, which mean that clinically it is unlikely to be of any use.

There are many factors that may affect the development of sepsis. It was decided therefore to examine some of the characteristics of the patients and injuries to determine their potential influence on sepsis development.

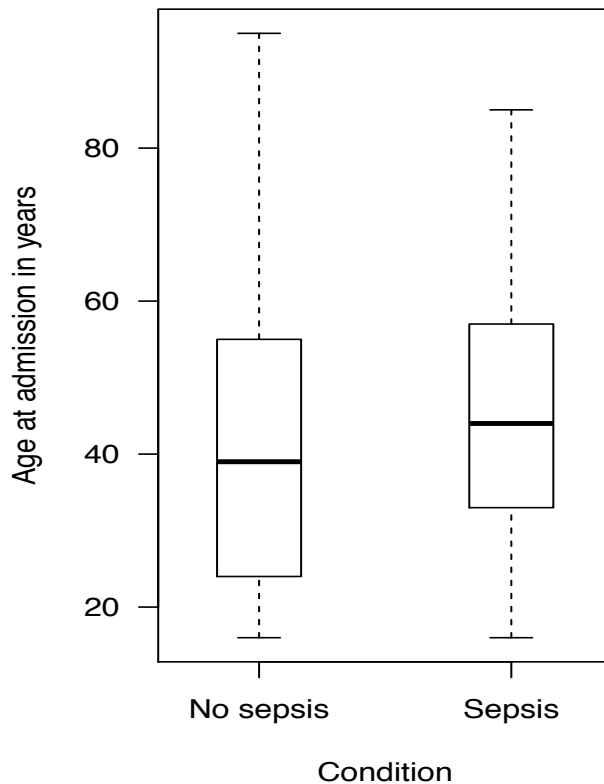


Figure 3.6 A boxplot graph of the age in years for the septic and non-septic patients. The length of the box covers the interquartile range, the lower edge of the box being the lower quartile, and the upper edge of the box being the upper quartile. The line across the box represents the median values (no sepsis 39 years, sepsis 44 years with mean values being 42.4 and 45 respectively). The dotted lines represent the range of values. The difference between the means for the two groups is 2.6 with 95% confidence limits 43.5 (41.12, 45.88). This is statistically significant.

The first aspect to be examined was age. See appendix 2 table 2 for summary statistics used for comparing the age of septic and non-septic patients. Figure 3.6 below shows there is a statistically significant difference in the age of the patients in the two groups. One explanation for this is that the response to injury is attenuated in the older patients making them less able to mount a response to the challenge posed by a burn and thus more likely to develop sepsis. Another reason could be that the older patients sustained worse burns making sepsis more likely. The TBSA% burnt and the development of sepsis was therefore examined to see if there was any correlation.

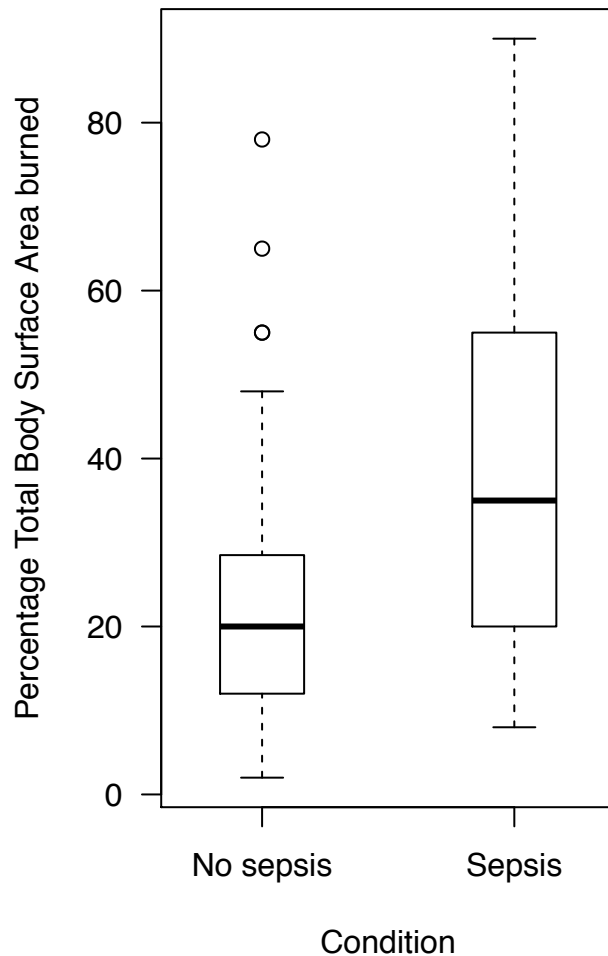


Figure 3.7. A boxplot graph demonstrating the percentage TBSA burned for the septic and non-septic patients. The length of the box covers the interquartile range, the lower edge of the box being the lower quartile, and the upper edge of the box being the upper quartile. The line across the box represents the median. The dotted lines represent the range of values. The difference between the means of % TBSA for the two groups is 18.4 with bootstrap 95% confidence limits (13.8, 23.0). This is statistically significant.

Figure 3.7 illustrates that there is a significant difference between the means of % TBSA for the two groups is 18.4 with bootstrap 95% confidence limits (13.8, 23.0). The patients who subsequently developed sepsis had significantly larger burns. This is exactly what one would expect. In general a larger wound surface area tends to mean a more significant injury (although this may not always be the case as the depth of the wound is also important, not just the surface area). Larger injuries as well as causing a larger systemic response also allows greater

opportunity for infection as there is a greater exposed area of skin that is compromised. Also the patients generally require more operations and more interventions (such as line changes for monitoring), which are again an opportunity for infection. Other factors examined were sex of the patient, the presence of inhalation injury.

Sex	Condition		Overall	Percentage with sepsis
	No sepsis	Sepsis		
Male	93	63	156	40.4
Female	43	40	83	48.2
Overall	136	103	239	43.1

Table 3.5. Effect of sex of patient on development of sepsis (Condition) Data demonstrates the number and sex of the patients and whether they developed sepsis or not. The difference between the percentages is 7.8% with 95% confidence limits (- 5.4%, 20.8%). This is not statistically significant.

Inhalation	Condition		Overall	Percentage with sepsis
	No sepsis	Sepsis		
No	116	54	170	31.8
Yes	20	49	69	71.0
Overall	136	103	239	43.1

Table 3.6. Effect of inhalation injury on the development of sepsis.

Data demonstrates the influence an inhalation injury has on the subsequent development of sepsis. Inhalation appears to make a large difference in the likelihood of developing sepsis. The difference between the percentages is 39.2% with 95% confidence limits (25.5%, 50.7%). This is statistically significant.

Many studies in burn patients have found that there are variations in outcome according to sex of the patient (Brusselaers et al., 2010). In our study we found shows that sex made no difference in the development of sepsis (table 3.5). Far more important in developing sepsis was the presence of an inhalation injury as shown in table 3.6. The next consideration was the effect sepsis had on outcome (death or survival).

Outcome	Condition		Overall	Percentage with sepsis
	No sepsis	Sepsis		
Alive	130	71	201	35.3
Died	6	32	38	84.2
Overall	136	103	239	43.1

Table 3.7. Effect of sepsis on outcome. Data shows the consequences of developing sepsis. The difference between the percentages of those that died versus those that survived is 48.9% with 95% confidence limits (32.7%, 59.3%).

Table 3.7 illustrates the profound influence sepsis has on outcome with almost half of the patients who developed sepsis dying. Of all the deaths in the study 84% were sepsis related. In total the number of patients in the whole study group dying from sepsis is 32 (13.4%) which is comparable to European data presented by Brusselaers et al., 2010).

Flame burns have been associated with a higher mortality rate (Kraft et al., 2011). This may be explained by the fact that flame burns have also been associated with more-extensive, deeper burns and the presence of inhalation injury. So it may also be possible that the mechanism of the burn may play a role in determining the development of sepsis.

Mechanism	Condition		Overall	Percentage with sepsis
	No sepsis	Sepsis		
Chemical	5	2	7	28.6
Contact	2	1	3	33.3
Electrical	9	5	14	35.7
Flame	100	89	189	47.1
Scald	20	6	26	23.1
Overall	136	103	239	43.1

Table 3.8. Effect of Mechanism of injury in developing sepsis. Data demonstrates how many people developed sepsis for each mechanism of burn.

Table 3.8 demonstrates the influence of the mechanism of the injury on the development of sepsis. The first three categories in Table 3.8 contain small numbers and would mean that if the variable were included in a regression model some regression coefficient estimates would be unreliable. To avoid this, these three categories (contact, chemical and electrical) have been combined have been combined in a derived variable- Other (demonstrated in table 3.9). This decision was made after consultation with a medical statistician (Dr Mike Parker, Anglia Ruskin University)

Mechanism	Condition		Overall	Percentage with sepsis
	No sepsis	Sepsis		
Flame	100	89	189	47.1
Scald	20	6	26	23.1
Other	16	8	24	33.3
Overall	136	103	239	43.1

Table 3.9. Effect of Mechanism of injury in developing sepsis (revised). Data demonstrates how many people developed sepsis for each mechanism of burn with contact, flame and chemical burns being combined into a single group (labelled ‘Other’) to aid statistical analysis.

Table 3.9 shows that flame burns not only are the most common mechanism of injury but also are the most likely to cause sepsis. These is not surprising, as flame burns result in a higher proportion of cases of multi-organ failure; and also have a higher mortality rate (Kraft et al., 2011).

For the data presented above a far stronger indicator of the development of sepsis rather than ACR was the TBSA% sustained by the patients. We therefore investigated whether there was a size of injury or cut-off point (as determined by TBSA %) that would make the identification of sepsis patients clearer.

Prediction of sepsis

This section examines the use of a simple cut-off value for diagnostic prediction of sepsis. For the set up considered below the receiver operating characteristic (ROC) curve shows the optimum cut-off value according to the maximum Youden Index. The analysis has been performed using the function ROC from the R package DiagnosisMed (Brasil P 2010). The bootstrap confidence limits have been obtained using 9999 bootstrap replicates with the function roc from the R package pROC (Robin et al., 2011).

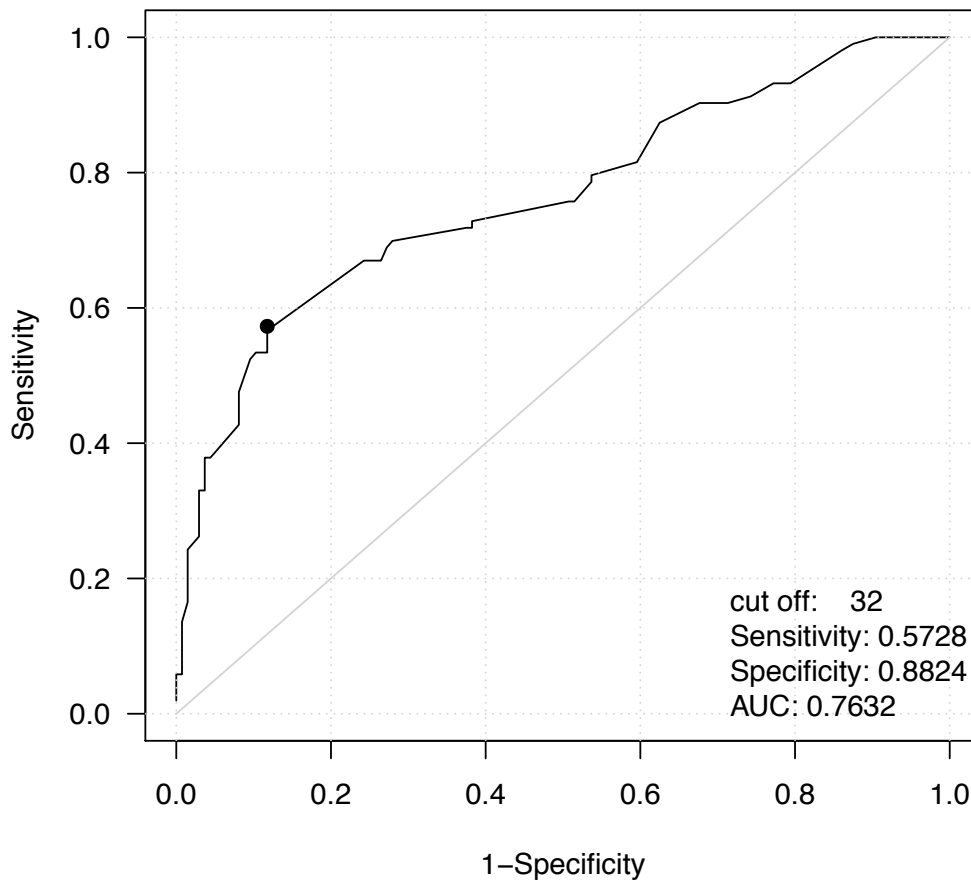


Figure 3.8 ROC curve for condition (sepsis/no sepsis) versus TBSA%. The cut off was estimated by maximizing the Youden Index. Data shows a graphical plot of sensitivity against 1-specificity, (true positive against false positive) the ROC curve for outcome (sepsis) against TBSA% of burn sustained. Using a cut-off TBSA % of 32, sepsis can be predicted with a sensitivity of 57.2% and a specificity of 88%. The analysis has been performed using the function ROC from the R package DiagnosisMed (Brasil P 2010). The AUC show this is a good test for determining sepsis.

		Predicted outcome		Total	Percentage correctly classified
		No sepsis	Sepsis		
Observed outcome	No sepsis	120	16	136	88.2
	Sepsis	44	59	103	57.3
Total		164	75	239	74.9

Table 3.10 Diagnostic classification table for using a cut-off value for % TBSA. The data used to derive the contingency table from which the sensitivity, specificity, PPV and NPV were determined. Diagnostic performance values obtained from the above table and their 95% confidence intervals are: sensitivity 57.3% (CI: 47.6%, 66.4%), specificity 88.2% (CI: 81.7%, 92.6%), PPV 78.7% (CI: 68.1%, 86.4%), and NPV 73.2% (CI: 65.9%, 86.4%). The area under the curve with bootstrap 95% confidence interval is 0.7632 (CI: 0.6998, 0.8225). This show the test to be good at determining sepsis when the % TBSA is greater than or equal to 32%.

Figure 3.8 demonstrates that a cut-off TBSA% of 32 (by cut-off we mean a TBSA% of 32 or greater) can be used to predict sepsis. The AUC is .76, which shows that it is a good test for determining sepsis. The data in summary table 3.10 show that the sensitivity, specificity, PPV and NPV are all superior to when the ACR on admission was used to determine sepsis (see figure 3.5 and table 3.4). Whilst this is a better test for determining the development of sepsis it may be argued that a cut off TBSA% of 32 is quite high and thus the clinical utility may be limited as many patients have smaller burns which can still be life threatening. The data presented in this chapter demonstrates that there are many factors that strongly influence the development of sepsis (particularly, TBSA%, inhalation injury and to a lesser extent age of the patient). To see if ACR has a role in sepsis prediction further analysis by logistical regression was performed.

Binary logistical regression to determine whether ACR can predict sepsis

In order to determine if ACR has a role as a predictive tool for sepsis binary logistic regression models can be used. Regression analysis measures the strength of a relationship between a dependent variable (in this case sepsis) and one or more independent variables (such as TBSA% and inhalation injury). Then knowledge of the values of the independent variables enables prediction of the value of the dependent variable or likelihood of the occurrence of an event (sepsis).

Binary (also known as binomial) logistic regression is used when the observed outcome can have only two possible types (e.g., "dead" vs. "alive", "success" vs. "failure", or in this case "sepsis" vs. "no sepsis") compared to multinomial logistic regression where the outcome can have three or more possible outcomes (such as better, worse and no change). The outcome is coded as "0" and "1" in binary logistic regression with the target group usually coded as "1" and the reference group as "0".

Regression is often to make predictions, and using standard linear regression might make predictions that make no sense for dichotomous dependent variables. For instance if you code sepsis as 1 and no sepsis as 0, then linear regression might predict 0.5. This makes no sense, as a patient cannot partially have sepsis. To get around this instead of trying to model the dichotomy itself (sepsis/no sepsis) we transform it into the ODDS of getting sepsis. The odds are defined as the probability of a case (sepsis) divided by the probability of a non-case (no sepsis).

In addition by using the odds ratio (OR) it tells us how the odds change when an independent variable changes. For instance how the odds change as the TBSA% increases.

The odds ratio is the primary measure of effect size in logistic regression and can be worked out to compare the odds that membership in one group will lead to a case outcome with the odds that membership in some other group will lead to a case outcome. An odds ratio of one indicates that the odds of a case outcome are equally likely for both groups under comparison. The further the odds deviate from one, the stronger the relationship.

Binary logistic regression was used to assess what variables (including ACR) a patient's Condition (sepsis or no sepsis) is related to. Therefore, it allows the

The first model is shown in table 3.11 and uses a single predictor for sepsis. The second model uses two predictor variables for modelling, table 3.12 (Dr Mike Parker).

The tables provide the following goodness of fit statistics for each model.

1. p The number of parameters fitted. The more parameters fitted the more complex the model is, and is therefore the more likely that it will not to be generalisable to other groups of patients.
2. R2 In statistical modelling using ordinary multiple regression with a continuous response variable the R-square indicates the proportion of the variance explained by the model. There is no goodness of fitness statistic that is exactly equivalent for binary logistic models, but the Nagelkerke R-square is sometimes regarded as analogous. It is often called a pseudo-R-square. Higher values are sought.
3. AIC Akaike Information Criterion. This penalises models for every additional parameter that they estimate. Lower values are sought.
4. CC Percentage of subjects correctly classified. This uses the fitted model to classify each subject. As the same data set is used to both fit the model and classify the subjects it can be expected to be over optimistic. Higher values are sought.
5. CCC A cross-validated estimate of the percentage of subjects correctly classified. Each subject is itself excluded from the model used to classify it. The intention is that it should not be so over-optimistic. Higher values are sought.
6. AUC The area under the receiver operating characteristic curve. Higher values are sought.
7. Brier CCC only corrects the over optimism in certain circumstances. The Brier score does not have this problem. The Briar score measures the accuracy of probabilistic predictions. Lower values are sought.
8. BrierS The Brier score does not have a constant scale, and so the Scaled Brier score remedies this and it is expressed as a percentage. Higher values are sought.

Model	Predictors	p	R2	AIC	CC	CCC	AUC	Brier	BrierS
1.1	TBSA%	1	29.8	240	75.1	74.6	0.7665	0.1852	23.6
1.2	Inhalation	1	15.9	266	69.5	69.5	0.6626	0.2120	12.6
1.3	Mechanism3	2	5.2	287	58.7	58.7	0.5768	0.2337	3.6
1.4	Log ACR	1	2.5	289	59.2	58.7	0.5549	0.2378	1.9
1.5	Sex	1	1.0	291	58.7	58.7	0.5429	0.2406	0.8
1.6	Age	1	0.5	292	58.7	56.8	0.5595	0.2416	0.4

Table 3.11. Summary statistics for the single-predictor models (6 possible models). The table of fitted models is sorted according to descending BrierS. The model has been fitted using the standard function glm in the computer program R. The data confirms that TBSA% is important for predicting sepsis.

Table 3.11 confirms the importance of TBSA% in predicting sepsis. The AUC (.76) confirms that it is a good test and the CC confirms this as it show the percentage of subjects correctly classified as 75.1%.

Table 3.11 also shows that inhalation has a role in predicting sepsis. As it is clear more than one variable can predict sepsis it may be that using a combination of the predictor variables may provide an even more accurate way of predicting sepsis. Table 3.12 show the summary statistics for the model to determine sepsis using a combination of two predictors. Table 3.12 show that the combination of TBSA% and inhalation injury are more useful in predicting sepsis than either of the variables, TBSA% or inhalation injury in by themselves. The AUC, (0.81) shows that it is a good test.

Model	Predictors	p	R2	AIC	CC	CCC	AUC	Brier	BrierS
2.1	TBSA% +Inhalation	2	37.8	225	75.6	74.6	0.8168	0.1683	30.6
2.2	TBSA% +Mechanism3	3	32.8	237	75.6	75.6	0.7897	0.1787	26.3
2.3	TBSA% +Age	2	31.6	238	75.6	74.6	0.7877	0.1806	25.5
2.4	TBSA% +log ACR	2	30.5	240	74.6	73.7	0.7764	0.1827	24.7
2.5	TBSA% +Sex	2	31.1	239	73.7	72.8	0.7707	0.1828	24.6
2.6	Inhalation +log ACR	2	17.7	265	70.0	69.0	0.7069	0.2086	14.0
2.7	Mechanism3+Inhalation	3	17.8	267	70.0	69.5	0.6899	0.2087	13.9
2.8	Sex +Inhalation	2	16.8	266	69.5	69.5	0.6958	0.2103	13.3
2.9	Age +Inhalation	2	16.4	267	69.5	69.5	0.7018	0.2111	12.9
2.10	Mechanism3+log_ACR	3	7.5	285	62.9	62.0	0.6240	0.2289	5.6

Table 3.12 Summary statistics for two-predictor variables model. Data is shown summarising the statistics for the model using two variables for the prediction of sepsis. The table of fitted models is sorted according to descending BrierS. The model has been fitted using the standard function glm in the computer program R. TBSA% in combination with sepsis is the best predictor for developing sepsis.

Further analysis of selected binary regression models is given below to estimate the probability of sepsis. As explained previously the odds ratio (OR) is given as it tells us how the odds change when an independent variable changes. For instance how the odds change as the TBSA% increases. An odds ratio greater than 1 indicates that the odds in favour of sepsis are increased by that factor. An odds ratio less than 1 indicates that the odds in favour of sepsis are reduced by the factor.

The factor Inhalation is a categorical factor with two levels Yes and No (inhalation injury and no inhalation injury). The model has been set up so that the reference category is “No”. This means that the odds of 5.16 indicates that “Yes” increases the odds in comparison with “No” by more than five times. In other words an inhalation injury makes sepsis five times more likely. For continuous predictors there is no reference category so the odds ratio indicates

the change in the odds for each change of one unit in the predictor. For example, for TBSA% (OR of 1.065) the odds ratio in favour of sepsis increases by 6.5% for each additional one percent TBSA. That is, the greater the percentage area burned increases the probability of sepsis.

Predictor	Odds ratio	95% confidence limits	
		Lower	Upper
TBSA%	1.065	1.046	1.088
Inhalation (reference category No)	5.16	2.85	9.63
Mechanism3 (reference category Flame)			
Scald	0.356	0.130	0.858
Other	0.578	0.231	1.357
Log ACR	1.21	0.98	1.51
Sex (reference category Female)	0.729	0.427	1.244
Age	1.007	0.994	1.021

Table 3.13. The probability of sepsis using single predictor models. Data shows the odds ratios for predicting sepsis using a single variable. The model has been re-fitted using the package `logistf` (Ploner et al., 2010) for the computer program R. This uses Firth’s biased-reduced logistic regression approach that aims to reduce the statistical bias in the model fitting. The 95% confidence limits for the odds ratios indicate the range of plausible values for the odds ratio therefore only TBSA and an inhalation injury are useful in predicting sepsis.

When looking at a range of variables (Age, TBSA%, Sex, Inhalation, Mechanism, log ACR on admission) in isolation only the TBSA% sustained and whether the patient had sustained an inhalation injury were of use in predicting sepsis, see table 3.13. When the confidence limits enclose the value 1 this indicates that we should not draw a firm conclusion about the effect of the factor. This is the case for log ACR, Sex, and Age.

As noted before combining the two variables (TBSA and inhalation) showed even better ability to predict sepsis so a regression model was created to predict sepsis based on the two-predictor model.

Predictors	Odds ratio	95% confidence limits	
		Lower	Upper
TBSA% Inhalation (reference category: No inhalation)	1.065 4.47	1.043 2.27	1.089 9.03

Table 3.14. Two-predictor model with TBSA% and Inhalation demonstrating the odds ratio and 95% confidence intervals. Data shows the odds ratios for predicting sepsis using two variables. The model has been re-fitted using the package `logistf` (Ploner et al., 2010) for the computer program R. This uses Firth’s biased-reduced logistic regression approach that aims to reduce the statistical bias in the model fitting.

Table 3.14 summarises the odds ratio for both TBSA% and inhalation injury when using both variables in a single model to predict sepsis. The factor Inhalation is a categorical factor with two levels (“Yes” and “No” which signify inhalation injury and no inhalation injury). The model has been set up so that the reference category is “No”. This means that the odds of 4.47 indicates that “Yes” increases the odds in comparison with “No” by more than four times. This means the probability of sepsis is four times higher when an inhalation is present (“Yes”). For continuous predictors there is no reference category so the odds ratio indicates the change in the odds for each change of one unit in the predictor. For example for TBSA% the odds ratio of 1.065 indicates that the odds ratio in favour of sepsis increases by 6.5% for each additional one percent TBSA. In essence, that means the greater percentage area burned increases the probability of sepsis.

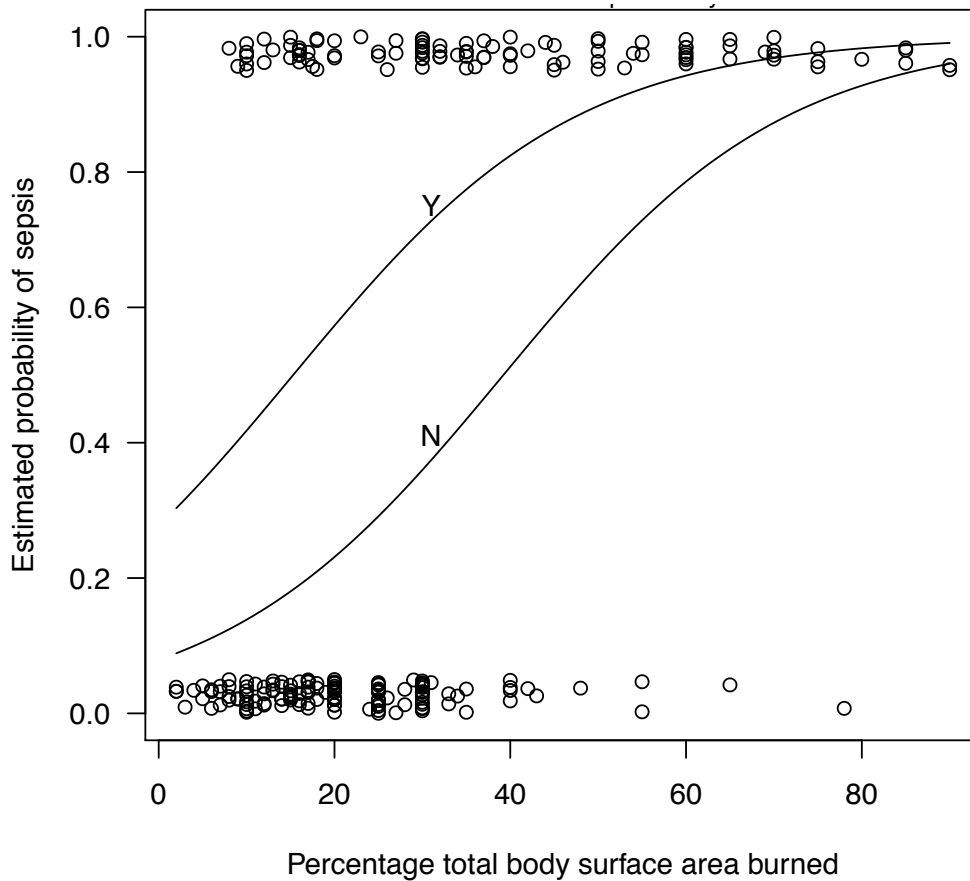


Figure 3.9 Binary logistic model of TBSA% and inhalation. The data shows the influence of the two variables (TBSA% and inhalation injury) on having sepsis. The fitted lines show the estimated probability of the condition (Y line = inhalation, N line =no inhalation). The observed successes are indicated with points at the probability of 1 and the failures are indicated at the probability of 0.

A binary regression logistic model of TBSA% and inhalation was produced as shown in figure 3.9 to clearly demonstrate the effect of the two variables on sepsis development. The points have been jittered to enable them to be seen more easily when there are a lot of them clustered together. It is clear that for values of TBSA% a little above 30% or more there is a greatly increased chance of a patient having sepsis as the probability increases from just over a 30% chance of having sepsis to a 70% chance of developing sepsis.

The estimated probability from a fitted binary logistic model can be used to produce a diagnostic indicator, as shown in the ROC curve below.

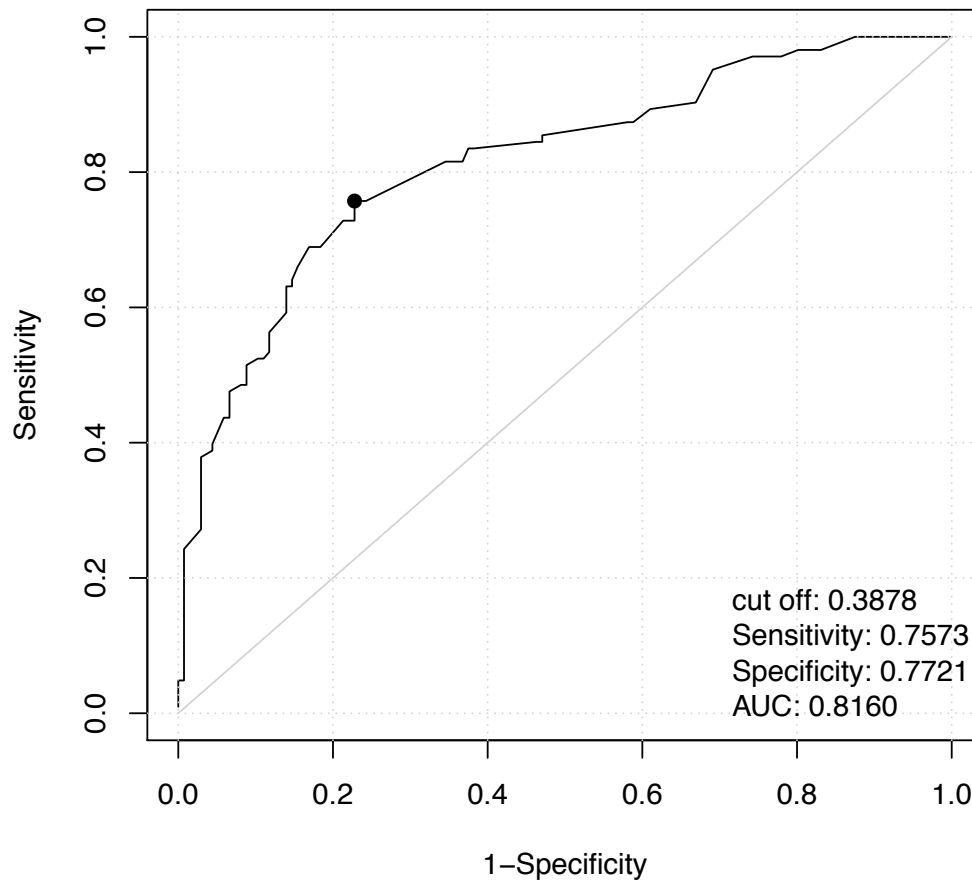


Figure 3.10 ROC curve for the logistic model: TBSA% and inhalation. Using a cut-off of .38 the sensitivity is 75% and the specificity 77%. Data shows a graphical plot of sensitivity against 1-specificity, (true positive against false positive) the ROC curve for the logistic model of TBSA% and inhalation. The cut off value of 0.387 is estimated by maximizing the Youden Index. The sensitivity for this test is 75 % and the specificity 77%. The AUC is 0.81, which shows this to be an excellent test for determining sepsis with a cut off value of 0.387. Analysis performed by computer program R (R Development Core Team, 2011).

The ROC curve above in figure 3.10 displays an optimum cut off value of 0.387. This represents an estimated probability of sepsis, and the displayed values of sensitivity and specificity have been derived using this cut off. When you have a single variable such as TBSA% then a cut-off value on the TBSA% scale can be used to predict an outcome e.g. sepsis. This means a patient with a value higher than that cut-off predicts sepsis and a lower value predicts freedom from sepsis. When you have more than one variable they need to be used in some way to

produce a prediction. One way is to use them as predictor variables in a binary logistic multiple regression. The fitted model estimates the probability of an outcome (sepsis) and the cut off estimated probability can be used to predict sepsis. Normally one uses a cut-off estimated probability of 0.5, so that a patient with a higher probability is predicted to have sepsis, and a patient with a lower probability is predicted to be sepsis-free. The computer program used to produce the ROC curve automatically displays an optimum estimated probability to use as a cut-off. This is 0.3878. That means a patient for whom the binary logistic model estimated a probability of sepsis greater than 0.3878 is put in the prediction class of having sepsis. In this case the 0.387 is a number that works within the model but does not directly translate to a clinically derived figure such as TBSA% for example. This represents an estimated probability of sepsis, and the displayed values of sensitivity and specificity have been derived using this cut off. However, it is more usual to use the standard cut off probability of 0.5, so that patients are classified simply according to whether they are more likely to be in one category than another. For the model being considered here a cut off probability of 0.5 produces the following classification table (Dr Mike Paker).

		Predicted outcome		Total	Percentage correctly classified
		No sepsis	Sepsis		
Observed outcome	No sepsis	117	19	136	86.0
	Sepsis	42	61	103	59.2
Total		159	80	239	74.5

Table 3.15. Diagnostic classification table using a cut-off probability of 0.5 for the two-predictor binary logistic model for sepsis. Data shows that the model predicts sepsis when the estimated probability is greater than or equal to 0.5. Diagnostic performance values obtained from the above table and their 95% confidence intervals are: sensitivity 59.2% (CI: 49.6%, 68.2%), specificity 86.0% (CI: 79.2%, 90.9%), PPV 76.3% (CI: 65.9%, 84.2%), and NPV 73.6% (CI: 66.2%, 79.8%). The area under the curve with bootstrap 95% confidence interval is 0.816 (CI: 0.759, 0.869).

Table 3.15 shows the cut-off being 0.5, the probability that they will fall into the sepsis category or the non-sepsis category. Whilst the area under the curve shows this to be an excellent test, by not choosing the optimum value (established in figure 3.10) the test loses both sensitivity and specificity.

In summary the admission ACR is not of use in predicting the patients who will develop sepsis. More useful factors in predicting sepsis are the TBSA% and the presence or not, of an inhalation injury.

3.4 Discussion

This study of adults admitted to a UK burns ICU and requiring resuscitation shows that admission ACR cannot be used to identify patients that will go on to develop sepsis.

Table 3.3 compares the two study groups, those that developed sepsis and those that did not. The most recent review of severe burns injuries in Europe presented figures of between 2 % and 14% for the number of burn patients dying from sepsis (Brusselaers et al., 2010). The rate of sepsis in the study population is 43% and the death rate in those suffering from sepsis is 13.3%. Although this figure falls within the range published by Brusselaers the actual figure for the unit is lower as this is a study of adults only. The paediatric population suffer more frequently from scalds and have a lower rate of sepsis. In addition although the patients suffered from sepsis without access to the post mortem results it cannot always be determined that sepsis was definitely the cause of death. Many burn patients suffer from multiple co-morbidities and some will have died from other causes such as myocardial infarcts for instance.

The impact that sepsis can have on a unit is clear by comparing the mean length of stay for the two groups. The septic patients stay over five and a half times longer than the non-septic patients (5.54 days vs. 32.65 days) resulting in a large difference in the amount of medical intervention required and costs incurred.

The mean ACR on admission (Non-septic, 4.49 mg mmol⁻¹ vs. septic 10.43 mg mmol⁻¹) shows a pronounced difference and the difference in the log ACR (figure 3.4) is statistically significant. A study by Basu et al., (2010) suggests that ACR has a role in predicting sepsis. Looking at a mixed population of medical and surgical patients (that did not include burn patients) they found the median ACR on admission being significantly higher in the sepsis group. The admission ACR showed a sensitivity of 80% and specificity of 64.1% however the cut off ACR value to determine this was much higher (14 mg mmol⁻¹) than in our study (2.35 mg mmol⁻¹), which had a sensitivity of 47.7% and specificity of 68.0%. Using such a high ACR cut off would undoubtedly improve the sensitivity and specificity in our study but at the expense of its use in the clinical scenario.

Although there was a significant difference between the means one cannot necessarily interpret this to infer that log ACR predicts sepsis.

Many factors may affect the development of sepsis so a binary logistical regression was performed to assess which variables sepsis may be related to. Both TBSA% and inhalation injury were found to be the strongest factors whilst admission ACR was not.

In conclusion, although ACR may have a role in demonstrating sepsis currently in burns care it appears to be limited. It may be that because of the systemic impact that burns injuries are more complex than many other medical illnesses and looking at ACR in isolation is inadequate to reflect the inflammatory changes going on. Perhaps sampling ACR in combination with another marker of inflammation such as CRP or procalcitonin would provide more accurate results.

Chapter 4 Multi-organ failure and ACR in burns

4.1 Introduction

Despite decreases in mortality rate over the last four decades, burns trauma still results in a high morbidity and mortality (Brusselaers et al., 2010). Although some burns are obviously non-survivable and the patients die early after the injury a significant number of burns patients have prolonged in-patient stays interrupted by setbacks and complications.

Infection and organ dysfunction is frequent and may ultimately lead to death; particularly in patients with larger burns but these factors also have an impact on the length of stay in hospital and the cost of care.

Multi organ failure and sepsis are the most frequently reported causes of death with multi-organ failure responsible for 25-65% of all burn deaths (in Europe) and sepsis for 2%-14% of all burns deaths (again in Europe) (Brusselaers et al., 2010).

4.1.2 SIRS and Multi-organ dysfunction syndrome (MODS)

Definition of SIRS and MODS

Inflammation is an essential part of normal healing. In a small burn this may manifest itself as a mild rise in cytokines with no observable clinical signs. The inflammatory response that occurs after a large burn can manifest itself systemically with microcirculatory failure of capillaries supplying individual organs, acute respiratory syndrome, severe coagulopathy, and the eventual development of multi-organ failure (Shankar et al., 2007).

After any large injury a number of biological cascades are activated in a temporal fashion. There is initially an acute phase of a hyper-reactive immune response followed by a hyporeactive phase.

The hyperactive phase is called the 'Systemic Inflammatory Response Syndrome,' or SIRS and the hyporeactive phase is called 'Counter Anti-inflammatory Response Syndrome' or CARS. The current definition of SIRS was introduced in the early 1990s at a consensus conference of critical care and trauma (Bone et al., 1992). It was designed to describe the widespread

inflammation (or clinical response to that inflammation) that can occur in patients with a range of disorders such as pancreatitis, ischaemia, trauma, hemorrhagic shock and immunologically mediated organ injury. Although sepsis can occur with SIRS the term sepsis was is to be used only if the patient has documented infection.

4.1.3 Criteria for SIRS

SIRS is considered to be present when a patients has two or more of the following problems:

1. Temperature above 38°C or below 36°C.
2. Heart rate >90 beats per minute (bpm)
3. Respiratory rate >20/min or maintenance of PaCO₂ <32mm Hg.
4. WBC count >12,000/mm³ or <4000/mm³.

The extent of organ dysfunction in SIRS patients can vary depending on several factors. Firstly the magnitude and duration of the initial injury and also host response factors which are partially determined by genetic variations in the magnitude of the inflammation (Bone et al., 1992). Thus the degree of organ dysfunction varies between patients and within the same patient over time. The term ‘Multiple Organ Dysfunction Syndrome’ (MODS) was therefore created to reflect the wide range of severity and changing nature of SIRS. MODS can develop via two pathways. First is a direct insult to the organ that shows an inflammatory response that is at least, initially confined to the affected organ. The second pathway is by infection or trauma in one part of the system resulting in systemic inflammatory responses and dysfunctions of organs elsewhere (Bone et al., 1992).

4.1.4 Appropriateness of SIRS definition in burns

Some studies have looked at SIRS on admission and found it to be predictive of mortality and ICU admission in trauma patients (Malone et al., 2001). In addition the admission SIRS score has been used to predict infection and outcome in

blunt trauma (Bochicchio et al., 2001). However there has been no study looking at SIRS on admission or during in-patient stay in burns patients.

After a major thermal injury the first barrier to microbial invasion is compromised and the patient is constantly exposed to the outside environment. In response to this, inflammatory mediators that change the baseline metabolic profile are continuously released. This results in a re-setting of the baseline temperature to around 38.5°C with co-existing tachycardia and tachypnea that can last for months in severe burns patients.

As can be seen from the criteria for SIRS (referred to overleaf) a lot of burns patients will fulfil these criteria and yet be within the normal boundaries expected after an extensive burn.

As most moderate sized burns will fulfil the criteria for SIRS (albeit fleetingly) perhaps the length of time that SIRS is manifested may be of more value? This has been found to be the case with trauma patients. SIRS for 3 days or more was found to be a strong determinant of developing MODS (Gando et al., 1999). Although not using the SIRS definition, a study by Sheridan et al., (1998) showed that there had been a prolonged period of organ failure in burns patients that eventually died. As well as characterizing the sequence of organ failure in those that died they also noted that those dying of multiple organ failure had most of their burn wounds closed. Also they were usually not suffering from uncontrolled infection at the time of their death, although they had suffered multiple infections prior to their death, each of which has been controlled. As noted above even after the acute inflammatory phase of the injury has settled the hypermetabolic phase can persist for weeks (even in the absence of infection) and no study has shown that in burns there is a period (either pre or post resuscitation) where the SIRS criteria becomes clinically valuable (Greenhalgh et al., 2007).

Two parameters that have been shown to be related directly to mortality in burns are, the size of the burn (in percentage of total body surface area affected) and the presence of an inhalation injury (Miller et al., 2006). However there are no studies that look at SIRS and the relationship to burn size. In addition the SIRS criteria do not reflect the impact that an inhalation injury has on outcome. It has therefore been recommended that routine use of SIRS in burns should not be used at least until further studies have been performed (Greenhalgh et al 2007).

4.1.5 Illness severity scores and measuring organ dysfunction.

The SIRS criteria have formed the basis of other scoring systems that are used in the general intensive care or critical care units. Many scoring systems have been designed but some of the most commonly used are the Acute Physiology and Chronic Health Evaluation scores (APACHE I-III) (Knaus et al., 1981, Knaus et al., 1985, Knaus et al., 1991) and Simplified Acute Physiology Score (SAPS) (LeGall et al., 1984). These allow measurements to be made that can then be used in predicting probability of survival and therefore outcome and mortality. The Apache III score developed in 1991 scores patients for acute severity of illness by weighting physiological derangement using 27 physiological variables, which are scored 0 to 4 to reflect degree of derangement. The calculation is proprietary, and hospitals must 'enroll' with the APACHE system to access the decision support instrument (the programme used to calculate outcomes from the variables examined). The physiologic points are assigned for the worst value recorded in the first 24 hrs in the ICU.

The SAPS score was developed to simplify the diagnostic data collection in the APACHE system, focusing on only physiologic measures easily and typically measured in ICU patients. The score is calculated from 12 routine physiological measurements during the first 24 hours and includes information about previous health status. It is designed to measure the severity of disease for patients admitted to the Intensive Care Unit and therefore is only calculated once during the admission.

Burns patients were excluded from the original design of the APACHE scoring systems (II and III), however a few studies have found the APACHE III score to be able to predict mortality in burns patients (Moore et al., 2010, Tanaka et al., 2007). Looking at the studies in more depth their results have to be interpreted with some caution.

Firstly both looked retrospectively at patients admitted to their unit over a seven-year and eleven year period. Over this time there may have been changes in practice due to technical developments or changes in technique or management. This would obviously mean different patients might have received different

standards or levels of care. This seems likely, as over the study period there was a decline in observed mortality accompanied by a parallel reduction in predicted risk of death (Moore et al., 2010), which suggests either an improvement in care or another factor such as a change in referral patterns.

By applying the APACHE criteria all patients under the age of sixteen were excluded and therefore this data cannot be extrapolated to the paediatric population without further studies. Both studies applied slightly different inclusion criteria with Tanaka et al., (2007) excluding those patients who were on the unit for less than 24 hours and Moore et al., (2010) excluding all patients who were on the unit for less than 4 hours.

Most importantly is the role of inhalation injuries and their assessment. Tanaka et al., recorded 31.4% of their study population receiving inhalation injuries. They excluded 5 of the patients with inhalation injuries who died, as their APACHE scores worsened rapidly over time although were not excessively high when admitted. No diagnostic criteria for inhalation injury were established in the study by Tanaka et al., (2007). In addition there was no data with which to quantify the severity of inhalation injury. They felt that the effect of inhalation injury might be under-estimated physiologically by the APACHE score.

In contrast Moore et al., state 86% of their patients had airway involvement. This is a higher figure than most other studies. Brusselaers et al., (2010) in their systematic review report inhalation rates between 0.3% - 43% of all patients hospitalized due to severe burns. Other studies have linked inhalation injury to mortality (Brusselaers et al., 2009) but Moore et al., (2010) found this was not the case in their population. Firstly their population may not be representative of other burns populations. Alternatively their interpretation of the diagnosis of airway injury may be different to other units resulting in an over diagnosis of airway injury and thus a subsequent underestimation of the physiological effects of inhalation injuries.

There have been several criticisms of the APACHE and SAPS scoring systems with the SAPS system never having been validated in a burns population. APACHE is complex to compute and the proprietary nature of APACHE III and its associated costs means that it is not used in all Intensive Care Units. Both systems suffer from the same fault of not being able to provide a score on admission as the scoring criteria include the worst measure of the physiological

parameters in the first 24 hours. By using the worst score in 24 hours there is also a question of the sensitivity of the score to precision of measuring the variables; one abnormal result throughout the day may not reflect the underlying trend therefore those ICUs using continuous electronic monitoring typically evaluate patients as being more severely ill.

There are many other systems that aim to evaluate organ dysfunction. Some look at the presence or absence of organ failure whilst others grade organ failure by the severity of the degree of organ dysfunction (in general the greater the organ dysfunction the higher the assigned score). There is variability between the scoring systems as some evaluate different organs systems. Many predicting formulas are limited because they only give specific levels of mortality risk, instead of a continuous range. Furthermore the weighting given to degrees of dysfunction in a particular organ varies between scoring systems. Once more like the APACHE score, some systems are calculated from the worst score obtained in a 24-hour period or during the admission whilst others use the scores obtained at certain times of day or on admission.

As both the SIRS criteria and the conventional scoring systems used in ICUs have significant failings the American Burn Association offered an alternative suggestion that the Marshall MODS Scoring System (Marshall et al., 1995) (as modified by Cook et al., 2001) be used to monitor and assess organ dysfunction in burns patients.

A modified version of the Marshall MODS score has been used in burns patients in two separate studies (Cumming et al., 2001, Fitzwater et al., 2003). The first of these by Cumming et al., (2001) looked at 104 adult patients with burns greater than 20%. Those patients for compassionate care and with inhalation injuries but burns less than 20% were excluded. They concluded that severe MOF was related to burn size, age and male sex in addition to intensive care unit length of stay and duration of mechanical ventilation. Several factors need to be noted when looking at their results. Only 17.6 % of the patients suffered inhalation injuries, a figure much lower than the studies mentioned above (Tanaka et al., 2007 and Moore et al., 2010). Secondly their definition of severe MOF (which they defined as a MOF score ≥ 6) was an arbitrary figure and they

admit that by considering the severe MOF patients as a group may not identify the factors that contribute to the most severe expression of organ dysfunction or failure.

In their modification they omit the central nervous system (CNS) category from their assessment because of its subjectivity. They reason that is difficult to score the Glasgow Coma Scale (GCS) precisely because of the frequent concurrent use of neuromuscular blocking agents, sedatives and blocking agents. It is therefore unclear if CNS dysfunction is the result of CNS-active medications or the effect of systemic inflammation or hemodynamic changes in organ function.

One important factor that is taken into account by the modification by Cumming et al is that measurements taken in the first 48 hours after admission were excluded. This period during burns resuscitation displays many acute but rapidly reversible changes in organ function and therefore may give a falsely worse MOF score and prognosis than is actually the case.

4.2 Aim: By a retrospective analysis of patients admitted to the Burn ICU between November 2003-September 2009 to determine whether ACR could predict the development of multi-organ failure and outcome in the burn ITU setting.

4.3 Methods

4.3.1 Clinical Setting

The Burns Service comprises twenty beds including four intensive care and four high dependency beds. There are twelve low dependency / rehabilitation beds. The unit is a tertiary referral centre and accepts patients from a population of over 6 million people. Patients are also taken from out of traditional catchment areas and occasionally internationally.

A multidisciplinary team including microbiologists, pharmacists, anaesthetists and burn surgeons reviews all major burns daily. The Parkland formula is used in the initial resuscitation to maintain a urine output of 0.5 ml/kg/h in adults and 1 ml/kg/h in children. Nutritional supplementation is via nasoduodenal enteral nutrition with high calorie, low fat formulas. As part of the burn treatment protocol gastric ulcer prophylaxis is performed with H2 antagonists. Low molecular weight heparin (40mgs/day) is prescribed daily. Early excision and grafting is routine performed for deep burns that would take more than two weeks to heal without surgical intervention using allografting, auto-grafting or dermal substitutes depending on the individual characteristics of burn injuries.

Retrospective collection of data regarding demographic details, mode of injury, examination findings was from clinical records. All other results were obtained from the Burns ITU computer system MetaVision (*iMDsoft*, Massachusetts, USA).

All adult patients admitted to the burns unit between November 2003 and September 2009 that fulfilled the unit's admission criteria were included in the study.

4.3.2 Exclusions

Exclusions were those patients with non-thermal processes including desquamating skin disorders, meningococcal septicaemia, necrotising fasciitis and purpura fulminans; patients who stayed in the ITU for less than 24 hours were excluded; patients who were transferred from other hospitals more than 5 days post injury; patients with multiple injuries (where the burn was clinically judged not to be the main injury); patients for compassionate care only; patients with no or inadequately recorded ACR results.

Patients with a diagnosis of Type I or Type II diabetes mellitus were included.

Parameters recorded included patient characteristic data such as age, gender, co-morbidities, injury data including time of injury, mechanism and TBSA. ACR was routinely measured on admission and recorded daily.

Inhalation injuries were determined by appearance on bronchoscopy.

Multi-organ failure was determined by using the Marshall score as modified by Cook et al., (2003) (see below table 4.1).

Organ system	0	1	2	3	4
Cardiovascular (heart rate, inotropes, lactate)	≤120	120-140	>140	Inotropes	Lactate >5
Respiratory, P _{O₂} /F _I O ₂	>300	226-300	151-225	76-150	≤75
Renal (creatinine, umol/L)	≤100	101-200	201-350	351-500	>500
Central nervous system (Glasgow Coma Score)	15	13-14	10-12	7-9	≤6
Hepatic (total bilirubin, umol/L)	≤20	21-60	61-120	121-240	>240
Hematologic (platelet count ×10 ³)	>120	81-120	51-80	21-50	≤20

Six domains of the MODS. The original MODS cardiovascular component was defined by the heart rate × right atrial pressure/mean arterial pressure. The modified cardiovascular component of MODS is defined as follows: 0, heart rate <120 beats per minute (bpm); 1, heart rate 120-140 bpm; 2, heart rate >140 bpm; 4, need for inotropes more than dopamine >3 ug/kg/min; 5, serum lactate >5 mmol/L.

Table 4.1. The Modified multiple organ dysfunction (MODS) score (Cook et al., 2003).

All blood samples (to obtain the creatinine, bilirubin and platelet counts) were taken at 06.00 in the morning (the same time the urinary analysis of ACR was undertaken) and GCS, cardiovascular and respiratory system status were recorded at the same time.

4.3 Results

Demographics

A total of 547 patients were admitted to the unit within the study period for the study from which 304 were excluded. The reasons for exclusion are summarised in Figure 4.1.

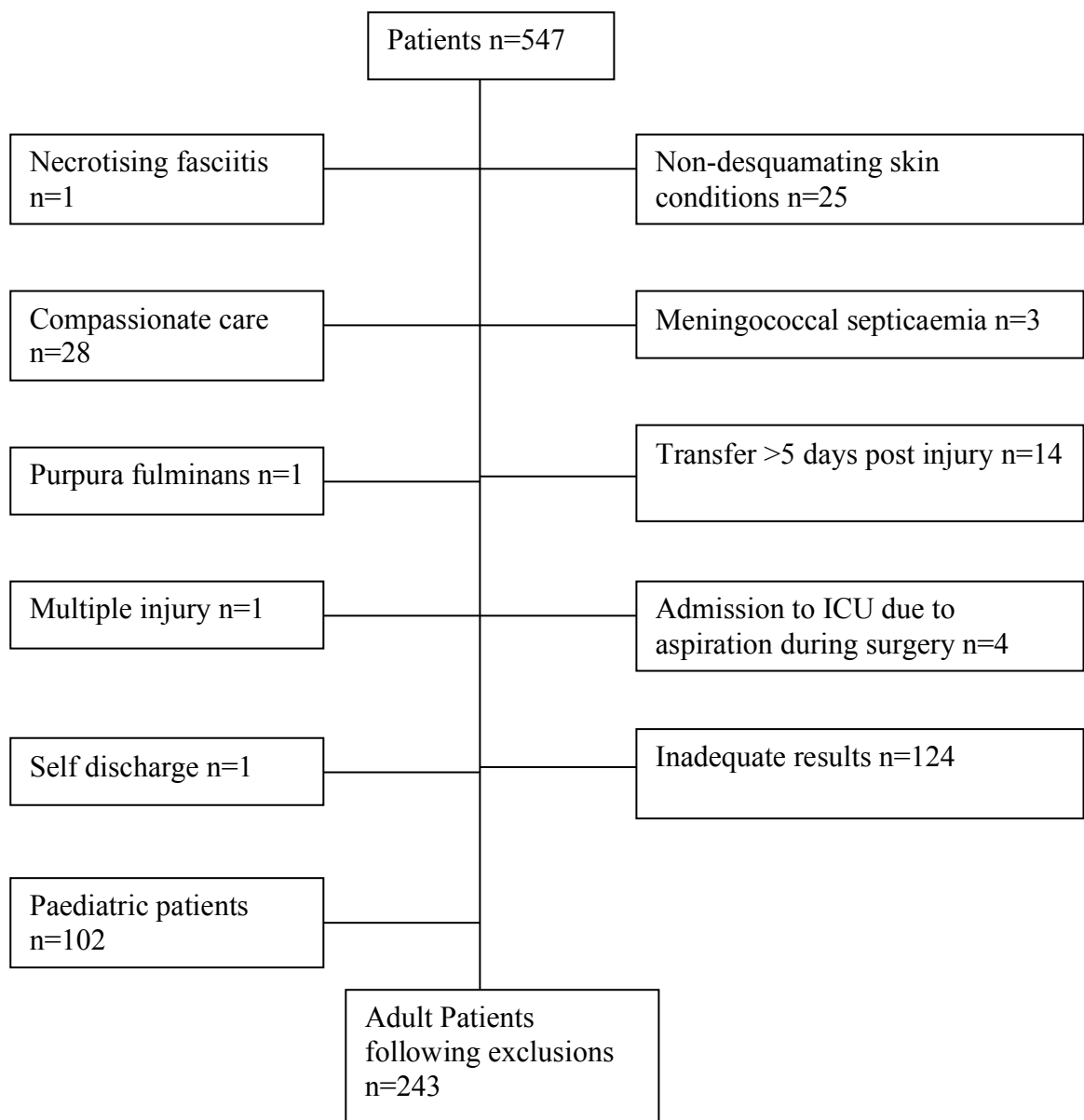


Figure 4.1. A flow chart showing the number of burns patients recruited and reasons for exclusions. 547 patients admitted to the burn ICU between November 2003-September 2009 were eligible for the study. Following exclusions 243 adult patients were eligible.

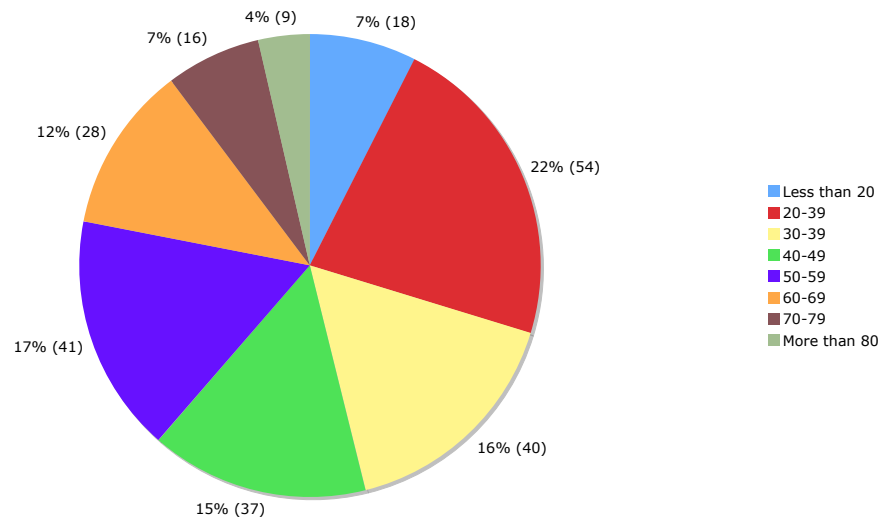


Figure 4.2. Demonstrates the number of patients in each age range admitted to the burns ITU. The data demonstrates the age in years, of the patients admitted to the burn unit in the study period, November 2003-September 2009. The data shows the percentage of the total each group contributes with the total number in the group in brackets. Each age range is a decade except for the patients aged 80 years or greater and those aged less than 20, which form separate groups.

Out of the 243 patients studied the mean age (range) 43.8 years (16-95 years old). Figure 4.2 demonstrates that the majority were aged between 20 and 59. This is a typical spread of ages to a UK burn unit when servicing a mainly urban population.

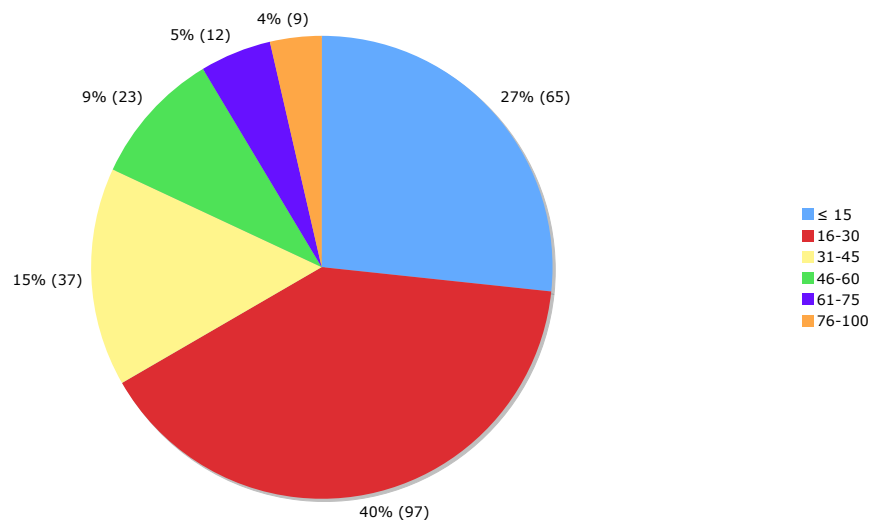


Figure 4.3 Demonstrates the percentage of patients within each range of TBSA% burned. The data shows the percentage and number (in brackets) of patients within each range of TBSA% burned admitted to the burn unit in the study period, November 2003-September 2009. Each group is a range of 15% except for the group representing burns of 75-100%.

Roughly three quarters of the patients had burns 45% TBSA or lower. This figure is in keeping with expectation given the admission criteria for burns ITU. Not only are higher TBSA% burns less frequent but also burns that affect a large TBSA are more likely to die at the scene. It should be noted that a number of burns with high TBSA% involvement were excluded from the study because they were for compassionate care only (n=28, see figure 4.1). The mean TBSA% sustained (range) was 29.5% (2-90%). The number of patients burned in each range of TBSA% is shown in figure 4.3.

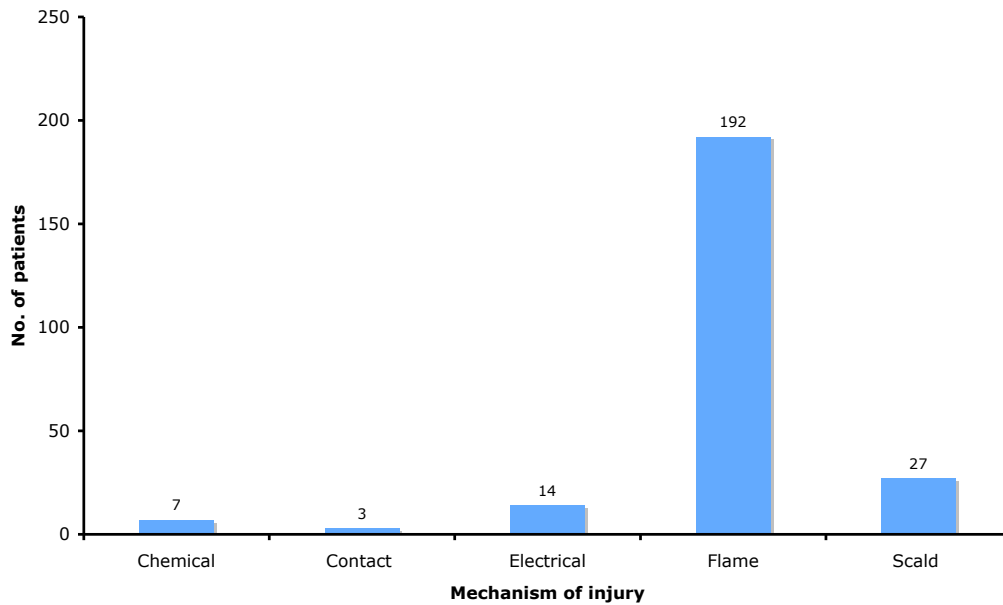


Figure 4.4. The mechanism of injury resulting in the patients being admitted to the burns ITU. The data shows the cause of the injury leading to admission for the adult population.

Within the 243 patients 74 patients' sustained inhalation injuries (30.4%) and 40 patients died (16.5%). There were 159 male patients and 84 female patients.

The mean length of stay was 17.3% (1-175 days) with the mean stay per percentage burn being 0.76-days/%burn. In keeping with a standard UK burn unit the majority of adult admissions were due to flame burns. Table 4.4 shows that 192 (79%) patients suffered a flame burn as their primary reason for admission. The next highest category was scald injuries accounting for 27 (11%) of patients in the study.

On admission 217 patients had a urinary sample taken and ACR recorded on admission (defined as being taken within 6 hours of coming to the unit). Twenty-six patients did not have an ACR recorded within 6 hours of admission (10.6%). The mean ACR on admission (range) was 7.3 mg mmol⁻¹ (0.3-120 mg mmol⁻¹). Post resuscitation (defined as 48 hours post admission) the mean ACR was 4.1 (0.34-77.1 mg mmol⁻¹) and the mean peak ACR (range) was 18.85 mg mmol⁻¹ (0.47-231).

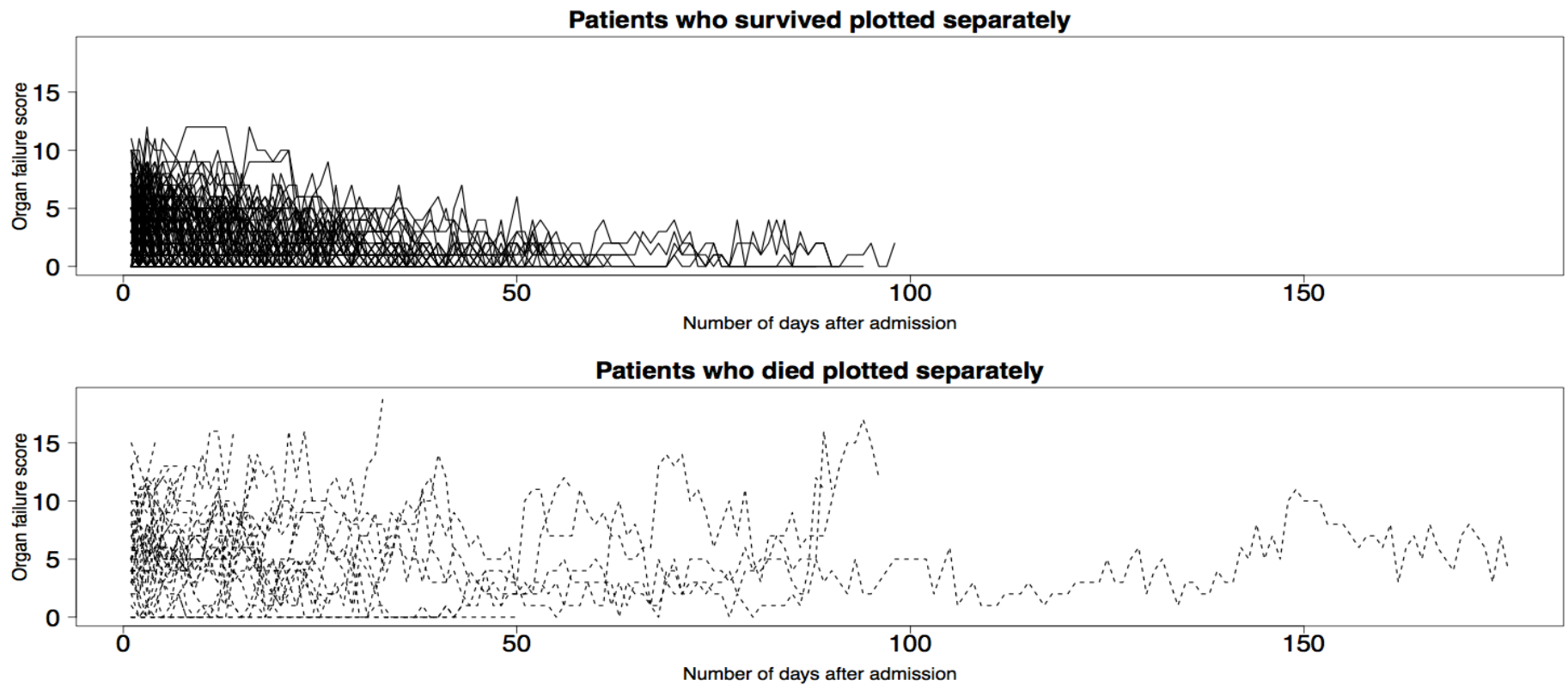


Figure 4.5. Organ failure score on each day of admission for all patients. The two graphs above show the organ failure score on each day of admission for the patients that survived (upper graph) and the patients that died (lower graph)

Figure 4.5 was performed to see if there were any obvious trends in MOF score over time comparing those patients that had died and survived. It demonstrates that the patients that died tended to have higher MOF scores and it can also be seen that some had protracted in-patient stays often with highly variable MOF scores. Therefore to determine the relationship the ACR was plotted against MOF for all patients (survivors and non-survivors) on all days.

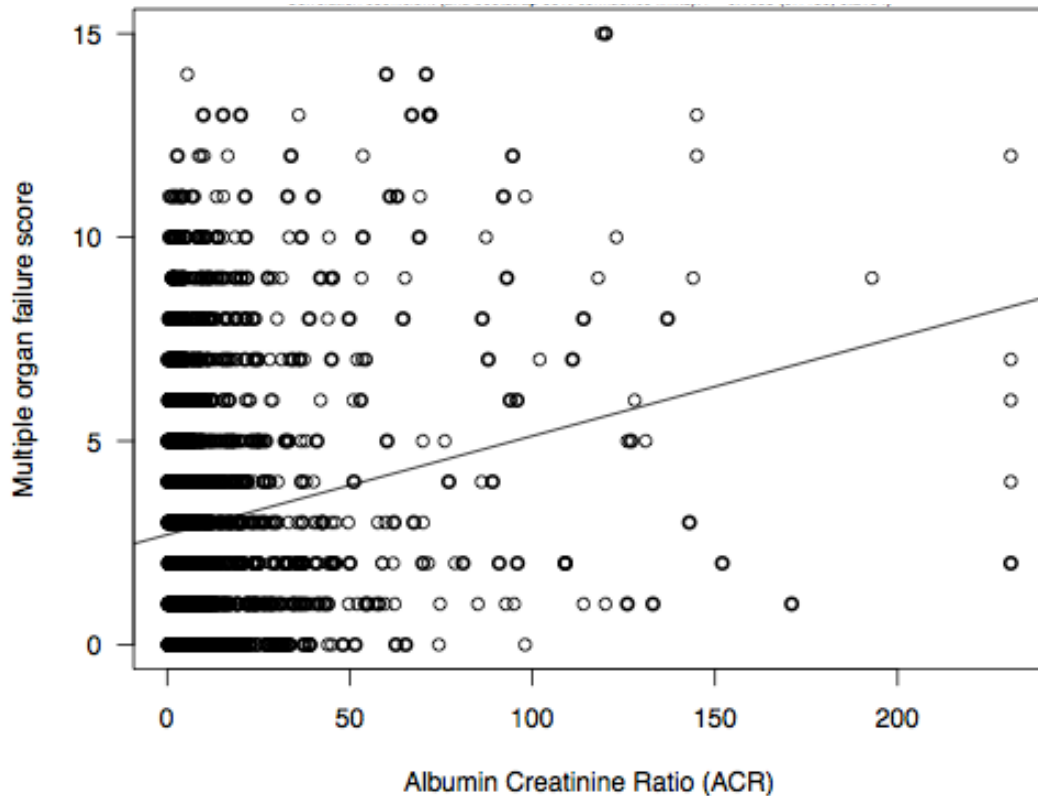


Figure 4.6. Multiple organ failure score against Albumin Creatinine Ratio (ACR). Data shows a scatter graph plotting combined data for all patients and all days of admission. The regression slope obtained was 0.024; Bootstrap CI (95%) (0.01682, 0.08247). The Correlation coefficient (r) obtained was 0.1658; Bootstrap CI (0.1139, 0.2154). There is a correlation between the MOF score and ACR score. The bootstrap confidence interval shows this is statistically significant.

Figure 4.6 show there is a relationship between ACR and MOF score, the higher the ACR value the higher the MOF score. The correlation coefficient, r is a measure of the strength and direction of the linear relationship between the two variables ACR and MOF. The ACR and MOF scores were then examined

to see if ACR could be used to differentiate patient outcomes in those patients suffering from multi-organ failure.

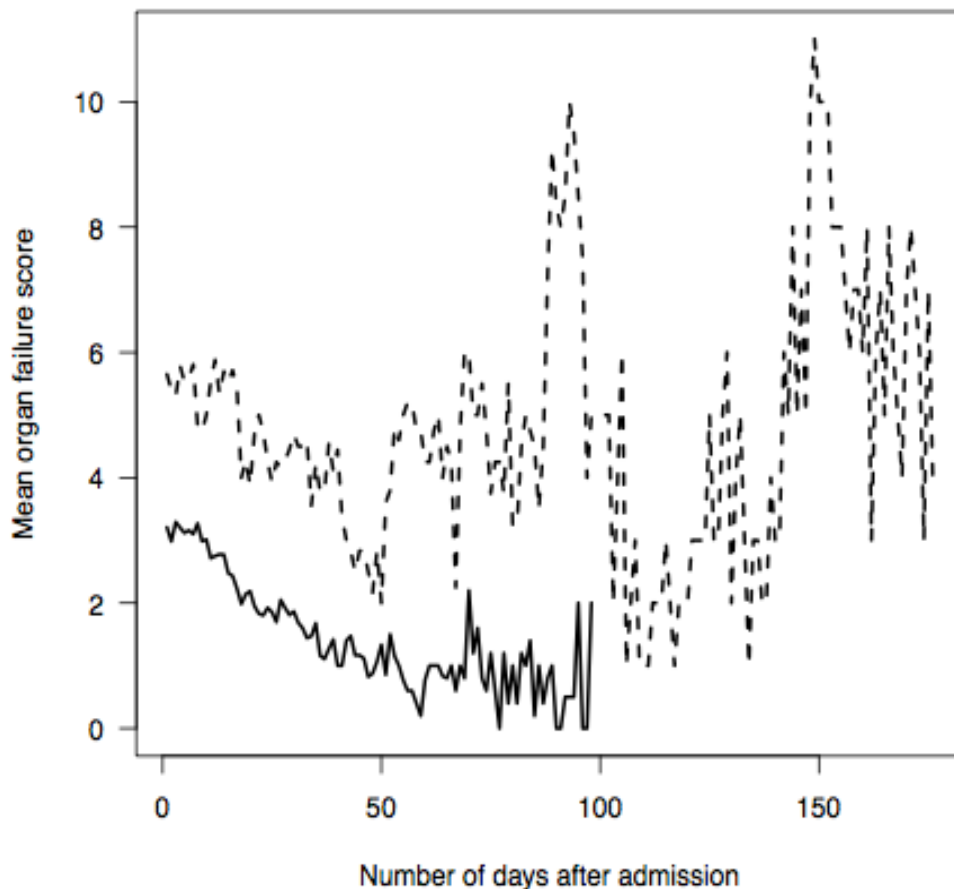


Figure 4.7. Daily mean MOF scores. The data compares the daily mean MOF score for those that survived and those that died with the number of days after admission. The patients that lived are represented by the solid line and the patients that died by the dashed line.

Because of the large numbers of patients (especially in the first weeks) it is difficult to determine any trends between the two groups in figure 4.5 so the daily mean MOF score was plotted for survivors and non-survivors. It is clearer to see in figure 4.7 that the mean MOF scores for those that die are higher than the survivors. Because of the small numbers of patients who stayed in hospital more than 75 days some caution must be used in drawing any conclusions from the trends seen from day 75 onwards.

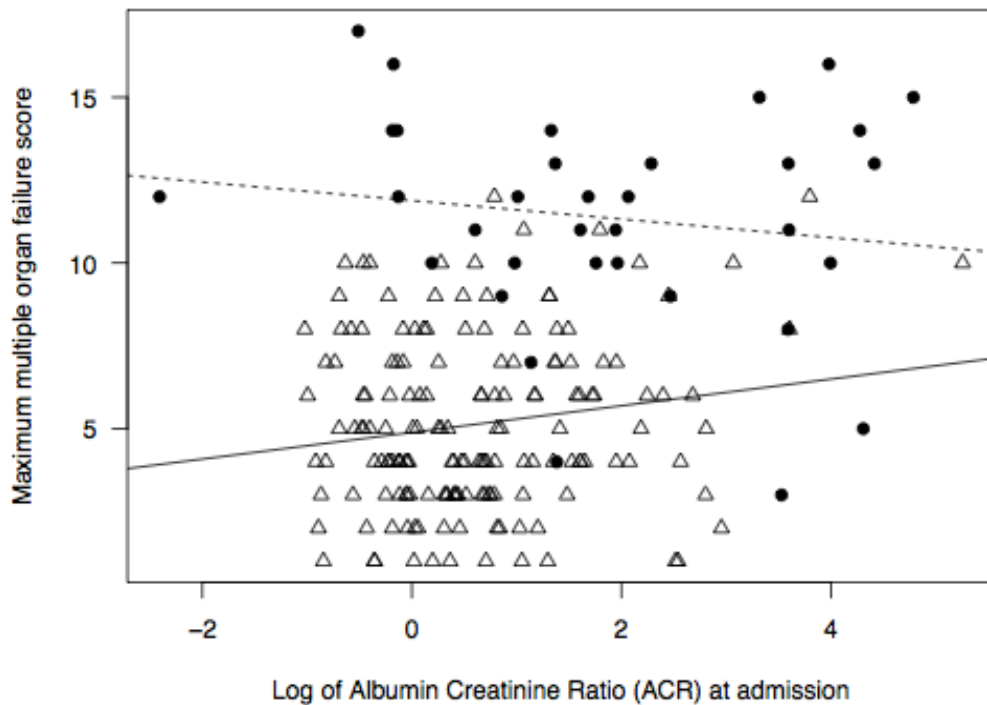


Figure 4.8. Maximum MOF score and ACR at admission. The scatter graph demonstrates the relationship between maximum multiple organ failure score against Log of Albumin Creatinine Ratio (ACR) at admission. The regression slope obtained for the Alive group was 0.402; bootstrap 95% CI (0.032, 0.79) Died group regression slope was -0.279 ; bootstrap 95% CI (-0.886 , 0.397). Alive group, open triangles and solid line, died group closed circles and dashed line.

To determine if the ACR at admission was related to how sick the patients became (measured by their maximal MOF score) the log ACR at admission was related to the patient's maximal MOF score in a scatter plot shown in figure 4.8. This showed that the maximum MOF score increases with increasing ACR for the patients that survived 0.402 (CI: 0.032, 0.79), suggesting there is a relationship between a high admission ACR and eventual degree of ill health (as measured by the maximal MOF score). From this you might expect therefore that the same trend would also be seen in the patients that eventually died. This was not the case; there was no relationship between log admission ACR and the maximal MOF score of those patients that died -0.279 (CI: -0.886 , 0.397).

One problem with looking at the maximal score is that incorrect recording of just one of the one of the organ failure categories could easily skew the results. Another point is that a high score may be achieved on a particular day that may not be a reflection of their general state of health. We therefore looked to see if there was any relationships between log ACR at admission and the median MOF score as this may eliminate the potential sources of bias.

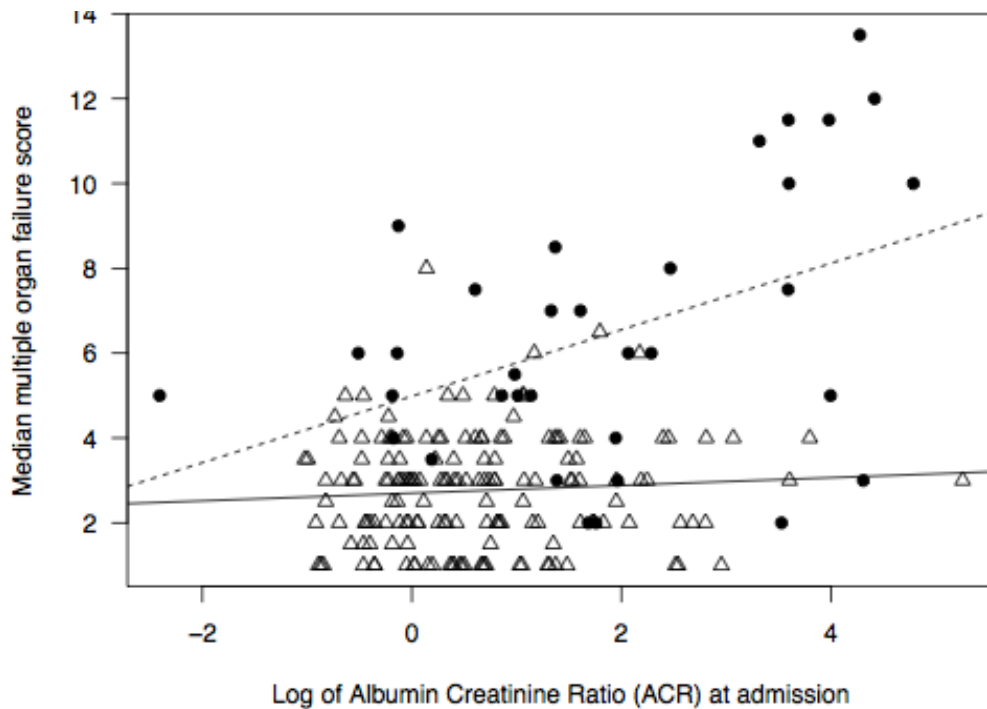


Figure 4.9. Median MOF score and ACR on admission. The data demonstrates the relationship between the median multiple organ failure score against Log of Albumin Creatinine Ratio (ACR) at admission. The regression slope obtained for the Alive group (solid line) was 0.091; bootstrap 95% CI (-0.095, 0.287). Died group regression slope, dashed line (and bootstrap 95% confidence limits): 0.783 (0.236, 1.34). There is no correlation between median MOF score and ACR on admission for the patients that lived but there is for those that died.

Figure 4.9 shows that for the patients that died the log ACR scores at admission are related to the median MOF score. It shows the higher the ACR on admission, the higher the median MOF scores. This method is probably a more sensible way of analysing multi-organ failure as it is more likely to represent the status of the

patient throughout their stay rather than a snapshot in time. However there is no relationship between the median MOF scores and admission ACR for the patients that lived.

Because large burns can cause such a rapid and profound systematic response it is argued that the physiological response seen in the first twenty-four to forty eight hours may not be representative of the true nature of the injury. With adequate resuscitation many of the early physiological derangements can be correctable presenting a very different picture of the patients' physiological state and potential prognosis. The MOF score may therefore change rapidly over the first few days of admission whilst resuscitation is ongoing. Daily plots of the MOF score against ACR were performed for the first 96 days to see if the relationship changes with time. Only the results for days 1-3 are shown below to illustrate the analysis in figure 4.10-4.12.

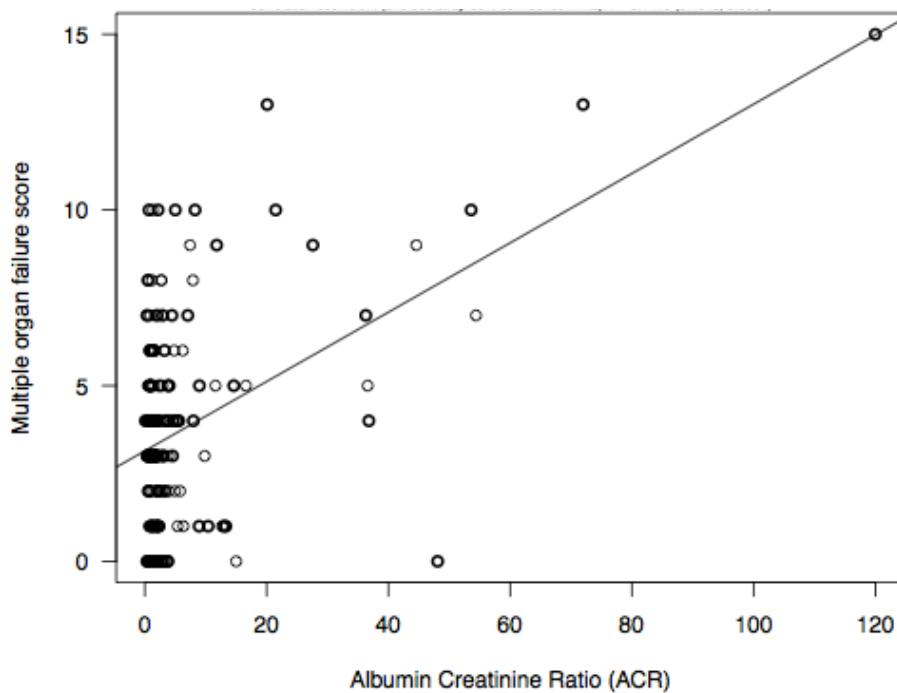


Figure 4.10. Multiple organ failure score against ACR, Day 1. Data shows MOF Day 1 plotted against ACR Day 1. The regression slope obtained was 0.09867; bootstrap 95% CI (0.05164, 0.13303) Correlation coefficient $r = 0.4416$; bootstrap 95% CI (0.1848, 0.6004).

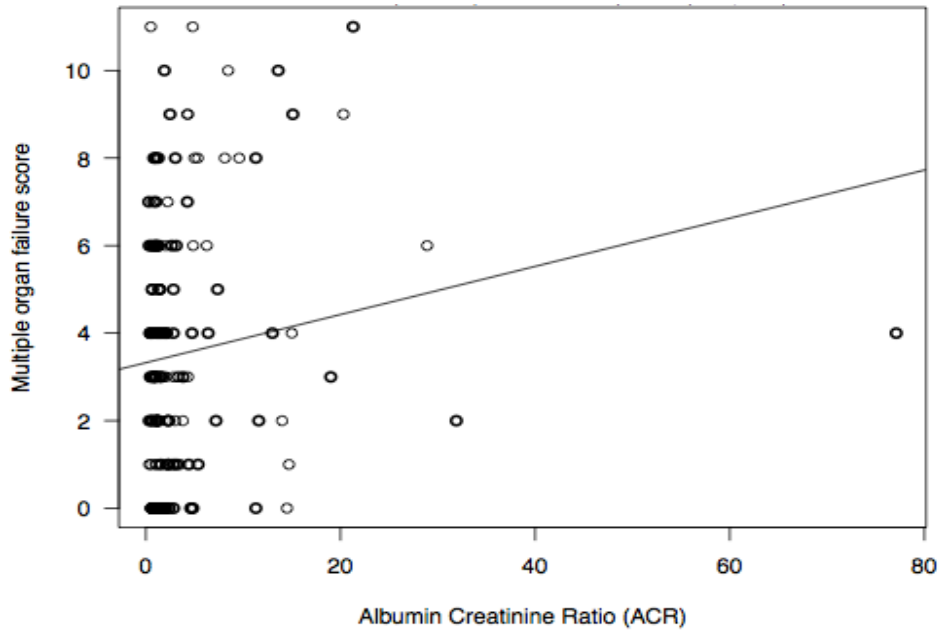


Figure 4.11. Multiple organ failure score against ACR, Day 2. Data shows MOF Day 2 plotted against ACR Day 2. The regression slope obtained was 0.05501; bootstrap 95% CI (0.01067, 0.21416). Correlation coefficient $r = 0.142$; bootstrap 95% CI (0.0278, 0.3459).

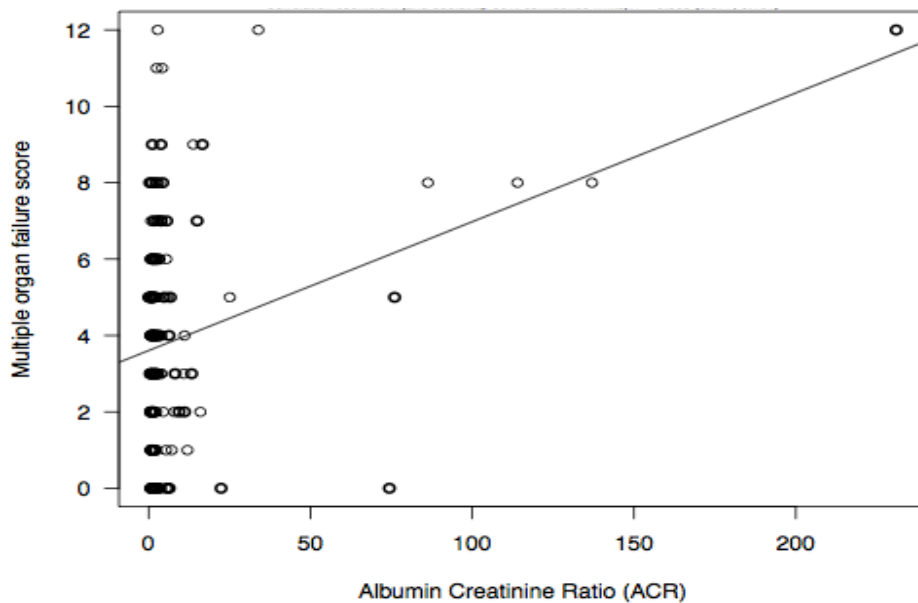


Figure 4.12. Multiple organ failure score against ACR, Day 3. Data shows MOF Day 3 plotted against ACR Day 3. The regression slope obtained was 0.03373; bootstrap 95% CI (0.01241, 0.04869) Correlation coefficient $r=0.305$; bootstrap 95% CI (0.077, 0.461).

Figures 4.10, 4.11 and 4.12 show the multiorgan failure score plotted against ACR for each day. All graphs show that there is a correlation between the MOF score and the ACR; the higher the MOF score the higher the ACR. To get an idea of the strength of the relationship between the two variables the correlation coefficient was also measured. The correlation coefficient is measured between 0 and 1 with 1 being a perfect correlation and 0 being no correlation. It can be seen from the figures 4.10-4.12 that there is a weak correlation. As the first days post injury involve resuscitation and or surgery the physiological changes are dynamic and it may be possible that the ACR or MOF score do not reflect what is going on when resuscitation has been completed and the patient has been stabilized. It was then decided to examine if the correlation coefficient changed over time and if there were any periods when there was a greater correlation between the two variables, figure 4.13.

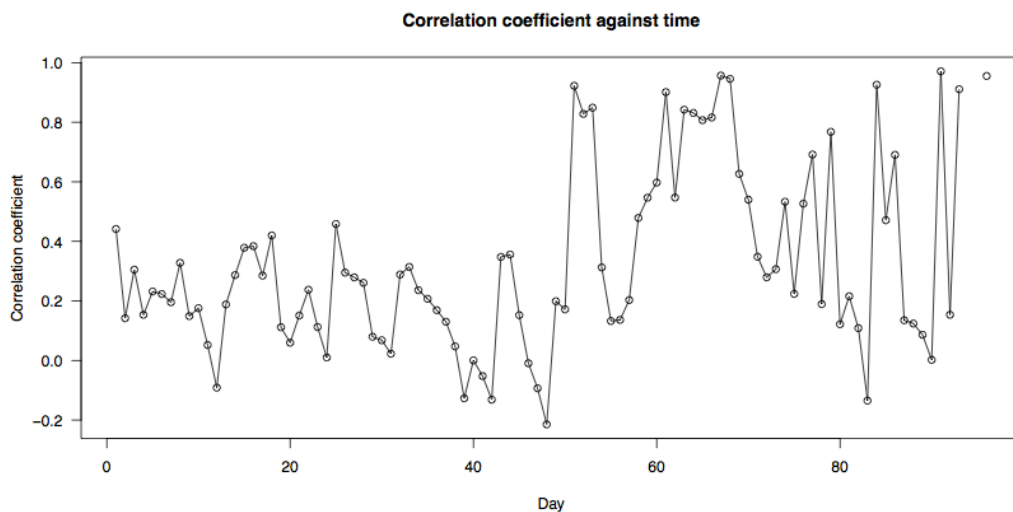


Figure 4.13. A summary chart of how the correlation coefficient varies with time. The data plotted shows the correlation coefficient against time (days). It can be seen that from day 50 the plots get more erratic as the number of patients diminishes.

Over time the correlation coefficient (the measure of the strength of the linear relationship between two variables) appears to improve as shown in figure 4.13. Unfortunately this period (from around day 50 onwards) is when the number of patients diminishes and the correlation coefficient gets increasingly erratic. It is

difficult therefore to ascribe the higher correlation coefficient scores seen in the later part of the graph to a genuine trend rather than a small number of individual patient scores skewing the data.

To look more closely at the relationship between MOF score and the ACR the regression line intercept was plotted against time, figure 4.14.

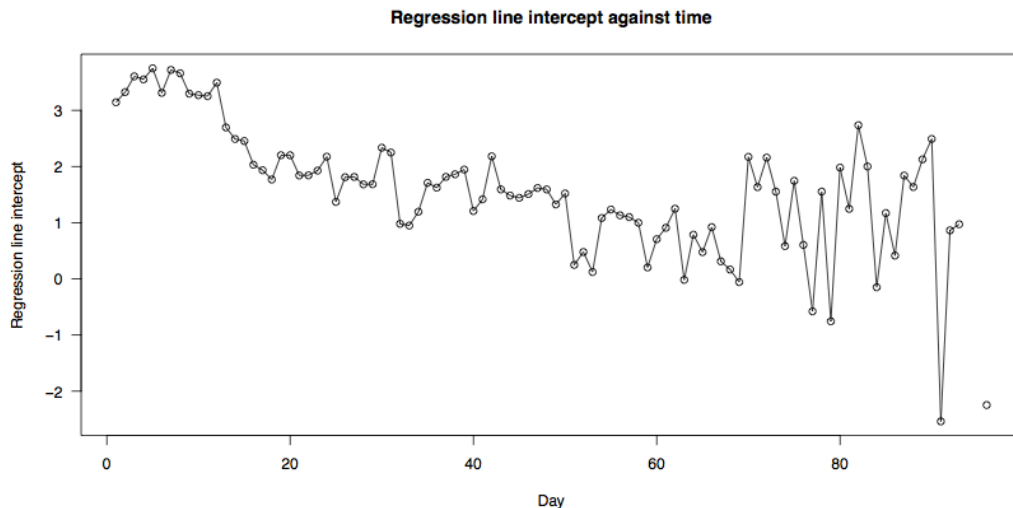


Figure 4.14. Plot of the regression line intercept against time. To examine the relationship between the two variables (MOF score and ACR) and how this changes with time the intercept point was plotted against time (days). It shows a downward trend over time.

From figure 4.13 and 4.14 it seems clear that that the relationship between the MOF score and ACR within a day is rather weak. Certainly in the first few days there does not seem to be a good correlation between the MOF score and the ACR value (as seen in figure 4.13) and shown by the higher values of the regression line intercept point in figure 4.14. However with time there is a weak trend of improved correlation coefficient and the intercept point moves towards zero. This seems to arise because in the early days there are a lot of patients with high MOF scores that also have low values of ACR, so that the regression line intercept is high. In later days the patients with high MOF scores and low ACR have disappeared so that the regression line intercept is low.

4.4 Discussion

Patients sustaining burns injuries are at a high risk of systemic inflammatory response syndrome (SIRS). When severe, SIRS leads onto organ failure. The inflammatory response to the injury, trauma or infection is wide spread and may damage organs distant from the initial insult. Although the sequence of inflammation, organ failure and sometimes ultimately death is well recognised the exact mechanisms are still poorly understood.

In burn unit's multi-organ failure is one of the most frequently reported causes of death, responsible for 25-65% of all burn deaths in Europe (Brusselaers et al., 2010).

One difficulty encountered when caring for the patients is identifying at an early enough stage those that may develop multi-organ failure in order that the clinician may try and modulate the inflammatory and immune response.

This study of admissions to a UK burns ICU show that ACR does predict organ failure. Figure 4.6 demonstrates the relationship between ACR and MOF. With an increasing ACR the MOF score tends to be higher.

Previous studies have suggested that ACR may have a role to play in identifying those patients that may suffer from organ failure. Gosling (1995) has reported that in both surgical and trauma patients whose microalbuminuria is maintained and does not return to normal within 4 and 8 hours (respectively) post insult it has been possible to predict subsequent organ failure. Smith et al., (1994) showed the urinary albumin levels taken within 4 hours of the start of aortic surgery have been found to be able to predict those patients who would later develop pulmonary complications that were non cardiogenic in origin

De Gaudio et al., (2000) showed an increase of the ACR was also positively correlated to the increase in the sepsis related organ failure assessment (SOFA) score (This is a scoring system based on physiological parameters that determines the degree of organ failure a patient may be suffering from).

A similar pilot study by MacKinnon et al., (2000) looking at a similar number but broader range of patients demonstrated that microalbuminuria 6 hours after admission to a general ICU could identify patients at risk of developing multi-organ failure and those at a greater risk of death.

There is very little information on the ability of ACR to predict MOF in the burn population. Gosling et al., (2006) examined a mixed cohort of medical (205 patients) and surgical (225) patients. In this study ACR was measured on admission and after 4-6 hours. It was found that ACR predicted ICU mortality and inotrope requirements as well or better than APACHE II and SOFA scores. This study had a small number of burns patients (n=21) and the results of the surgical, trauma and burns patients were grouped together so it is difficult to draw any conclusions.

From our study although the relationship is clear between ACR and MOF it does not offer clear a clinical utility at the moment to identify those patients that may suffer from multi-organ failure. It may be that burn injuries are too complex and there are too many confounding factors in multi-organ failure for ACR to be able to distinguish those patients who will achieve higher MOF scores.

One other intriguing aspect that came out of the study by Sarti et al., (2001) was that two patients showed a rise in ACR after the initial postoperative value returned to normal but before the clinical appearance of a surgical complication (peritonitis and a large subcutaneous haematoma). A further patient showed a persistent rise in ACR before the appearance of a septic complication. It may be that there is a time lag between a rise in ACR and development of MOF at a later date.

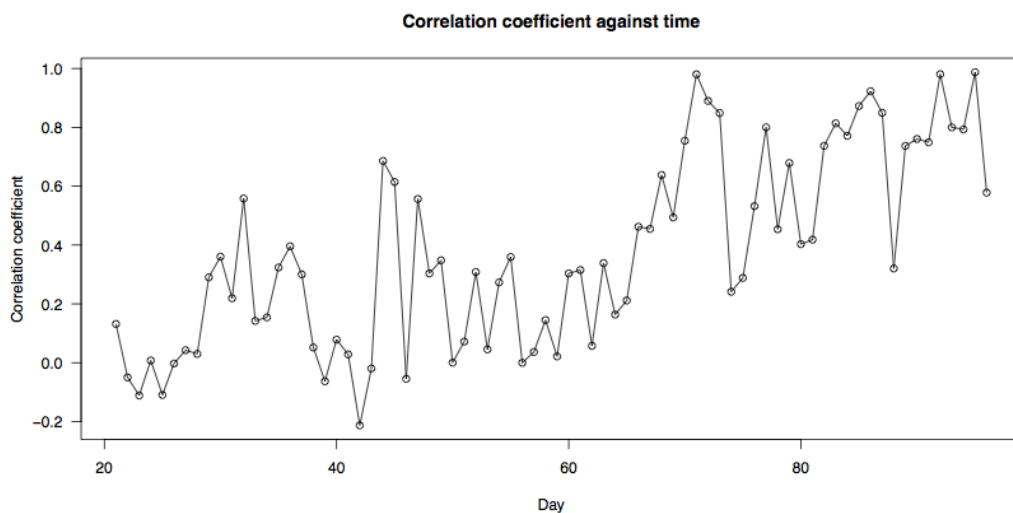


Figure 4.15. MOF score with ACR from 20 days earlier. The graph shows the correlation of the MOF score with the ACR from 20 days earlier.

To test whether there is a time lag between an ACR rise and MOF the correlation of the MOF score with the ACR from 20 days earlier was analysed, figure 4.15. It appears that as patients remain in the study for a longer period there is an increasing trend in the correlation coefficient. This would mean that for the patients remaining later their MOF scores become more closely related to their ACR from 20 days earlier. Put another way, for the patients who stay longer the ACR can predict the MOF score 20 days ahead, and this prediction gets better the longer they stay.

Unfortunately the number of patients that remain gets quite small at the later stages, so one cannot be very confident that this is a reproducible result. It could be an artefact arising from the few patients that have both high MOF scores and high ACR (perhaps for unrelated reasons) and remain in the unit.

In conclusion whilst the ACR value is closely related to the MOF score further studies will be required before it can be demonstrated whether it has a role in the acute setting or can predict events at a later date.

Chapter 5

The Impact of *Acinetobacter baumannii* on a UK Burn Unit and its relationship to patient outcomes

5.1 Introduction

Hospital acquired infections constitute a large and persistent problem in the UK and throughout the world. About 9% of inpatients have a hospital-acquired infection at any one time, equivalent to at least 100,000 infections a year (National Audit Office 2000 UK). The consequences to the patient may vary from discomfort or inconvenience to permanent ill health or even death in a small proportion of patients. Repercussions for the hospital are no less serious as hospital acquired infections result in increased length of inpatient stay, increased pressures on staff and a large financial burden. Government figures estimate the cost to the NHS to be as much as £1000 million a year (National Patient Safety Agency; The Economic Case 2004).

Infection is a common complication in burns patients, and burns patients are particularly prone to nosocomial infections (Wertz et al., 1995). This can result in increased morbidity and mortality for the patient (Weber et al., 1997) or loss of grafted skin necessitating further operations and a subsequently prolonged hospital stay (Unal et al., 2005).

In recent years, certainly in the mainstream media, the focus of attention has been on those organisms that have achieved multi drug resistance to standard antibiotic treatment for instance, MRSA (Multi Resistant *Staphylococcus Aureus*) and *C. Diff* (*Clostridium Difficile*). There are a wide variety of organisms causing significant problems in hospitals at the moment however. One that is increasingly found to have a clinical impact, especially in the critical care setting is *Acinetobacter baumannii*.

The impact of multi drug resistant organisms is striking. In a study by Williams et al., (2009) into the causes of death in a paediatric population they found that from 1989 to 1999, sepsis accounted for 35% of deaths but increased to 54% from 1999 to 2009, which they attributed a significant increase in that proportion to antibiotic resistant organisms.

Acinetobacter baumannii

Acinetobacter baumannii is an aerobic, gram negative, oxidase negative non-fermenting bacterium (Appleman et al., 2000) that is normally a commensal organism but due to growing antibiotic resistance is now a key opportunistic pathogen in burns and intensive care units. Nosocomial outbreaks of *A. baumannii* have previously been described in the literature and many of the outbreaks have been associated with multiresistant strains (Lyytikainen et al., 1995, Mulin et al., 1995 and Simor et al., 2002). Multiresistant strains of these species causing bacteraemia, pneumonia, meningitis, urinary tract infections and surgical wound infections have been isolated from hospitalised patients worldwide (Van Looveren et al. 2004). The overall incidence of *A. baumannii* as a nosocomial infection was 9% in Europe in 1995 (Vincent et al., 1995) but a higher reported incidence has been found in burn units, 13% in a Canadian burns unit (Simor et al., 2002) and 14% in a Brazilian unit (Santucci 2003).

A number of factors have been identified for the risk of acquisition and subsequent mortality of multi resistant *Acinetobacter baumannii* (MRAB). (*Acinetobacter baumannii* that is resistant to carbapenem has been termed MRAB-C). The factors for acquisition of MRAB include receipt of blood products and duration of mechanical ventilation (Simor et al., 2002), Acute Physiology and Chronic Health Evaluation (APACHE II) score on admission (a measure of physiological variables to determine severity of disease) and number of intravascular lines placed (Wong et al., 2002). Previous broad-spectrum antibiotics have been found to play an important role in MRAB acquisition (Mulin et al., 1995, Villiers et al., 1998, Ayan et al., 2003). Factors associated with mortality include immunosuppression, shock, recent surgery, invasive procedures such as central venous or pulmonary catheterization and mechanical ventilation (Chen et al., 2005). In the same study mortality was significantly associated with lower platelet count and lower serum albumin concentration.

In addition many outbreaks have been linked to equipment or the inanimate hospital environment. This is partly due to the ability of *Acinetobacter* to survive for long periods on dry surfaces (Wendt et al., 1997). Catalano et al., (1999) describe the survival of *Acinetobacter* on hospital bed rails causing sporadic

outbreaks whilst Neely et al., (1999) report the presence of *Acinetobacter* on computer keyboards in the burns unit. Beck-Sague et al., (1990) again found higher rates of *Acinetobacter* in ventilated patients although found no link to the equipment.

One important aspect not to be forgotten is that transmission via the hands of hospital staff can be a frequent cause of spread of infection. *Acinetobacter* is considered a low-grade pathogen and can remain on or in the human body without causing illness, and for this reason dissemination via the hands of hospital staff may remain undetected (Joly-Guillou 2005). Close adherence to standard levels of hand hygiene can have an important impact on reducing or eliminating outbreaks of colonization or infection (Chan et al., 2007). Other factors that have been implicated in the transmission and spread of infection within a hospital or unit include bed occupancy and therefore the turnover of patients. The number of procedures or points of contact between the patient and members of staff as well as the level of training of the staff involved may also be important.

For those patients with infections possibly caused by *Acinetobacter*, a β -lactam alone or in combination with an aminoglycoside or fluoroquinolone was the initial empirical therapy (Meyer 2005). This regimen has become less effective due to the appearance of β -lactamases (Rahal and Urban 2000), extended spectrum β -lactamases (ESBLs) and enzymes that inactivate aminoglycosides (Bergogne-Bérézin and Towner 1996).

The speed in which *Acinetobacter baumannii* has become a significant problem in hospitals is illustrated by the rise in cases resistant to carbapenem in one unit increasing from 0% to 55% between 1998 and 2006. This overall increase coincides with the emergence of the MRAB-C OXA-23 clone-1 as the most prevalent strain in London and South East England (Wareham et al., 2008). There appears to be little clinical difference in clinical outcome in patients with bacteraemia from *Acinetobacter baumannii* and other *Acinetobacter* species (Choi et al., 2006) but it may be that different clones of MRAB result in different patient morbidity or mortality rates.

5.2 Aim: By using retrospective case control study of all admissions to the burns ITU/HDU over a twenty-nine month period (2004-2006) to determine the impact of *Acinetobacter baumannii* on a UK burn unit and its relationship to patient outcomes and to look at possible influencing factors.

5.3 Method

5.3.1 Clinical setting

The Burns Service comprises twenty beds including four intensive care and four high dependency beds. There are twelve low dependency / rehabilitation beds. The unit is a tertiary referral centre and accepts patients from a population of over 6 million people. Patients are also taken from outside of traditional catchment areas and occasionally internationally.

A multidisciplinary team including microbiologists, pharmacists, anaesthetists and burn surgeons review all major burns daily. The Parkland formula is used in the initial resuscitation to maintain a urine output of 0.5 ml/kg/h in adults and 1 ml/kg/h in children. Nutritional supplementation is via nasoduodenal enteral nutrition with high calorie, low fat formulas. As part of the burn treatment protocol, gastric ulcer prophylaxis is performed with H₂ antagonists. Low molecular weight heparin (40mgs/day) is prescribed daily. Early excision and grafting is routinely performed for deep burns that would take more than two weeks to heal without surgical intervention using allografting, auto-grafting or dermal substitutes depending on the individual characteristics of burn injuries.

Retrospective collection of data regarding demographic details, mode of injury, examination findings was from clinical records. All other results were obtained from the Burns ITU computer system MetaVision (*iMDsoft*, Massachusetts, USA).

All patients admitted to the Burns ITU of the St Andrew's Centre for Burns and Plastic Surgery over a 29-month period from were eligible for the study (2004-2006). There were no omissions in this series.

Records of *Acinetobacter baumannii* acquisition from 2000 -2004 were also examined to provide comparative data.

5.3.2 Definitions

Acinetobacter baumannii resistance definition

The UK Working Party Guidance on the Control of Multi-Resistant *Acinetobacter* outbreaks was used to define resistance. The working party defined multi-resistant *Acinetobacter spp.* “MRAB” as *Acinetobacter spp.* isolates that are resistant to any aminoglycoside (e.g. gentamicin) and to any third generation cephalosporin (e.g. ceftazidime, cefotaxime). Those isolates that are additionally resistant to imipenem and/or meropenem are designated “MRAB-C” (Cookson et al., 2007). During this study MRA has in been used to refer to both MRAB and MRAB-C unless otherwise stated.

Multi-organ failure definition-score.

Multiorgan failure in patients was assessed by using the **Organ Dysfunction** and/or **INfection Score (ODIN)**.

Dysfunctions	Variables
Respiratory	PaO ₂ < 60mmHg (FiO ₂ =0,21) or: Need for ventilatory support
Cardiovascular (in the absence of hypovolemia with CVP< 5 mmHg)	Systolic Arterial Pressure < 90 mmHg with signs of peripheral hypoperfusion or : Continuous infusion of vasopressor or inotropic agent to maintain SBP > 90 mmHg
Renal (excluding chronic dialysis)	Serum creatinine > 300 micromol /L or :Urine output < 500mL/24 h or <180 mL/8 h or : Need for hemodialysis or peritoneal dialysis
Neurologic	Glasgow < 6 (in absence of sedation at any time in the day) or : Sudden onset of confusion or psychosis
Hepatic	Serum bilirubin > 100 micromol /L or : Alkalines phosphatases > 3 x N
Hematologic	Hematocrit ≤ 20% o:WBC < 2.000 /mm ³ or : Platelet Count < 40.000 /mm³
Infection (with clinical evidence of infection)	2 positive blood cultures or: Presence of gross pus in a closed space or: Source of the infection determined during hospitalization or at autopsy in case of death within the 24 h

Table 5.1 Organ Dysfunctions and/or Infection Score. Used as a measure of severity of organ dysfunction in a patient (Fagon et al., 1993).

This is a scoring system with a range from 0-7. If a patient achieved one of the parameters listed above they got a score of one for that system. Patients cannot score more than one in any category. It assesses five organ systems as well as the

haematological response and response to infection. For consistency, each patient was scored using the same parameters for each system assessed. This scoring system was chosen after discussion with the lead surgical and anaesthetic consultants in the unit. Several scoring systems were looked at and the ODIN score was thought to be the most comprehensive and all the data to provide the scores was routinely recorded. Other scoring systems were more complicated and required information that was not routinely recorded in our unit therefore there would have been holes in the data set.

5.3.3 Study design

5.3.3 a) Study design for multi-organ failure study

A retrospective case control study of all admissions to the burns ITU/HDU over a twenty-nine month period (2004-2006). All patients presenting within this period were eligible for the study if they met the criteria for admission to the burns unit ITU/HDU:

- Total burn surface area (TBSA) >10% in adults, >5% in children
- Extremes of age <5 years or >60 years
- Inhalation injury
- Special sites: face, hands, feet, perineum, flexure surfaces, circumferential burns
- Chemical, electrical or radiation injuries

Group 1 contained all the patients with multi drug resistant *Acinetobacter baumannii*. Group 2 contained all the patients with drug sensitive *Acinetobacter baumannii*. Group 3 was a control group of patients who had not contracted any form of *Acinetobacter baumannii*.

5.3.3 b) Study design for analysing MRA and bed occupancy rates and the use of agency nursing staff

A retrospective cases control study of all admissions to the burns ITU/HDU during the main outbreak of MRA (April 2004-March 2005). All patients presenting within this period were eligible for the study if they met the criteria for admission to the burns unit ITU/HDU as outlined above.

5.4 Results

5.4.1 *Acinetobacter baumannii* prevalence

The prevalence of *Acinetobacter baumannii* was examined during an outbreak of MRA in 2004 onwards and then retrospectively to August 2000 when the first data was collected.

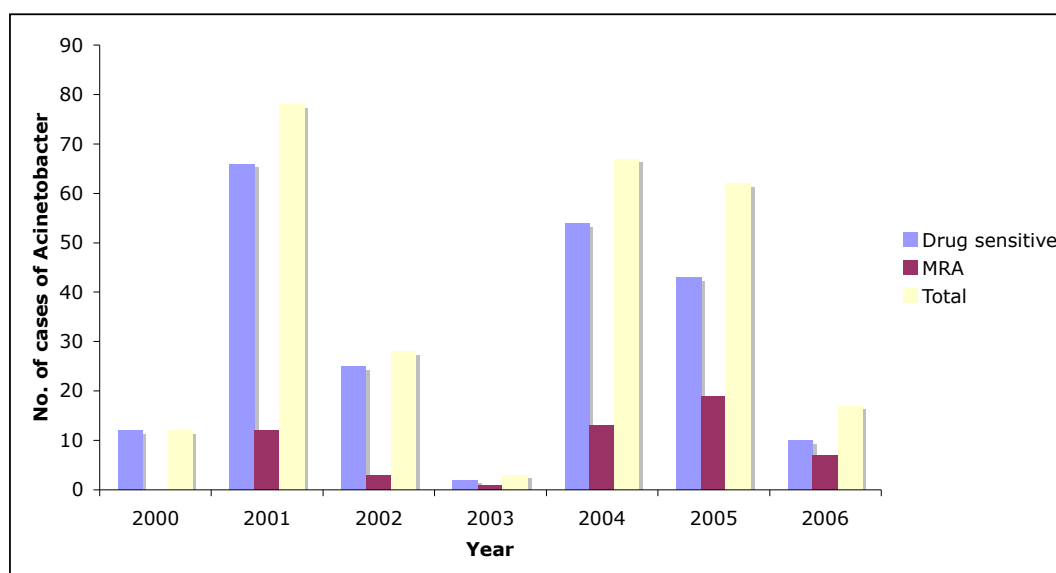


Figure 5.1. *Acinetobacter baumannii* per year from August 2000-June 2006

Data shows the total number of cases (both adult and paediatric) in the burns unit (this includes Burn ICU, HDU and rehabilitation ward). It includes both multi resistant *Acinetobacter baumannii* (MRAB) and Drug Sensitive *Acinetobacter baumannii* cases.

The number of cases of *Acinetobacter baumannii* isolated in the burns unit varies from year to year. Figure 5.1 shows the number of patients through out the unit (including the ICU, HDU and rehabilitation wards but excluding the out patient clinic, which is geographically separated from the wards). It can be seen that *Acinetobacter baumannii* decreases in prevalence from 2001 to 2003. It is thought this is because after an outbreak of drug resistant *Pseudomonas Aeruginosa* in 2001 a strict hand washing policy was instituted in the unit, which appears to have had the knock-on effect of reducing the rate of *Acinetobacter baumannii* infection over the subsequent two years. An outbreak of MRA occurred early in 2004 and continued into 2006. One further case (of drug

sensitive *Acinetobacter baumannii*) not included in figure 5.1 is an environmental isolate from a mechanical arm used on some equipment in the ICU.

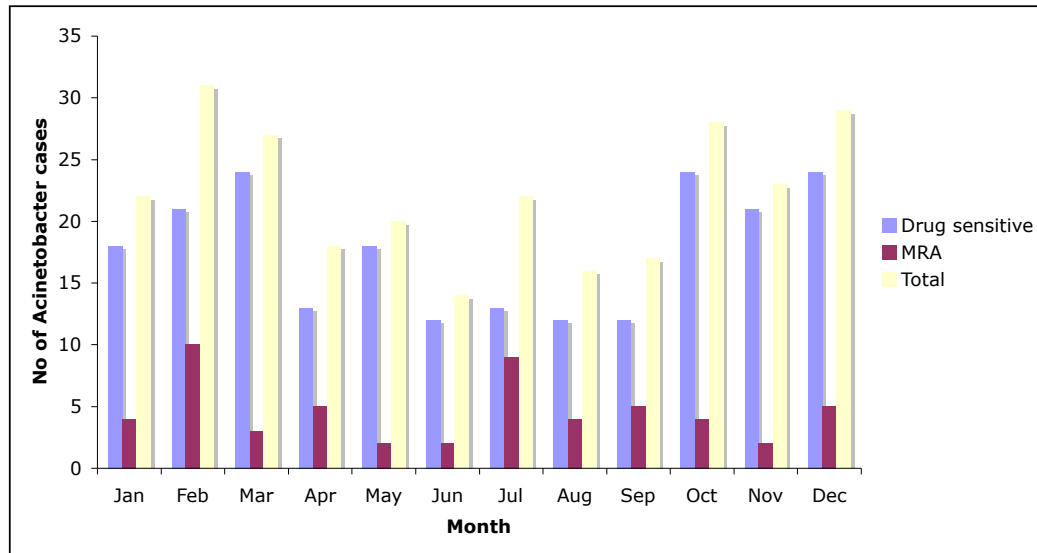


Figure 5.2. Monthly variation in the total number of cases of *Acinetobacter baumannii*. This data shows the isolates per year from August 2000-June 2006 in both the adult and paediatric population. This includes Burn ICU, HDU and the rehabilitation ward. It includes both multi resistant *Acinetobacter baumannii* (MRAB) and Drug Sensitive *Acinetobacter baumannii* cases.

Looking at all the cases of *Acinetobacter baumannii* (both drug resistant and drug sensitive) it can be seen from Figure 5.2 that there appears to be a seasonal variation with a preponderance of cases between October and March. Other studies have had mixed results with some demonstrating a seasonal variation whilst others have found no variation. As there was no change in operating policy from month it is presumed the variation is due to an as yet unidentified environmental factor.

To investigate the extent of the problem MRAB might have on a burn unit the proportion of MRAB was looked at, table 5.2. It can be seen that there is a general trend for MRAB cases to make up a greater proportion of all the *Acinetobacter* isolates. In 2004 the number of cases of *Acinetobacter baumannii*

positive swabs isolated from patients started to increase and the number of MRA cases doubled when compared with 2002 as can be seen in table 5.2. There are so few cases in 2003 that the proportion of MRA cases should be ignored. It is unclear from the data why the total number of cases in 2003 is so low (despite the implementation of the hand washing regimen in 2001). Whilst the hand-washing regimen may explain the reduction in isolates from 2001 it does not explain the substantial increase in 2004. It must be noted that there were no dramatic changes in operating policy during this period.

Year	Total Acinetobacter cases	MRA cases (%)
2000	12	0
2001	78	15.3
2002	28	10.7
2003	3	33.3
2004	67	19.4
2005	62	30.6
2006	17	41.1

Table 5.2. The percentage of multi-resistant *Acinetobacter baumannii* cases per year for the whole unit (ICU/HDU and the rehabilitation ward). It must be noted that the results from 2000 are from August to December and the results from 2006 are to the end of June only.

5.4.2 Multi-organ failure and *Acinetobacter baumannii* acquisition.

To look at MOF in burn patients all admissions over a twenty-nine month period were studied (April 2004-June 2006).

Two hundred and eighty patients were admitted via ITU/HDU of which 53 patients acquired *Acinetobacter baumannii* (18.9%) and of these cases 30 were MRA (10.7%). Three patient groups were identified. Group 1 had MRA (n=30), Group 2 had drug sensitive *Acinetobacter* (n=23) and Group 3 were a control group who had not contracted any form of *Acinetobacter* (n=31).

Tables 5.3, 5.4 and 5.5 detail the individual patient results for each group and further summary data is shown in Appendix III, section 1.

Table 5.3 Group 1 MRA patients

Patient	Sex M=1	Days on unit	Died=1	Age	TBSA % burn	Total MOF score	Theatre visits	Days until acquisition	Blood transfused before acquisition	Blood after acquisition	Blood total	Inhalation injury =1
1	1	36.3	0	33	30	4	2	11	2196	2044	4240	1
2	0	37	0	41	30	1	3	2	3884	2291	6175	1
3	1	18.83	0	1	40	2	4	7	510	483	993	0
4	1	23.12	0	61	85	5	4	12	4970	9637	14607	1
5	0	154.3	1	52	45	3	9	95	28222	4045	33971	1
6	0	33.96	1	32	95	5	2	6	2080	3706	5786	0
7	0	30.84	0	53	32	3	6	8	1200	7326	8526	0
8	1	14.83	0	74	35	3	2	5	2258	2051	4309	0
9	0	16.33	0	44	36	3	3	3	316	4624	4940	0
10	1	20.23	0	28	40	3	3	5	4321	2061	6382	0
11	1	32.36	0	45	80	2	6	1	0	8673	8673	1
12	1	34.67	0	17	50	3	12	2	3590	3590	7180	0
13	1	13.6	0	71	55	5	2	2	2849	2575	5424	1
14	1	12.65	0	16	48	2	2	11	1200	220	1423	0
15	1	22.16	0	14	23	3	2	9	2386	1898	4284	1
16	1	46.01	0	35	35	4	6	23	5223	2226	5484	1
17	1	82.18	1	55	37	5	7	9	2196	26135	28331	1
18	1	26.82	0	38	30	4	5	26	7464	0	7464	1
19	0	53.07	0	17	50	4	9	1	15075	0	15075	0
20	1	27	0	1	37	1	6	9	710	450	1160	0
21	0	94.8	1	52	30	5	2	8	821	9224	10045	1
22	0	21.44	0	76	16	3	1	10	1395	2654	4049	0
23	0	63.72	0	26	37	3	4	10	1817	6259	8076	1
24	1	20.77	0	56	40	2	1	7	447	1538	1985	1
25	0	15.13	0	15	42	3	2	9	2026	781	2807	0
26	0	15.75	1	39	90	2	2	4	1440	5597	7037	0
27	1	17.53	0	33	37	2	8	7	4193	0	4193	0
28	0	19.58	0	26	46	3	3	20	3894	0	3894	0
29	1	17.96	1	47	70	3	6	5	8139	3121	11260	0
30	0	21.89	1	43	40	3	4	7	5178	3961	9139	0

Table 5.4 Group 2 Drug sensitive *Acinetobacter baumannii* patients

Patient	Sex M=1	Days on unit	Died=1	Age	% burn	Total MOF score	Theatre visits	Days until acquisition	Blood transfused before acquisition	Blood after acquisition	Blood total	Inhalation injury=1
1	0	60.89	0	58	60	3	3	9	0	5467	5467	0
2	1	51.88	0	20	28	3	3	15	1241	6190	7431	1
3	1	0.42	0	18	7	0	0	1	0	0	0	0
4	0	15.85	1	70	30	5	3	8	20402	8465	28867	0
5	1	24.64	0	34	32	2	5	4	458	2149	3890	0
6	0	0.84	0	8	14	0	1	1	0	0	0	0
7	1	23.04	0	44	10	1	1	4	0	1402	1402	0
8	1	0.83	1	35	13	0	2	1	0	0	0	0
9	0	12.2	0	11	25	1	2	5	868	610	1478	0
10	1	16.23	0	45	40	3	2	10	4241	0	4241	1
11	0	2.14	0	62	8	0	2	1	0	0	0	0
12	1	23.55	0	13	50	1	4	6	2919	2015	4934	0
13	1	37.18	0	72	30	3	2	28	2630	600	3230	0
14	0	23.4	1	58	45	2	4	3	4589	5669	10258	0
15	1	22.42	0	26	45	1	13	16	3450	931	4381	0
16	0	3.27	0	1.25	60	0	1	1	0	0	0	0
17	1	25.38	0	31	8	2	2	2	329	853	1182	0
18	1	5.9	0	53	25	0	1	3	775	0	775	0
19	0	7.79	0	18	30	0	0	8	0	0	0	1
20	1	32.28	0	43	40	3	4	29	2097	2506	4603	0
21	0	89.39	0	44	60	4	9	15	7085	10359	17444	1
22	1	23.94	0	60	37	2	2	7	778	3811	4589	0
23	0	11.71	0	31	60	4	8	1	0	2532	2532	0

Table 5.5 Group 3 Control group. Matched as closely as possible by age and sex to group 1

Patient	Sex M=1	Days on unit	Died=1	Age	% burn	Total MOF score	Theatre visits	Blood total	Inhalation injury
1	1	14.68	0	37	44	1	2	1493	0
2	0	24.61	0	53	35	1	3	5358	0
3	1	3.86	0	1	40	0	4	250	0
4	1	12.34	1	48	70	2	3	4107	1
5	1	3.41	1	38	60	2	1	500	1
6	1	42.54	1	33	85	3	6	12792	1
7	0	2.42	0	45	25	0	1	0	0
8	1	44.65	0	60	32	2	1	6129	1
9	0	27.4	0	60	17	1	4	1203	1
10	0	25.74	0	42	40	1	3	3324	0
11	1	1.82	0	27	30	1	1	0	0
12	1	3.23	0	44	30	1	1	0	0
13	1	19.65	0	17	45	3	3	4292	0
14	0	1.24	1	70	32	1	0	0	1
15	1	9.95	0	15	47	1	3	1292	0
16	1	1.88	0	21	25	1	1	0	0
17	1	3.01	0	37	27	2	2	0	1
18	1	1.27	1	56	35	1	0	575	1
19	1	12.56	0	68	28	0	2	928	0
20	1	6.53	0	17	40	1	3	300	0
21	0	2.2	0	22	20	0	1	0	0
22	1	39.89	0	36	33	2	5	6196	1

23	1	5.79	0	4	50	0	3	0	0
24	1	26.65	0	50	35	1	3	1070	0
25	0	6.17	0	36	42	2	1	0	0
26	1	2	0	69	30	0	1	0	0
27	0	87.2	0	28	65	2	11	32365	1
28	1	10.67	1	29	90	2	4	10869	1
29	1	10.93	0	13	25	1	2	1200	1
30	1	12.38	0	39	30	0	2	500	0
31	1	53.97	0	22	70	1	9	18474	1

	Group 1 n=30	Group 2 n=23	Group 3 n=31
Sex M: F	17:13	13:10	23:8
Days on unit, mean (range)	34.8 (12-154)	22.39 (1-89)	16.8 (2-53)
No. of patients that died	7	3	6
Age, mean (range)	38 (1-76)	37.18 (1-72)	36.67 (1-70)
TBSA%, mean (range)	45.3 (16-95)	32.91 (7-60)	41.19 (17-90)
MOF score, mean (range)	3.13 (1-5)	1.73 (0-5)	1.16 (0-3)
Theatre visits	4.26 (1-12)	3.21 (0-13)	2.77 (0-11)
Days unit acquisition of <i>Acinetobacter</i>	11.13 (1-95)	7.73 (1-29)	N/A
Volume of blood transfused pre <i>Acinetobacter</i> acquisition (mls)	4000 (0-28222)	2254 (0-20402)	N/A
Volume of blood transfused post <i>Acinetobacter</i> acquisition (mls)	3905 (0-26135)	2328 (0-10359)	N/A
Total blood volume transfused (mls)	7897 (993-33971)	4639 (0-28867)	3652 (0-18474)
Inhalation injury	13	4	13

Table 5.6. Demographic and statistical data to compare groups 1, 2 and 3.

To allow an easier comparison between the *Acinetobacter* and control groups the results from table 5.3, 5.4 and 5.5 have been summarised above.

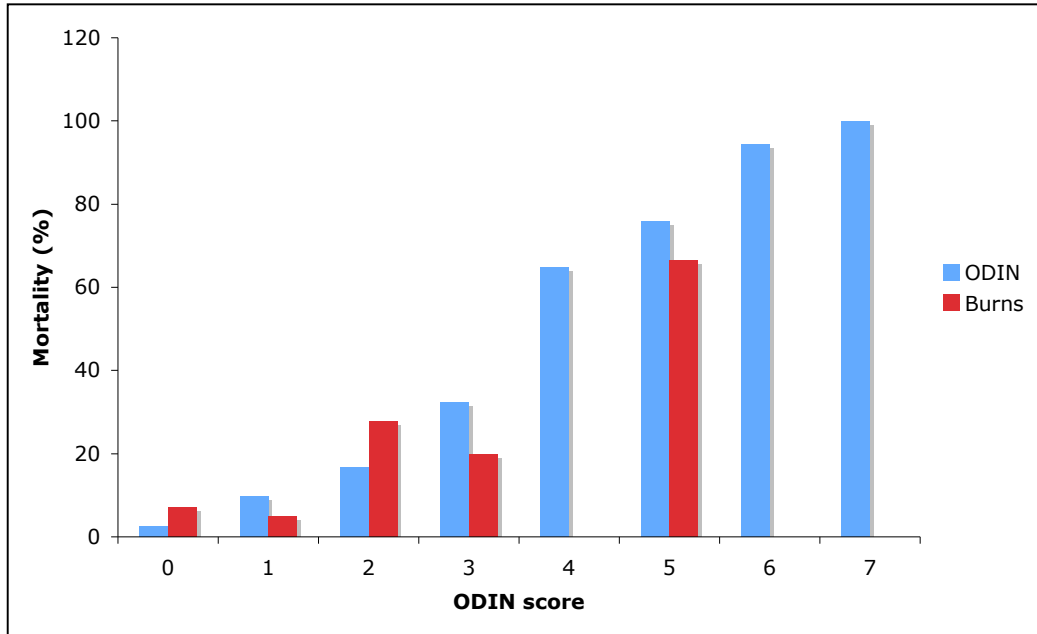


Figure 5.3. Mortality and Multi-organ Failure compared with the original study by Fagin et al., (2003). A table comparing the mortality rate for each MOF score in the study (in red, labelled burns) with the original study by Fagon et al., (1993), in blue labelled ODIN.

Figure 5.3 compares the mortality for each MOF score in our study with that in the original study by Fagon et al., (1993). Although no patient in our study obtained an MOF score of 6 or 7 and no patient with a score of 4 in our study died the results show the same progression; as the MOF score increases so does the mortality. This is one way of attempting to validate the use of the ODIN score as our study appears to identify the number of patients dying for each MOF score in roughly similar proportions as the original study by Fagon et al., (1993).

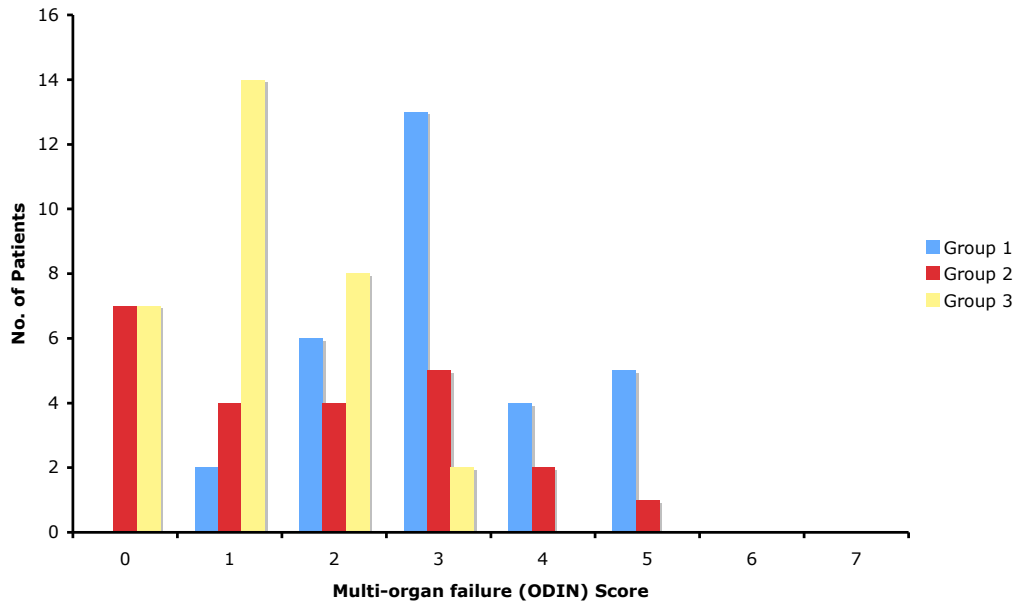


Figure 5.4. A graph showing the ODIN scores for all three groups. The data shows the number of patients getting an individual ODIN score for each group studied.

It can be seen in figure 5.4 that in group 1, no patient ever obtains an MOF score of one and the majority of patients have a score of three or over. The maximal score is 5. Group 2 also has a maximal MOF score of 5 but seven patients have an MOF score of 0 and the scores are more evenly distributed. Finally, the majority of patients in Group 3 have low MOF scores with only two patients achieving a score of three. The data suggests that the severity of injury/illness is greatest in Group 1 patients and is less in Group 2, which in itself displays greater severity of injury than Group 3 (the least injured group).

To determine whether there was a relationship between the acquisition of *Acinetobacter* or not and the development of multi-organ failure the multiple organ failure means were compared for the three groups, MRA, drug sensitive *Acinetobacter* and the control group (Groups 1, 2 and 3 respectively).

Analyses have been performed using the computer program R (R development core team). Bootstrapping has been performed using functions `one.boot` and `two.boot` from R package `simpleboot` (Peng 2008). Bias-corrected and accelerated confidence limits have been obtained using function `boot.ci` from R

package boot (Canty and Ripley 2011, Davidson and Hinckley 1997). The permutation test for one-way analysis of variance has been done using function `aovp` from R package `lmPerm`.

The main analysis used here is analysis of variance, and also multiple regressions to produce an analysis of covariance (performed by Dr Mike Parker).

Firstly the three groups were compared without any attempt to allow for any differences between the groups (Appendix III, section 2) and then an analysis of covariance (Appendix III, section 3 and section 4) was performed to provide the estimated differences between the groups adjusted for the covariates. This is because many of the other covariates, sex, age TBSA%, blood volume transfused and inhalation injury are factors that may explain any differences between the groups rather than the presence or not of *Acinetobacter*.

The details of the analysis are presented in Appendix III

In summary the bootstrap confidence limits for the differences between the means indicate that the mean MOF score is higher for the MRA group (group 1) 1.394 (CI 95%: 0.617, 2.155) than for the Drug Sensitive group (group 2). This is statistically significant. The mean MOF score is also higher for the MRA group than for the no infection group (group 3) 1.972 (CI 95%: 1.230, 2.640). Again this is statistically significant. The difference between the means for the group 2 and group 3 is not statistically significant 0.578 (CI: -0.195, 1.359).

The covariate, which explains most variation in the MOF score, is the volume of blood transfused. In Appendix III section 3, blood volume transfused is used as a covariate to adjust the differences between the means of the total number of organ failures. The adjusted differences between the means are all smaller than the unadjusted differences shown in Appendix III, Section 2, and table 3. Despite this, the conclusions are the same.

An analysis was then performed examining all the covariates (Appendix III, Section 4). The problem with this is that some of the covariates might not contribute to the model and would therefore increase the standard errors in the

model and would produce a more complex model. This now demonstrates that there was a statistical difference between all three groups.

A final model was created looking at the covariates that produced the greatest variance in the results, the total volume transfused and the number of theatre visits (Appendix III, Section 5). This analysis confirms the initial findings of the differences between the groups as detailed in Appendix III tables 3 and 6.

In summary the analysis of covariance provided estimated differences between the groups means adjusted for the covariates. However, the size of the differences is determined by which set of covariates is used. The best fit is provided using the covariates blood volume transfused, number of operating theatre visits, and the interaction between them. This accounts for 48.0% of the variance. When adding the factor for groups this goes up to 60.0%. The sizes of the differences between the means adjusted for the covariates, with their bootstrap multiplicity-corrected 95% confidence intervals (CI) are MRA – Drug-Sensitive 1.001 (CI: 0.395, 1.588), MRA – No-infection 1.229 (CI: 0.615, 1.811), and Drug-Sensitive – No-infection 0.228 (CI: – 0.351, 0.829). Thus the first two differences are statistically significant, but the third one is not.

Therefore Multi-drug resistant *Acinetobacter baumannii* does cause multi-organ failure in the burn ICU population but *Acinetobacter* infection (drug sensitive) in itself does not.

One of the interesting points that came out of the analysis was the influence of the total volume of blood transfused. Blood transfusions have previously been described as a risk factor for infections in burns patients (Graves et al., 1989). In a general ICU setting, multivariate analysis showed transfusion as an independent risk factor for infection, mortality, hospital and ICU length of stay. In addition *A. baumannii* accounted for a disproportionate share of infections among transfused patients ($P < 0.001$) (Rachoin et al., 2009).

5.4.3 Volumes of blood transfused and the relationship to multi-organ failure

Further analysis was performed to investigate the influence of blood transfusion before and after the acquisition of infection. The summary tables of the statistical data used to analyse blood volume and the relationship to Acinetobacter acquisition are in Appendix III section 6, tables 11-14.

The recommended method for the comparison of groups in terms of a change from pre- to post-event is to regress the post-event values on the pre-event values (Dr Mike Parker, Anglia Ruskin University). This has been done for pre- and post-acquisition of infection in the graph below.

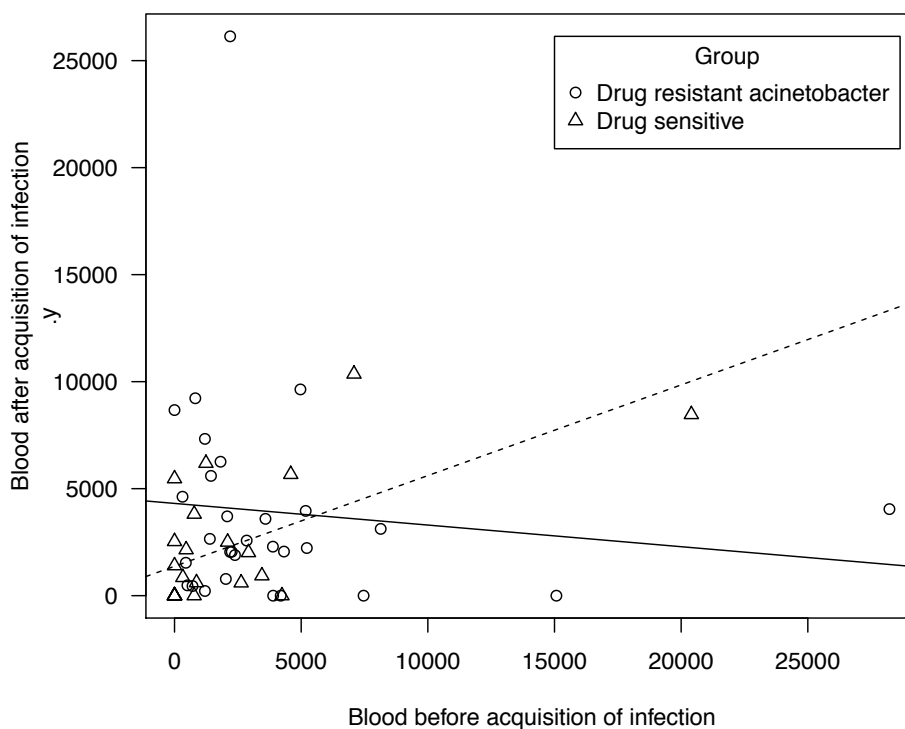


Figure 5.5 Scatter plot showing the regression lines for blood volume transfused after acquisition of infection against blood volume transfused before acquisition of infection. The data shows the regression slope for Drug resistant Acinetobacter was -0.01; boot strap 95% CI (-0.285, 0.575) shown by the solid line. The drug sensitive Acinetobacter regression slope (dashed line) was 0.424; boot strap 95% CI (0.286, 0.729) Analyses have been performed using the computer program R (R development core team). Bootstrapping has been performed using functions one.boot and two.boot from R package simpleboot (Peng 2008).

Whilst figure 5.5 appears to show that the regression is statistically significant for the drug sensitive group the slope of this line is largely dependent in this data set on a single point and so the regression relationship is not very convincing. Therefore looking at the graph one must conclude that the blood volume transfused before and after the acquisition of infection is not related.

A further way of looking at this is by looking at the difference between the means of the blood volumes pre-acquisition of *Acinetobacter* and the means of the blood volumes post acquisition of *Acinetobacter*, this is shown below in tables 5.7 and 5.8.

Group difference	Difference between means	Standard error	95% confidence limits	
			Lower	Upper
Resistant – Sensitive	1745	1359	- 1196	4266

Table 5.7 Difference between means of pre-acquisition blood volume with bootstrap standard error and 95% confidence limits. The means of blood volume transfused prior to *Acinetobacter* acquisition are compared between the drug resistant and drug sensitive groups. Bootstrapping shows the 95% confidence limits enclose zero, which confirms that the difference is not statistically significant (function `boot.ci` from R package `boot` (Canty and Ripley 2011, Davidson and Hinckley 1997)).

Group difference	Difference between means	Standard error	95% confidence limits	
			Lower	Upper
Resistant – Sensitive	1577	1157	- 785	3750

Table 5.8 Difference between means of post-acquisition blood volume with bootstrap standard error and 95% confidence limits. The means of blood volume transfused post *Acinetobacter* acquisition is compared between the drug resistant and drug sensitive groups. The bootstrapped 95% confidence limits enclose zero, which confirms that the difference is not statistically significant.

Tables 5.7 and 5.8 show that there is no statistical significance between the means of the blood volumes transfused prior to and post *Acinetobacter* acquisition. This confirms the earlier analysis shown in figure 5.5.

Further analysis looking at the variance between the groups is given in appendix III, Section 7 and could be used for more detailed analysis (not performed). In addition post acquisition blood volumes and post- minus pre-acquisition blood volumes found no difference between the groups. Full analysis is detailed in Appendix III section 4.

5.4.4. Organisms isolated concurrently with *Acinetobacter baumannii*

A growing field of research is into competitive strategies between species of bacteria. It has revealed that there are diverse mechanisms by which bacterial species can coexist with, or dominate, other organisms competing for the same pool of resources. As well as engaging in intraspecies competition bacteria can also participate in cooperative behaviours (Hibbing et al., 2010). To see if there was any evidence that *Acinetobacter* may be involved in either cooperative or competitive behaviour with any other microorganisms we examined the organisms that were isolated at the same time as the *Acinetobacter* samples, figure 5.6. At a simple level it was thought that isolation of a particular organism at high frequency at the same time that *Acinetobacter* was isolated might suggest that they work in cooperation for example. It may be that any cooperative or competitive behaviour is different for drug sensitive and drug resistant *Acinetobacter*,

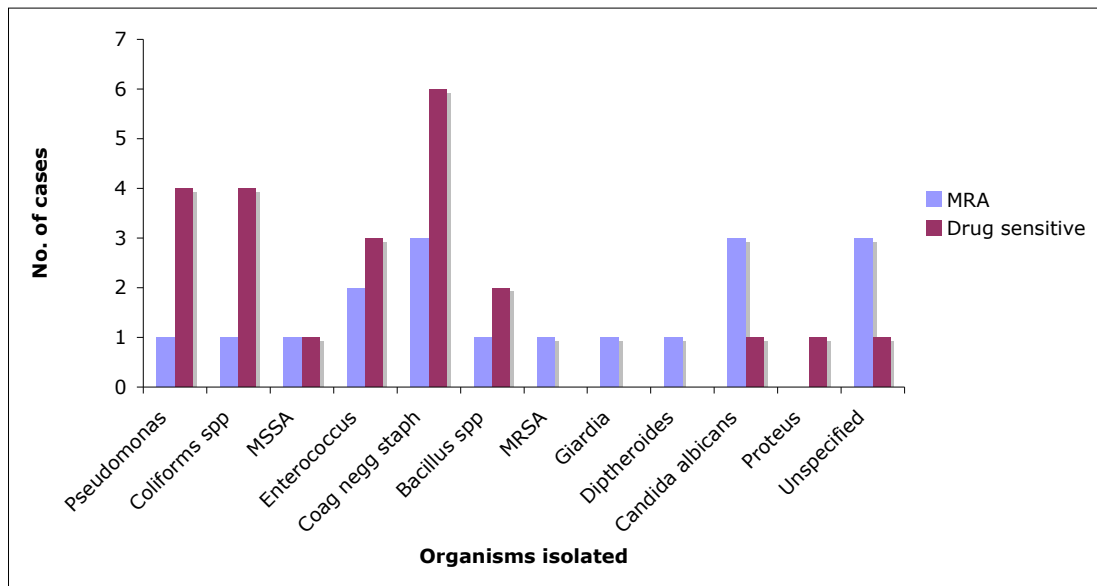


Figure 5.6 Organisms isolated at the same time as *Acinetobacter* samples.

The data shows the frequency and type of organisms isolated at the same time as positive *Acinetobacter* samples in the MRAB group (blue) and Drug sensitive group (purple). The data was obtained from the *Acinetobacter baumannii* database.

To see if the presence of other organisms had any influence on *Acinetobacter* acquisition all bacteria isolated at the same time as *Acinetobacter* were recorded, figure 5.6. Of the 30 patients within group 1, seventeen had one or more organisms isolated at the same time. Within this group of 20 patients ten different organisms or species were identified. Within group 2, eighteen patients had one or more organisms isolated at the same time. The numbers are too small to draw any meaningful conclusions but there is no obvious pattern or difference between the two groups. A larger sample size is required to detect any potential links between *Acinetobacter* and other microorganisms.

5.4.5 Agency Nursing Staff as a contributing factor to MRA acquisition.

From the start of what was considered the beginning of the *Acinetobacter* outbreak until the end of the main outbreak other factors were examined that may explain the persistence of *Acinetobacter* on the unit despite measures to prevent transmission and increased vigilance.

In a one year period eighty four patients from the burns unit had a positive wound swab, sputum or blood culture for *Acinetobacter baumannii* and of these, 19 were found to be multi-resistant (Table 5.9).

	Percentage of Agency staff employed on the burns unit (%)	No. of cases of Multi Resistant <i>Acinetobacter Baumannii</i>
April-June 2004	42	4
July-Sept 2004	43	5
Oct-Dec 2004	27	3
Jan-March 2005	64	7

Table 5.9. MRA and agency staff. The data shows the number of MRAB cases during each quarter of the study period compared with the number of agency staff.

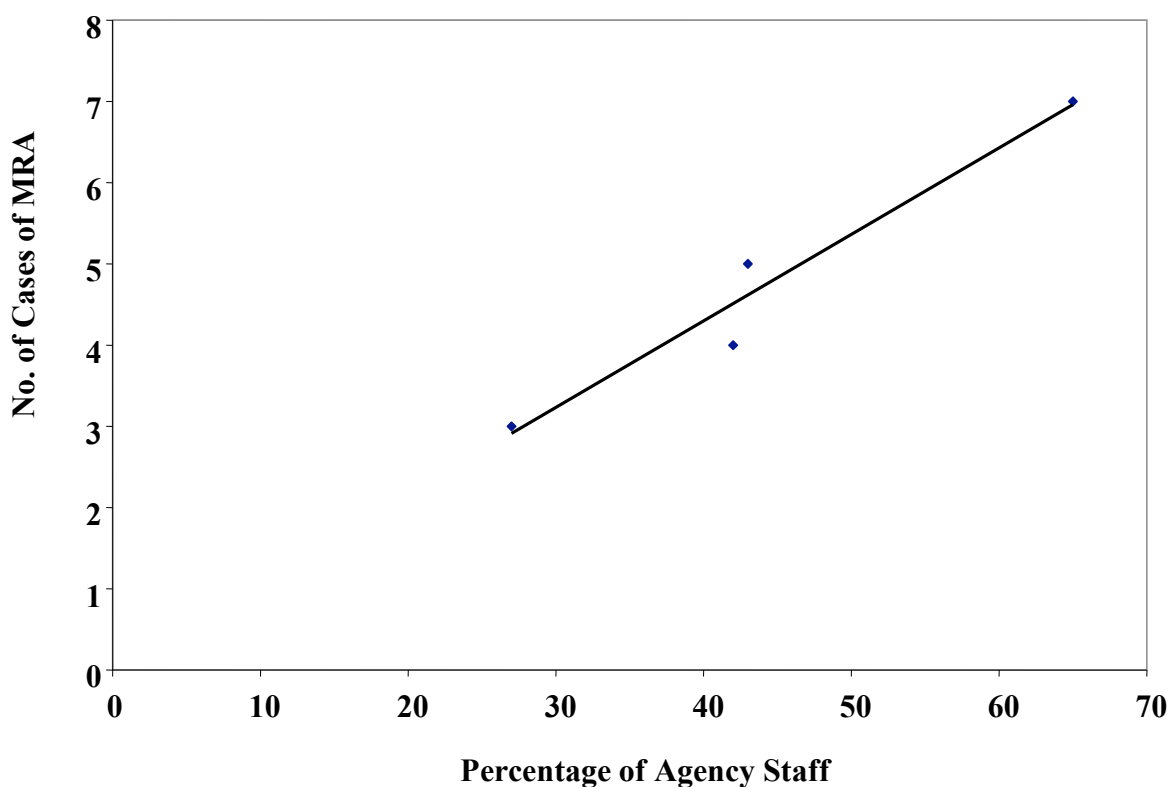


Figure 5.7. A scatter plot of the number of cases of Multi-resistant *Acinetobacter Baumannii* and percentage of agency staff. This graph looks at the number of cases of MRAB between April 2004 and March 2005 in each quarter of that year compared with the percentage of agency nursing staff used on the unit. The trendline shows the correlation coefficient r to be 0.95 ($p < 0.05$). This is statistically significant. (Excel 2007)

To see if there was any link between the MRA cases and use of agency staff the data from table 5.9 was plotted and the correlation coefficient plotted in figure 5.7. Investigating staffing levels between April 2004 and Mar 2005 established a direct correlation between the number of agency staff employed and the rate of infection of patients with multi-resistant *Acinetobacter baumannii*. Data was analysed using Pearson Correlation Coefficient. Correlation is a technique for investigating the relationship between two quantitative, continuous variables, for example, age and blood pressure. Pearson's correlation coefficient (r) is a measure of the strength of the association between the two variables. Pearson's correlation coefficient (r) for continuous data ranges from -1 to +1. A value of 0

would indicate no correlation between the variables and 1 would indicate a perfect correlation between the variables. An r-value of 0.95 shows a strong correlation between the number of cases of MRA and the number of agency staff used. The square of the sample correlation coefficient, denoted r^2 is called the coefficient of determination and estimates the fraction of the variance in Y that is explained by X in a simple linear regression. The r^2 for the above data is 0.89. This means we can say that 89% (0.95^2) of the variation in MRAB rates is explained by the percentage of agency staff.

5.4.5 Bed Occupancy on Unit as a contributing factor to MRA acquisition.

Another factor identified that may influence MRA acquisition was bed occupancy. Table 5.10 shows the quarterly data for bed occupancy and the number of cases of MRA during the study period.

Study Period	Percentage of Occupied Beds (%)	No. Of Cases of multi-resistant <i>Acinetobacter</i>
April-June 2004	46	4
July-Sept 2004	58	5
Oct-Dec 2004	43	3
Jan-March 2005	61	7

Table 5.10. The number of cases of MRAB compared with the bed occupancy. The data shows the quarterly number of cases of MRAB and the percentage of occupied beds from April 2004 to March 2005.

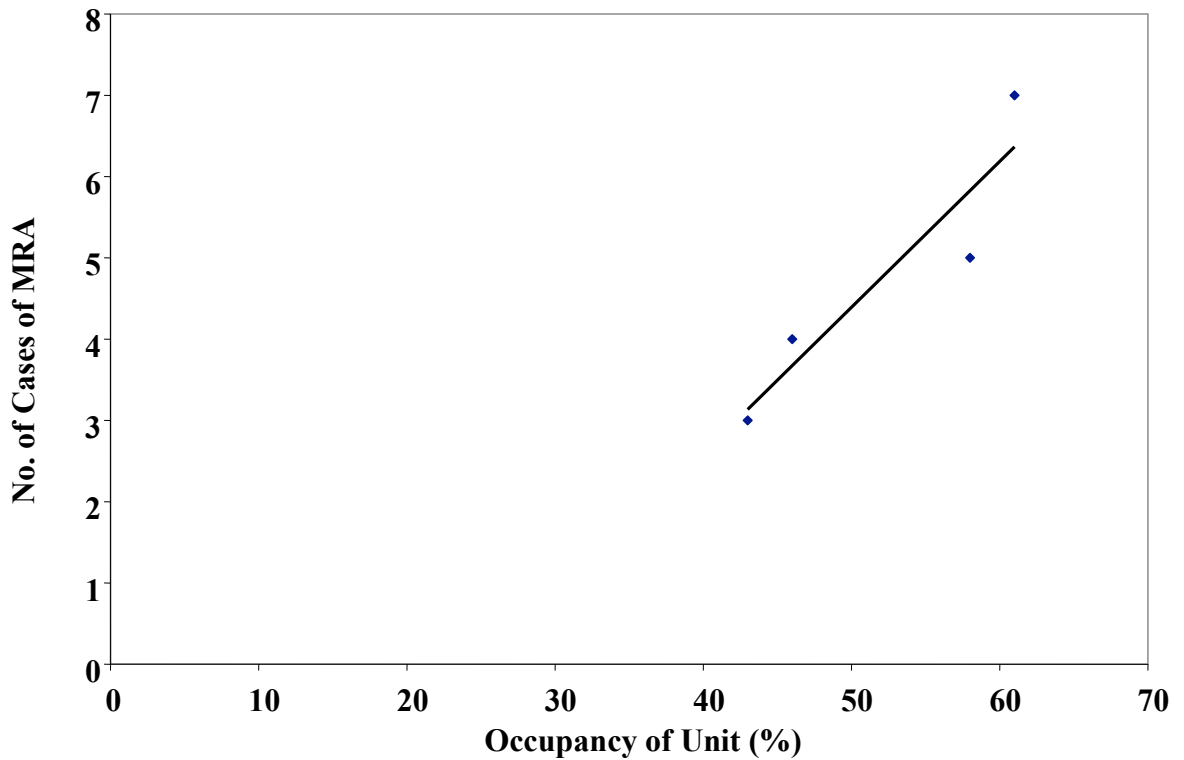


Figure 5.8. A scatter plot of the number of cases of Multi-resistant *Acinetobacter Baumannii* against bed occupancy. This graph looks at the number of cases of MRAB between April 2004 and March 2005 in each quarter of that year compared with the occupancy of the unit (%). Data was analysed using Pearson Correlation The trend line shows the correlation coefficient r to be 0.92, ($p < 0.05$) which is statistically significant. (Excel 2007).

The trendline in figure 5.8 shows the correlation coefficient r to be 0.92. This demonstrates there is a strong positive correlation between the number of cases of MRAB and the occupancy ($p < 0.05$). As the occupancy increases the number of cases of MRAB also increase. The coefficient of determination (r^2) is 0.86 and shows that 86% of the variance in the number of MRAB cases is explained by the occupancy of the unit. We can therefore see that both the number of agency staff and how busy the unit is play a strong role influencing the rate of MRAB.

5.5 Discussion

Burn wound infection and *Acinetobacter baumannii*.

One of the main complications of burn wounds is infection. Sepsis and multi-organ can potentially lead to death of the patient but more frequently results in prolonged hospital stays and possible poorer outcome in terms of function, psychological aspects and aesthetics. Despite advances in assessment, management, surgical treatment and vital antibiotic therapies over the last 40 years infections and their sequelae are still a major contributor to the mortality figures for a burns unit.

Without the patient developing sepsis, infections can still have a large impact on patient outcomes. Wound infections can increase the depth of superficial burns resulting in a greater likelihood of surgery or scarring. A wound bed that is infected and inadequately prepared will result in grafts not 'taking' and wound infections can destroy established grafted skin both of which result in longer in-patient stays.

Whilst improvements in burn care have been made, new hurdles have arisen that continually challenge the physician. Indiscriminate use of antibiotics both in hospitals and the community has resulted in a generation of organisms that are resistant to most mainstream antibiotics. The status of nosocomial infections has risen to national importance with frequent reports being in the media. The combination of these factors has resulted in major problems for both the hospitals and patients.

Although methicillin resistant *Staphylococcus aureus* (MRSA) and *Clostridium Difficile* have received the majority of the press attention it is clear that a number of other organisms frequently acquired in the hospital setting are having a significant impact on patient care due to their bacterial resistance.

One organism that is achieving a more prominent profile in hospitals and particularly burns units and ITUs is the gram-negative bacteria *Acinetobacter baumannii*. Its importance has only been realised relatively recently and few studies have actually assessed what impact it is having on patient morbidity and mortality in burn patients.

This study is therefore both topical and important as it sheds new light on the potential severity of a multi resistant *Acinetobacter* outbreak and demonstrates its association with multi-organ failure.

5.5.1 Epidemiology

Acinetobacter baumannii has been found on the burns unit for a number of years but the number of cases that were MRA numbered few and the majority were drug sensitive. Figure 5.1 shows that only 12 patients had *Acinetobacter* isolated in 2000 but that this had risen to 78 cases by 2001, 12 of which were MRAB.

In figure 5.1 1 it can be seen that there is a significant drop in the number of cases of *Acinetobacter*. During 2001 there was a strain of multi-resistant *Pseudomonas* isolated from several patients on the burns unit. After investigation it was thought that staff hand hygiene was a major contributing factor to transmission of the organism. A strict hand washing policy was implemented and in time the *Pseudomonas* outbreak was stopped. A by-product of the strict hand-washing regime was the reduction in the number of cases of *Acinetobacter* suggesting that the continued presence of *Acinetobacter* on the unit could be due to transmission by members of staff rather than multiple sporadic outbreaks. It can be seen that by 2003 there was an almost complete eradication of *Acinetobacter* from the unit, however without knowing the total number of patients admitted each year one cannot establish the true incidence per year. It is possible that there was a reduction in the number of patients admitted over this time period. A further outbreak of MRA occurred in 2004-2005, this will be discussed later.

5.5.2 Seasonal Variation

Throughout the study period (2000-2006) there was a marked seasonal variation with *Acinetobacter* being more frequent between October and March than the summer months (Figure 5.2). This is in contrast to previous studies. In the Spain, Pedraza et al., (1993) found no seasonal variation after a two and a half year study period; this picture was echoed in America by Seifert et al., (1995) who also found no seasonal variation. Elsewhere in America studies showed a greater number of cases in the summer months (Christie et al., 1995) and during July to October (McDonald et al., 1999).

It is unclear from our current data why this might be. Further analysis may show whether a particular strain of *Acinetobacter* is responsible or if there were any factors within the hospital that may have contributed i.e. changing in staffing patterns or characteristics of the patients admitted to the unit, for instance more patients from war zones at certain times of the year.

5.5.3 MRA Incidence and Acquisition

This study does not tell us where the strains originated. There are several potential agents. Firstly there may be a member of staff or a piece of equipment acting as a reservoir of infection. Secondly *Acinetobacter* may be introduced to the unit by a patient. Patients are not only admitted directly to the unit but are also transferred from other hospitals where surveillance of such organisms may not have been conducted. In addition patients are sometimes transferred from abroad and these patients tend to be from either war zones or as a result of industrial accidents (sometimes in remote areas), which increase the chance of acquisition of *Acinetobacter* as normal patterns of emergency care may not be instituted. *Acinetobacter* is the most common organism isolated from infected wounds in US army personnel in the most recent war in Iraq and with many isolates being multi-drug resistant (Petersen et al., 2007).

The total incidence of MRA between April 2004 and June 2006 was 10.7%, which compares favourably with the rates found in other burn units or in the ITU setting, 11.5% (Sengupta et al., 2001), 13% (Simor et al., 2002) and 14% (Santucci 2003). In previous studies a range of factors have been linked to the acquisition of MRA including peripheral artery catheters, mechanical ventilation, or pulmonary artery catheters (Beck-Sague et al., 1990). Mortality due to *Acinetobacter baumannii* has been associated with recent surgery, urinary catheterization, nasogastric tubes and well as the factors listed above (Chen et al., 2005) Whilst this study was not able to analyse all these factors that contribute to acquisition or mortality it looked at MRA and multi-organ failure.

It has been shown that the mortality rate is increased in patients with MRA (Cisneros et al., 1996) with mortality being higher in those infected rather than colonized (Rodríguez-Baño et al., 2004). This study is the first to show an association between MRA and multi-organ failure. This is an important finding

because multi-organ failure is associated with a greater morbidity and mortality (Cumming et al., 2001). If the patient does not die, multi-organ failure means not only poorer outcomes for the patient (physically and psychologically) but has important financial implications in the short term for the hospital and in the long term for the patient and the community. Wilson et al., (2004) established that the mean hospital costs of patients that acquired MRAB was \$98,575 higher than that of control patients who had identical burn severity of illness indices.

The average mortality of patients with *Acinetobacter* was 18.86% (Group 1 23.33%, Group 2 13.04%) which is lower than that reported in some other studies. Trottier et al., (2007) describe a 33% mortality rate in patients with *Acinetobacter* (a 30% rate is attributed to patients with active infection as one patient died of a gastrointestinal bleed). In other studies reported in burns units the mortality rates vary from 22% (Chen et al., 2005) to 4.42% (Sengupta 2001). It is impossible to compare these figures with the mortality rate in group 3 as this groups was matched as closely as possible to group 1 (in terms of age of patient and TBSA% sustained) and therefore may represent a skewed populations of those patients with no *Acinetobacter* infection.

Data shown in figure 5.3 compares the mortality of the patients in the original ODIN study (Fagon et al., 1993) with the patients in all three groups of the current study. It can be seen that the mortality figures approximately follow that of the original study. It may be argued that a major burn wound is unique in its characteristic and thus a scoring system used for general medical problems is not appropriate. There are many different scoring systems used to assess multi-organ failure, injury or trauma or indeed combinations of these, at the time of the study the consensus paper by the American Burn Association (Greenhalgh et al., 2007) had not been published. Therefore commonly used systems were considered in consultation with both one of the lead anaesthetists of the burns ITU and the lead burns surgeon. These were generally thought to be firstly too complicated and that some of the data required would be too difficult to retrieve retrospectively from the patient records.

In the end a balance was struck for a system that looked at all the major organ systems of the body and would allow collection of data that would be found in the records of all patients being admitted to the unit. The ODIN scoring system was therefore selected for this study.

5.5.4 Transmission of *Acinetobacter* - possible contributing factors

In our unit, a previous outbreak of multiresistant *Pseudomonas aeruginosa* in 2001 was thought, after extensive investigation, to be partly due to transmission via the hands of the staff. A strict hand washing policy was implemented using senior staff as opinion leaders (those who influence the behaviour of others) and as a result the unit had no further significant outbreak of multiresistant *Pseudomonas* for two years. This policy appears to have had the knock-on effect of reducing the rate of *Acinetobacter baumannii* infection over the subsequent two years.

Unfortunately in April 2004 to March 2005 there were a number of cases of MRA in the unit despite the fact that the hand-washing regime implemented was still in place and the 'culture' of hand washing was firmly established. This study looked at other possible factors that might explain the increased number of cases of MRA over this period. Since nurses provide most of the care and manipulations to a patient on a burns unit, the level of care provided and adherence to protocol may be critical for the prevention of nosocomial infections. Previous reports have linked staffing grade and staffing levels with a greater number of infections and a higher mortality. We looked at the number of 'agency' or 'bank' health care workers employed in comparison to the number of full time regular staff. In addition this study looked at how busy the unit was. This was judged by the occupancy of the unit, as it was thought that when the unit was very busy staff might be more prone to error or more likely to let standards slip.

There are many factors that may be responsible nosocomial infections in the hospital setting. Our study suggests that in addition to the recognised factors associated with the spread of infection agency or bank nurses may be associated with a high rate of multiresistant *Acinetobacter* infections in a burns unit. Research on the relationship between staffing levels of nurses in hospitals and patients' outcomes however, are conflicting.

A number of studies have found that nurse staffing levels have a direct impact on a number of patient outcomes. Understaffing has been identified as a risk factor

for increased rates of healthcare associated infections (Arnow et al., 1982, Fridkin et al., 1996, Garfield et al., 2000). Conversely Mark et al., (2004) found increased nursing staff levels were associated with lower rates of urinary tract infections, decubitus ulcers, pneumonia and mortality. Other studies have also found the higher the ratio of registered nurses per bed is associated with lower mortality and failure to rescue (Silber et al., 2000) and that an inverse relationship between nurse staffing and mortality (Sasichay-Akkadechanunt et al., 2003) and failure to rescue exists (defined as the 30 day mortality in patients with complications plus deaths without a recorded complication) (Aitkin et al., 2002).

Further studies have established the importance of the experience level of the staff. McGillis et al., (2004) found that the lower the proportion of registered nurses on a unit and the less experienced the nurses, the higher the number of medication errors and wound infections. However a study by Jarman et al., (1999), like a number of other studies did not find a correlation between nursing numbers and any measure of mortality.

This is the first time a correlation has been found between infection rates and the use of agency or 'bank' nurses in a burns unit. The only comparative study in the literature examined a surgical intensive care unit. Robert et al., (2000) found that increased use of 'pooled nurses' (the American equivalent of bank or agency nurses) may be an important risk factor for nosocomial blood stream infections in a surgical intensive care unit despite the nurse to patient ratio being not significantly different between case patients and controls.

In our case there may be a number of reasons why agency staff may be associated with an increased risk of MRA acquisition. After a previous outbreak of MRA on the unit several years before a stringent policy of hand washing and hygiene was instituted. It may be that the increased MRA rate is due to the fact that agency staff may be less familiar and aware of departmental procedures that have maintained high standards. Even with limited training prior to working on the unit it is hard to seamlessly integrate into the ingrained 'culture' already prevalent on the unit.

The use of agency staff most frequently occurs when the unit is busy. It is during these times of increased workload that all staff are put under increased pressure. Pittet et al., (1999) found a number of factors that altered compliance with hand

washing in a teaching hospital. They found that procedures that generated most opportunities for hand washing (after patient care, drug preparation, after intravenous care and during house keeping) and procedures associated with a high risk of transmission (before intravenous or respiratory care and care between clean and dirty body sites) were associated with low levels of hand washing compliance. In addition hand-washing compliance was lower when the activity level was high. It may be that during times when there are increased agency staff compared with regular staff the work load may increase for the regular staff thus mimicking the effect of understaffing despite the nurse to patient ratio being unchanged.

This study also showed that there was an association between the occupancy of the unit and number of MRA cases. On some days the unit was running at 120% occupancy as patients were moved off the unit and new patients moved onto the unit.

Some of the possible reasons for the number of MRA cases increasing with the occupancy have been covered already, hand washing compliance being lower when activity is high and use of agency staff. Other potential reasons may include inadequate washing of the rooms between patients or staff not changing clothing between seeing a patient on the unit and a subsequent emergency admission.

5.6 Conclusions

Study limitations

One of the limitations of this study into *Acinetobacter baumannii* is that it is only looking at one unit within the hospital. It would be useful to see if this picture is replicated throughout the whole hospital or similar units. Furthermore, to obtain a more accurate picture it may be useful to know the ratio of agency to regular staff that worked with the individuals that became infected to more closely identify any trend. Unfortunately this level of information was not available. Another factor that could have been looked at was the data regarding other healthcare staff such as doctors. Although the number of doctors working in the unit over the time period studied are relatively few there are still times when locum staff are used and may therefore suffer the same problems with unfamiliarity with the unit procedures and policies. So many factors affect the acquisition of nosocomial infections that we cannot exclude some as yet undiscovered confounding factor that may explain our results.

Staff training is crucial to prevention of this problem to minimise the risk associated with increased use of agency staff. Burn Unit specific teaching for agency staff is essential. Other strategies to tackle this could involve targeting the patients at greatest risk of MRA and assigning the regular staff to these patients with agency staff being assigned to lower risk patients.

In conclusion this study demonstrates an association between MRA and multi-organ failure in a burns unit. In addition patients who acquired MRAB had more theatre visits, stayed longer in ITU and greater blood transfusion requirements. The consequences of MRAB acquisition and the development of MOF have serious implications for the unit in terms of workload and medical and nursing care but also financially for the hospital.

Continued strategies to combat nosocomial infections are required and the identification of high-risk patients may be ways of tackling the growing problem of MRAB in the burns ITU.

Chapter 6

Antimicrobial peptides and the role of the cathelicidin LL-37

6.1 Introduction

Mammals are constantly exposed to a variety of microorganisms and yet in healthy individuals rarely become infected because of the barrier function of the skin and epithelia. It is not only the physical barrier of skin prevents microbial entry, but also the presence of antimicrobial substances.

Antimicrobial peptides (AMPs) make up one of the most ancient defence systems within the animal and plant kingdoms. It has been suggested that their widespread distribution and function have been fundamental in the evolution of complex multi-cellular organisms. Their ancient lineage and continued effectiveness may contradict the theory that bacteria, fungi and viruses eventually develop resistance to all substances (Zasloff 2002).

In mammals, two forms of immunity have evolved. The first, the innate system is non-clonal and non-specific and the second is adaptive and antigen specific. Innate immunity is the first line of defence that is rapidly mobilized following the detection of microbial invasion. The effector branch of innate immunity consists of two major components: the recruitment and/or activation of leukocytes (e.g., phagocytic granulocytes, monocytes/macrophages, etc.) capable of combating the invading pathogens and the release and/or activation of a variety of extracellular humoral mediators (complement, cytokines and antimicrobial substances).

Adaptive immunity is induced when lymphocytes are activated in response to antigen (Ag) presented by antigen-presenting cells (APCs). T cell antigen receptors recognize antigenic epitopes bound to the major histocompatibility complex (MHC) on the surface of APCs. CD8⁺ T cells, once activated differentiate into cytotoxic T cells that directly kill cells infected by intracellular pathogens. The complex of MHC class II and antigenic epitope triggers the activation of CD4⁺, generating T-helper cells that, by producing various cytokines, promote B cell activation and enhance the efficiency of phagocytes to eliminate pathogens (Yang et al., 2004). The combination of these two systems

working together and in parallel is an effective solution in eliminating microbial invaders.

6.1.1 Antimicrobial peptides

AMPs were initially discovered on the basis of their antibiotic activity but they have been found to have additional biological properties that assist in modulating the host immune system including amongst others, stimulation of chemotaxis, suppression of proinflammatory cytokine production, promotion of angiogenesis and wound healing (Schauber and Gallo 2008 B). Because of these other properties, these peptides are now sometimes referred to as host-defence peptides (HDPs).

6.1.2 Antimicrobial peptide structure

There are now more than 1000 recognised AMPs (Mangoni 2011) and the range of AMPs is so great that it has been difficult to categorize them except in general terms on the basis of their secondary structure and amino acid composition. AMPs are 8–50 amino acids long and there are three main AMP structural groups: α -helical peptides, β -sheet peptides and extended peptides as illustrated in Figure 6.1

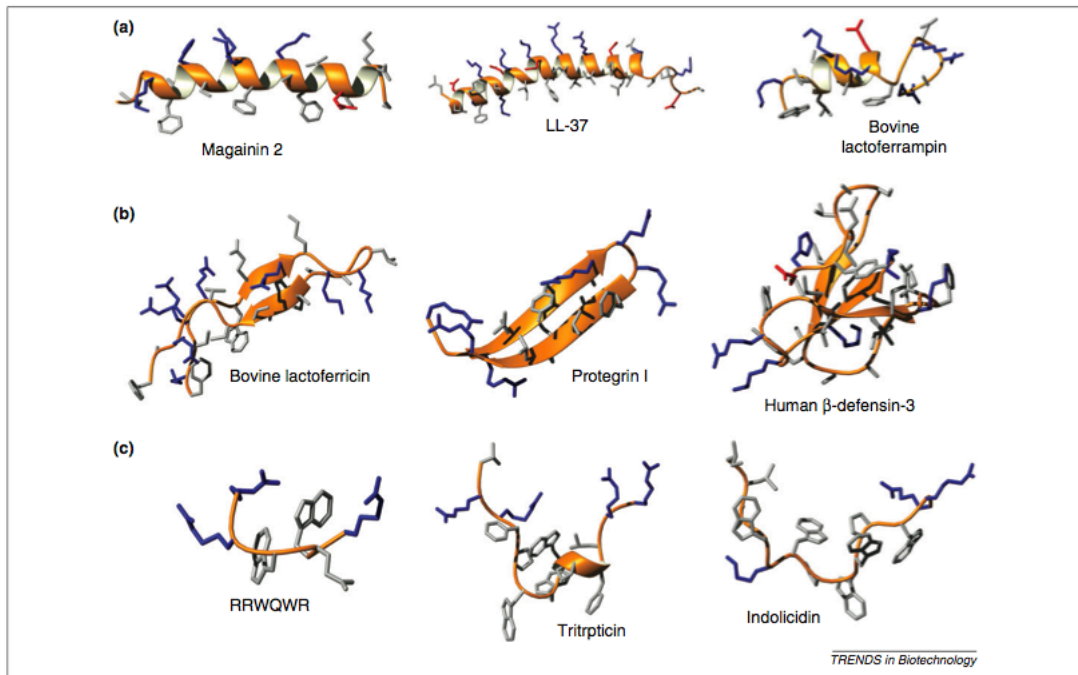


Figure 6.1. An overview of the major structural classes of antimicrobial peptides (AMPs). (a) α -Helical peptides, (b) β -sheet peptides and (c) extended peptides. Positively charged side chains are coloured in blue, negatively charged side chains in red and remaining side chains in grey (Nguyen et al., 2011).

6.1.3 Antimicrobial peptide mechanism of action

One of the reasons that AMPs are so effective is down to the physical characteristics of AMPs and the difference between the design of the membranes of microbes and multicellular animals.

Bacterial membranes have a lot of lipids and anionic components in their cell wall (e.g., endotoxin or lipo- polysaccharide, LPS, in gram-negative bacteria, and lipoteichoic acid, LTA, in gram-positive bacteria) with negatively charged phospholipids in their membranes (Mangoni 2011).

Lipids with no net charge, however, mainly populate the outer part of the membrane of plants and animals and most of the lipids with negatively charged headgroups are in the inner part of the bilayer (facing the cytoplasm) as shown in Figure 6.2.

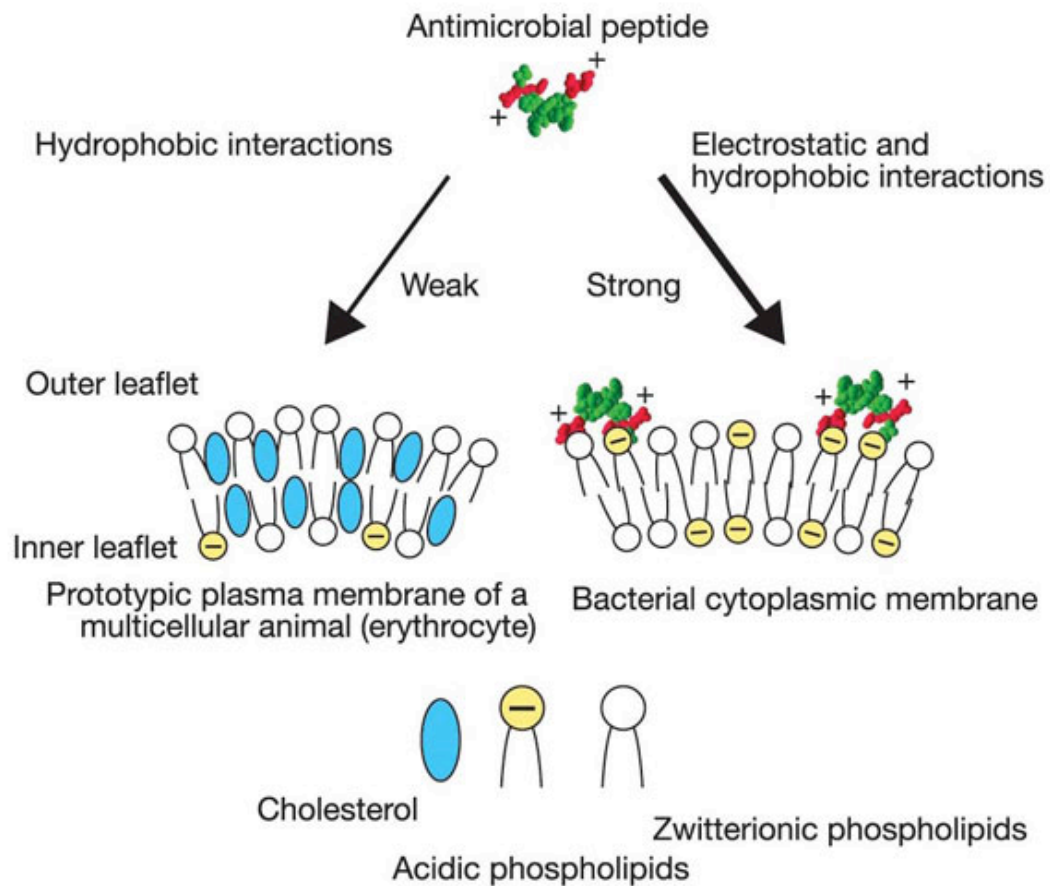


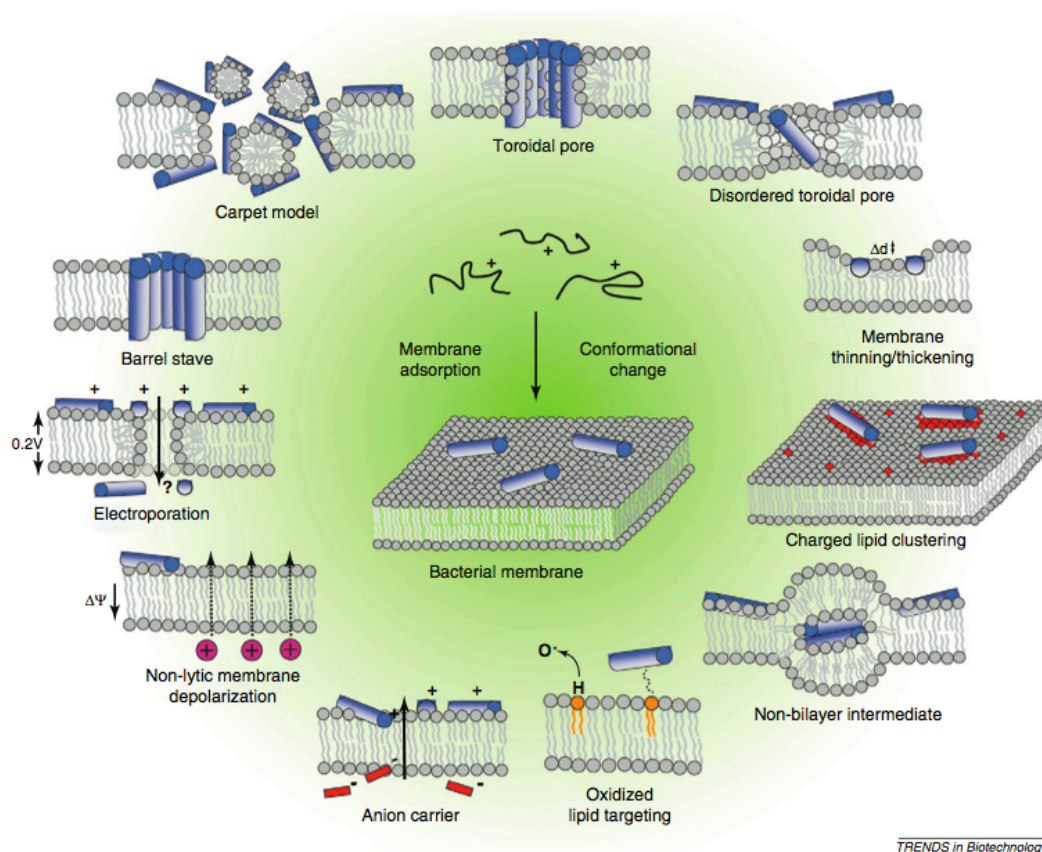
Figure 6.2. The membrane target of antimicrobial peptides of multicellular organisms and the basis of specificity (Zasloff 2002).

AMPs use this to their advantage. They possess firstly a cationic charge and secondly a significant proportion of hydrophobic residues. The positive cationic charge promotes selectivity for negatively charged microbial cytoplasmic membranes over zwitterionic mammalian membranes whereas the hydrophobic residues facilitate interactions with the fatty acyl chains (Nguyen et al., 2011).

The majority of AMPs work by killing microbes by disrupting their membrane and causing irreversible damage although other mechanisms have now been characterized that target key cellular processes including DNA and protein synthesis, protein folding, enzymatic activity and cell wall synthesis (Nicolas 2009).

The classical description of the mechanism of action of membrane disruption is the Shai-Matsuzaki-Huang (SMH) model (Zasloff 2002). In this the outer part of the bilayer membrane becomes covered with the peptide. The peptide becomes

integrated into the membrane resulting in displacement of lipids. This results in the outer part of the bilayer increasing in size compared to the inner layer (curvature strain), which can allow an intracellular influx of AMPs in some cases or fragmentation and physical disruption of the membrane. (Zaslhoff 2002, Nicholas 2009). A variety of other models have been proposed (as illustrated in figure 6.3) and it may be that individual AMPs may utilize different methods.



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Figure 6.3 Demonstrates the variety of models of membrane disruption that AMPs may use to fragment or physically disrupt the bilayer membrane.

Events occurring at the bacterial cytoplasmic membrane following initial antimicrobial peptide (AMP) adsorption. These events are not necessarily exclusive of each other. In the classical models of membrane disruption, the peptides lying on the membrane reach a threshold concentration and insert themselves across the membrane to form either peptide-lined pores in the barrel-stave model, solubilize the membrane into micellar structures in the carpet model, or form peptide-and-lipid-lined pores in the toroidal pore model. In the revised disordered toroidal pore model, pore formation is more stochastic and involves fewer peptides. The thickness of the bilayer can be affected by the presence of the peptides, or the membrane itself can be remodelled to form domains rich in anionic lipids surrounding the peptides. In more specific cases, non-bilayer intermediates in the membrane can be induced; peptide adsorption to the membrane can be enhanced by targeting them to oxidized phospholipids; a peptide may couple with small anions across the bilayer, resulting in their efflux;

the membrane potential can be dissipated without other noticeable damage; or conversely, in the molecular electroporation model, the accumulation of peptide on the outer leaflet increases the membrane potential above a threshold that renders the membrane transiently permeable to various molecules including the peptides themselves.(Nguyen et al., 2011)

6.1.4 AMPs in humans

AMPs are present at all human body sites normally exposed to microbes such as the skin and mucosal surfaces. Furthermore, some blood cell types including neutrophils, eosinophils and platelets contain large amounts of AMPs (Wiesner and Vilcinskas 2010). Production of AMPs may be constitutive, or frequently induced by inflammation or injury. AMPs are produced in varying amounts in different parts of the body as each tissue has its own profile of different AMPs that may vary significantly depending on physiological conditions. Certain AMPs predominate at specific body sites, but only a few of them are exclusively produced by a certain tissue or cell type.

Whilst a number of endogenous AMPs that play an important role in the defense of the skin have now been discovered (Table 6.1) two particular classes of peptide have been the subjects of much research, defensins and cathelicidins.

Peptide	Cellular source	Susceptible organisms	Comments
Dermcidin	Eccrine sweat glands	Broad-spectrum	principal sweat antimicrobial peptide; -not inducible by injury or inflammation
Psoriasin	Keratinocytes, sebocytes	Gram-negatives <i>E. coli</i>	-most abundant antimicrobial peptide in healthy skin; -induced by <i>E. coli</i> flagellin
rNase 7	Keratinocytes	<i>Enterococcus faecium</i> broad-spectrum	-antimicrobial activity independent of rNase activity
Cathelicidin (LL-37)	Keratinocytes, sebocytes	Gram-positives Gram-negatives	-induced by injury or inflammation; -also plays a role in wound healing
hBD-1	Keratinocytes, sebocytes	Gram-negatives	-constitutively produced at low amounts
hBD-2	Keratinocytes	Gram-negatives	-induced by injury or inflammation
hBD-3	Keratinocytes	Broad-spectrum	-induced by injury or inflammation
hBD-4	Keratinocytes	Gram-positives Gram-negatives	-inducible in primary keratinocytes in vitro
SLPi	Keratinocytes, hair follicles, eccrine and apocrine sweat glands, sebocytes	Broad-spectrum	-upregulated during inflammation; -also functions as an inhibitor of neutrophil elastase and cathepsin G; -plays a role in wound healing probably dependent on its antiprotease activity
Adrenomedullin	Keratinocytes, sebocytes, hair bulb cells	Gram-positives Gram-negatives	-pluripotent peptide also involved in wound healing and various other processes

Table 6.1. Antimicrobial peptides of human skin (Wiesner and Vilcinskas 2010).

6.1.5 Defensins

Defensins are a widely dispersed family of anti-microbial peptides (AMPs) that like cathelicidins are expressed by keratinocytes and mucosal epithelial cells (including those of gastrointestinal, genitourinary and pulmonary epithelia). They are cationic peptides containing 6-8 cysteine residues that form disulfide bridges (Milner and Ortega 1999) The alignment of the bridges determines the division of defensins into three subfamilies α -defensins, β -defensins (only present in humans) and θ -defensins.

α -defensins

α -defensins were first discovered in the granules of white cells. Human neutrophils express 4 distinct α -defensins (1-4) that are also known as human neutrophil peptides 1 to 4 (Liu et al., 1998). These peptides alter the host innate immune response to pathogens in addition to their anti-microbial properties.

β -defensins

Have 6 cysteine motifs connected by 3 disulfide bridges. The difference in spacing of these disulfide bridges determines the classes of defensins. The disulfide bonds of β -defensins are C1-C5, C2-C4 and C3-C6 (Izadpanah and Gallo 2003).

β -defensins have been identified in many cell types, including epithelial cells and neutrophils. Four types have been identified in humans HBD-1 to -4. HBD-1 expression appears predominately constitutive; HBD-2 expression is increased with inflammatory insults (Liu et al., 2002).

β -defensins have multiple other functions in addition to their anti-microbial properties. HBD-1 and 4 promote histamine release and prostaglandin D2 production in mast cells (Befus et al., 1999) and like LL-37 chemoattract monocytes and T-cells (Chertov et al., 1996). Niyonsaba et al., (2007) have shown a key role human beta defensins play stimulating epidermal keratinocyte migration, proliferation and production of pro-inflammatory cytokines and chemokines. Defensins -2, -3 and -4 increased the protein production of IL-6 and IL-10.

The normal levels of β -defensins are minimal but they increase in skin affected by inflammatory diseases.

6.1.6 Cathelicidins

Similar to defensins, cathelicidins act as pre-cursor molecules that can release an antimicrobial peptide after proteolytic cleavage. A key characteristic of the cathelicidin family of proteins is the presence of a highly conserved cathelin domain (Zanetti 2004). The cathelicidin proteins are characterized by a highly conserved N-terminal domain of about 100 amino acid residues. This 14 kDa cathelin-like domain is flanked by a signal peptide domain (approximately 30 residues long) on its N-terminus, and by an antimicrobial peptide region on its C-terminus (see figure 6.4). The single 16 kDa human cathelicidin is denoted as hCAP18.

The primary translation product is called the 'preprotein', since it contains a signal sequence as a prelude to the cathelicidin holo-protein. This signal peptide is cleaved off once it has fulfilled its purpose of targeting the cathelicidin to storage granules or to the exterior of the cell. The formation of two disulfide-bridges finalizes post-transcriptional processing. The cathelicidin holo-protein is then referred to as the 'pro- protein', since it does not represent the active form, but a storage form instead. Only after cleavage of the proprotein into the cathelin domain and the cathelicidin-derived AMP, does it unfold its host of activities (Durr et al., 2006).

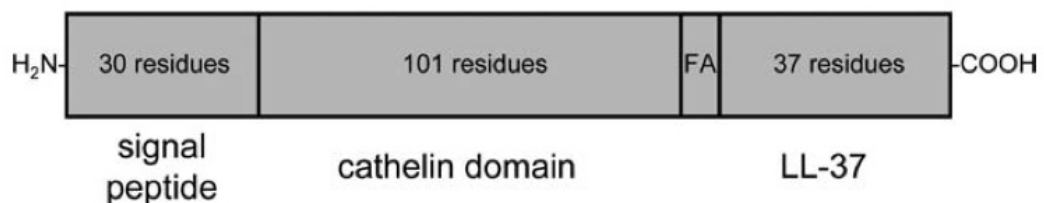


Figure 6.4. A simplified representation of the only human cathelicidin hCAP18 (Durr et al., 2006).

LL-37 is the only human cathelicidin-derived AMP, it belongs to the class of α -helical AMPs. LL-37 owes its name to its 37 amino acid overall length with the two leading residues being leucines.

6.1.7 Presence of LL-37 in the human body

LL-37 and its precursor, hCAP18, are found at different concentrations in very different cell and tissue types and body fluids, this probably reflects the fact that it acts not only as a peptide antibiotic but also as a signaling molecule.

LL-37 was first described in 1995 by two separate teams, in the testis by Agerberth et al., (1995) and in leukocytes by Cowland et al., (1995). Subsequently LL-37 has been found to be constitutively expressed in many different tissues, cells and fluids in the human body. In general these seem to be epithelial lined areas that will have close contact with the environment such as the skin (Marchini et al., 2002), lung epithelia, (Bals et al., 1998), salivary glands (Woo et al., 2003) and colonic mucosa (Tollin et al., 2003) for example. Of particular interest to this study is the presence of LL-37 in wound and blister fluid (Frohm et al., 1996).

LL-37 is up regulated in some disease states and down regulated in others. In a number of skin disorders such as psoriasis LL-37 is up regulated, but down regulated in both acute and chronic atopic dermatitis and there is an inverse correlation between severity of the disease and the level of AMP production (Ong et al., 2002). It has been postulated by Ong et al., (2002) that a deficiency in the expression of antimicrobial peptides may account for the susceptibility of patients with atopic dermatitis to skin infection with *S. aureus*.

6.1.8 LL-37 expression

At the cellular level, cathelicidins are most abundant in granules of neutrophils of (Turner et al., 1998); however, other cell types also express them, either constitutively or in an inducible manner. LL-37/hCAP18 is expressed by various epithelial cells and keratinocytes, (Dorschner et al., 2001, Frohm et al., 1999, Sorensen et al., 2003) as well as in monocytes, NK cells, B cells, and $\gamma\delta$ T cells (Agerberth et al., 2000), mast cells (Di Nardo et al., 2003), and eccrine glands (Murakami et al., 2002). Furthermore, LL-37 is induced in keratinocytes in response to inflammatory stimuli (Bals et al., 1998 and Frohm et al., 1997) or injury (Dorschner et al., 2001). Di Nardo's work (2003) found not only the

presence of cathelicidins in human mast cells, but the expression could also be up regulated by bacterial components such as LPS or lipoteichoic acid.

6.1.9 Cathelicidins and direct microbial activity.

LL-37 was initially recognised for its antimicrobial properties. It exhibits a broad spectrum of antimicrobial activity against bacteria, fungi, and viral pathogens, with microbicidal activity varying against different species and strains. Its activity against *Streptococcus*, *Staphylococcus aureus* and *Escherichia coli* has been demonstrated by Dorschner et al., (2001). Bals et al., 1998 has shown its activity against *Pseudomonas aeruginosa*. In addition it is effective against Spirochaetes (Sambri et al., 2002) and *Candida albicans* (Larrick et al., 1995) as well as many other organisms.

Nizet et al., (2001) provided some of the evidence for the antimicrobial properties of cathelicidin by creating knockout mice in which the gene coding for CRAMP, the mouse analogue of LL-37 was disrupted. These animals demonstrated diminished resistance to skin infections caused by Group A Streptococcus. With the finding of Ong et al., (2002) that in atopic dermatitis there is an inverse correlation between severity of the disease and the level of AMP production LL-37 seems to play an important defensive role in the skin due to its antimicrobial properties.

LL-37 shows a strong binding affinity for lipopolysaccharides (LPS) (Turner et al., 1998). LPS form the outermost membrane leaflet of Gram-negative bacteria. Released from those bacteria in sepsis, LPS is called 'endotoxin' and causes adverse effects like septic shock by inducing the production of higher concentration of systemic pro-inflammatory cytokines. There is evidence that LL-37 can neutralize the biological activity of LPS by binding with it and may therefore have a protective role against gram-negative sepsis.

Cirioni et al., (2006) used an animal model of gram-negative sepsis in which rats were given an intraperitoneal injection of *Escherichia coli*. The animals then received either LL-37 or antibiotics. Endotoxin and TNF-alpha plasma levels were significantly higher in conventional antibiotic-treated rats than in LL-37- and polymyxin B-treated animals.

6.1.10 Immunomodulatory functions of LL-37

In addition to its antimicrobial activity LL-37 has also been found to be involved in the modulation of the inflammatory response. LL-37 can exert both pro- and anti-inflammatory effects and can mediate these effects either directly by stimulating cells or indirectly by modulating the cellular response to a particular cytokine or signal. Alalwani et al., (2010) exposed human neutrophils were to LPS, *Staphylococcus aureus* and *Pseudomonas aeruginosa* subsequent to incubation with LL-37. The incubation with LL-37 significantly decreased the release of proinflammatory cytokines from stimulated human neutrophils as well as increasing the neutrophil generation of reactive oxygen species (ROS) and phagocytosis.

LL-37 can promote the recruitment of inflammatory cells directly by stimulating leukocyte chemotaxis (Yang et al., 2001) or indirectly by inducing IL-8 release by bronchial epithelial cells and airway smooth muscle cells (Filewod et al., 2009). LL-37 also induces IL-8, IL-18 and IL-20 production by human keratinocytes through MAP kinase pathway (Niyonsaba et al., 2005).

Scott et al., (2002) used gene expression profiling to identify macrophage functions that might be modulated by LL-37. Their studies revealed that LL-37 directly up-regulates 29 genes and down-regulated another 20 genes. Among the genes predicted to be up-regulated by LL-37 were those encoding chemokines and chemokine receptors. Consistent with this, LL-37 up-regulated the expression of chemokines in macrophages and whole human blood (monocyte chemoattractant protein 1 and IL-8), without stimulating the proinflammatory cytokine, TNF α . LL-37 also up-regulated the chemokine receptors CXCR-4, CCR2, and IL-8RB.

Brown et al., (2011) suggested that the effect of LL- 37 could differ with the type of tissue macrophage examined. They found the potent inhibitory effect of LL-37 was restricted to specific functions of classically activated (LPS or LPS/IFN-g) macrophages, (mainly TNF-a and NO production), but did not affect the ability of M1-BMDM (Bone marrow derived macrophages) to phagocytose and kill bacteria or the ability of M2-BMDM to phagocytose apoptotic neutrophils, events that are essential for wound healing and inflammatory resolution.

LL-37 has been found to have many other immunomodulatory effects and all these findings tend to indicate that LL-37 may contribute to the immune response by limiting the damage caused by bacterial products and by recruiting immune cells to the site of infection so that they can clear the infection.

6.1.11 LL-37 and wound healing.

LL-37 may also have a key role in wound healing. Koczulla et al., (2003) showed LL-37 induced angiogenesis, mediated by formyl peptide receptor-like 1 expressed on endothelial cells, and in a rabbit model of hind-limb ischemia application of LL-37 resulted in neovascularization. Furthermore using mice deficient for CRAMP, the murine homologue of LL-37/hCAP-18, decreased vascularization during wound repair was shown. In the same year Heilborn et al., (2003) found that high levels of hCAP18 are produced in skin in vivo upon wounding. The highest hCAP18 levels are attained at 48 h post-injury, declining to pre-injury levels upon wound closure. hCAP18 was detected in the inflammatory infiltrate and in the epithelium migrating over the wound bed. In chronic ulcers, however, hCAP18 levels are low and immunoreactivity for hCAP18/LL-37 is absent in ulcer edge epithelium, which may be one of the factors responsible for their slow healing.

6.1.12 Control of LL-37 expression in the skin

Since it has been found that LL-37 is up regulated in response to bacterial skin infections and cutaneous mechanical disruption the mechanism that controls this has been sought.

A number of studies have found that cathelicidin is a direct target of vitamin D3 in keratinocytes (Wang et al., 2004 and Weber et al., 2005). On skin injury or bacterial infection, there is a local increase in expression of 1 α -hydroxylase (CYP27B1), and as a direct consequence, more vitamin D3 is activated to induce cathelicidin expression and function (Schauber and Gallo 2008 A, Schauber and Gallo 2008 B). This may lead to therapies that can target the vitamin D3 pathway and thereby alter the response of LL-37 to infectious and inflammatory skin diseases.

6.2 AMPs in burn wounds

6.2.1 Defensins in burns

Studies of both the defensins and LL-37 have been limited in burn wounds. The first study looking at B defensins showed reduced anti-microbial peptide expression in human burn wounds. Milner and Ortega (1999) took samples of normal and burned skin from four patients with full thickness burns at time of wound excision and skin grafting. Total RNA was isolated and purified from the samples and then mRNA was subjected to reverse transcriptase polymerase chain reactions (RT-PCR) for hBD-1, hBD-2 and 2 control genes (γ -actin and cyclophilin) using specific primers.

Their results showed that hBD-2 was present in normal skin but profoundly reduced in burnt tissues (1 patient had low hBD-1 in burnt and normal skin). HBD-1 expression was preserved in all the burn wounds and was identified in all normal skin samples (Milner and Ortega 1999).

Using a different technique Milner identified hBD-2 protein in skin samples of partial and full thickness burns (6 patients) and normal skin using fluorescence deconvolution microscopy.

Their study showed decreased hBD-1 staining in partial thickness burns compared to normal skin, but no hBD-2 staining in full thickness burns although there was some staining in a number of subcutaneous structures and reticular dermis, most notably in eccrine and apocrine gland acini (Milner et al., 2004). In a further study the same group looked at blister fluid from partial thickness burns. No hBD-2 was found in the burn blister fluids of the 5 patients' analysed (Ortega et al., 2000)

Bick et al., (2004) examined the effects of pro-inflammatory cytokines, interleukin-1 β (IL-1 β), gamma interferon (IFN γ) and tumour necrosis factor α (TNF α) on human β -defensin 2 levels in cultured keratinocytes as well as the effect of heat shock (at 42 c). Their results showed only TNF α showed significant induction of hBD-2 but that this was not sustained in the long-term. The endogenous levels of hBD-2 were significantly reduced by exposure to heat shock (Bick et al., 2004)

A further study looked at hBD-1, hBD-2 and hBD-3 in cultured keratinocytes and CSS (cultured skin substitutes) in uninjured donors and burn patients. They found that hBD-1 was expressed in all keratinocyte strains analysed. HBD-2 expression in keratinocyte monocyte layers was highly variable but did not correlate with burn injury. HBD-3 was expressed at variable levels in all but one keratinocyte strain (Supp et al., 2004). Poindexter et al., (2006) found that after burns (of a depth that disrupted or destroyed most of the epidermis) HBD-1 was localized to dermal glandular structures and hair shafts but HBD-2 and HBD-3 was found in the upper portions of the remaining keratin layers. They concluded that although the upper layers of skin are destroyed and disrupted by burn, cells in the lower portions of the skin could demonstrate an ability to synthesize most of the AMPs, and thereby maintain some barrier against infection.

6.2.2 LL-37 in burns and trauma

Kaus et al., (2007) examined LL-37 in addition to defensins in burn wounds. They found no differences in hCAP-18/LL-37 expression levels when comparing burned skin with healthy controls. Unburned sections of the wound edge however, showed a 10-fold decrease of mRNA levels of hCAP-18/LL-37 ($p < 0.05$) compared with healthy and burned skin. The unburned skin was skin taken from adjacent to the burned skin and not from other parts of the body.

Most of the analysed host defence peptides and proteins showed higher mRNA levels in partial-thickness burns than in unburned tissue. In situ hybridisation revealed expression of hCAP-18/LL-37, hBD2 and hBD3 at the surface of burns was independent of burn depth. They found however that higher host defence peptide gene expression rates did not correlate with the incidence of wound infection in burns.

Lippross et al., (2011) examined the serum profiles of defensins, cathelicidins and pro-inflammatory cytokines post trauma and found that hBD-2, hBD-3 and LL-37 concentrations were significantly elevated after trauma and followed different characteristic concentration curves. In addition, similar patterns of concentration profiles were recorded for hBD-2/IL-6 and hBD-3/IFN-gamma.

6.3 Aim

To determine the presence of LL-37 in skin after a burn injury and subsequently in the healing phase, these samples will be compared with compared with normal, healthy controls firstly by immunohistochemistry and then rt PCR.

LL-37 has been found in the serum of patients who have sustained trauma and developed sepsis. Concurrently with the skin samples blood samples will also be taken to determine the presence of serum levels of LL-37 by ELISA to see if there is any relationship with the size of the burn injury. Significant pro-inflammatory cytokines will be also be measured at the same time as LL-37 to determine if there is any correlation between the molecules.

LL-37 is known to have antimicrobial activity against many organisms. With growing antibiotic drug resistance LL-37 may have role to play as an alternative to treatment when conventional antibiotics do not work. The activity of LL-37 against multi drug resistant strains of *Acinetobacter baumannii* that have been isolated from the burn ICU will be tested to determine if it may have useful clinical application.

6.4 Methods

6.4.1 Collection and preparation of clinical samples

The Burns Service comprises twenty beds including four intensive care and four high dependency beds. There are twelve low dependency / rehabilitation beds. The unit is a tertiary referral centre and accepts patients from a population of over 6 million people. Patients are also taken from out of traditional catchment areas and occasionally internationally.

A multidisciplinary team including microbiologists, pharmacists, anaesthetists and burn surgeons review all major burns daily. The Parkland formula is used in the initial resuscitation to maintain a urine output of 0.5 ml/kg/h in adults and 1 ml/kg/h in children. Nutritional supplementation is via nasoduodenal enteral nutrition with high calorie, low fat formulas. As part of the burn treatment protocol, gastric ulcer prophylaxis is performed with H₂ antagonists. Low molecular weight heparin (40mgs/day) is prescribed daily. Early excision and grafting is routinely performed for deep burns that would take more than two

weeks to heal without surgical intervention using allografting, auto-grafting or dermal substitutes depending on the individual characteristics of burn injuries.

All research was carried out under Local Research Ethical Committee approval (North and Mid Essex Local Research Ethical Committee-Ref 04/Q0303/9) with approval from the local Research and Development Departments. All procedures were performed under written informed consent from the patients if the patients were conscious or from their parents when the patient was under the age of consent. If the patient was unconscious and unable to consent, ethical approval was granted to collect samples and gain consent when the patient was conscious. In the event of the patients death before gaining consent all samples for that patient were to be destroyed.

Patients included in the study were those sustaining deep dermal or full thickness burns that required hospital treatment and admission.

Exclusions were those patients with non-thermal processes including desquamating skin disorders, meningococcal septicaemia, necrotising fasciitis and purpura fulminans; patients who stayed in the ITU for less than 24 hours were excluded; patients who were transferred from other hospitals where treatment other than resuscitation had been commenced. Healthy controls were patients undergoing plastic surgery procedures such as abdominoplasty and breast reduction or procedures where a full thickness portion of skin would otherwise be discarded.

Both sets of patients attended St Andrew's Hospital for Burns and Plastic Surgery in Chelmsford, Essex.

Skin samples

Punch biopsies of 3 or 4mm diameter were taken from areas of deep dermal or full thickness burn at the time of operation. The burn was assessed clinically by appearance, its response to pressure and degree of sensation prior to the operation in those patients who were conscious before the operation.

In patients who were unconscious prior to admission the burn was assessed by appearance and response to pressure.

The first sample was taken on the day of the first operation; this (unless stated otherwise) was the day after admission in keeping with the unit policy of early

excision and treatment of deep dermal and full thickness burns. The samples were taken prior to any wound débridement or surgery or other wound manipulations bar removal of dressings. Subsequent punch biopsies were taken in the first week (typically days 3-6), the second week and the last sample at 21 days or greater (or as close to these dates as possible). Once again these samples were taken prior to any wound manipulation.

All punch biopsies were taken under sterile conditions and were sutured closed afterwards. The samples were placed in formaldehyde and processed into paraffin wax blocks for immunohistochemistry or frozen in liquid nitrogen until need for PCR work.

Table 6.2 shows the details of the patients recruited to the study thus far.

There were 21 patients in the burn arm of the study three of whom died whilst inpatients of the burns unit and before regaining consciousness. This means that although samples were gathered it was impossible to consent the patients. In accordance with Ethic Committee requirements these samples have been disposed of according to hospital policy and these patients are no longer in the study.

Of the remaining 18 patients the male to female ratio is 13:5. The age range is 2-77 with a mean of 27.3. The range percentage of burn (of total body surface area) was 1-46% with a mean of 20.38.

The majority of the burns were caused by contact with flames (n=12) with 2 scalds caused by hot water, 2 scalds caused by food (normally a combination of hot water and oil), one electrical flash burn and one caused by prolonged contact with hot metal (a cooker).

There were 10 patients in the control arm of the study. The male to female ratio was 1:1. The age range was 37-86 with a mean of 58.1 years.

Burn patients	Age in years	Sex	TBSA% Burn	Mode of Injury	Other
B001	17	♂	4	Flame	
B002	77	♂	15	Flame	
B003					Died
B004	2	♀	30	Scald	
B005	17	♂	3	Flame-petrol	
B006	3	♂	15	Hot fat	
B007					Died
B008	32	♂	19	Flash –electrical burn	
B009	26	♀	40	Flame	
B010	3	♂	8	Scald	
B011	27	♂	46	Flame	
B012	33	♂	7	Hot oil/flame	
B013	60	♀	17	Flame	
B014	23	♂	18	Gas explosion	
B015					Died
B016	42/12 months	♀	22	Scald-oil	
B017	37	♂	44	Flame	
B018	18	♂	1% FT 11% PT	Flame-petrol	
B019	32/12 months	♂	40	Scald-food/oil	
B020	53	♀	35	Flame	
B021	59	♂	3	Contact burn	Hot metal, 5 mins
Non-burn control patients				Site of skin sample	
FT01	50	♀		DIEP	
FT02	48	♀		DIEP	
FT03	37	♀		DIEP	
FT04	49	♀		DIEP	
FT05	47	♂		Arm	
FT06	70	♂		Post auricular	
FT07	81	♀		Pre auricular	
FT08	63	♂		Pre auricular	
FT09	86	♂		Supra clavicular	
FT10	50	♂		Arm	

Table 6.2. Patients recruited to study LL-37 in human skin. The table shows the number of patients in both the investigation and control group who provided skin samples. For the patients in the burn group the TBSA% and mode of injury is also shown.

6.4.2 Immunohistochemistry

LL-37

The paraffin sections were firstly deparaffinised and hydrated this was done by sequentially dipping the slides in the following solutions

Xylene 2 x 5 minutes

100% ethanol 2 x 3 minutes

95% ethanol 1 x 3 minutes

70% ethanol 1 x 3 minutes

Excess endogenous peroxidases were destroyed by 15 minutes in 1:100 Peroxide: Methanol bath followed by

H₂O (running tap) 1 x 15 minutes

The slides were then microwaved at a high setting in citrate buffer (1L x 10 3.78g Citric acid (monohydrate), 23.78g Tri-sodium citrate, 3.7g EDTA disodium salt pH to 6.0) for 5 minutes, the buffer solution was changed to fresh citrate buffer and the slides were micro waved for a further 5 minutes and allowed to cool for 20 minutes prior to a wash in H₂O (running tap) for 5 minutes and then a TBS water bath for 10 minutes. (Tris Buffered Saline TBS (1 litre) 80g NaCl, 2g KCl, 30g TRIS pH to 7.4 to make 1L x10)

The slides were removed and the sample encircled with a PAP pen.

Blocking solution was added to the samples and the slides before incubating the slides in the humidity chamber for 20 minutes. Blocking solution 10mls TBS, 10µL 10% BSA (A8806, Sigma-Aldrich, Gillingham, Dorset, UK), 150µl goat serum (S-1000 Vector Laboratories, Peterborough, UK), 50µl human serum (from patient samples). The blocking solution was then removed and the sections were incubated with a rabbit anti-LL-37 polyclonal antibody (LL-37 PA-LL37-100, Innovogen, Sweden) diluted 1:100 at room temperature for 1 hour.

The slides were washed in TBS for 3 x 5 minutes and the secondary antibody applied (10mls TBS, 150µl goat serum, 50µl biotinylated anti rabbit serum).

The slides were incubated at room temperature for 45 minutes. The slides were then washed in TBS for 15 minutes before application of the 'ABC' complex (Vectastain Elite Kit PK-6100, Vector Laboratories, Peterborough, UK). The slides then underwent a further 15-minute wash in TBS before application of DAB solution for 2 minutes and then another wash in TBS and H₂O for 4 minutes each. The slides were counterstained with Mayer's Haemalum (Mayer's

Haemalum 350604 T, VWR, Belgium) before dehydration in the reverse process to that outlined at the beginning of the procedure.

Once the slides were removed from the xylene they had a few drops of DPX applied to the tissue and a cover slip mounted.

Controls included the substitution of primary or secondary antibody with TBS.

The positive control Erythropoietin receptor, EPOR was processed using the same protocol. The EPOR has been used as a positive control in previous experiments and was kindly donated by Dr Alan Cruchley (Centre for Clinical and Diagnostic Oral Sciences).

6.4.3 Data analysis of LL-37 immunohistochemistry

The staining data were analysed by visual assessment and quantitated by image analysis. Two observers visually assessed each slide, independently, and those showing positive staining were graded on a three-point equally weighted scale (+, ++ or +++) for staining in the basal layer and the suprabasal layers. A cumulative staining quotient (CSQ) was then calculated for LL-37, in normal and burn samples:

Cumulative staining quotient (CSQ)= Number of (+) in all samples/Number of samples x 100

Slides were photographed with a NanoZoomer 2.0-RS slide scanner (Hamamatsu Photonics, Hertfordshire, UK) and the images assembled using ADOBE PHOTOSHOP CS4 (Adobe Systems Inc., San Jose, CA, USA).

For quantification of staining showing a different pattern in normal and burn samples, pixel analysis was performed on the digital images, using the technique described by Lalli et al., (2008) which is a modification of the approach described by (Elie et al., 2003). Photographs were taken at 100x magnification with identical optical and digital zoom camera settings. In PHOTOSHOP, the epithelium was carefully selected from the tissue section using the Lasso tool and the total pixel count in the selected area recorded from the Histogram palette (Fig. 6.5). A new image was created by cutting and pasting the selected epithelium and from this the basal and suprabasal compartments carefully delineated by the Lasso tool to give the pixel count for each segment of the epithelium. To separate brown DAB staining from the blue haematoxylin counterstain, a colour range selection was performed using the same sampled colour with RGB values of 193, 157 and 137, respectively, as confirmed in the Colour palette of the software. The RGB values were determined by the staining colour produced in the positive control. The range of colours selected was set to 100 on the Fuzziness slider, which selects other parts of the image by the degree their colour is related to the sample colour. To confirm that all DAB-stained sections of the image were selected, these pixels were cut from the image to leave just the counterstained areas. Any DAB-stained areas remaining when the selected pixels were removed, for example, with very dark brown staining, were manually selected and employed for the evaluation using the Plus Eyedropper

tool of the software. The DAB pixel count in the basal and the suprabasal compartments of the epithelium was recorded and calculated as a percentage of the total basal or suprabasal pixel count (that will include DAB and haematoxylin).

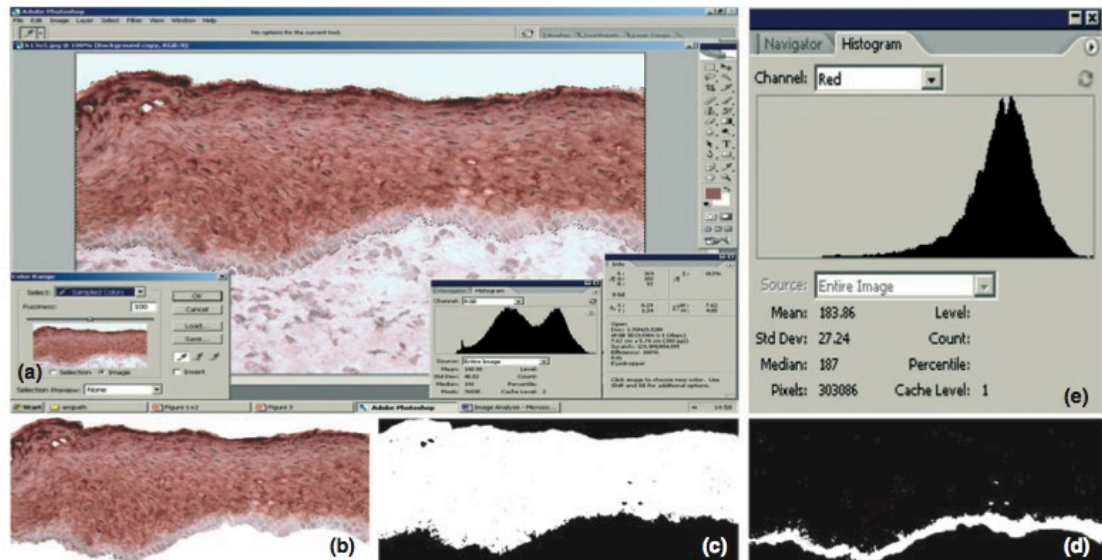


Figure 6.5. Quantitative analysis of 3,3'-diaminobenzidine (DAB)-immunostained and haematoxylin-counterstained tissue images. (a) Original images imported into ADOBE PHOTOSHOP CS2 v9.0.2 (Adobe Systems Inc.), (b) with epithelium delineated and (c) DAB staining specifically selected or (d) haematoxylin counterstain selected by adjustment of 'colour selection' properties. (e) The pixel count of the selected area is given in the Histogram palette. (Lalli et al., 2008)

6.4.4 Collection of Blood samples

Blood samples were collected from arterial lines in burns patients in the intensive care unit or prior to any subsequent surgical procedure in theatre. Whilst on the wards or in the follow-up clinic venous blood was collected from the patients. The first sample was taken on the day of the first operation; this (unless stated otherwise) was the day after admission in keeping with the unit policy of early excision and treatment of deep dermal and full thickness burns. The samples were taken prior to any wound débridement or surgery or other wound manipulations bar removal of dressings. Subsequent blood samples were taken in

the first week (typically days 3-6), the second week and the last sample at 21 days or greater (or as close to these dates as possible). Once again these samples were taken prior to any wound manipulation. All the blood samples were taken at the same time as the skin samples.

For the healthy controls venous or arterial blood was collected whilst the patients were under a general anaesthetic undergoing their in-patient treatment. This was taken prior to any procedure starting.

6.4.5 Isolation of plasma

Blood samples were collected in Vacutainer blood bottles (Becton Dickinson, Oxford, UK), containing EDTA (Ethylenediaminetetraacetic acid). The blood was returned to the laboratory and spun down at 1000 rpm for 15 minutes as per the instructions from the ELISA kits (R&D Systems, Abingdon, UK and HyCult biotechnology, The Netherlands). The plasma (top layer) was removed and stored at -20° C prior to experimental use.

6.4.6 Elisa Protocol for LL-37

Elisa Protocol Assay procedure (HyCult biotechnology Human Elisa LL-37 Kit HK 321)

All reagents were brought to room temperature (20 - 25°C) before use.

100µL of the sample or the standard (in duplicate) was added to each well. The plasma samples were diluted 20 times with wash/dilution buffer as per the protocol instructions. The plate was then covered and incubated for one hour at room temperature. The aspiration/wash was repeated four times with 200µL of wash/dilution buffer to each well. 100µL of diluted tracer was added to each well. The plate was then covered and incubated for one hour at room temperature. The washing procedure was repeated and 100µL of diluted streptavidin-peroxidase conjugate was added to each well. The plate was then covered and incubated for one hour at room temperature. The washing procedure was repeated and 100µL of TMB substrate solution was added to each well. The plate was then covered and incubated for thirty minutes at room temperature avoiding exposure to strong light. The reaction was stopped by adding 100µL of

stop solution to each well. The plate was read in a spectrophotometer with the absorbance measured at 450nm. All samples were tested in triplicate.

6.4.7 Elisa Protocol IL-6, IL-8 and IFN- γ

Commercial kits available from R&D Systems (Abingdon, UK) were used to measure IL-6 (DY-206), IL-8 (DY-208) and IFN- γ (DY-285) following the manufacturers protocol. However detailed below is a brief description of the method used. The standard curves for the ELISA plates are shown in Appendix IV, section 2.

Plate preparation

The capture antibody was diluted to the working concentration* in PBS and 100 μ L were added per well to a 96-well microplate. The plate was sealed and incubated overnight at room temperature. In the morning each plate was aspirated and washed a total of three times with 400 μ L of wash buffer (Wash buffer 0.05% Tween 20 in PBS).

Plates were then blocked by adding 300 μ L of reagent diluent to each well and incubating at room temperature for a minimum of an hour. (Reagent diluent 0.05% tween with PBS) The aspiration/wash as before was repeated three times.

Assay procedure

100 μ L of the sample or standard** in reagent diluent was then added to each well. The plate was then covered and incubated for two hours at room temperature. The aspiration/wash was repeated as before.

After the final wash 100 μ L of the detection antibody^S (diluted in reagent diluent) was added to each well. The plate was then covered and incubated for two hours at room temperature. The aspiration/wash was repeated as before.

After the final wash 100 μ L of the working dilution of Streptavidin –HRP was added to each well. The plate was cover and placed out of direct sunlight for 20 minutes. The aspiration/wash was repeated as before.

After the last wash 100 μ L of substrate solution was added to each well. The plate was cover and placed out of direct sunlight for 20 minutes.

Finally 50 μ L of stop solution was added to each well. The plate was tapped gently to ensure thorough mixing of the stop solution.

The optical density was then determined using a microplate reader at 450 nm. Wavelength correction was available which meant readings taken at 570nm were subtracted from those taken at 450nm to correct for optical imperfections in the plate. All samples were tested in triplicate. The standard curves for each ELISA plate are found in Appendix IV.

*** Capture antibody**

IFN- γ 720 μ g/mL of mouse anti-human IFN- γ reconstituted with 1.0 mL of PBS. Diluted to a working concentration of 4.0 μ g/mL in PBS.

IL-6 360 μ g/mL of mouse anti-human IL-6 reconstituted with 1.0 mL of PBS. Diluted to a working concentration of 2.0 μ g/mL in PBS.

IL-8 720 μ g/mL of mouse anti-human IL-8 reconstituted with 1.0 mL of PBS. Diluted to a working concentration of 4.0 μ g/mL in PBS

**** Standard**

IFN- γ 30ng/mL of recombinant human IFN- γ when reconstituted with 0.5 mL of distilled water. A seven point standard curve using 2-fold serial dilutions in reagent diluent was performed. High standard 1000pg/mL.

IL-6 70ng/mL of recombinant human IL-6 when reconstituted with 0.5 mL of distilled water. A seven point standard curve using 2-fold serial dilutions in reagent diluent was performed. High standard 600pg/mL.

IL-8 100ng/mL of recombinant human IL-8 when reconstituted with 0.5 mL of distilled water. A seven point standard curve using 2-fold serial dilutions in reagent diluent was performed. High standard 2000pg/mL.

\$ Detection antibody

IFN- γ 18 μ g/mL of goat anti-human IFN- γ reconstituted with 1.0 mL of Reagent Diluent. Diluted to a working concentration of 100 ng/mL in reagent diluent.

IL-6 36 μ g/mL of goat anti-human IL-6 reconstituted with 1.0 mL of Reagent Diluent. Diluted to a working concentration of 200 μ g/mL in Reagent Diluent.

IL-8 3.6 mg/mL of biotinylated goat anti-human IL-8 when reconstituted with 1.0 mL of Reagent Diluent. Dilute to a working concentration of 20 ng/mL in Reagent Diluent.

6.4.8 PCR

Extraction of RNA

Homogenization

The skin samples were homogenized with Ultraspec™ (Biotecx, Houston, USA) RNA reagent (2ml per 0.1g of tissue).

RNA Extraction

The skin samples were homogenized by grinding them in a pestle and mortar in liquid nitrogen. Once homogenised, 0.2 ml of chloroform was added (per 1 ml of Ultraspec™ RNA), the samples covered, vortexed for 15 seconds and placed on ice at 4' C for 10 minutes. The homogenate was then centrifuged at 12,000 g (4 C) for 10 minutes (Beckman J2 centrifuge, GMI, USA).

RNA Precipitation

The aqueous phase was transferred to a new eppendorf and an equal volume of isopropanol was added the samples were then stored for 10 minutes at 4' C. The samples were centrifuged as before and the supernatant removed.

RNA Wash

The RNA pellet was washed twice with cold 75% ethanol (1 ml of 75% ethanol/1 ml of reagent used) vortexed briefly and was subsequently centrifuged as before. At the end of procedure, the pellet was aired dried on ice for 30-60 minutes. The pellet was dissolved in 25ul of DEPC treated water. To ensure the RNA dissolved it was heated for 10-15 minutes at 55-60 C.

TURBO DNase Digestion

To clear any DNA contamination from RNA samples prior to RT-PCR the process outlined below was used as even a few copies of DNA can lead to false

positive outcomes by PCR. This process was performed using reagents supplied in the Turbo DNase kit produced by Ambion (Applied Biosystems, Carlsbad, California, Cat # AM1907).

After defrosting the buffer, the mastermix was prepared combining DNase reaction components as shown below in table 6.3: (Buffer, DNase enzyme, RNase Out (optional) and/or H₂O). It was briefly mixed by vortex and the RNA sample added.

Treatment Components	X 1 reaction (µl)
10X TURBO Buffer	5.00
H ₂ O	8.00
TURBO Dnase* (2U /10ug RNA)	1.00
RNA (gDNA) sample	36.00
Total Rxn Vol	50.00

***1 µl DNase (2U) removes maximum 2 µg genomic DNA in 50 µl rxn**

Table 6.3. Example calculation for 1 sample treated with the Turbo DNase mastermix.

The resulting mastermix was placed on PCR heat block: 37°C, 30 min. When complete, the samples were taken off the heat block and 5µl of DNase Inactivation Reagent added (for a 50µl volume reaction). This was incubated for 5 min at room temperature and then centrifuged at 10,000g for 1.5 min. The purified RNA, a clear fluid was removed taking care not to disturb the pelleted material. The RNA yield (ng/µl) was measured by absorbance at 260nm using NanoDrop® spectrophotometer (Thermo Fisher Scientific, Wilmington USA). The samples were stored at -70 C until required.

Reverse transcription protocol

Reverse Transcription Reaction (First-Strand cDNA Synthesis)

cDNA was obtained by reverse transcription using reagents supplied in the Promega reverse transcription system (Promega, Southampton, UK) shown in table 6.3.

1µg (2µl) of total RNA was in a microcentrifuge tube, and incubated at 70°C for 10 minutes, centrifuged briefly in a microcentrifuge, then place on ice.

A 20µl volume of the following reaction was created by adding the following reagents in the order listed in table 6.4. The resulting solution was incubated at 42°C for 15 minutes.

The sample was heated at 95°C for 5 minutes, then incubated at 0–5°C for 5 minutes then stored at -20°C until required.

Component	Amount
MgCl ₂ , 25mM	4µl
Reverse Transcription 10X Buffer	2µl
dNTP Mixture, 10mM	2µl
Recombinant RNasin® Ribonuclease Inhibitor	0.5µl
AMV Reverse Transcriptase (High Conc.)	15u
Oligo(dT)15 Primer	0.5µg
Total RNA	1µg
Nuclease-Free Water to a final volume of	20µl

Table 6.4. Master mix for one reverse transcription reaction. The above components were used to make the mastermix for one reverse transcription reaction.

Testing qPCR primer design and specificity

Before qPCR analysis of LL-37 gene expression in skin samples it was necessary to confirm primer specificity. The forward (Fw) and reverse (Rw) primers for qPCR for L-37 were designed online to produce an amplification product <150bp in the primer design tool of the Roche Applied Science website

(<https://www.roche-applied-science.com/sis/rtPCR/upl/index.jsp?id=UP030000>).

Primers were supplied by Sigma-Aldrich (Dorset, UK), see table 6.5 for sequences.

<http://www.sigmaaldrich.com/configurator/servlet/DesignCenter>

Primer name	Primer sequence	Primer Tm	Product size
LL-37_F	TCGGATGCTAACCTCTACCG	60 °C	
LL-37_R	GTCTGGGTCCCCATCCAT	60 °C	
PPIA_F	TCATCTGCACTGCCAAGACTG	66.6 °C	71 bp
PPIA_R	CATGCCTTCTTTCACCTTGCC	65.7 °C	

Table 6.5 Primer sequences for LL-37 and the reference gene PPIA.

Primers were tested for gene specificity by production of a single PCR product from RNA from hTERT cells supplied by Dr Eleni Pantazi (CDOS, QMUL). PCR was carried out using SYBR Green I Master Mix (5ml; LightCycler® 480 SYBR Green I Master, Roche Applied Biosciences, Burgess Hill, UK) containing 30µl of 0.001% bromophenol blue (BPB; Table 6.6) in a LightCycler® 480 multi-well plate 96 (Roche Applied Biosciences). The plate was sealed with sealing foil and centrifuged for 10 seconds to allow through mixing at the bottom of the well. The plate was transferred to the LightCycler® LC480 and amplification carried out using the following protocol:

- 1) Initial denaturation step to activate the Taq polymerase enzyme for 5 minutes at 95°C.

2) 40-45 amplification cycles consisting of a) double strand melting for 10seconds at 95°C, b) annealing for 10 seconds at 60 °C and c) extension at 72 °C for 10 seconds with acquisition of the fluorescence signal.

Reagent	Volume per reaction well
2X SYBR Green I Master Mix	20µl
dH ₂ O	20µl
Template cDNA	5µl
5 mM Fw/Rw Primer	7µl
Total volume/well	50µl (+/-2µl)

Table 6.6. PCR mixture using LC480 Sybr Green I. The above mixture of components was used to assess the specificity of primer pairs resulting in a single peak (i.e. a single PCR product). The Fw (forward) and Rw (reverse) pair (25µl each) of primers were from a 100µM stock solution dissolved in 450µl dH₂O to give 5µM working strength (final concentration of primers in the 50µl mix was 0.7µM).

Following amplification, the number of PCR products in each well was determined by initial melting for at 95°C for 10 seconds, followed by x45 cycles of an annealing phase at 67 °C for 12 seconds and extention phase of 72 °C for 10 seconds. The melting curve analysis should show a single narrow peak, indicating a single PCR product and therefore primer specificity.

In order to determine DNA copy number of the purified amplicon (for creating a standard curve), the formula was completed which takes into account the length in base pairs (bp) and quantity of the DNA. First, the molecular weight (MW) was determined by multiplying the number of bp in the amplicon by 665 (which is the assumed average weight of a bair pair). The quantity of DNA (ng) was measured by Nanodrop® (260nm, Thermo Fisher Scientific, Wilmington USA) on 1 µl of sample and was incorporated into the formula below in order to determine DNA copy number.

Formula: $[(\text{ng}/\text{MW})/1 \times 10^9] \times 6.02 \times 10^{23} \text{ molecules/mole} = \text{number of copies (per } \mu\text{l)}$.

Determining the reference gene PPIA

The housekeeping (reference gene) was determined using the Real Time ready Human Reference Gene Panel kit (Cat No. 05467675001 Roche, Burgess Hill, UK). The reference gene is used to normalize for possible variations in the amount and quality of RNA between different samples. The Real Time ready Human Reference Gene Panel allows quantification of the expression levels of 19 human genes that can be used as reference genes. This panel can be assayed directly on a LightCycler® 480 Instrument. A reference gene is used to determine expression levels of other genes by relative quantification. If one of the reference genes in the panel is expressed at a level similar to the gene being studied, it can be used for relative quantification of the gene.

Three samples were used in the Reference gene panel kit an early burn, a healing burn and a control patient (unburnt skin).

To perform the analysis the stock solutions from the kit were thawed, mixed carefully and stored on ice. The PCR mix for 96 reactions is detailed below, table 6.7.

Component	Volume	Final Conc.
Water (PCR grade)	480 µl	
LightCycler 480 probes Master, 2x conc	960 µl	X1
Total volume	1440 µl	

Table 6.7 PCR mix for 96 reactions in the Real Time ready Human Reference Gene Panel

The solution was mixed carefully by pipetting only. 15µl was placed into each well of the LightCycler 480 Multiwell Plate. 5µl of the cDNA template was placed into each well (except well H12 which contained the negative control, 5 µl of the untranscribed RNA was placed in well H12). The plate was sealed and with sealing foil and centrifuged for 2 minutes at 1500 x g. The plate was transferred to the LightCycler 480 and the programme started. The run protocol was imported directly into the LightCycler 480 software via the Roche website <http://www.roche-applied-science.com>.

geNorm analysis was performed on the results. geNorm is an algorithm to determine the most stable reference (housekeeping) genes from a set of tested candidate reference genes in a given sample panel. From this, a gene expression normalization factor can be calculated for each sample based on the geometric mean of a user-defined number of reference genes. This data was analysed using Biogazelle qbasePlus, which incorporates the geNorm program (Biogazelle, Zwijnaarde, Belgium). The geNorm software ranks the reference (house-keeping) genes in order of stability of expression (see appendix IV section 3 for results). Melting peak and standard curves for genes analysed Appendix IV, figure 4 and figure 5.

Reference gene work was performed by Tanya Novak.

6.4.9 The activity of LL-37 against *Acinetobacter baumannii*-determining the MIC and LD 50.

Minimum inhibitory concentrations (MICs) are considered the 'gold standard' for determining the susceptibility of organisms to antimicrobials and are therefore used to judge the performance of all other methods of susceptibility testing (Andrews 2001). Minimum inhibitory concentrations (MICs) are defined as the lowest concentration of antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation. The minimum bactericidal concentration (MBC) is the lowest concentration that kills 99.9% of the population (Collins et al., 2004). The term half maximal effective concentration (EC₅₀) refers to the concentration of a drug or antibody, which induces a response halfway between the baseline and maximum after some specified exposure time. It is commonly used as a measure of drug's potency.

The range of antibiotic concentrations used for determining MICs is normally performed in doubling dilution steps, up and down from 1 mg/L as required. Although the MIC is defined as the lowest concentration of a drug that will inhibit the visible growth of an organism after overnight incubation, this period is extended for organisms such as anaerobes, which require prolonged incubation for growth (Andrews 2001). The protocol used followed the method of Chromek et al., (2006) using a microtiter plate method (modified according to The Swedish Reference Group for Antibiotics- subcommittee on methodology, www.srga.org).

Preparation of antibiotic dilution range and MIC broth

LL-37 peptide (SP-LL37-1 Innovogen, Sweden) was diluted in 0.1% trifluoroacetic acid and a peptide range of concentrations was produced from 2 μ M to 32 μ M.

The organism suspension was prepared by growing the bacteria on ISO-Sensitest Agar (CM0471, Oxoid Basingstoke, UK). Four or five colonies of pure culture (to avoid selecting an atypical variant) were then suspended in 10 ml of Mueller Hinton broth (CM0405 Oxoid Basingstoke, UK). This was grown overnight and the resulting solution was diluted to produce an inoculum of 10⁵ cfu/ml. Using a 96 well plate the bacterial suspension (90 μ l) was grown in the presence of

different concentrations of the peptides diluted in 0.1% trifluoroacetic acid (10 μ l). As a control both inoculated and uninoculated wells of antibiotic-free broth were included on the plate (the first controls the adequacy of the broth to support the growth of the organism, the second is a check of sterility). Each inoculum was in duplicate and the experiment repeated three times. The plates were covered with sealing tape and incubate at 35-37⁰C for 18-20 h in air. The MIC endpoint was the lowest concentration of antibiotic at which there is no visible growth. The control strain of bacteria used was *Pseudomonas aeruginosa* (supplied by Dr R Wade). This was chosen because the MIC for the control strain should be within one two-fold dilution of the expected MIC for the bacteria being tested. All of the *Acinetobacter baumannii* samples were supplied by Dr Jane Turton, (Laboratory of HealthCare Associated Infection, Centre for Infections, Health Protection Agency, Colindale, UK). They are all multi resistant strains isolated from the unit and at the time of the study prevalent in a number of hospitals and critical care units in the south east of England. The strains used were Southeast Clone AB 11, Southeast Clone AB 12 and Oxa 23 Clone 2. (See Appendix IV, section 1 for the dendrograms).

EC₅₀ was determined by analyzing plated serial dilutions of the bacteria-peptide *Acinetobacter baumannii* solutions (in Mueller Hinton broth) on ISO agar plates after 60 minutes of growth.

6.5 Results

6.5.1 Immunohistochemistry

The presence of LL-37 was determined in skin samples of patients on the first day after admission to a burn ICU.

The positive control tissue was salivary gland tissue where it has been shown that LL-37 can be demonstrated in the ducts (Woo et al., 2003).

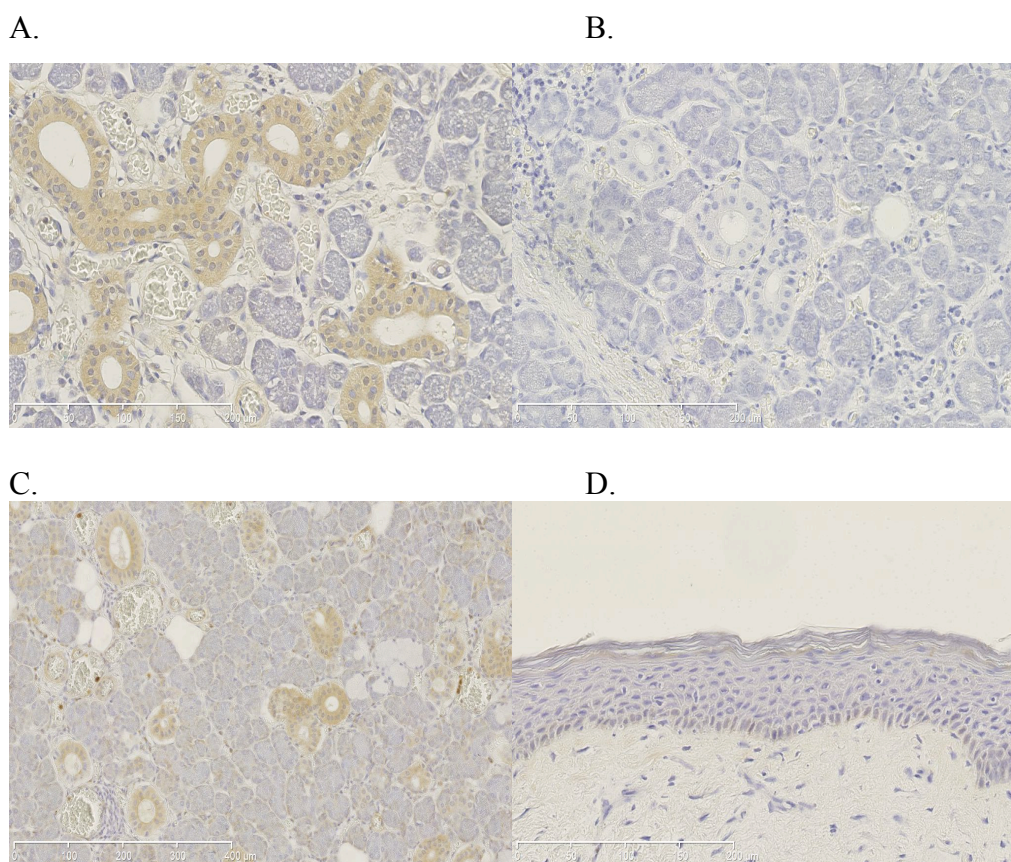


Figure 6.6. LL-37 in salivary gland tissue. Panel A demonstrates the presence of LL-37 (1:100) in the ductal tissue. Panel B is the negative control with no primary antibody. Panel C shows EPOR staining of the ductal tissue (1:500). Panel D is a skin sample with no primary antibody (all pictures magnification x200).

To ensure the protocol for immunohistochemistry worked, salivary tissue was tested with an antibody known to be present in salivary glands, EPOR (supplied by Dr Alan Cruchley, CDOS) shown in Figure 6.6, Panel C. The LL-37 antibody has also been found in the salivary glands and these tissue samples were used as a positive control when all of the skin samples were analysed. Panel D demonstrates that there was no staining present when the primary antibody was not used in both salivary tissue and skin.

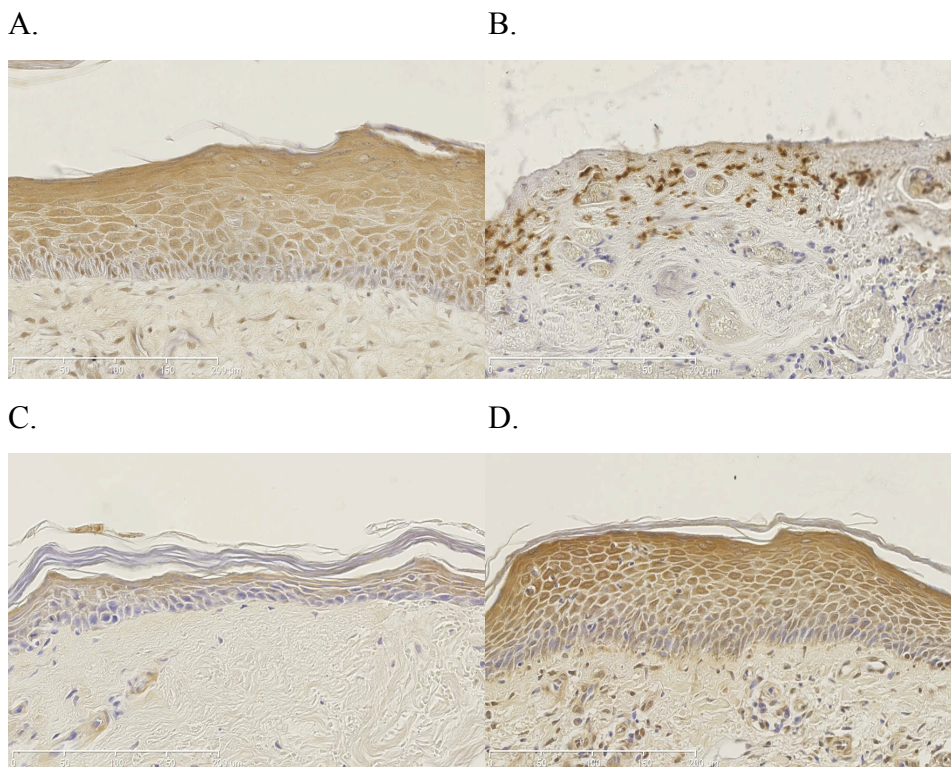


Figure 6.7 The presence of LL-37 in skin samples. Panel A LL-37 in normal unburnt skin. Panel B shows LL-37 in a burnt skin sample taken on day 1 after admission to the burn ICU. Panel C shows a tissue sample taken a few days after surgery where the burnt skin has been tangentially excised and a split thickness skin graft has been placed. Panel D demonstrates the positive staining seen in a healing grafted area 46 days after the initial injury (magnification x 200).

In figure 6.7 panel A shows there is staining through out the epidermis but the greatest concentration is in the more superficial layers. Panel B demonstrates the

destruction of the epidermis with an ill-defined layer above the dermis with loss of the normal architecture of the epidermis. There is scattered staining throughout this layer. Panel C shows recently grafted tissue. The epidermis is only a few cells in height and yet the upper layers show some marked positive staining. Finally panel D is a healed grafted area with a thick epidermis, possibly showing some hyperproliferation. The staining is more like the control samples with less basal staining and increased staining more superficially.

A.

B.

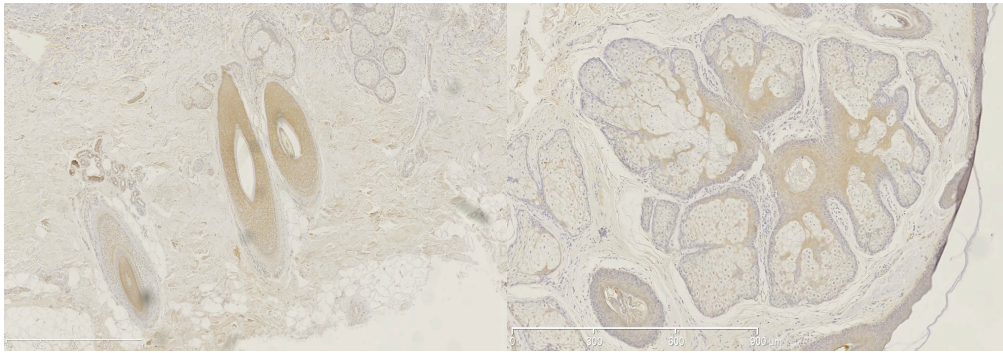


Figure 6.8 The presence of LL-37 in the dermal adnexal structures. Panel A, A hair follicle in transection. Panel B Sebaceous gland (magnification x200). Figure 6.8 shows positive staining in deeper dermal structures.

The burnt skin samples of day one invariably had the majority of the epidermis destroyed with variable amounts of the dermis. In general there was minimal positive staining due to LL-37 in the epidermal remnants but it was often found that there was some positive staining on the surface of the resultant wound. This echoes the findings of Kaus et al., (2008) who through detection of hCAP-18/LL-37 mRNA by *In situ* hybridisation determined that the LL-37 was localised to the wound surface in burns. The areas of LL-37 they detected were located in the cytoplasm of the remaining intact cells, whereas the majority of the surrounding cells were disrupted.

In the healthy controls, LL-37 was observed throughout the epidermal layer from the stratum basale, throughout the stratum spinosum and corneal layers. This contrary to many other studies that have found little or no LL-37 in the skin of the normal, healthy, controls (Frohman et al., 1997).

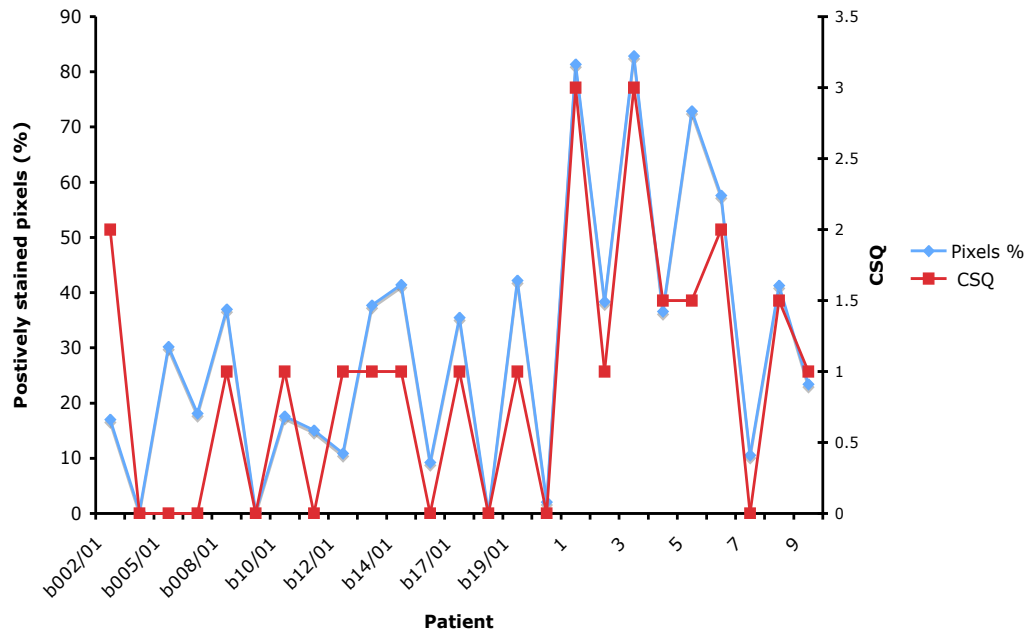


Figure 6.9. Correlation between visual assessment grading and image analysis. Healthy controls labeled 1-10 and burn samples coded for individual patients on the day 1. The blue line represents image analysis and shows the number of positively stained pixels (as seen on the left sided y-axis). The red line represents visual assessment as determined by CSQ (as seen on the right sided y-axis). There is close correlation between visual assessment grading and image analysis of 3,3'-diaminobenzidine immunostaining for LL-37 in the samples of burnt skin and control healthy patients on day one ($r=0.69$).

In an attempt to quantify the amount of staining present the CSQ (Cumulative Staining Quotient) was utilized as described by Lalli et al., (2008).

Quantification in this manner permitted use of the Mann-Whitney U-test to determine statistically significant differences in the DAB staining between normal and diseased samples. The validity of the pixel analysis was confirmed by correlation coefficient $r = 0.69$ ($P < 0.005$; Figure 6.9) with our visual assessment grading, even with all non-stained remnants of the epithelium disregarded. Figure 6.9 is used to give as a visual aid to demonstrate the close correlation between the visual assessment grading and the image analysis. The same graph as used by Lalli et al., (2008) has the data points connected to make this visual aid clearer as in reality the data points are not connected. This graph was chosen over a histogram because it makes the correlation clearer. Because of

the difference in units between the image analysis and visual grading scale it is not so easy to appreciate the correlation when looking at a histogram.

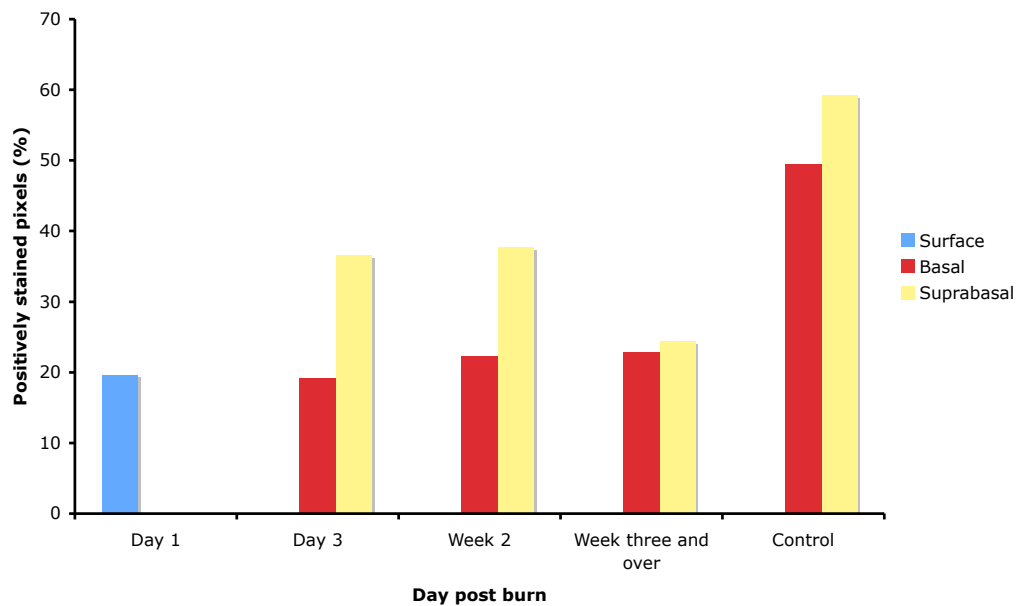


Figure 6.10. Percentage of positively stained pixels in the skin samples. This graph demonstrates the percentage of positively stained pixels in the skin samples in both the basal and suprabasal layers of the epidermis over time compared with the control group.

It must be noted when examining the data in figure 6.10 that for the day one burn sample the epidermis has been variably destroyed and it is difficult to differentiate the layers of the epidermis. The surface staining therefore reflects the amount of staining above the dermis. This staining may represent LL-37 that has been released from the cells due to the thermal damage (as found by Kaus et al., 2008) or non-specific staining of denatured proteins. In addition at day three the skin graft does not have a clearly defined basal layer so the deepest layer of skin graft was considered the basal layer.

The LL-37 staining in the samples of the burn patients taken in the subsequent time periods showed a mixed pattern of distribution but consistently had reduced LL-37 in both the basal and supra basal areas compared with the control patients (see figure 6.10). As time progressed there was a gradual increase in the amount of LL-37 staining (both basal and supra basal) although it never reached the amounts seen in the healthy controls.

6.5.2 qPCR

The reference gene PPIA (peptidylprolyl isomerase A) was present in all the samples. The raw data is shown in Appendix IV, section 4 with the melt point, amplification and standard curve graphs for both PPIA and LL-37.

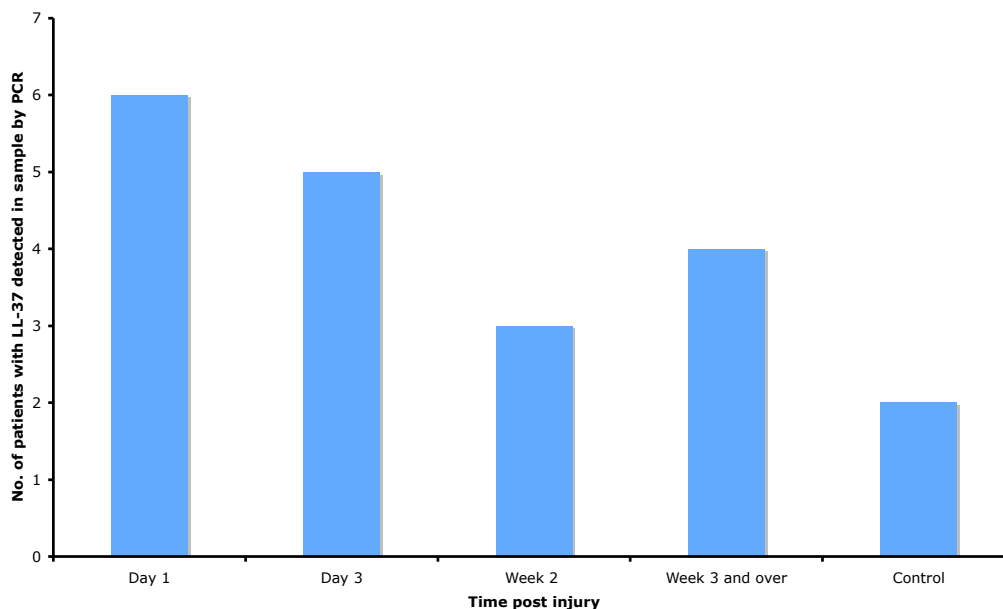


Figure 6.11. The number of patients with LL-37 detected in the skin sample by rtPCR. The data shows the number of patients with LL-37 detected in their sample on each day of analysis. In total there were 42 burn patient samples and six control patient samples for the LL-37 analysis. For day 1 there were 17 samples, for day 3 there were 11 samples, for week two 9 samples, for over week two 5 samples and six control samples.

LL-37 was detected in only twenty of the 48 patient skin samples analysed. Figure 6.11 suggests that LL-37 expression was more frequent in the earlier stages i.e. day 1 the acute burn, and in the immediate post-admission period. There appears to be a trend showing expression decreasing during the healing time towards levels seen in the control patients. An Anova analysis of the data shows there is no statistical difference in the patient samples, $p=0.054$. This result may not be accurate however. Firstly the total population size is small and sample size within each group is small. Drawing any firm conclusions from the statistical analysis is likely to be flawed. However looking at the raw data may allow some basic ideas about the results. Looking at the raw data (Appendix 4)

shows that only two of the patients showed a significant amount of LL-37 (one a burn patient on day three after the burn and one in a patient over three weeks after the burn injury). 15 patients had only one positive result out of the samples run in triplicate. Two patients had two positive results out of the three samples. Only one patient had three positive results.

In these 18 patients there are weak reactions that may indicate minimal amounts of nucleic acid. In a real time PCR assay a positive reaction is detected by accumulation of a fluorescent signal. The cycle threshold (Ct) or crossing point (Cp) is defined as the number of cycles required to reach a defined fluorescence intensity or for the fluorescent signal to cross the threshold (i.e. exceeds background level). Ct levels are inversely proportional to the amount of target nucleic acid in the sample (i.e. the lower the Ct level the greater the amount of target nucleic acid in the sample). Normally Cts < 29 are strong positive reactions indicative of abundant target nucleic acid in the sample. Cts of 30-37 are positive reactions indicative of moderate amounts of target nucleic acid but Cts of 38-40 are weak reactions indicating minimal amounts of target nucleic acid, which could represent an infection state or environmental contamination. The Cts for the two samples with large amounts of nucleic acid were between 21-24 whilst for the remaining 18 patients not only in the majority of these cases did only one sample out of the three test positive but with Cts between 38-40 it is possible that these results are due to contamination.

6.5.3 Serum levels of LL-37 and proinflammatory cytokines

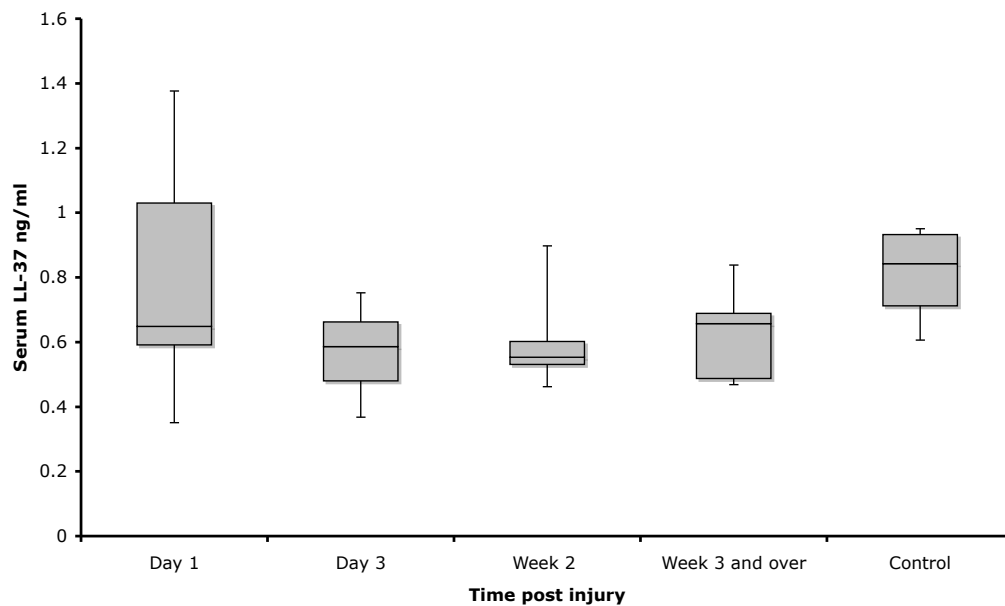


Figure 6.12 Box plot graph of serum levels of LL-37. The data show the serum levels of LL-37 at varying time periods compared with the control group. The length of the box covers the interquartile range, the lower edge of the box being the lower quartile, and the upper edge of the box being the upper quartile. The line across the box is at the median. The whiskers extending beyond the box go to the extremes of the data. There is no difference between the LL-37 levels in the serum on day 1 compared with the controls ($p=0.18$), Mann U Whitney.

It can be seen in figure 6.12 that the serum LL-37 levels are very low with a wide range in values in the samples taken on day 1 of admission to the unit. It can be seen that during the period of study from acute injury throughout the healing period that the LL-37 levels are depressed compared with the control subjects. Specifically there is no significant difference between the control group and the admission values ($p=0.18$, Mann U Whitney). Analysis of all the groups show there is no statistical difference between any of the groups ($p=0.054$ Anova).

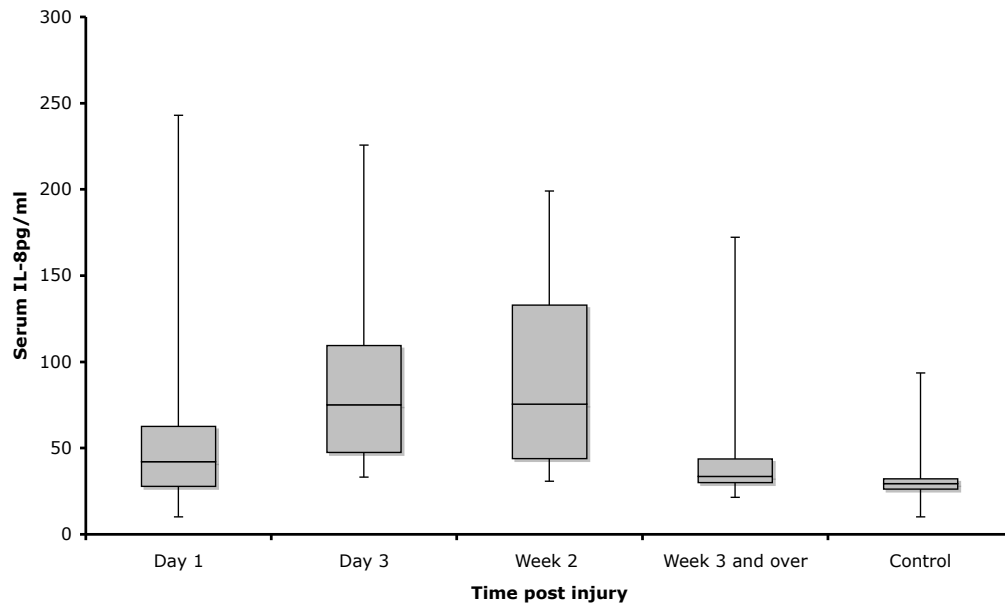


Figure 6.13 Box plot graph of serum levels of IL-8. The data show the serum levels of IL-8 at varying time periods compared with the control group. The length of the box covers the interquartile range, the lower edge of the box being the lower quartile, and the upper edge of the box being the upper quartile. The line across the box is at the median. The whiskers extending beyond the box go to the extremes of the data. There is no difference between the IL-8 levels in the serum on day 1 compared with the controls ($p=0.16$), Mann U Whitney.

Each group in figure 6.13 shows a wide range in the values which given the small sample size means any interpretation of the results should be done with caution. Statistically there is no difference between any of the groups ($p=0.096$ Anova), however the broad trend of IL-8 levels in the serum post injury seem to be slightly elevated levels in the acute stage after injury and then a rise in levels over the next two weeks. There is a gradual return towards normal levels (control group) as time passes. There is no difference between the serum IL-8 values on day 1 and the control group ($p=0.16$), Mann U Whitney.

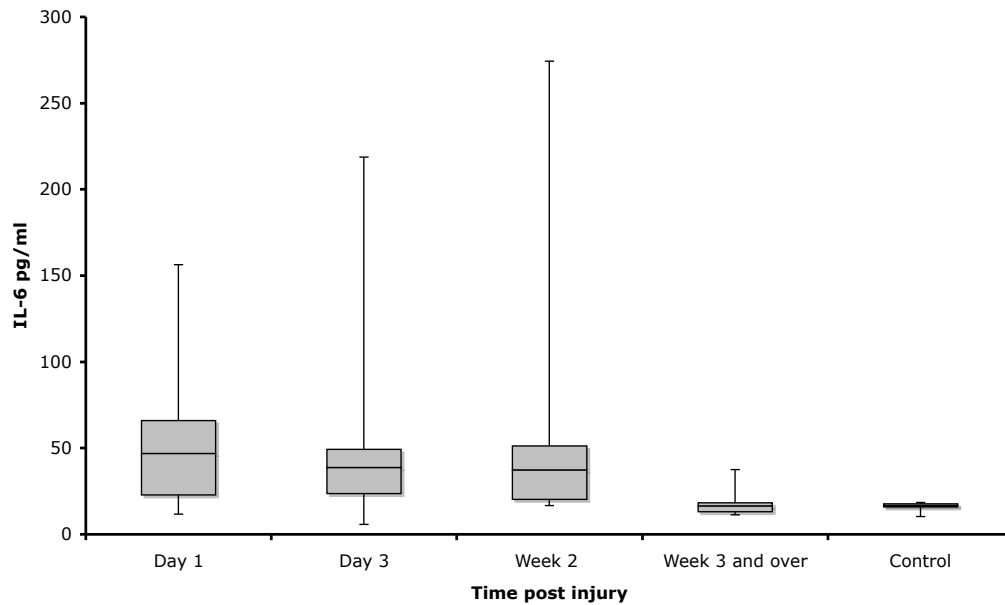


Figure 6.14 Box plot graph of serum levels of IL-6. The data show the serum levels of IL-6 at varying time periods compared with the control group. The length of the box covers the interquartile range, the lower edge of the box being the lower quartile, and the upper edge of the box being the upper quartile. The line across the box is at the median. The whiskers extending beyond the box go to the extremes of the data. There is no statistical difference between the groups ($p=0.31$ Anova).

Figure 6.14 shows that IL-6 serum levels are slightly elevated in the first two weeks after a burn injury but fairly rapidly return towards normal levels by week three. There is no difference however, between the IL-6 levels in the serum on day 1 compared with the controls ($p=0.0537$), Mann U Whitney. The wide range of results that are seen in the first two weeks are due to a small number of patients having very high values and suggests that these patients may have another inflammatory process, such as sepsis going on. Further more there is no statistical difference between any of the groups ($p=0.31$ Anova).

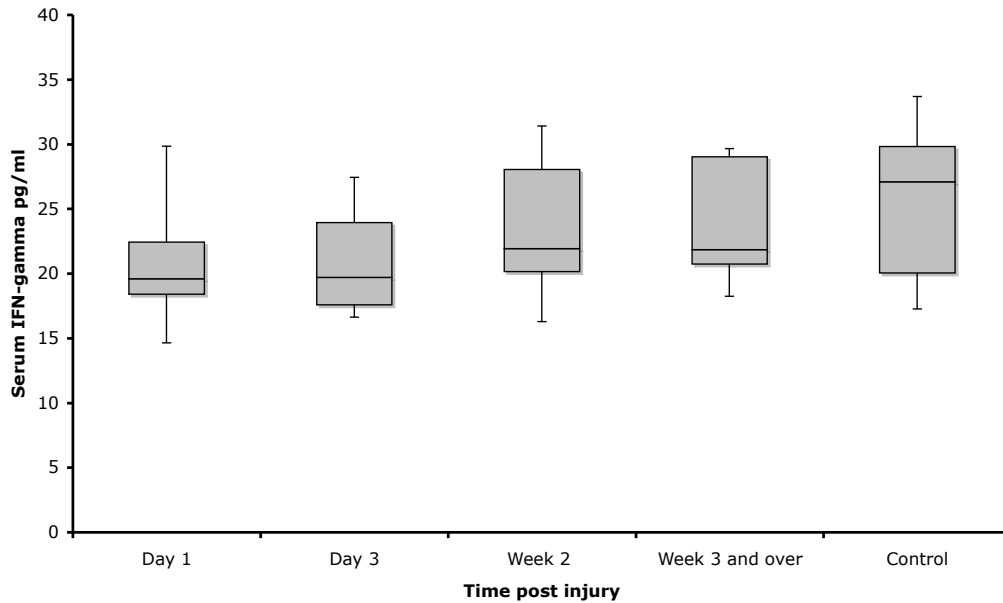


Figure 6.15 Box plot graph of serum levels of IFN-gamma at selected time periods post injury. The data show the serum levels of IFN-gamma at varying time periods compared with the control group. The length of the box covers the interquartile range, the lower edge of the box being the lower quartile, and the upper edge of the box being the upper quartile. The line across the box is at the median. The whiskers extending beyond the box go to the extremes of the data. There is no difference between the IFN-gamma levels in the serum on day 1 compared with the controls ($p=0.0582$), Mann U Whitney.

Again, with the box plot graphs the small sample group must be borne in mind when trying to infer from the results but figure 6.15 shows a broad trend of IFN-gamma being suppressed in the acute stage and gradually returning towards normal levels as time passes despite this there is no difference between the groups compared with the control group ($p=0.17$ Anova).

The relationship of LL-37 and proinflammatory cytokines to TBSA% burn

As LL-37 has been found to be elevated after multiple trauma (Lippross et al., 2011) LL-37 and the proinflammatory cytokines were examined to see if there was any relationship with the magnitude of injury (defined by TBSA % burn).

There is no relationship between the TBSA % and the serum levels of IL-6 and IFN-gamma and that there may be a weak association between TBSA % and IL-8 levels in this sample group (Results in Appendix IV, section 6 tables 6 A-D).

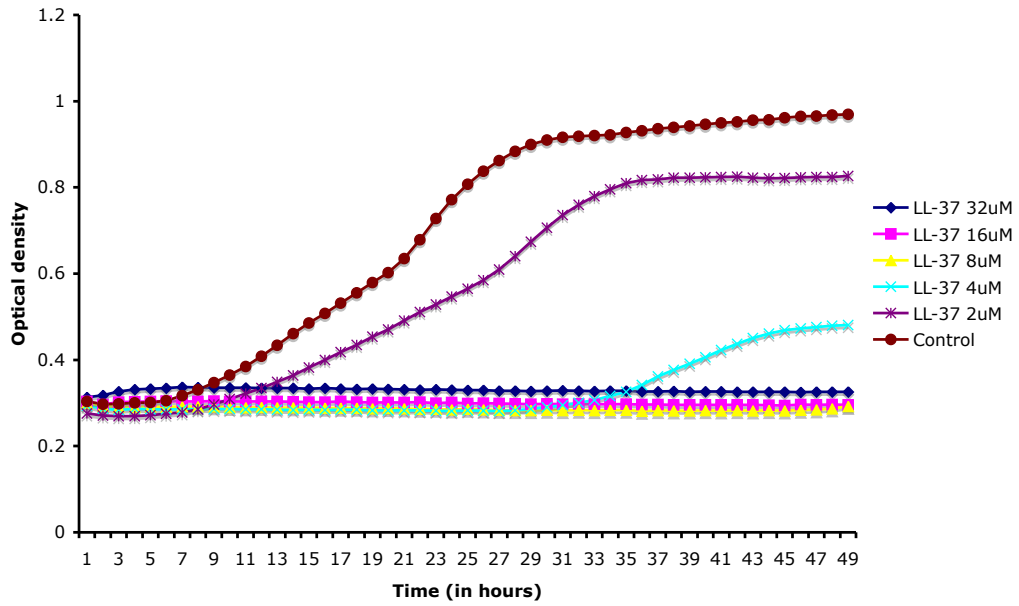
The relationship between serum levels of LL-37 and cytokines with age

As age can have an influence on the inflammatory response (Nomellini et al., 2009) the serum levels of LL-37 IFN γ and the proinflammatory cytokines IL-6 and IL-8 were examined to see if a relationship existed that might have influenced our results.

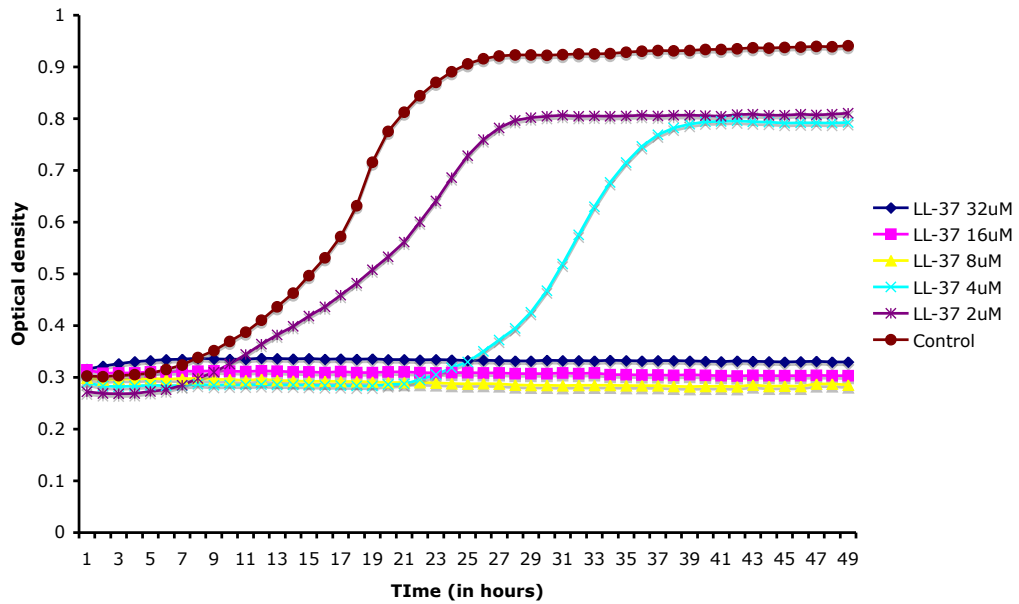
No relationship was found between age and serum levels of any of the molecules examined (Results in Appendix IV, section 5 tables 5 A-D).

6.5.4 Minimum inhibitory concentrations of LL-37

To determine whether LL-37 might have an effect on drug resistant *Acinetobacter baumannii* the minimum inhibitory concentrations were examined after an overnight growth of the organisms. Three strains of *Acinetobacter* were investigated, Southeast Clone AB 11, Southeast Clone AB 12 and Oxa 23 Clone 2. All three strains had been isolated in the unit and a number of other ICUs in the south east of England. They were all multi-drug resistant and were perceived to be difficult to eradicate (Personal correspondence, Dr L Teare, Broomfield Hospital, Essex and Dr J Turton, Health Protection Agency, Colindale, UK). The control had no LL-37.



A.

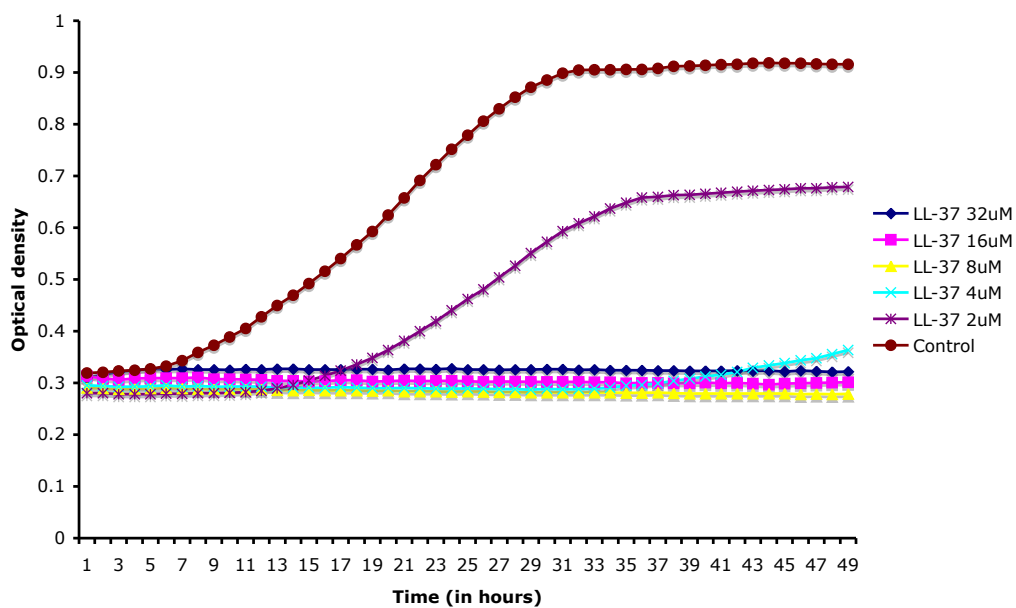


B.

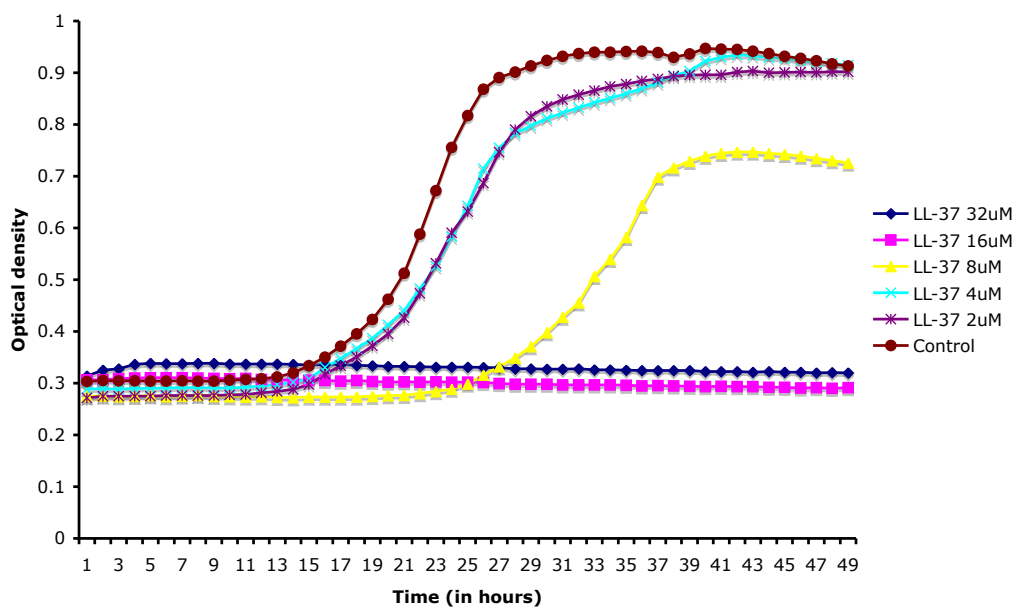
Figure 6.16. Minimum inhibitory concentrations for *Acinetobacter Baumannii* Southeast Clones AB11 and AB 12.

A. Southeast clone AB11, B. Southeast Clone AB 12.

Figure 6.16 shows that the MIC was $8\mu\text{M}$ (which corresponds to 36 g/ml LL-37) for both Southeast clones, AB 11 and AB 12. The time overnight culture was for a total of 16 hours. It can be seen that for both samples a concentration of $4\mu\text{M}$ appeared to inhibit bacterial growth but not ultimately stop it.



A.



B.

Figure 6.17. Minimum inhibitory concentrations for *Acinetobacter Baumannii* (Oxa 23 Clone 2) and *Pseudomonas Aeruginosa*.

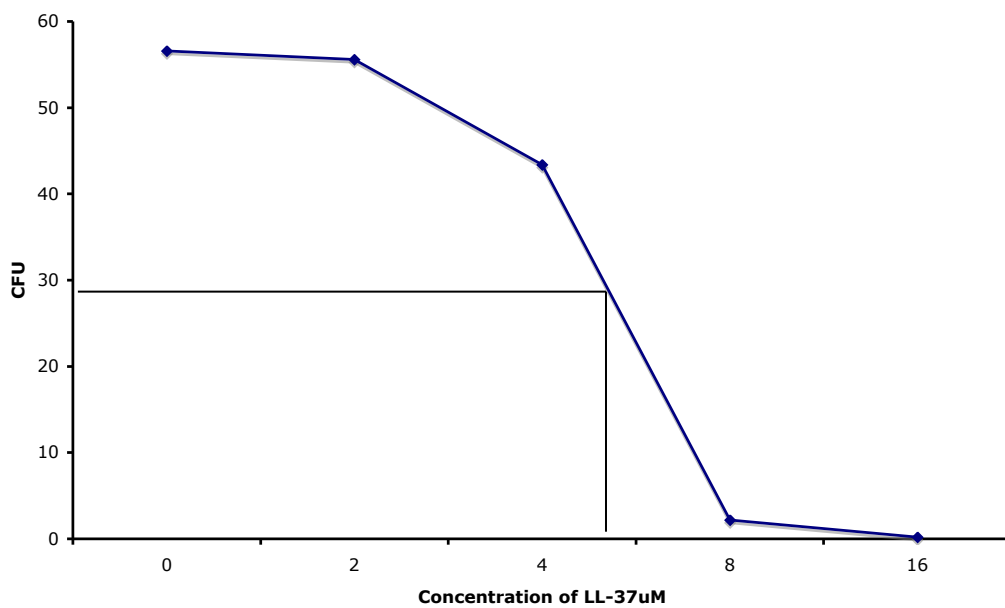
Figure 6.17 shows that the MIC was 8µM for Oxa 23 Clone 2 and 16µM for *Pseudomonas Aeruginosa*. Again it can be seen that for the *Acinetobacter* species a concentration of 4µM appeared to inhibit bacterial growth although in this case growth was minimal and only at the end of the study period and could represent a contaminant as well as inhibited *Acinetobacter* growth.

6.5.5 Determining the EC₅₀ for LL-37 against *Acinetobacter Baumannii*

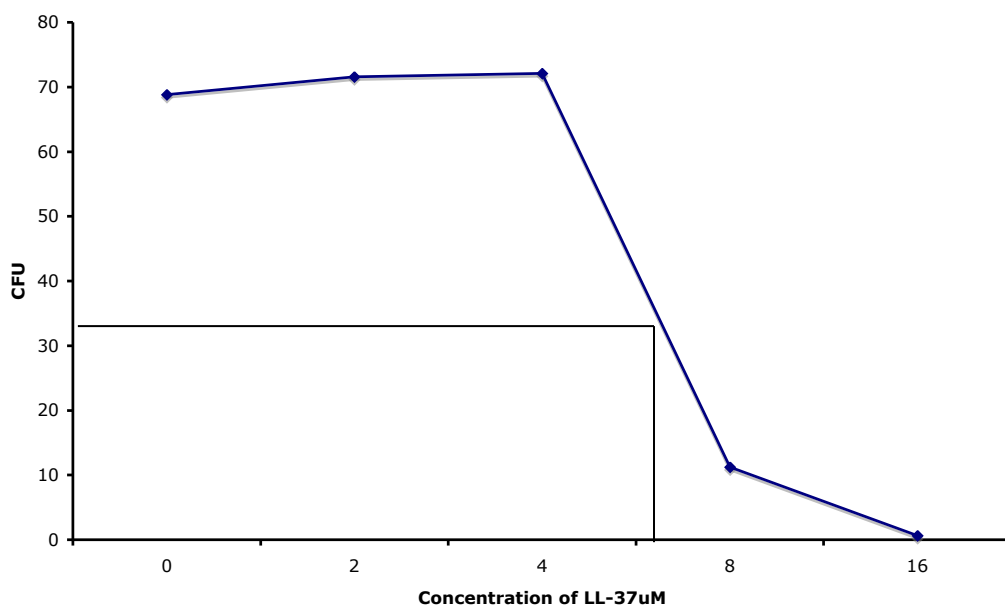
A way of giving some idea of the potency is to test the EC₅₀. This refers to the concentration that induces a response halfway between the baseline and maximum after some specified exposure time.

EC₅₀ was determined by analyzing plated serial dilutions of the bacteria-peptide *Acinetobacter baumannii* solutions on ISO agar plates after 60 minutes of growth. The numbers of colony forming units were counted after incubation.

A.



B.



C.

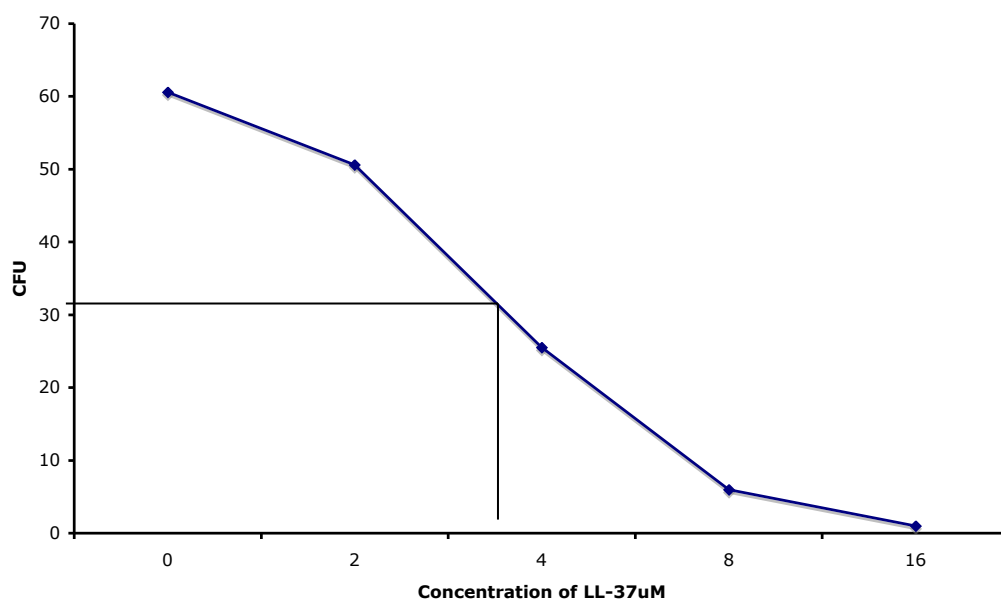


Figure 6.18 EC_{50} for the three strains of drug resistant *Acinetobacter baumannii* with EC_{50} shown on graph. The data shows the EC_{50} for the three strains of *Acinetobacter baumannii*. Graph A. demonstrates Southeast Clone AB 11, with an $EC_{50} < 6\mu M$, graph B shows Southeast Clone AB 12 ($EC_{50} 6\mu M$) and graph C. Oxa 23 Clone 2 ($EC_{50} 4\mu M$).

It can be seen in figure 6.18 that after 60 minutes of incubation of the bacteria-peptide solution a concentration of $6\mu M$ was sufficient to reduce the number of colony forming units by half. LL-37 is more effective against *Acinetobacter* than *Pseudomonas* as the EC_{50} for *Pseudomonas* was $10\mu M$ (results not shown) under the same conditions in our study.

6.6 Discussion

In mammals, two forms of immunity have evolved. The first, the innate system is non-clonal and non-specific and the second is adaptive and antigen specific.

Antimicrobial peptides (AMPs) are part of the innate system and make up one of the most ancient defence systems within the animal and plant kingdoms.

A growing number of AMPs have been found to play a role in the defence of the human skin. LL-37 is the only human cathelicidin-derived AMP; it belongs to the class of α -helical AMPs.

First found in the testis (Agerberth et al., 1995) and in leukocytes by Cowland et al., (1995), it has subsequently been found to be constitutively expressed in many different tissues, cells and fluids in the human body. In general these seem to be epithelial lined areas that will have close contact with the environment.

As well as having direct antimicrobial properties, LL-37 also displays a range of immunomodulatory functions as well as being involved in neovascularization and epithelial wound healing.

Its role in the defence of skin is particularly important, as in conditions where LL-37 has been down regulated (acute and chronic dermatitis) there is an inverse correlation between severity of the disease and the level of AMP production (Ong et al., 2002).

In burn patients not only has the protective mechanical barrier been disrupted but the immunomodulatory response has also been modulated making the patients susceptible to infection. The role of LL-37 in burn patients is not clear and the studies limited.

By immunohistochemistry this study has shown that LL-37 is present in the acute and healing burn wound. Like Kaus et al., (2008) LL-37 was shown at the surface of burned tissue (Panal 6.7, Picture B). In their study this was independent of burn depth. Our sample size was too small to draw any clear statistical conclusions but it was noted that if the whole of the epidermis was destroyed there was minimal LL-37 at the surface but even a small layer of epidermal remnants seemed to be associated with more LL-37 at the surface. This may be because the integrity of the cell wall has been disrupted by the thermal injury thereby allowing release of LL-37 to the wound surface. Few

studies have been conducted into other skin AMPs and it would be interesting to compare LL-37 with other defense peptides found in the skin.

Unlike other studies (Frohm et al., 1997) we found significant LL-37 in the healthy control patients. The reasons for this are unclear but could be due to the way in which the samples were collected. The speed in which LL-37 may be released is unknown and it could be that in the process of taking a punch biopsy and processing the sample some signaling and transcriptional factors may have been induced. As a result an increase in LL-37 levels may be seen. This would be true for both the control patients and the burn patients. Because the samples had to be transported from Chelmsford to London it may have allowed more time for signaling and transcriptional factors have been induced compared with other studies which may have been able to process their samples quicker.

Furthermore our data may not reflect the true level of LL-37 in “quiescent” normal skin. Unique phenotypes and variable responses to environmental stimuli mean that differential gene expression exists between individuals. This may manifest as different levels of LL-37 under the same conditions. For instance different patients may show a different response (i.e. release of LL-37) to the same stimuli (such as a punch biopsy) under the same conditions (an operating theatre). Such a small sample size is prone to pick up errors due to normal variation in normal skin, which may lead to false positive or false negative conclusions about affected tissue samples. Steps to reduce the variability include faster and more consistent processing or larger sample sizes to average out the results. A way to examine differential gene expression between individuals would be to take two samples from each patient, one of burned skin and a tissue sample of unaffected skin (a control sample). This may help elicit some of the variability between individuals however as Kaus et al., (2008) demonstrated, LL-37 levels were increased in non burnt tissue adjacent to the burn wound. Control samples would have to be taken from areas distant to any injury, which would involve scarring in a previously uninjured area (which may not be popular with patients).

To try and quantify the amount of staining seen, both the CSQ and total pixel count was used (as described by Lalli et al., 2008). Both visual assessment and pixel count showed good correlation with each other (correlation coefficient $r = 0.69$, $P < 0.005$; Figure 6.9). Figure 6.10 shows how the pixel count of positive

staining stained varied over time and showed that the LL-37 in the skin samples was initially low and gradually increased through the healing period. It is unclear how long it would take for levels to return to normal values.

From this result one might postulate that systemic LL-37 levels may also be depleted.

Serum samples of LL-37 also appear to be reduced in the acute phase and healing period (figure 6.12). The trend appears to suggest that LL-37 increases as time goes on towards that of the control patients; however statistically there is no difference between the expression on day 1 and the control patients. Whilst this may be a true finding with a small sample size firm conclusions cannot be made about the results.

In addition, like the work of Poindexter (2005) we found LL-37 tended to be found in the upper epidermal layers, especially the stratum corneum. Figure 6.10 shows most of the staining is suprabasal (clearly so in the control patients). In the early healing phases when skin has been grafted there is not a well-defined basal layer until later when the skin graft cells start proliferating and differentiating. Therefore the suprabasal layer staining that is seen may just reflect the transfer of the LL-37 in the skin graft rather than a change in the levels due to the injury. One way of determining this may be to try and define keratinocyte expression throughout the healing period to match keratinocyte activity to LL-37 levels.

As figure 6.8 shows LL-37 was also found in adnexal structures such as hair follicles and sweat glands. This confirms the work of Poindexter (2005) who has shown that other AMPs were present in dermal structures such as human beta defensin-1 in hair shafts; human beta defensin-2 and human beta defensin-3 in the glands of the lower dermis and LL-37 was evident in very high concentrations in the epithelium of sweat ducts.

The rt PCR showed significant results in only two of the samples (one a burn patient on day three after the burn and one in a patient over three weeks after the burn injury).

In another 18 patients there are weak reactions that may indicate minimal amounts of nucleic acid. Normally Cts < 29 are strong positive reactions indicative of abundant target nucleic acid in the sample. Cts of 30-37 are positive reactions indicative of moderate amounts of target nucleic acid but Cts of 38-40

are weak reactions indicating minimal amounts of target nucleic acid, which could represent an infection state or environmental contamination. As in the majority of these cases only one sample out of the three tested was positive it is possible that this is due to contamination.

There are a number of reasons for the PCR results. Firstly the processing of the samples may have influenced the results. When looking at the results in more detail it was clear that the two samples with a highest yield of nucleic acid were those that had the best yield of RNA (ng/μl) determined by measuring the absorbance at 260nm using NanoDrop® spectrophotometer. Most of the remaining samples had 260/280 ratios < 1.6, as determined by NanoDrop® spectrophotometer. This can be an indication of incomplete extraction or inefficient isolation. No yield or a small yield may be expected in the acute burn samples where the tissue and protein destruction is so great that all genomic material is destroyed but the poor yields in the healing wounds and control samples suggest that the processing could be a contributing factor. One part of the process where this is possible is the homogenization of the samples. Skin has evolved to withstand substantial shearing forces and as such trying to grind a full thickness sample may result in incomplete sample disruption (Berglund et al., 2006). The difficulty in disrupting the sample sufficiently also increases the risk that the sample will thaw. A study by Ambion (Tech notes 9.3) has shown that samples that are allowed to thaw result in more RNA degradation. Slow processing may allow endogenous RNases released internally resulting in RNA degradation. Another potential influence is the timing of the sample acquisition. Due to logistical reasons there was sometimes a delay in freezing the samples of over an hour. A study by Cole et al., (2001A) demonstrated how important timing of sample collection is to the final result. They collected samples of normal and injured skin from healthy patients undergoing surgery. Specimens were obtained at 30 minutes and 1 hour after the initial injury. RNA was extracted; reverse transcribed into cDNA and hybridized onto high-density cDNA microarray membranes of 4,000 genes. At 30 minutes, injury resulted in a consistent increase (> 2 standard deviations) in gene expression in 3% of genes. These genes were primarily involved in transcription and signaling. None of the 4,000 genes were decreased (< 2 standard deviations) at 30 minutes. At 1 hour however only 46 out of the 4,000 genes were increased in expression (1.15%) but

264 out of 4,000 (6.6%) genes were decreased greater than 2 fold. This is important as the delay in freezing the skin samples could result in reduced LL-37 expression. Furthermore Cole et al., (2001A) were only able to extract useable RNA in 56% of their samples (defined as A 260/280 ratios > 1.8). Using this criteria only one specimen in the skin samples was adequate quality for analysis. Even with rapid sample excision and freezing in liquid nitrogen there may be some problems as some signaling and transcriptional factors may be have been induced already and as noted before for the immunohistochemistry the data even for the control samples may not reflect the true level of gene expression of “quiescent” normal skin (Cole et al., 2001B). The speed with which cells on the sample border may respond to the excisional injury and influence AMP expression is unknown. This creates a difficult challenge: to accurately obtain a genomic expression profile for normal human skin under ideal quiescent conditions let alone acutely injured skin. Also in the majority of cases the patients were under general anesthesia, the potential variable of general anesthesia on baseline levels of gene expression is also unknown.

As well as its antimicrobial properties the immunomodulatory properties of LL-37 may mean it has a role in sepsis or the immune response seen after a burn. In a rat model of intra-abdominal sepsis, LL-37 therapy improved outcome and in combination with hyperthermic preconditioning the survival rate after sepsis was increased and the proinflammatory cytokine response was downgraded. A study of 31 sepsis patients in a general intensive care unit found that whilst some alpha-defensins and other AMPs were increased in these patients LL-37 was not (Berkestedt et al., 2010). Another study looking at LL-37 levels in the critically ill found that the mean plasma LL-37 levels were significantly lower in critically ill subjects compared to healthy controls (Jeng et al., 2009). In contrast Lippross et al., (2011) examined the serum profiles of defensins, cathelicidins and pro-inflammatory cytokines post trauma and found that hBD-2, hBD-3 and LL-37 concentrations were significantly elevated after trauma and followed different characteristic concentration curves. In addition, similar patterns of concentration profiles were recorded for hBD-2/IL-6 and hBD-3/IFN-gamma.

Burn patients not only frequently suffer concurrent trauma as well as the burn injury but also frequently develop complications such as infections or sepsis. The role of LL-37 in burns patients may therefore be complex.

As can be seen from figure 6.12, in our study, serum LL-37 levels were reduced in the acute and healing phase compared with the healthy controls. Initially low, the serum LL-37 dropped even more over the first few weeks before rising slightly again.

The hypermetabolic response after a major burn begins on the fifth day post injury and continues up to 24 months post burn. The increased metabolic requirements cause tissue catabolism, leading to nitrogen loss and a potentially lethal depletion of essential protein stores. The energy requirements are met by the mobilization of proteins and amino acids. As a consequence, the structure and function of essential organs, such as the heart, the liver, skeletal muscle, the skin, the immune system and cellular membrane transport functions, are compromised (Jeschke et al., 2007). It may be that the reduced LL-37 levels are a direct consequence of this catabolic state and therefore could potentially stay depleted for many months.

This is an important finding as reduced levels of LL-37 are associated with severity of diseases such as in atopic dermatitis. Therefore in the burn patients this may manifest as poor wound healing or may predispose to increase levels of sepsis and potentially increased morbidity.

Like Jeschke et al, (2007) we found that both IL-6 and IL-8 were increased after the burn injury (figures 6.13 and 6.14) but unlike their study we found there was no link to the burn size. In concordance with their results, IFN γ although affected by the burn was not affected by the burn size. There was no statistical difference between the pro-inflammatory cytokine levels on day 1 and the controls.

The relationship between AMPs and cytokines is complex. In the study by Lippross et al., (2011) IL-6 showed a similar concentration profile to hBD-2 in the trauma patients. LL-37 has been shown to induce the production of a number of cytokines (IL-8, IL-18 and IL-20) through the MAP kinase pathway (Niyonsaba et al., 2005) and in psoriasis patients' LL-37 levels have been correlated with interferon (IFN)- γ and IL-10 levels (Kanda et al., 2010). Although the possibility of LL-37 influencing IL-8 or (IFN)- γ in burns patients

exists the sample size was too small and the variability in the results too great to see if any such relationships is present.

The role of LL-37 as immune response modulator is complex and it may be that its antimicrobial abilities may be more useful in the immediate clinical setting. The rapid emergence of bacterial strains with growing resistance to conventionally used antibiotics mean alternative solutions to treating infection should be sought.

The antimicrobial activity of LL-37 has been shown against a variety of organisms, but no studies have been conducted to determine its direct action on *Acinetobacter baumannii*. After obtaining clinical isolates of three multi drug resistant strains that were prevalent in the area the MIC of LL-37 against the strains was determined.

For all three strains the MIC was 8 μ M (Figures 6.16 and 6.17) showing it to be slightly more effective than against *Pseudomonas aeruginosa*. The peptide concentration killing 50% of the bacteria, EC₅₀ was around 6 μ M. The study by Chromek et al., (2006) when investigating the role of cathelicidin in protecting the urinary tract against invasive bacterial infection found similar levels of activity against *E.coli*.

The levels of LL-37 found in these studies are low and reduced by the burn injury therefore to be of clinical use levels would have to be increased. There are several factors that have to be considered however.

To be of clinical use, any increase in LL-37 must reach a concentration that is sufficient not only to effectively kill bacteria but also then maintain that effective killing concentration for a time period needed to keep the bacteria from re-growing.

Whilst the MIC is the concentration that will kill the microbe in vitro, the MBC (minimum bacteriological concentration) is the concentration that will effectively kill the microbe in vivo. If an antimicrobial agent produces its activity in a concentration-dependent manner, it must reach the effective MBC in order to kill the microbe by producing a peak concentration that is generally about 8–10 times the MIC.

If an antimicrobial agent produces its activity in a concentration-independent or time-dependent manner, it must maintain a concentration at least 1–2 times the

MIC for the entire period between dosing intervals in order to kill the microbe and prevent its re-growth (Herndon 2007). These factors determine in which way LL-37 may be utilized.

Some efforts have been made in testing various ways of delivering or increasing LL-37. Bals et al., (1999) used a murine model of infection and sepsis and demonstrated that systemic gene transfer resulted in high concentrations of the mature peptide in the serum, and this over expression of LL-37/hCAP-18 improved the survival of mice after injection of LPS or *E.Coli*.

Transient cutaneous adenoviral transfection with hCAP-18/LL-37 into infected burn wounds (in rats) was shown to be more effective than using a synthetic LL-37 in bacterial inhibition in a study by Jacobsen et al., (2005).

Other methods to use LL-37 would be to determine if there are there drugs or chemicals that could target specific cell types to induce increased synthesis of defensins and LL-37 in the remaining skin elements following burn injury. Alternatively when cells are cultured for CEA (cultured epithelial autograft) the defensins or LL-37 could be combined with keratinocytes other cell types to provide a form of topical application in which AMPs can be delivered to the wound bed.

The role of AMPs in burns is complex and needs further investigation but in an era of growing bacterial drug resistance offers an exciting alternative strategy to improve care and combat the constant threat of wound infection and sepsis.

7.0 Conclusions

Since humans have suffered thermal injuries the battle has always been to keep the wound clean and prevent infection, the consequence of uncontrolled infection may be sepsis, multi-organ failure and death.

The treatment of sepsis and multi-organ failure is facilitated by the early detection of those patients that may suffer from these conditions so treatment can be initiated as soon as possible and the right patients targeted effectively.

What is required is a relatively cheap easily performed test that would identify those patients that will develop sepsis and multi-organ failure.

We identified the urinary ACR as a possible candidate for this role. Severe burn causes a systemic inflammatory response, endothelial dysfunction and a microvascular leak of fluid and protein into the interstitium. Almost 40 years ago Parving et al (1974) showed that there was a correlation between microalbuminuria and the increased rate of transcapillary escape of radiolabelled albumin in hypertensive and diabetic patients. A link between increased systemic vascular permeability to albumin and microalbuminuria was therefore made.

In the intervening years microalbuminuria has been used as a surrogate marker for systemic endothelial dysfunction and a predictor of outcome in a variety of surgical and intensive care settings (Vlachou et al 2006).

There have been limited studies looking at ACR in the burn ICU setting. The three most relevant studies (Yew and Pal 2006, Vlachou et al., 2006 and Vlachou et al., 2008) look at specific populations in this setting.

This study demonstrates firstly the prevalence of microalbuminuria in a general burn ICU population (including all types of thermal injuries, sizes and age ranges) for the first time with 45.5% having an elevated ACR (>2.3 mg mmol⁻¹) on admission to the unit. It was found the peak ACR is related to the length of stay of the patients who survive, (which suggesting it may have some predictive value in patient outcome as those with longer in-patient stays tend to have a more complicated stay and recovery). The peak ACR has little clinical benefit, as one cannot predict when the peak may be. Therefore, the admission ACR and ACR after resuscitation (at 48 hours) was examined. No relationship was found between the TBSA% length of stay and either of these time periods.

In addition no link was found between the log admission ACR and the TBSA% sustained. This is in contradiction to the work of Vlachou et al., (2006). This showed a positive correlation between urinary ACR and TBSA at 3 and 7 hours post injury and with the percentage of full thickness burn between 3 and 7 hours. Vlachou's later work (2008) showed per-operative ACR was associated with % TBSA excised. One reason for this may be in the recording of the surface area burned. Only recording the TBSA % and not consistently differentiating the amount of full thickness burn as opposed to more superficial burns makes the results difficult to interpret. As noted before a 10% full thickness burn may be a more significant injury or cause a greater inflammatory response than a 20% partial thickness burn. Secondly the timing of the samples is important. In Vlachou's study (2006) all patients that presented to the unit 6 hours after the injury were excluded. It is likely these results offer a more accurate idea of whether ACR on admission and TBSA% are related. Due to the geographical position of the unit in our study and nature of the referrals many patients' samples were not collected until after 6 hours.

The ACR at admission did predict outcome (death) with a sensitivity and specificity comparable to some other studies that have examined ACR in the critical care setting (although not burns) at a low level of ACR, $3.78 \text{ mg mmol}^{-1}$. This predicts outcome at a far lower level than previously described by Yew and Pal (2006) who had determined that a mean ACR of 20 mg mmol^{-1} was associated with poorer outcomes. Despite this, the sensitivity and specificity are not high enough to use it as a clinical tool in our ICU. It may be that its diagnostic accuracy could be improved by using it in conjunction with another marker of inflammatory response (such as C-reactive protein or procalcitonin).

Because ACR was related to the length of stay (in survivors) the relationship between ACR and both sepsis and multi-organ failure (complications that may increase length of stay) was examined. Whilst on the ICU many patients suffer from one or both of these conditions and the concurrent inflammatory response may be reflected in the degree of endothelial dysfunction.

Our analysis demonstrated that there is a clear relationship between the admission ACR and the development of sepsis (figure 3.4 and 3.5). Again however the sensitivity and specificity make its utility poor in the clinical

scenario. More important factors for developing sepsis were the TBSA% and the presence of an inhalation injury.

This study showed that the ACR value was directly related to the multi-organ failure score which we analysed further to see if ACR could be used to differentiate patient outcomes in those patients suffering from multi-organ failure. From our study although there was a clear relationship between ACR and MOF it does not offer clear a clinical utility at the moment to identify those patients that may suffer from multi-organ failure. It may be that burn injuries are too complex and there are too many confounding factors in multi-organ failure for ACR to be able to distinguish those patients who will achieve higher MOF scores. An interesting result that appeared to come of the analysis was a correlation between MOF score and ACR value from 20 days before which suggest that the relationship may be far more complex than suspected from the results in both this and previous studies of ACR. It is unclear why there may be a correlation between MOF score and ACR from a number of weeks before. It may be related to the pattern of “two cascades of organ failure” described by Yew and Pal (2006) who noted deaths in the first week post injury result from failure of reversal of the burn shock and then a second group of patients dying several weeks after the burn injury typically from an infectious process usually associated with pneumonia. In the initial injury the same process that causes endothelial dysfunction (and therefore an ACR rise) also affects the immune system with the result that the body is immunosuppressed. By the third week post injury with adequate support most patients are able to mount an effective immune response. It may be that the ACR picks up on those patients that are not so able to mount an immune response. This becomes more obvious at around the 20-day post injury stage with the result that these patients suffer from more complications and organ failure.

It must be noted however, that this apparent link could be spurious. The number of patients this data is based on gets smaller and smaller as generally the number of patients remaining on the unit for a long period reduces. It could therefore be an artefact arising from the few patients that have both high MOF scores and high ACR (perhaps for unrelated reasons) and remain in the unit.

In conclusion, although ACR may have a role in demonstrating outcome and the development of sepsis and multiorgan failure, currently in burns care it appears to be limited. It may be that because of the systemic impact that burns injuries are more complex than many other medical illnesses and looking at ACR in isolation is inadequate to reflect the inflammatory changes going on. Perhaps sampling ACR in combination with another marker of inflammation such as CRP or procalcitonin would provide more accurate results.

Despite all the technical advances in burn care in the last 50 years one of the reasons that sepsis and multi-organ failure are the most frequently reported causes of death in the burns units throughout Europe (Brusselsaers et al 2010) and the US is the rise of multi drug resistant organisms. A striking example of this is in a study of five-thousand two-hundred-sixty paediatric patients over a twenty years period in Galveston, Texas. The leading causes of death over 20 years were sepsis (47%). From 1989 to 1999, sepsis accounted for 35% of deaths but increased to 54% from 1999 to 2009, with a significant increase in the proportion due to antibiotic resistant organisms (William et al., 2009).

One of the multi-drug resistant bacteria that have caused problems in the UK is *Acinetobacter baumannii* (MRAB), illustrated by the rise in cases resistant to carbapenem in one unit increasing from 0% to 55% between 1998 and 2006. This overall increase coincides with the emergence of the MRAB-C OXA-23 clone-1 as the most prevalent strain in London and South East England (Wareham et al., 2008).

Anecdotally it was felt (by the surgical and anaesthetic consultants running the unit) that those patients with the multi-drug resistant strain of *Acinetobacter baumannii* tended to have a greater morbidity than those with drug sensitive *Acinetobacter* or no infection, this was not based on any data however.

We therefore investigated this by examining acquisition of MRAB and drug sensitive *Acinetobacter* and the development of multi-organ failure during an outbreak of *Acinetobacter* in the ICU. MRAB was shown to be related to the acquisition of *Acinetobacter baumannii* and in table 5.6 it can be seen the impact this had. The patients in this group not only had more visits to theatre, but also spent longer on the unit. It is difficult to determine whether MRAB was the cause of the MOF or a consequence of it, but the regression analysis and use of

matched groups (who had drug sensitive *Acinetobacter baumannii* and no *Acinetobacter* infection) shows that the group with MRAB had a higher rate of multi-organ failure despite the other parameters remaining similar between the groups. We therefore concluded that MRAB was responsible for the multiorgan failure.

This study is the first to show an association between MRA and multi-organ failure. This is an important finding because multi-organ failure is associated with a greater morbidity and mortality (Cumming et al 2001). If the patient does not die, multi-organ failure means not only poorer outcomes for the patient (physically and psychologically) but has important financial implications in the short term for the hospital and in the long term for the patient and the community. Wilson et al (2004) established that the mean hospital costs of patients that acquired MRAB was \$98,575 higher than that of control patients who had identical burn severity of illness indices.

The isolation of multi resistant *Acinetobacter* should result in an aggressive policy of management to identify the source, educate the staff and ensure good hand hygiene to avoid transmission to other patients and potentially life threatening problems and a large extra cost.

Even with all these measures in place the main problem is the emergence of multi resistance to conventional antibiotic treatment. New therapies are required that can help the fight against these organisms. One such group of molecules with this potential are the AMPs, part of the innate immune system that are increasingly found to play an important role in the defence of the body. Studies have shown not only direct antimicrobial properties but also indirect antimicrobial ability through their modulation of the immune response by interaction with a number of cytokines and cells. The presence of the main AMPs in skin, both defensins and cathelicidin has been established in burns.

Our study demonstrated how the LL-37 is reduced in the acute burn and for some time afterwards in the healing wound although statistical significance was not achieved in the analysis of serum levels of LL-37 and LL-37 levels in the skin through rtPCR analysis. Unlike Frohm et al., 1997 we found significant LL-37 in the healthy control patients in the immunohistochemical analysis. The reasons for this are unclear but could be due to the way in which the samples were collected. The speed in which LL-37 may be released is unknown and it could be

that in the process of taking a punch biopsy and processing the sample some signaling and transcriptional factors may have been induced. The time taken for the samples to be transported to London for analysis may have allowed for this process to occur whereas in other studies the samples are processed immediately perhaps before these processes occur.

Another factor to consider is that unique phenotypes and variable responses to environmental stimuli may mean that differential gene expression exists between individuals. This may manifest as patients having different levels of LL-37 under the same conditions. The number of samples in this study may be too small to determine abnormal LL-37 expression especially if there is a very variable expression in the standard population.

In general the immunohistochemistry results showed that LL-37 was reduced in the post injury period but began to return towards normal as healing commenced. To try and quantify the amount of staining seen, both the CSQ and total pixel count was used (as described by Lalli et al., 2008). Both visual assessment and pixel count showed good correlation with each other (correlation coefficient $r = 0.69$, $P < 0.005$; Figure 6.9). Figure 6.10 shows how the pixel count of positive staining stained varied over time and showed that the LL-37 in the skin samples was initially low and gradually increased through the healing period (towards values seen in the control patients). It is unclear how long it would take for levels to return to normal values or indeed if LL-37 returns to normal in the injured areas of skin.

The rtPCR showed significant results in only two of the samples (one a burn patient on day three after the burn and one in a patient over three weeks after the burn injury). In another 18 patients there are weak reactions that may indicate minimal amounts of nucleic acid. Again these results maybe a result of the processing. It was clear that the two samples with a highest yield of nucleic acid were those that had the best yield of RNA (ng/ μ l) determined using the spectrophotometer. This can be an indication of incomplete extraction or inefficient isolation. Studies by Cole et al., (2001A) found useable RNA in only 56% of their samples (a useable sample was defined as having a 260/280 ratio > 1.8). Using these criteria only one of the burn or control samples was suitable for analysis. As noted above even with rapid sampling, some signaling and

transcriptional factors may have been induced already by the process required to take the sample.

With results such as these it is difficult to draw clear conclusions regarding the significance of the presence of LL-37 in burns without further studies but irrespective of this LL-37 may yet have a role in the treatment of burns by modulation of the immune system or by its direct antimicrobial action. With the rapid emergence of bacterial strains with growing resistance to conventionally used antibiotics it is imperative that alternative solutions to treating infection should be sought.

Whilst the antimicrobial activity of LL-37 has been shown against a variety of organisms, no studies have been conducted to determine its direct action on *Acinetobacter baumannii*. The last part of this study demonstrated LL-37 had bactericidal activity against *Acinetobacter baumannii* and therefore has potential to be used in a clinical setting such as the burn ICU. Three strains of drug resistant *Acinetobacter baumannii* that are prevalent in hospitals in the southeast of England were studied and LL-37 had similar MIC and EC₅₀ values for all three strains.

This is important because studies both in the laboratory and in the clinic confirm that emergence of resistance against antimicrobial peptides is less probable than observed for conventional antibiotics (Zasloff 2002). This has provided the impetus to develop antimicrobial peptides, both natural and laboratory conceived, into therapeutically useful agents. These are several ways in which LL-37 and other antimicrobial peptides may have a therapeutic role: (1) as single anti-infective agents, (2) in combination with conventional antibiotics or antivirals to promote any additive or synergistic effects, (3) as immunostimulatory agents that enhance natural innate immunity, and (4) as endotoxin-neutralizing agents to prevent the potentially fatal complications associated with bacterial virulence factors that cause septic shock (Gordon et al., 2005).

Studies have so far been limited in the burns world. Bals et al., (1999) tried to increase LL-37 availability in a murine model by systemic gene transfer. Over expression of LL-37/hCAP-18 improved the survival of mice after injection of LPS or *E.Coli*. Jacobsen et al., (2005) found that transient cutaneous adenoviral

transfection of hCAP-18/LL-37 in infected burn wounds (using a rat model) was more effective than using synthetic LL-37.

There are a number of difficulties in developing antimicrobial peptides as anti-infective drugs, which include systemic and local toxicity, costs of synthesis and economic viability in production, natural resistance and confounding biological functions of LL-37. The potential advantages of broad-spectrum activity, potentially low levels of induced resistance and concomitant broad anti-inflammatory properties remain an attractive proposition.

So whilst AMPs will not be the magic bullet to solve all issues associated with increasing antimicrobial resistance and overwhelming immune response in sepsis they have the potential to be significant reinforcements to the currently available therapeutic options.

8.0 Future work

ACR

The study into ACR was broad based and inclusive. It may be that there has been a loss of discriminatory power by such an approach and that by focusing on a more selective patient group may clarify the role of ACR. The patient cohort is therefore currently being analyzed further with all patient co-morbidities included (i.e. diabetes, high blood pressure and other illnesses that may influence ACR). A separate analysis of children with regards to sepsis and MOF is also being undertaken. The work by Vlachou et al., (2006 and 2008) showed a correlation between ACR and TBSA%, which was not seen in this study. One possible reason is their more accurate measurement of the surface area involved in the burn as they measured the percentage of full thickness burn (%FTB) as well as TBSA%. Further studies will include the % FTB as well as the TBSA % to clarify the relationship between ACR and burn size. In addition Vlachou's studies also highlighted the importance of the timing of the urine analysis. Future studies need to ensure there is a consistent approach and like Vlachou ensure all specimens are taken within six hours of injury. One way in which this maybe achieved is by requesting all referring hospitals take a urine sample at the time of the first assessment and send it to the burn unit for analysis.

There is yet no investigation to determine if the urine samples can deteriorate if left for a number of hours so a small pilot would have to be performed to see if the urine samples give consistent ACR results overtime. This could be achieved by testing the same urine sample of 20 different patients on an hourly basis to see if the ACR varies.

An intriguing part of the analysis of ACR was the correlation of the MOF score with the ACR from 20 days before at this stage it is unclear if this is of significance. Further analysis is required of those patients staying for long periods (greater than 30 days). As the number of patients staying for long periods is small this may be best achieved by collaboration with one or more other large burn units.

Acinetobacter baumannii

One of the most intriguing results of the *Acinetobacter baumannii* study was the correlation between MRAB transmission and the number of agency staff on the unit i.e. it acted as a flag for potential underlying problems.

If transmission of *Acinetobacter baumannii* is increased because of staff being unsure of unit protocols or through inadequate training then other mistakes may also be made for the same reasons. It is therefore planned to see retrospectively if there is any link to overall patient outcomes (assessed by patient death or increased length of stay) during these periods.

As the number of cases of *Acinetobacter baumannii* varies seasonally and is not a problem in many other critical care units it would be useful to know if there are any other infective organisms that can be used as such a flag instead of *Acinetobacter baumannii*. This would allow the results to be extrapolated to other units or allow other units to look at their staffing levels and the consequences for patient care.

LL-37

Whilst the LL-37 immunohistochemistry results fit in with current understanding of AMPs in thermal injury the rt PCR does not endorse this work. It may be a fault of the processing of the samples but needs confirmation by repeating the analysis with refinement of the technique. This can firstly be approached by speeding up the process of sample collection with quicker freezing of the samples in liquid nitrogen. The grinding down of the skin samples in a pestle and mortar is also a potential source of RNA degradation due to thawing and endogenous RNase release. An alternative technique as described by Berglund et al., 2007 will be tried to provide improved RNA yield and also provide protein for more in depth analysis.

Unique phenotypes and variable responses to environmental stimuli means that differential gene expression exists between individuals. As the control patients showed greater staining of LL-37 compared to other studies it is clear there may be greater variability in the normal uninjured population than has previously been understood. To examine this further we will examine whether there is any variation in LL-37 expression with age and racial background and whether there is any variation in LL-37 skin in different areas of the body. In addition samples

of unburnt skin would be collected at the same time as the burnt samples from areas distant from the wound site (pending ethical approval) to see the patients normal baseline level to get a comparative idea of how much a burn injury affects the skin locally.

The immunohistochemistry results showed that the LL-37 was initially reduced and then returned towards normal levels. In future work it is hoped to follow the patients for a longer time to see if the LL-37 does return back to normal or if the levels in the damaged or grafted skin are always suppressed.

A further question that became apparent is that when taking samples from the healing wounds that had been grafted it was impossible to tell if the LL-37 that was stained was due to LL-37 being transferred with the skin graft or because of local factors increasing expression in the healing wound. To examine this a sample of the donor skin should be analysed to see how much LL-37 is transferred and if there is any relationship with the depth of the skin graft taken.

Although the levels of pro-inflammatory cytokines studied showed no significant findings they do echo the results of larger studies and the results probably reflect the small sample size. The finding of Lippross et al., 2011 that LL-37 was significantly elevated after trauma and that hBD levels were related to IL-6 levels show that the relationship between AMPs and cytokines may be complex. The collection of serum samples will be continued with the targeting of those patients who develop sepsis.

The work of Vlachou et al., (2006 and 2008) demonstrated how sensitive ACR was to wound manipulation. Papini et al., (1997) also showed that cleaning or debriding a wound altered serum IL-6 levels. It is feasible that LL-37 could be equally sensitive to wound manipulation. It is therefore planned to see how serum LL-37 levels are influenced by these factors by taking serial samples before, during and after a range of procedures and interventions including, changing a dressing, cleaning a wound, surgical débridement of a wound and a non wound related intervention such as changing an intravenous line.

LL-37 has been used to treat sepsis in the animal model, raising the possibility that it or an analogue could be used in some form in humans. Before this, a clearer understanding of LL-37 is needed and I hope my work contributes to this.

Appendix I

Chapter 2 details the role of ACR in the burns ICU and these results relate to the study of both adults and children.

Section 1

	Mean	Standard deviation	Percentiles				
			Smallest 0%	25%	Median 50%	75%	Largest 100%
Peak ACR	17.7	33.3	0.5	2.6	6.3	16.6	231.0

Table 1. Summary statistics of peak ACR (see p92)

	Mean	Standard deviation	Percentiles					Number of values
			Smallest 0%	25%	Median 50%	75%	Largest 100%	
Admission ACR	0.599	0.931	0.020	0.130	0.310	0.690	8.800	301

Table 2. Summary statistics of length of stay per percent TBSA by ACR at admission (see p97)

Section 2

Log ACR at admission and other variables

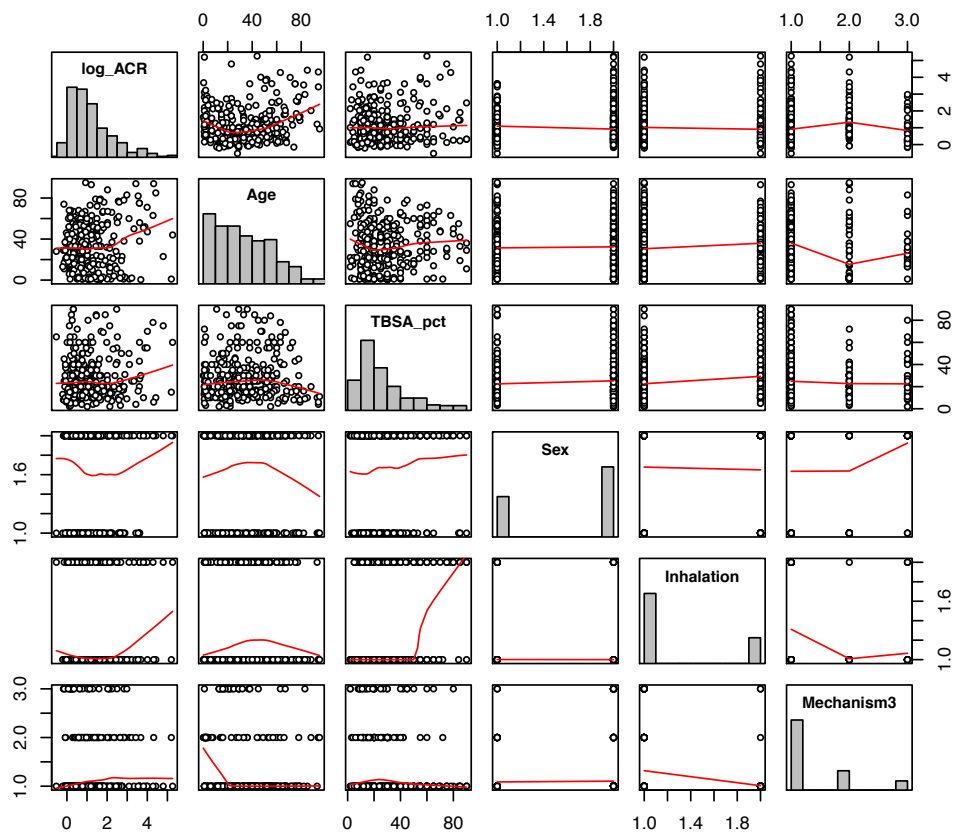


Figure 1. The scatterplot matrix above shows all the variables plotted against each other in both directions (y vs. x and x vs. y), and also the histograms of the individual variables. There are no clear relationships. Table created by Dr Mike Parker, Anglia Ruskin University.

Section 3. Multiple regression models (see p89) Performed by Dr Mike Parker, Anglia Ruskin University.

Model response variable.

Log_ACR Log ACR at admission

Potential model predictor variables

1. Age Age at admission in years
2. TBSA_pct Percentage of total body surface area burned
3. Sex
4. Inhalation A categorical variable (Yes, No)
5. Mechanism3 A categorical variable (Flame, Scald, Other)

Model goodness of fit summary statistics

The tables below list the following statistics for each model.

1. p The number of parameters fitted. The more parameters fitted the more complex the model is, and is therefore more likely not to be generalisable to other groups of patients.
2. AdjR2 In modelling using ordinary multiple regression with a continuous response models the adjusted R-square indicates the proportion of the variance explained by the model.
3. AIC Akaike Information Criterion. This penalises models for every additional parameter that they estimate. Lower values are sought.
4. PRESS Prediction error sum of squares. Smaller values are sought.
5. CVE Cross-validation estimate of the Mean Square Prediction Error (MSPE) and is an unbiased estimate of the residual variance.

Over fitting

Models can be found that will fit all the observations in a data set perfectly, but this model will be of no value for a different data set. The more complicated a model is the more likely it is that it will apply only to the current data set and be poor at prediction for other data sets.

Models with all possible combinations of predictors

The tables below should be treated with some caution. If any one of the models were to be examined in detail it might be found to be unsuitable because of large standard errors. The search for multiple regression models involves 301 cases.

Table 3 Single-predictor models (5 possible models)

Model	Predictors	p	AdjR2%	AIC	PRESS	CVE
1.1	Age	1	2.1	885	332	1.1
1.2	Mechanism3	2	2.0	886	332	1.1
1.3	TBSA_pct	1	0.4	890	338	1.1
1.4	Inhalation	1	-0.2	892	339	1.1
1.5	Sex	1	-0.3	892	339	1.1

The table above shows that the fit of the models is very poor.

Table 4 Two-predictor models (10 possible models)

Model	Predictors	p	AdjR2	AIC	PRESS	CVE
2.1	Mechanism3+Age	3	5.3	876	322	1.1
2.2	TBSA_pct+Mechanism3	3	2.7	885	332	1.1
2.3	TBSA_pct+Age	2	2.4	884	333	1.1
2.4	Mechanism3+Inhalation	3	2.3	886	333	1.1
2.5	Age+Inhalation	2	1.8	886	334	1.1
2.6	Sex+Age	2	1.7	887	334	1.1
2.7	Mechanism3+Sex	3	1.7	888	334	1.1
2.8	TBSA_pct+Sex	2	0.1	892	340	1.1
2.9	TBSA_pct+Inhalation	2	0.1	892	340	1.1
2.10	Sex+Inhalation	2	-0.5	894	341	1.1

The two-predictor models still show a very poor fit.

Table 5 Two-predictor models with interaction (20 possible models)

Model	Predictors	p	AdjR²	AIC	PRESS	CV E
2.1i	Mechanism3+Age+Mechanism3:Age	5	6.2	875.6	324.5	1.1
2.2i	Mechanism3+Age	3	5.3	876.4	322.4	1.1
2.3i	TBSA_pct+Mechanism3+TBSA_pct: Mechanism3	5	5.3	878.4	324.3	1.1
2.4i	TBSA_pct+Age	2	2.4	884.4	332.7	1.1
2.5i	TBSA_pct+Mechanism3	3	2.7	884.5	331.9	1.1
2.6i	Mechanism3+Inhalation+Mechanism 3:Inhalation	5	3.2	885.1	Inf	Inf
2.7i	Mechanism3+Inhalation	3	2.3	885.9	332.7	1.1
2.8i	Age+Inhalation+Age:Inhalation	3	2.2	886.2	334.9	1.1
2.9i	TBSA_pct+Age+TBSA_pct:Age	3	2.1	886.3	336.3	1.1
2.10i	Age+Inhalation	2	1.8	886.4	334.1	1.1

The models with interaction still have not improved the goodness of fit to useful levels. Further analysis (not shown) reveals that the best three-predictor models only explain 6.1% of the variance, and the best three-predictor model with interactions only explains 11.2% of the variance. It is clear that the predictors considered here are not useful in a model of log ACR.

Appendix II

Chapter 3 details the investigation into whether ACR has a predictive role in identifying sepsis in burn patients admitted to the ICU. Statistical analysis performed by Dr Mike Parker, Anglia Ruskin University.

Section 1

Condition	Mean	Standard deviation	Percentiles					Number of values
			Smallest 0%	25%	Median 50%	75%	Largest 100%	
No sepsis	0.983	0.911	-0.174	0.372	0.693	1.322	4.316	125
Sepsis	1.281	1.248	-0.528	0.320	0.978	1.722	5.260	88
Overall	1.107	1.071	-0.528	0.329	0.846	1.502	5.260	213

Table 1. Log ACR at admission by condition (see p139)

Condition	Mean	Standard deviation	Percentiles					Number of values
			Smallest 0%	25%	Median 50%	75%	Largest 100%	
No sepsis	42.4	20.2	16	24	39	55	95	136
Sepsis	45.0	16.8	16	33	44	57	85	103
Overall	43.5	18.8	16	28	42	56	95	239

Table 2 Summary of statistics used comparing age at admission by condition (see p142)

The difference between the means for the two groups is 2.6 with 95% confidence limits 43.5 (41.12, 45.88).

Appendix III

Chapter 5 examines the role of Acinetobacter on burn ICU outcomes and the relationship to multi-organ failure. Statistical analysis performed by Dr Mike Parker, Anglia Ruskin University.

Section 1

Infection group	Mean	Standard deviation	Percentiles					Number of values
			Smallest 0%	25%	Median 50%	75%	Largest 100%	
Drug resistant	3.13	1.14	1	2	3	4	5	30
Drug sensitive	1.74	1.54	0	0	2	3	5	23
No infection	1.16	0.86	0	1	1	2	3	31
Overall	2.02	1.45	0	1	2	3	5	84

Table 1: Total number of organ failures by infection group (see p 197)

Section 2

Analysis of variance of the total number of organ failures

In this section the group mean total number of organ failures are compared without any attempt to allow for any imbalance of the groups (see p205).

Source of variation	Degrees of freedom	Sum of squares	Mean Square	Variance ratio	Permutation test P-value
Group	2	61.857	30.929	22.35	< 0.00001
Residual	81	112.095	1.384		
Total	83	173.952			

Table 2: Analysis of variance summary table of organ failures

The residual variance is 1.384 and its bootstrap 95% confidence limits are (1.093, 1.895).

The residual standard deviation is 1.176 and its bootstrap 95% confidence limits are (1.045, 1.376).

The model underlying the analysis of variance involving just the Groups factor has an adjusted R-square times 100 of 34.0%. This is the percentage variance accounted for by the model.

The usual F test invokes Normal distribution theory, but permutation tests do not. An exact permutation test finds all possible permutations of the data to find the probability of the observed result or any result more extreme. This is not

practical for any but very small samples, so the approach used is to generate a large number of random permutations. This has been done here and the P-value obtained is presented in Table 2. The conclusion is the same for the Normal theory P-value and the permutation test P-value.

Normal distribution theory is usually invoked to calculate confidence limits. The required assumptions can be avoided using bootstrap analyses. This involves taking repeated random samples of the data and performing calculations of the resulting distribution of the estimate of interest. In the bootstrap analyses here 9999 samples have been selected. The confidence limits below have been adjusted using the Dunn-Sidak adjustment for multiplicity so that a nominal 95% confidence level has become a 98.3% confidence level.

Group difference	Difference between means	Standard error	98.3% confidence limits	
			Lower	Upper
Resistant – Sensitive	1.394	0.320	0.617	2.155
Resistant – No infection	1.972	0.296	1.230	2.640
Sensitive – No infection	0.578	0.319	- 0.195	1.359

Table 3: Differences between the means of organ failures with bootstrap standard errors and 98.3% confidence limits

The bootstrap confidence limits for the differences between the means indicate that the mean number of total organ failures is higher for the Resistant group than for the Sensitive group. The mean number of total organ failures is also higher for the Resistant group than for the No-infection group. The difference between the means for the Sensitive and No-infection groups is not statistically significant.

Section 3 Statistical analysis performed by Dr Mike Parker, Anglia Ruskin University.

Analysis of variance of the total number of organ failures with covariates

In this section an attempt is made to adjust for imbalance between the groups by adjusting the differences between the means using covariates (see p205).

Blood volume transfused as a single covariate

The potential covariate which, on its own, explains most variation in the total number of organ failures is the volume of blood transfused. In this section this variable is used as a covariate to adjust the differences between the means of the total number of organ failures.

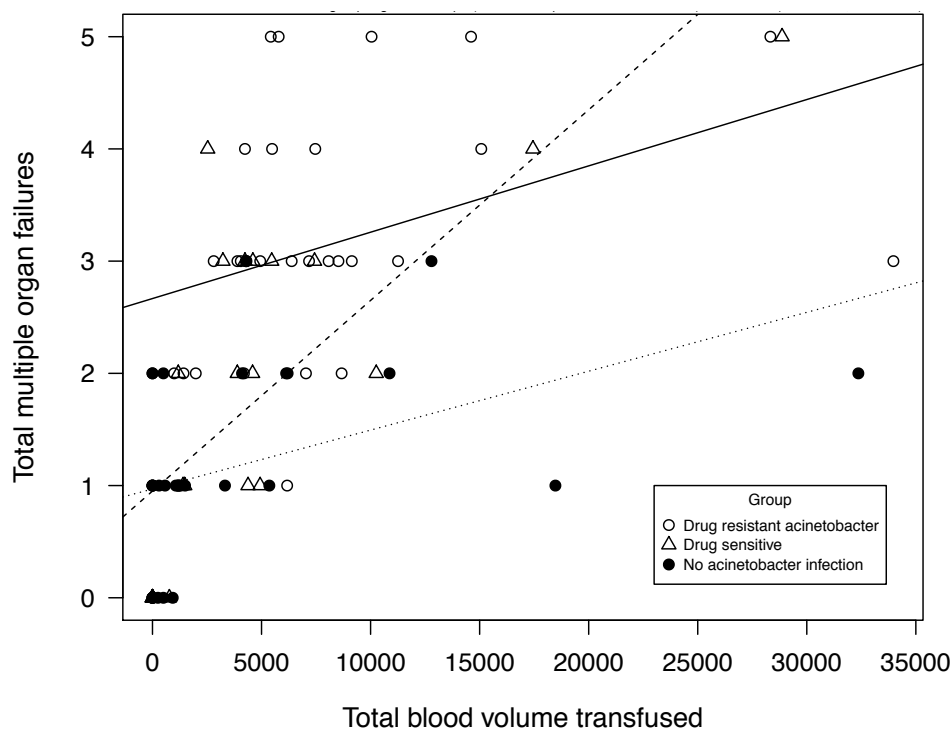


Figure 1. A scatter plot of total organ failure against total blood volume transfused with scatter plot regression lines fitted separately to groups. MRAB solid line, Drug sensitive dashed, no Acinetobacter infection, dotted. MRAB regression slope (and bootstrap 95% CI) 5.911e-05 (8.04e-06, 0.00011163). Drug sensitive regression slope .0001699 (0.00011589, 0.0002425). No Acinetobacter group regression slope 5.247e-05 (1.561e-05, 9.491e-05)

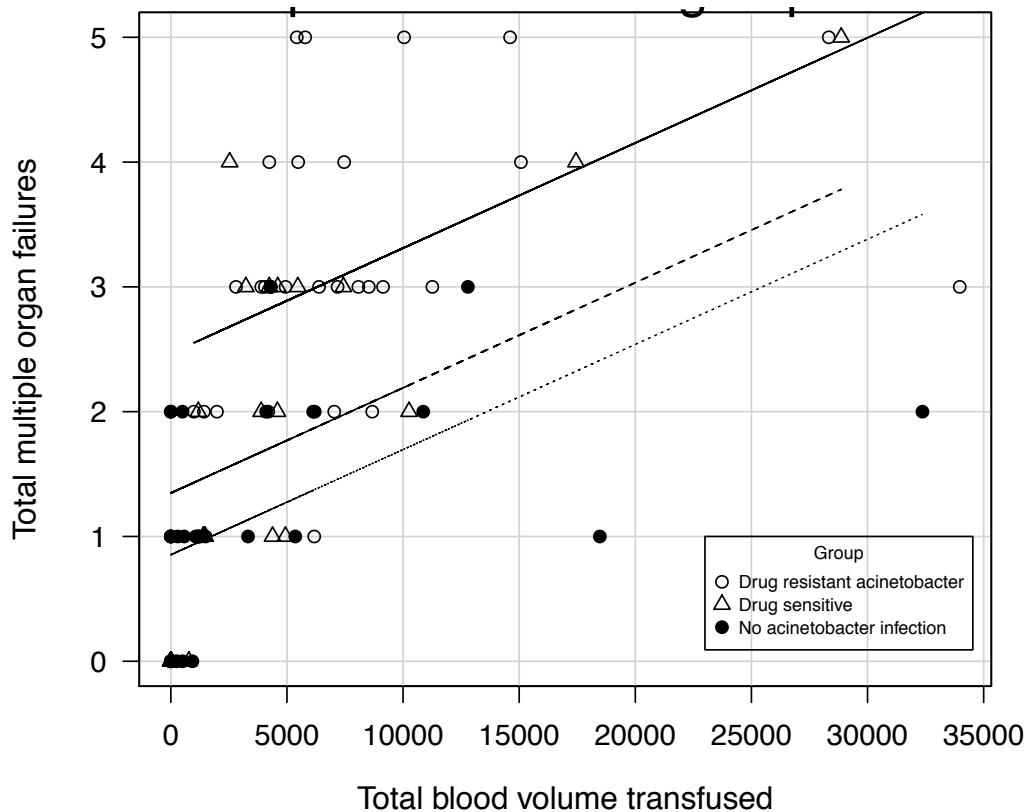
The graph above suggests that the regression line for the drug sensitive patients differs from the regression lines for the other two groups. However, there is a

great deal of scatter and it might be that parallel lines provide an adequate description of the data.

Source of variation	Degrees of freedom	Sum of squares	Mean Square	Variance ratio	Permutation test P-value
Group	2	61.857			
Blood vol	1	27.821			
Slopes	2	9.546	4.773	4.98	0.005
Residual	78	74.728	0.958		
Total	83	173.952			

Table 4: Analysis of variance summary table for testing parallelism

The adjusted R-square or percentage variance accounted for by the model with separate regression lines is 54.3%. For the model with parallel regression lines it is 49.7%. Although there is some evidence of a lack of parallelism the simplification in the analysis achieved by using the model with parallel regression lines makes this model much more useful and is the one generally adopted except in cases where there is a marked reduction in the goodness of fit of the model with parallel regression lines.



Line styles: Drug resistant acinetobacter = solid, Drug sensitive = dashed, No acinetobacter infection = dotted

Figure 2 Scatterplot with parallel lines for groups. The adjusted differences between the means of the total numbers of organ failures are the vertical distances between the parallel regression lines. The analysis of variance for testing these adjusted differences is as follows.

Source of variation	Degrees of freedom	Sum of squares	Mean Square	Variance ratio	Permutation test P-value
Group	2	38.325	19.177	18.20	< 0.00001
Blood vol	1	51.325	51.325	48.72	< 0.00001
Residual	80	84.274	1.053		
Total	83	173.952			

Table 5: Analysis of variance summary table for adjusted means

The residual variance is 1.053 and its bootstrap 95% confidence limits are (0.847, 1.441).

The residual standard deviation is 1.026 and its bootstrap 95% confidence limits are (0.920, 1.200).

The very small P-value indicates that there are statistically significant differences amongst the group means. The confidence limits below have been adjusted using the Dunn-Sidak adjustment for multiplicity so that a nominal 95% confidence level has become a 98.3% confidence level.

Group difference	Difference between means	Standard error	98.3% confidence limits	
			Lower	Upper
Resistant – Sensitive	1.120	0.284	0.428	1.792
Resistant – No infection	1.614	0.266	0.959	2.238
Sensitive – No infection	0.495	0.277	- 0.177	1.160

Table 6: Differences between the means of organ failures, adjusted for blood volume transfused, with bootstrap standard errors and 98.3% confidence limits

The adjusted differences between the means are all smaller than the unadjusted differences shown in Section 1, Table 3. However, the conclusions are the same. The bootstrap confidence limits for the adjusted differences between the means indicate that the mean number of total organ failures is higher for the resistant group than for the sensitive group. The mean number of total organ failures is also higher for the resistant group than for the No-infection group. The difference between the adjusted means for the sensitive and No-infection groups is not statistically significant.

Section 4

Adjustment using all available covariates (see p 208)

All of the six available covariates are used in this section in an attempt to adjust the differences between the group means using all the available information. This might not be a sensible approach as some of the covariates might not contribute the model and would therefore unnecessarily increase the standard errors in the model and its complexity. There also no attempt being made here to include interactions between the covariates which could also be important.

Source of variation	Degrees of freedom	Sum of squares	Mean Square	Variance ratio	Permutation test P-value
Group	2	40.271	20.135	21.55	< 0.00001
Sex	1	0.978	0.978	1.05	0.421
Age	1	10.552	10.552	11.30	0.001
TBSA	1	24.891	24.891	26.64	< 0.00001
Theatre vsts	1	9.994	9.994	10.70	0.003
Blood vol	1	16.050	16.050	17.18	0.00006
Inhalation	1	1.151	1.151	1.23	0.433
Residual	75	70.066	0.934		
Total	83	173.952			

Table 7: Analysis of variance summary table for adjusted means using all covariates

The residual variance is 0.934 and its bootstrap 95% confidence limits are (0.788, 1.490).

The residual standard deviation is 0.967 and its bootstrap 95% confidence limits are (0.888, 1.221).

The model with just the six covariates accounts for 31.6% of the variance. When the Groups factor is added the model accounts for 55.4% of the variance. The confidence limits below have been adjusted using the Dunn-Sidak adjustment for multiplicity so that a nominal 95% confidence level has become a 98.3% confidence level.

Group difference	Difference between means	Standard error	98.3% confidence limits	
			Lower	Upper
Resistant – Sensitive	0.948	0.270	0.311	1.591
Resistant – No infection	1.713	0.248	1.107	2.295
Sensitive – No infection	0.765	0.265	0.136	1.394

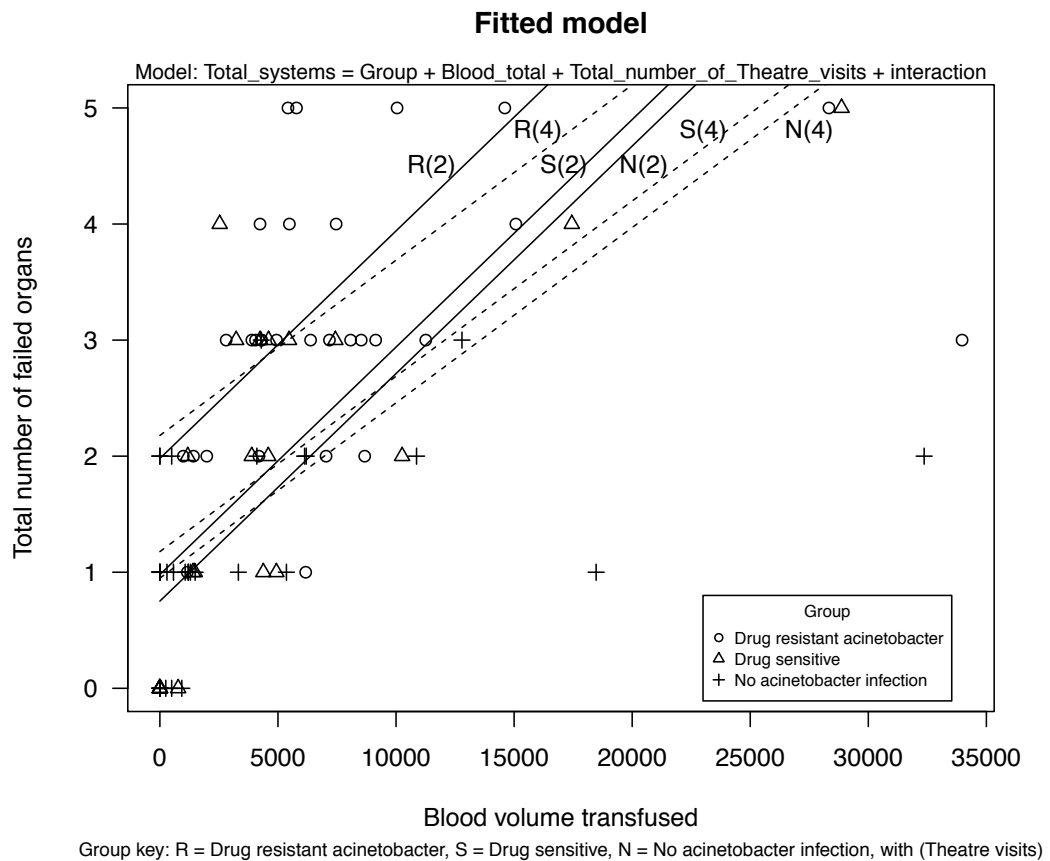
Table 8: Differences between the means of organ failures, adjusted for six covariates, with bootstrap standard errors and 98.3% confidence limits

When compared with the unadjusted differences shown in Table 3, the results in Table 8 show that the adjusted differences between the means are smaller when the resistant group is involved but the adjusted difference between the sensitive and No-infection group is larger. All the differences are now statistically significant.

Section 5

Adjustment using blood volume transfused and number of theatre visits (see p206)

The model involving just covariates which accounts for the largest percentage of variance has the blood volume transfused, the number of visits to the operating theatre, and their interaction.



Source of variation	Degrees of freedom	Sum of squares	Mean Square	Variance ratio	Permutation test P-value
Group	2	21.322	10.661	12.72	< 0.00001
Blood vol	1	17.476	17.476	20.85	< 0.00001
Theatre vsts	1	33.866	33.866	40.41	< 0.00001
Interaction	1	35.942	35.942	42.89	0.00001
Residual	78	65.346	0.838		
Total	83	173.952			

Table 9: Analysis of variance summary table for adjusted means

The residual variance is 0.837 and its bootstrap 95% confidence limits are (0.683, 1.232).

The residual standard deviation is 0.915 and its bootstrap 95% confidence limits are (0.827, 1.110).

The model with just the covariates accounts for 48.3% of the variance. When the Groups factor is added the model accounts for 60.0% of the variance. The confidence limits below have been adjusted using the Dunn-Sidak adjustment for multiplicity so that a nominal 95% confidence level has become a 98.3% confidence level.

Group difference	Difference between means	Standard error	98.3% confidence limits	
			Lower	Upper
Resistant – Sensitive	1.001	0.250	0.395	1.588
Resistant – No infection	1.229	0.249	0.615	1.811
Sensitive – No infection	0.228	0.249	- 0.351	0.829

Table 10: Differences between the means of organ failures, adjusted for covariates, with bootstrap standard errors and 98.3% confidence limits

When compared with the unadjusted differences shown in Table 3, the results in Table 10 show that the adjusted differences between the means are always smaller than the unadjusted differences. The bootstrap confidence limits for the

differences between the means in Table 10 indicate the same conclusions as in Table 3 and Table 6. The mean number of total organ failures is higher for the resistant group than for the sensitive group. The mean number of total organ failures is also higher for the resistant group than for the No-infection group. The difference between the means for the sensitive and No-infection groups is not statistically significant.

Section 6 (see p207)

Infection group	Mean	Standard deviation	Percentiles					Number of values
			Smallest 0%	25%	Median 50%	75%	Largest 100%	
Drug resistant	4000	5486	0	1249	2227	4289	28222	30
Drug sensitive	2255	4391	0	0	775	2775	20402	23
Overall	3243	5070	0	458	2026	3894	28222	53

Table 11 Summary table of blood volume by infection group pre-acquisition of *Acinetobacter*

Infection group	Mean	Standard deviation	Percentiles					Number of values
			Smallest 0%	25%	Median 50%	75%	Largest 100%	
Drug resistant	3906	5038	0	970	2433	4479	26135	30
Drug sensitive	2329	2975	0	0	931	3172	10359	23
Overall	3221	4304	0	450	2061	4045	26135	53

Table 12 Summary table of blood volume by infection group pre-acquisition of *Acinetobacter*

Infection group	Mean	Standard deviation	Percentiles					Number of values
			Smallest 0%	25%	Median 50%	75%	Largest 100%	
Drug resistant	-94.3	7846	-24177	2093	-234	3524	23939	30
Drug sensitive	73.8	3434	-11937	-517	0	1547	5467	23
Overall	-21.4	6271	-24177	1245	0	1691	23939	53

Table 13 Post- minus Pre-acquisition blood volume by infection group

Infection group	Mean	Standard deviation	Percentiles					Number of values
			Smallest 0%	25%	Median 50%	75%	Largest 100%	
Drug resistant	7897	7249	993	4205	5981	8636	33971	30
Drug sensitive	4639	6656	0	388	3230	4769	28867	23
No infection	3652	6875	0	0	928	4200	32365	31
Overall	5438	7123	0	725	4078	6546	33971	84

Table 14 Total blood volume by infection group

Tables 11-14 summarise the results used in the statistical analysis.

Section 7 (see p 209)

Pre-acquisition blood volumes

The comparison of the pre-acquisition blood volumes for the two infection groups is presented as an analysis of variance (ANOVA), which is equivalent to a two-sample t-test. A permutation test has been used to avoid the assumption of Normally distributed data.

Source of variation	Degrees of freedom	Sum of squares	Mean square	F ratio	Permutation test probability
Group	1	39648705	39648705	1.56	0.24
Residual	51	1296892785	25429270		
Total	52	1336541490			

Table 15 Analysis of variance summary table for pre-acquisition blood volume. The residual variance is 25429270 and its bootstrap 95% confidence limits are (8737554, 68753541). The residual standard deviation is 5043 and its bootstrap 95% confidence limits are (2925, 8284).

Post-acquisition blood volumes

Source of variation	Degrees of freedom	Sum of squares	Mean square	F ratio	Permutation test probability
Group	1	32377595	32377595	1.77	0.20
Residual	51	930898210	18252906		
Total	52	963275805			

Table 16 Analysis of variance summary table for post-acquisition blood volume. The residual variance is 18252906 and its bootstrap 95% confidence limits are (7546503, 64348638).

The residual standard deviation is 4272 and its bootstrap 95% confidence limits are (2746, 7935).

A claim for a difference between the group means is not supported by this statistical analysis.

Post- minus pre-acquisition blood volumes

Table 17 Analysis of variance summary table for post- minus pre-acquisition blood volume

Source of variation	Degrees of freedom	Sum of squares	Mean square	F ratio	Permutation test probability
Group	1	367952	367952	0.01	0.93
Residual	51	2044871037	40095511		
Total	52	2045238988			

The residual variance is 40095511 and its bootstrap 95% confidence limits are (18945199, 91465295).

The residual standard deviation is 6332 and its bootstrap 95% confidence limits are (4351, 9558).

A claim for a difference between the group means is not supported by this statistical analysis.

Table 18 Difference between means of post- minus pre-acquisition blood volume with bootstrap standard error and 95% confidence limits

Group difference	Difference between means	Standard error	95% confidence limits	
			Lower	Upper
Resistant – Sensitive	- 168	1711	- 3439	3370

The 95% confidence limits enclose zero which confirms that the difference is not statistically significant.

Appendix IV

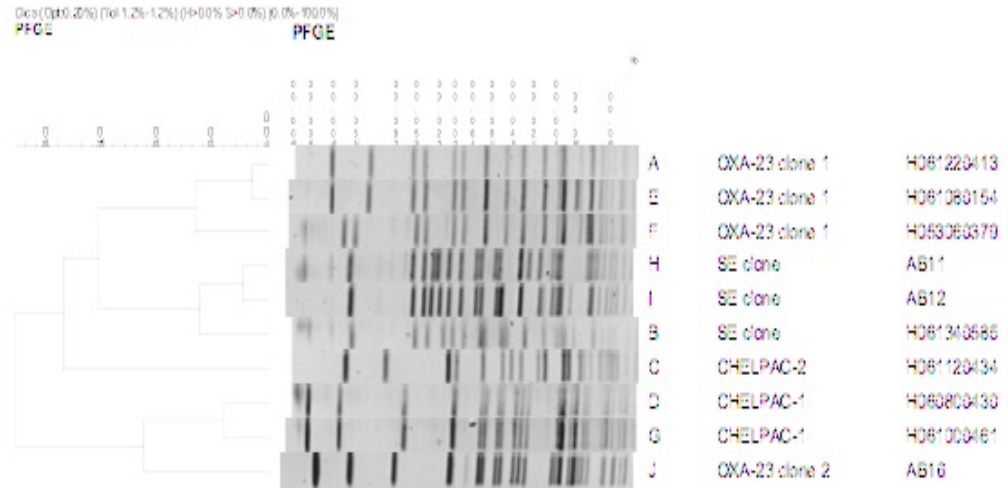
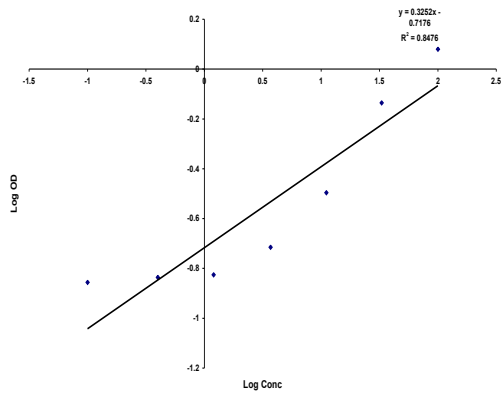


Figure 1. Dendrograms for *Acinetobacter baumannii* isolates

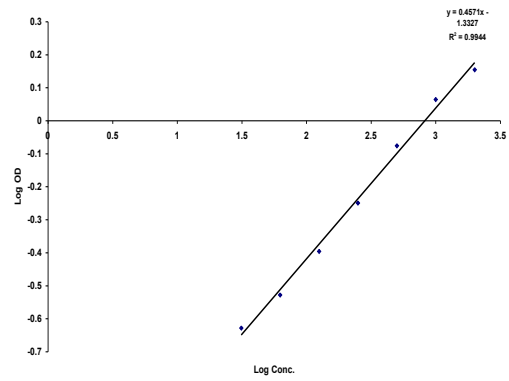
Supplied by Dr Jane Turton, (Laboratory of HealthCare Associated Infection, Centre for Infections, Health Protection Agency, Colindale, UK). The strains used were Southeast Clone AB 11, Southeast Clone AB 12 and Oxa 23 Clone 2. See p 258

Section 2 (see p248)

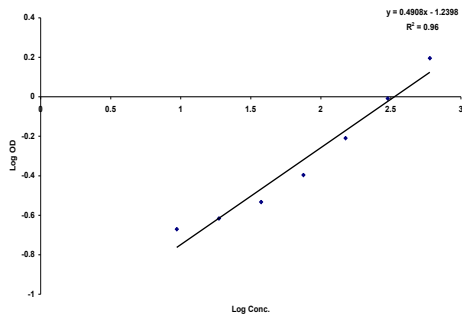
A.



B.



C.



D.

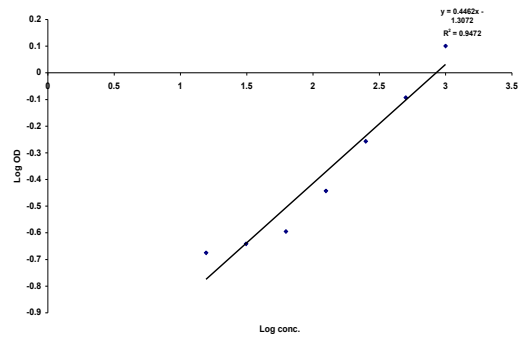
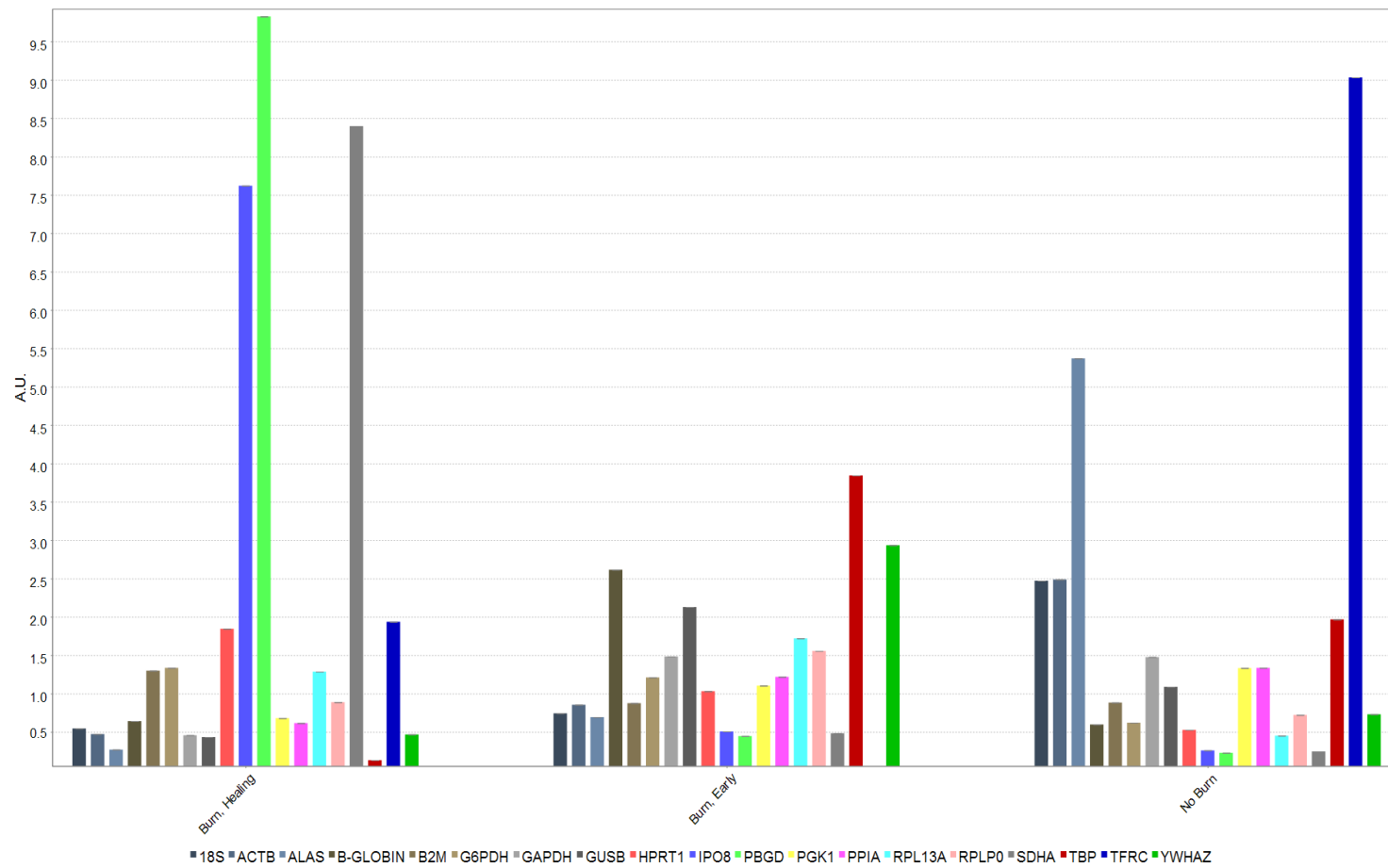
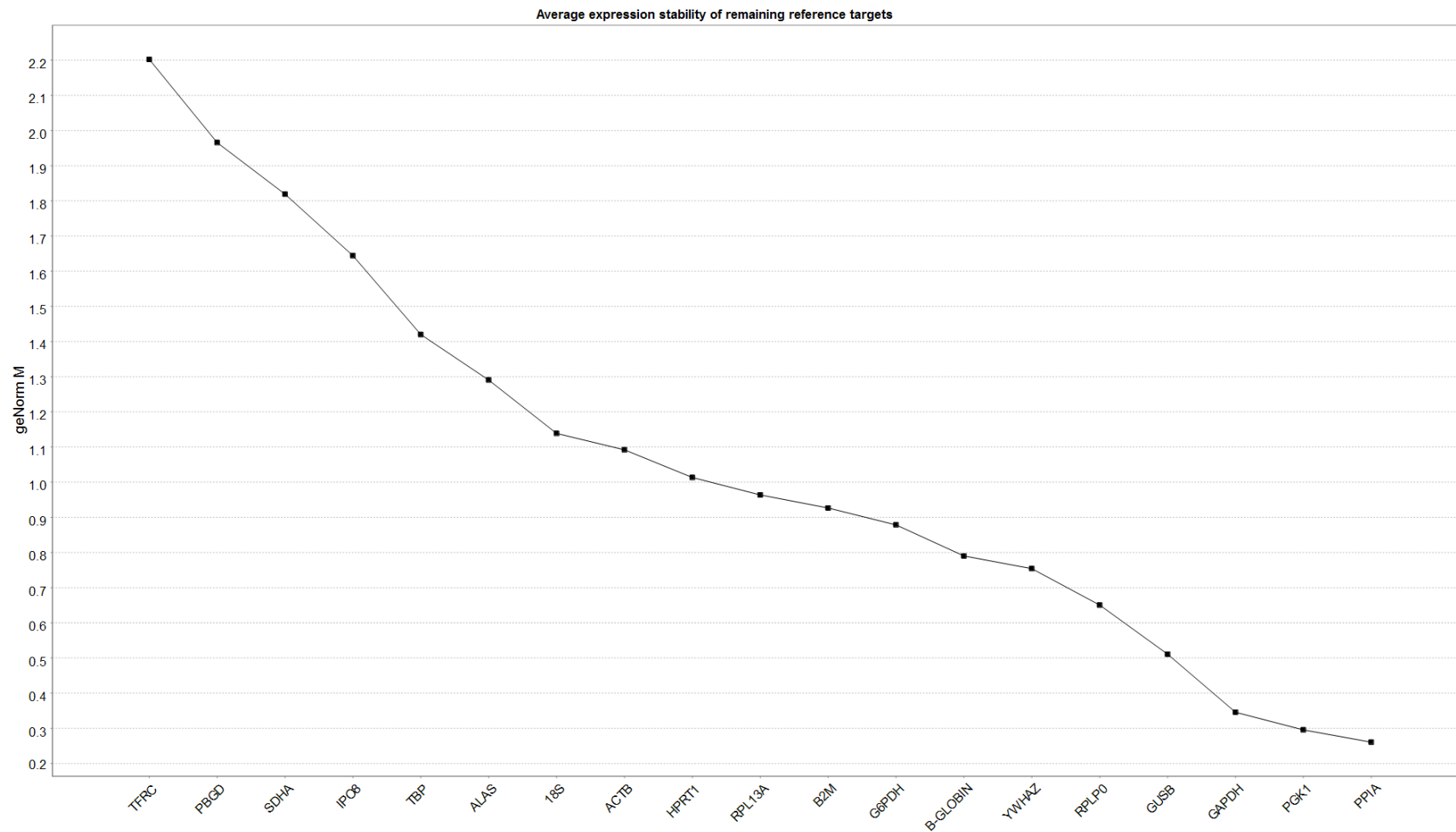


Figure 2. Standard curves for ELISA plates A. LL-37, B. IL-8, C. IL-6 and D. IFN-gamma



Human reference gene panel for three samples, healing burn , early burn and no burn (Control sample)
 Section 3 (see p 256)



The average expression stability of the reference genes. It can be seen that PPIA is the most stable for the three samples tested.

Section 4. Figure 3 A. Raw data for PPIA reference gene for analysis of LL-37 (see p 265)

Pos	Name	Cp	Concentration
A4	ft006	24.34	5.53E+06
A5	ft006	23.8	6.93E+06
A6	ft006	24.11	6.08E+06
A7	ft009	31.2	3.09E+05
A8	ft009	30.96	3.43E+05
A9	ft009	31.14	3.17E+05
A10	b20/03	24.02	6.33E+06
A11	b20/03	23.9	6.65E+06
A12	b20/03	23.73	7.14E+06
B4	ft004	32.45	1.83E+05
B5	ft004	32.01	2.20E+05
B6	ft004	32.66	1.68E+05
B7	b13/05	22.21	1.35E+07
B8	b13/05	22.23	1.34E+07
B9	b13/05	22.33	1.29E+07
B10	b14/02	21.03	2.22E+07
B11	b14/02	21.26	2.02E+07
B12	b14/02	21.02	2.22E+07
C4	b009/03	22.08	1.43E+07
C5	b009/03	22.34	1.28E+07
C6	b009/03	22.18	1.37E+07
C7	ft002	29.8	5.56E+05
C8	ft002	30.26	4.60E+05
C9	ft002	30.01	5.09E+05
C10	b19/01	23.74	7.12E+06
C11	b19/01	24.35	5.49E+06
C12	b19/01	24.09	6.14E+06
D4	b006/05	22.56	1.17E+07
D5	b006/05	22.75	1.08E+07
D6	b006/05	22.55	1.17E+07
D7	b14/03	22.72	1.09E+07
D8	b14/03	22.6	1.15E+07
D9	b14/03	22.47	1.21E+07
D10	b006/01	33.45	1.20E+05
D11	b006/01	32.81	1.58E+05
D12	b006/01	33.28	1.29E+05
E4	Water		
E5	b20/02	23.11	9.26E+06
E6	b20/02	22.99	9.72E+06
E7	b20/02	23.05	9.50E+06
E8	Water		

E9	b18/01	21.95	1.50E+07
E10	b18/01	22.01	1.47E+07
E11	b18/01	21.95	1.51E+07
E12	Water		
F4	b13/04	22.78	1.06E+07
F5	b13/04	23.05	9.52E+06
F6	b13/04		
F7	b005/01	23.66	7.35E+06
F8	b005/01	32.82	1.57E+05
F9	b005/01		
F10	ft010	23.88	6.71E+06
F11	ft010	23.81	6.89E+06
F12	ft010	24.01	6.35E+06
G4	b004/01	35.31	5.39E+04
G5	b004/01	35	6.27E+04
G6	b004/01	36.6	1.35E+04
G7	b002/01	27.56	1.43E+06
G8	b002/01	27.31	1.59E+06
G9	b002/01	27.23	1.64E+06
G10	b001/03	31.09	3.24E+05
G11	b001/03	31.07	3.27E+05
G12	b001/03	31.13	3.19E+05
H4	b006/03	23.56	7.66E+06
H5	b006/03	23.9	6.64E+06
H6	b006/03	23.7	7.24E+06
H7	b002/05	26.87	1.91E+06
H8	b002/05	26.64	2.10E+06
H9	b002/05	26.63	2.11E+06
H10	b14/01	22.9	1.01E+07
H11	b14/01	22.83	1.04E+07
H12	b14/01	22.71	1.10E+07

Figure 3. B. Raw data for PCR analysis of LL-37

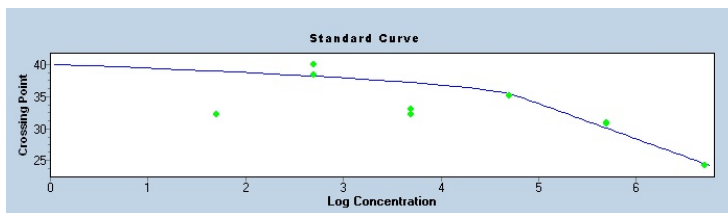
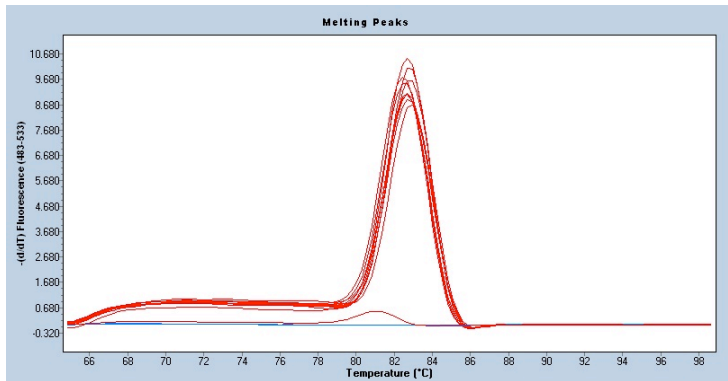
	CDNA Sample 1 in3 dilution and NegC		
Pos	Name	Cp	Concentration
A4	b19/01		
A5	b19/01		
A6	b19/01		
A7	ft009		
A8	ft009		

A9	ft009		
A10	ft004		
A11	ft004		
A12	ft004		
B4	ft006		
B5	ft006		
B6	ft006		
B7	b002/05		
B8	b002/05		
B9	b002/05		
B10	b001/03		
B11	b001/03	39.23	8.82E-50
B12	b001/03		
C4	b18/01		
C5	b18/01		
C6	b18/01		
C7	b006/01		
C8	b006/01		
C9	b006/01		
C10	b20/02		
C11	b20/02		
C12	b20/02		
D4	ft002		
D5	ft002	40	4.85E-57
D6	ft002		
D7	b14/01		
D8	b14/01		
D9	b14/01	40	4.85E-57
D10	b13/04		
D11	b13/04	40	4.85E-57
D12	b13/04	40	4.85E-57
E4	Water		
E5	ft010		
E6	ft010		
E7	ft010		
E8	Water		
E9	b14/02		
E10	b14/02	40	4.85E-57
E11	b14/02		
E12	Water		
F4	b002/01		
F5	b002/01		
F6	b002/01		
F7	b13/05		

F8	b13/05		
F9	b13/05	40	4.85E-57
F10	b006/03		
F11	b006/03		
F12	b006/03	40	4.85E-57
G4	b005/01	40	4.85E-57
G5	b005/01		
G6	b005/01		
G7	b004/01		
G8	b004/01		
G9	b004/01		
G10	b009/03		
G11	b009/03		
G12	b009/03	40	4.85E-57
H4	b14/03		
H5	b14/03		
H6	b14/03		
H7	b20/03		
H8	b20/03		
H9	b20/03		
H10	b006/05	40	4.85E-57
H11	b006/05		
H12	b006/05		
Pos	Name	Cp	Concentration
A4	b16/01		
A5	b16/01		
A6	b16/01		
A7	b001/02	40	6.81E-110
A8	b001/02		
A9	b001/02	40	6.81E-110
A10	b12/02		
A11	b12/02		
A12	b12/02		
B4	ft003		
B5	ft003		
B6	ft003		
B7	b12/02		
B8	b12/02	40	6.81E-110
B9	b12/02		
B10	b20/04		
B11	b20/04	22.52	1.40E+05
B12	b20/04	24.38	4.51E+04
C4	b002/04		
C5	b002/04		
C6	b002/04		

C7	b006/02		
C8	b006/02		
C9	b006/02		
C10	b13/01		
C11	b13/01		
C12	b13/01		
D4	b11/01		
D5	b11/01	40	6.81E-110
D6	b11/01		
D7	b17/02	23.47	7.84E+04
D8	b17/02	40	6.81E-110
D9	b17/02	21.24	3.04E+05
D10	b001/01		
D11	b001/01		
D12	b001/01		
E4	b002/03		
E5	b002/03		
E6	b002/03		
E7	ft008		
E8	ft008	40	6.81E-110
E9	ft008		
E10	b17/01	40	6.81E-110
E11	b17/01	40	6.81E-110
E12	b17/01	40	6.81E-110
F4	b20/01		
F5	b20/01		
F6	b20/01		
F7	b11/02		
F8	b11/02		
F9	b11/02		
F10	b21/01		
F11	b21/01	40	6.81E-110
F12	b21/01		
G4	ft007		
G5	ft007		
G6	ft007		
G7	b002/02		
G8	b002/02	40	6.81E-110
G9	b002/02		
G10	b13/02		
G11	b13/02		
G12	b13/02		
H4	b008/01		
H5	b008/01		
H6	b008/01		
H7	ft001		

H8	ft001		
H9	ft001		
H10	b009/01	40	6.81E-110
H11	b009/01		
H12	b009/01		



Error .00876
Efficiency 1.924

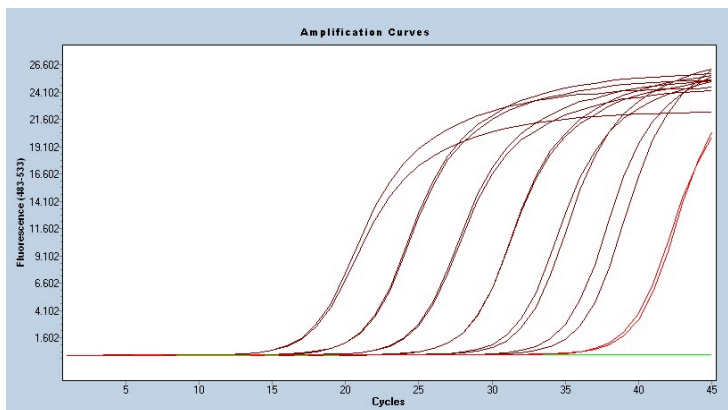
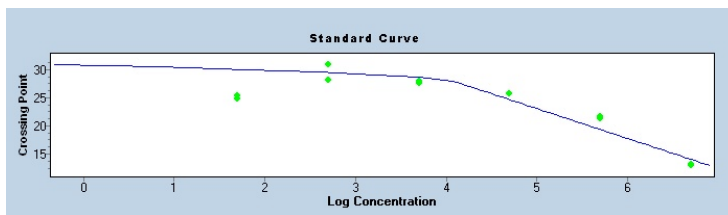
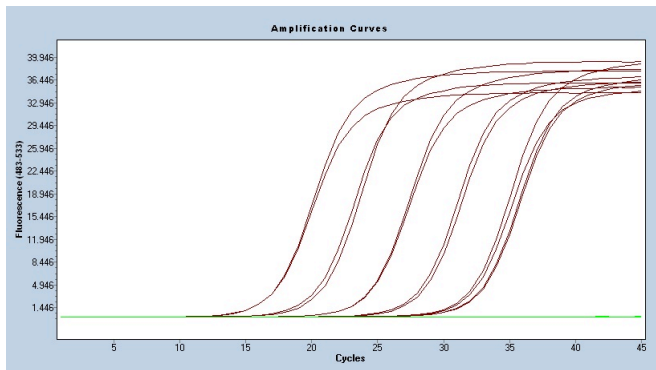


Figure 4. Melting peak and standard curve for genes analysed using PCR

PPIA

LL-37



Error 0.0134
Efficiency 1.839

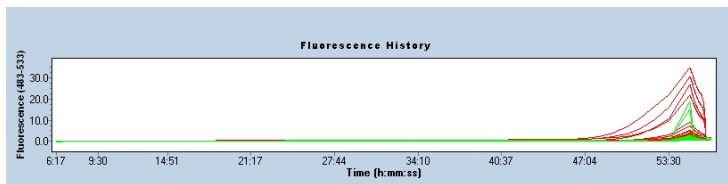


Figure 5. Melting peak and standard curve for genes analysed using PCR

LL-37

Section 5 (see p271)

Figure 5. Serum levels of LL-37 and proinflammatory cytokines related to age of patient. There is no relationship between age and any of the molecules studied.

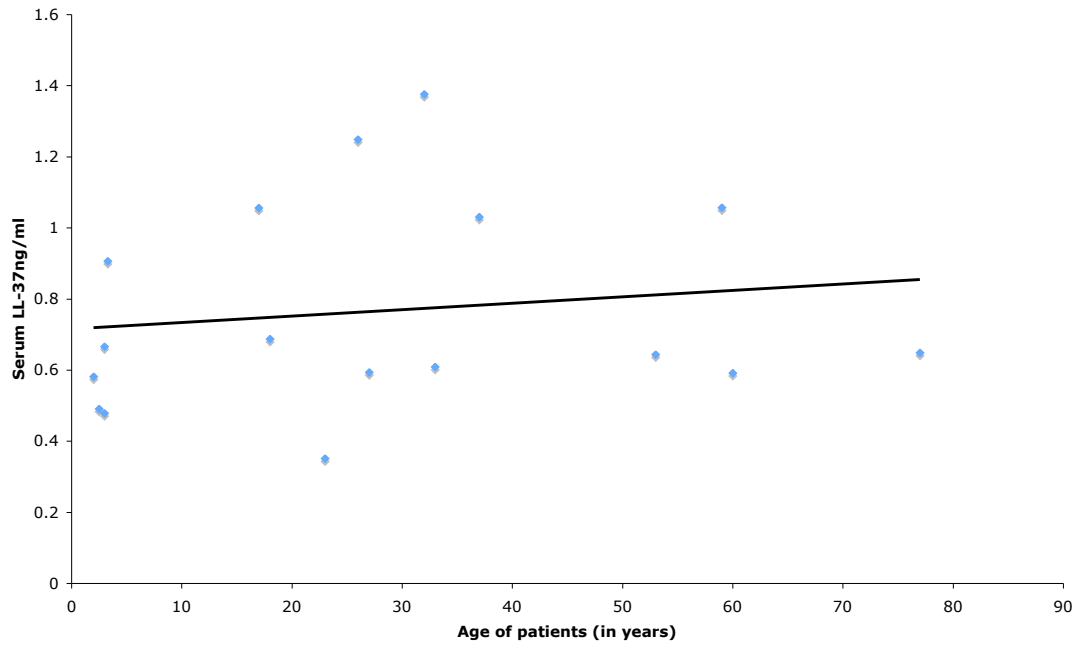


Figure 5.A. The relationship between serum LL-37 and age. ($R^2=0.02$)

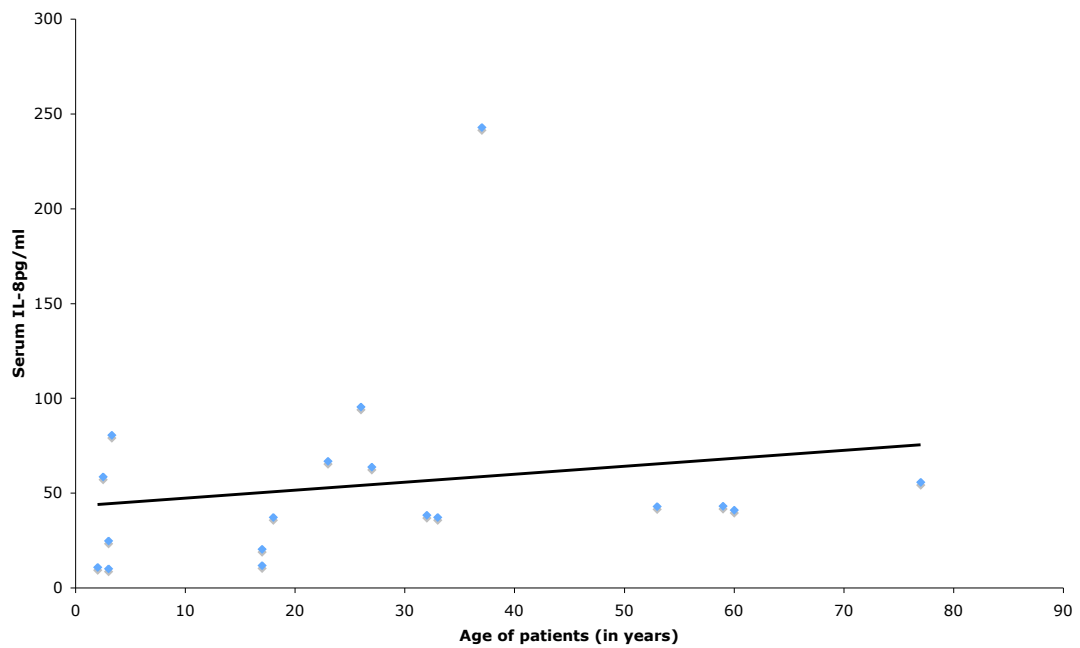


Figure 5.B. The relationship between serum IL-8 and age. ($R^2=0.03$)

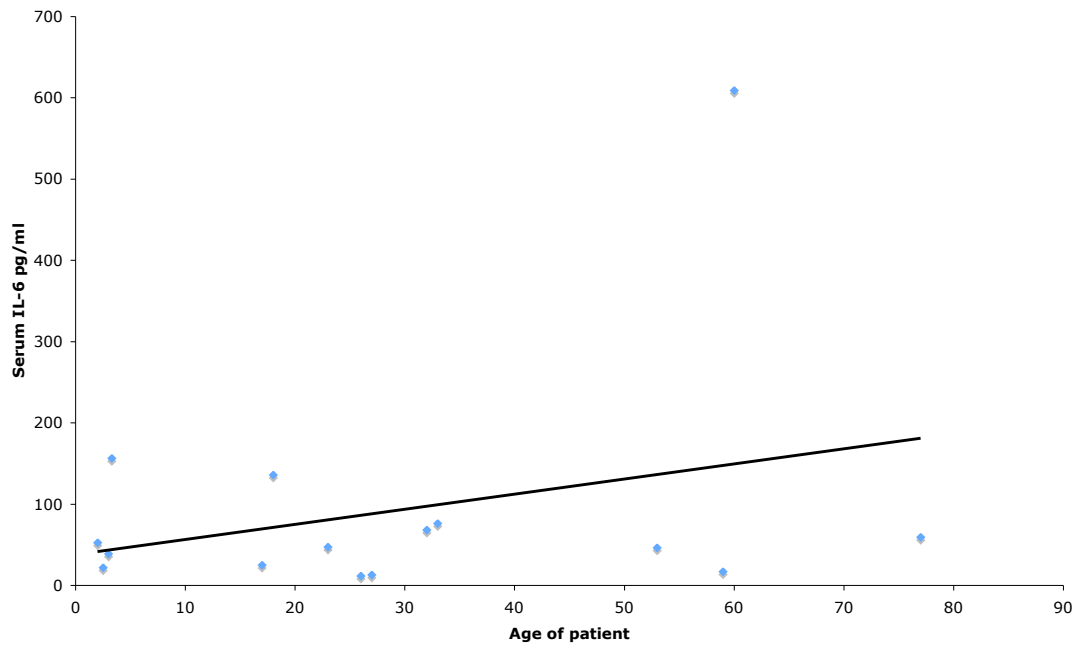


Figure 5.C. The relationship between serum IL-6 and age. ($R^2=0.08$)

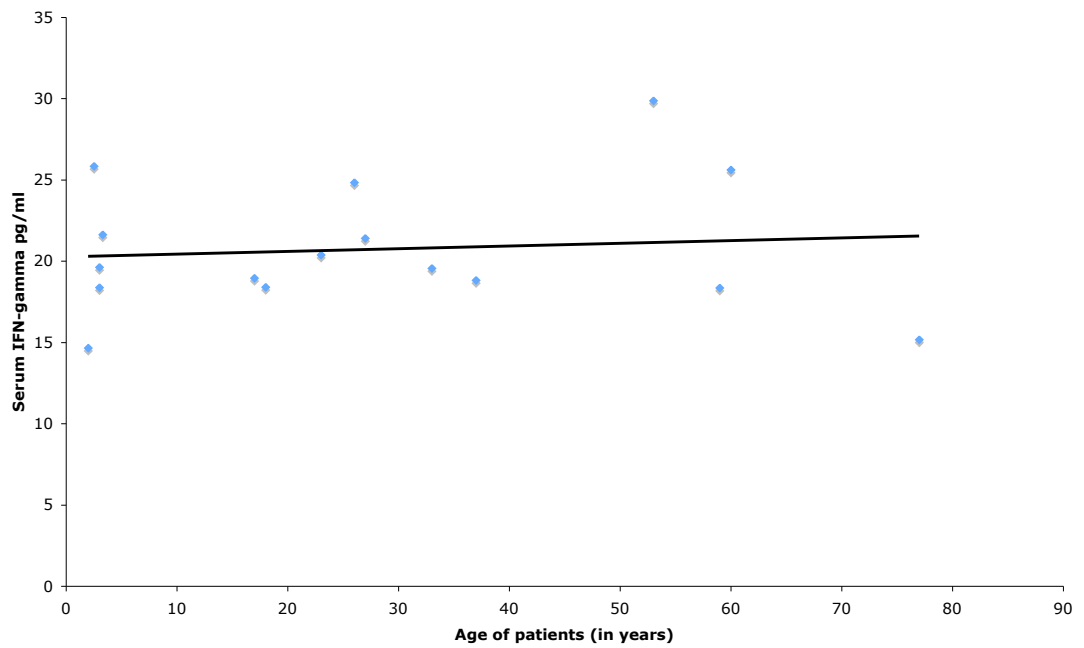


Figure 5.D. The relationship between serum LL-37 and age. ($R^2=0.009$)

Section 6 (see p 270)

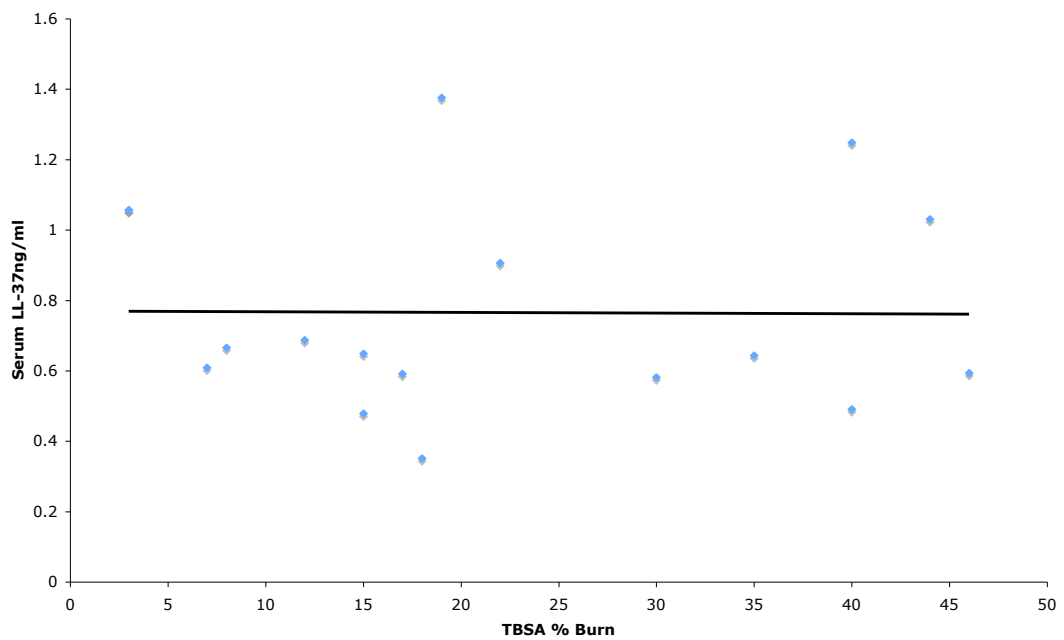


Figure 6.A. The relationship between serum LL-37 and TBSA%. ($R^2=0$)

Figure 6.A shows that there was no relationship between the size of the injury sustained (as determined by TBSA %) and serum LL-37.

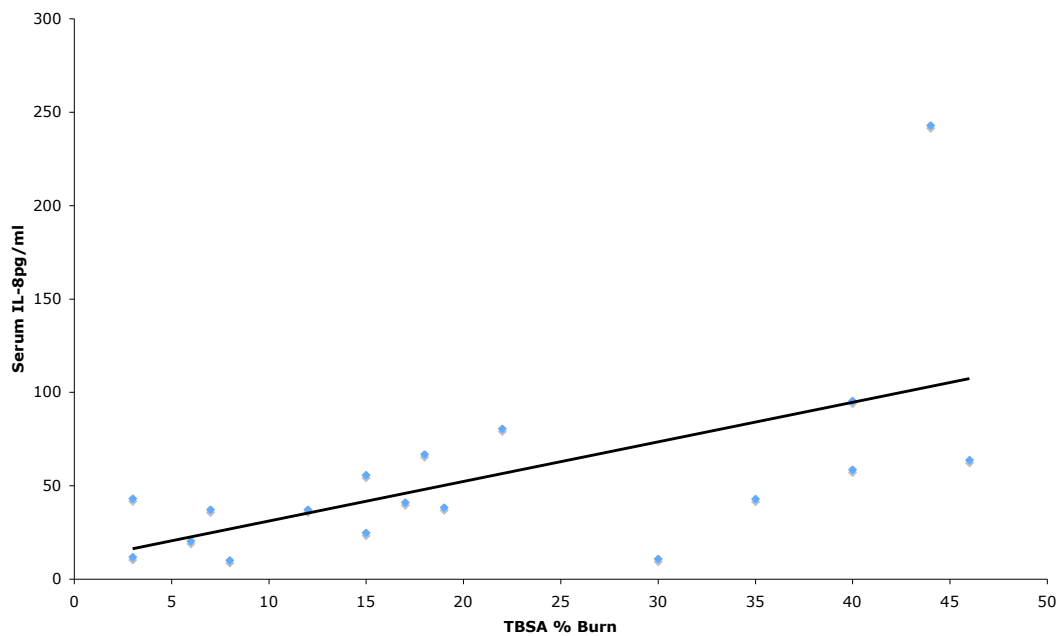


Figure 6.B. The relationship between serum IL-8 and TBSA%. ($R^2=0.34$)

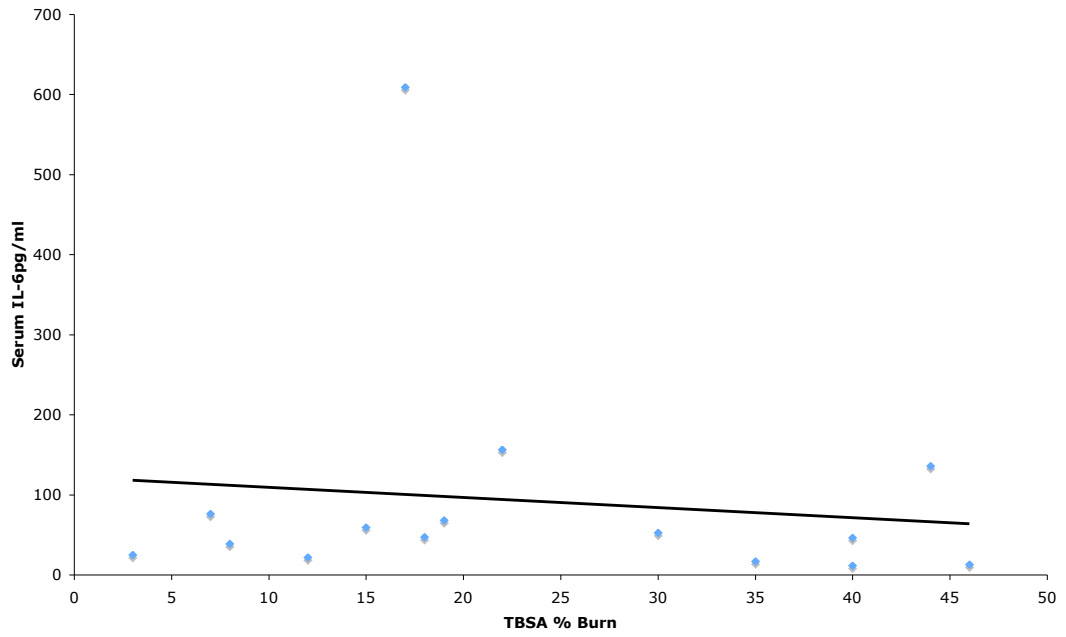


Figure 6.C. The relationship between serum IL-6 and TBSA%. ($R^2=0.01$)

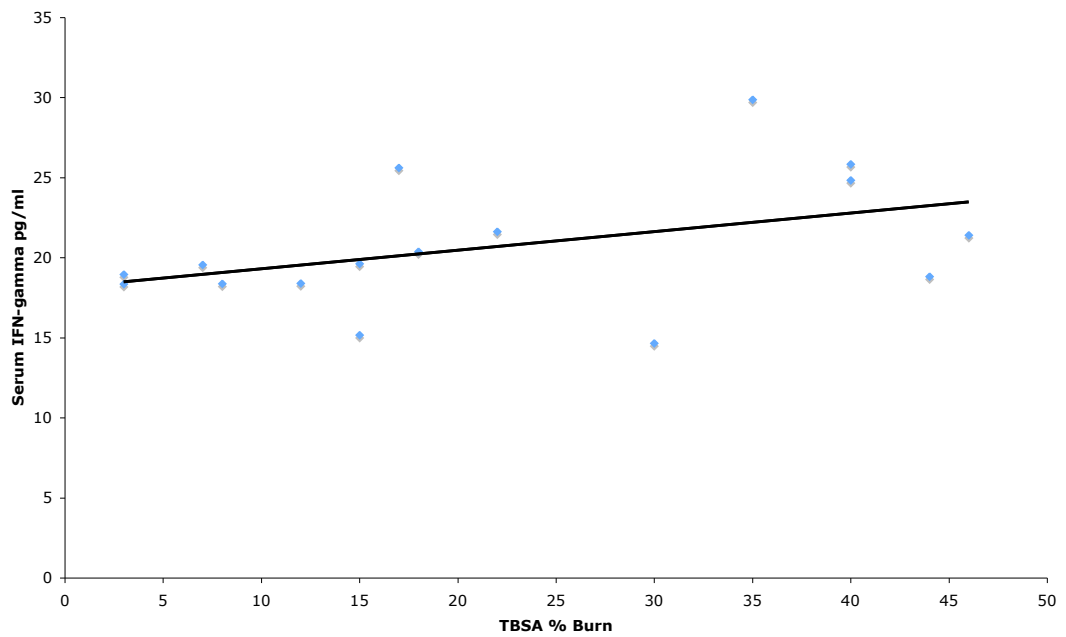


Figure 6.D. The relationship between serum IFN-gamma and TBSA%. ($R^2=0.18$)

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