PRODUCTION AND IMPAIRED REGULATION OF NEUTROPHIL EXTRACELLULAR TRAPS FOLLOWING SEVERE THERMAL INJURY, IMPLICATIONS FOR SEPSIS AND MULTIPLE ORGAN FAILURE

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Abstract

Advancements in burn care have improved immediate outcome, however, the prevalence of sepsis and multiple organ failure (MOF) remain significant. Although well characterised the mechanisms responsible for the pathogenesis of MOF and increased propensity to infection are poorly understood. Neutrophil extracellular traps (NETs) provide protection against invading pathogens but also contribute to thrombosis.

Sepsis is required for NET generation following severe thermal injury. Quantification of circulating NET biomarkers shows good discriminatory power for diagnosis of sepsis. Interestingly, neutrophils isolated from 24 patients with severe thermal injuries, ≥ 15% total body surface area, had a significantly reduced ability to form NETs ex vivo, potentially mediated by phenotypical changes of neutrophils and inhibitory effects of formyl peptides.

This thesis identified a major biological mechanism driving MOF after severe thermal injury, namely the compromise to the actin scavenging system which leads to reduced DNAse activity and a build-up of circulating DNA. Preliminary analysis suggests that DNAse activity can be restored by prehospital use of fresh frozen plasma following major trauma. Thus, administration of blood products or manipulation of the actin scavenging system is a potential therapeutic target.

This thesis has identified a number of novel mechanisms responsible for the regulation of NETs following severe thermal injuries and their implications for sepsis and MOF.
Acknowledgements

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Thank you to my greatest teacher the black dog, your intermittent barking has fuelled my determination and passion.

I would like to thank my whole family for always being there for me and supporting me throughout this whole process. Dad and lovely mummy, thank you for all of the support, love and advice throughout everything. Thank you for everything you have ever given me from the countless opportunities to the reassurance on top of the landing. I hope this work makes you proud. Grace, Maggie and Elsie, thank you for your companionship. To the members of my family who will never be able to read this, I hope it would have made you proud.

Thank you.
You'll never walk alone
Disclosures

All work presented in this thesis was performed as part of The Scientific Investigation of the Biological Pathways Following Thermal Injury in Adults and Children (SIFTI Study) and is therefore part of a collaboration between a number of investigators. Where applicable, credit has been given to persons responsible for data generation or collaboration. Importantly, all data presented in this thesis was analysed and presented by Robert J Dinsdale.

All patients recruited within Camp Bastion, Afghanistan, were recruited by Professor Midwinter, Dr Kirkman, Professor Woolley, Dr Watts and Dr Dalle Lucca. Again, all laboratory experiments and data analysis was performed by Robert J Dinsdale.
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a. **Dinsdale RJ**, Hampson P, Hazeldine J, Wearn CM, Brill A, Lord JM, Moiemen NS, Harrison P. *Neutrophil Extracellular Trap (NET) formation in patients with post-burn sepsis (oral).*

6. **British Burns Association, Birmingham, May 2015**
   a. **Dinsdale RJ**, Hampson P, Hazeldine J, Wearn CM, Lord JM, Moiemen NS, Harrison P. *Clinical applications of novel blood counter parameters in burn injury (poster).*

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   a. **Dinsdale RJ**, Hampson P, Hazeldine J, Wearn CM, Lord JM, Moiemen NS, Harrison P. *Neutrophil Extracellular Trap Formation in Patients with Post-Burn Sepsis. (poster).*

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   b. **Dinsdale RJ**, Hampson P, Hazeldine J, Wearn CM, Lord JM, Moiemen NS, Harrison P. *Evidence of Neutrophil Extracellular Trap (NET) formation in patients with post-burn sepsis (poster).*

**Awards**


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## Abbreviations

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<td>ABA</td>
<td>American Burn Association</td>
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<tr>
<td>ABSI</td>
<td>Abbreviated burn severity index</td>
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<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>APC</td>
<td>Allophycocyanin</td>
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<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>AUROC</td>
<td>Area under the receiver operator curve</td>
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<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>CARS</td>
<td>Compensatory anti-inflammatory response syndrome</td>
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<td>cfDNA</td>
<td>Cell-free deoxyribonucleic acid</td>
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<td>CGD</td>
<td>Chronic granulomatous disease</td>
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<td>Cit H3</td>
<td>Citrullinated histone H3</td>
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<td>Cecal ligation puncture</td>
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<td>CRP</td>
<td>C-reactive protein</td>
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<td>DAMPs</td>
<td>Damage associated molecular patterns</td>
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<td>DIC</td>
<td>Disseminated intravascular coagulation</td>
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<td>DMSO</td>
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<td>DNase</td>
<td>Deoxyribonuclease</td>
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<td>DVT</td>
<td>Deep vein thrombosis</td>
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<td>Escherichia coli</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<tr>
<td>FFP</td>
<td>Frozen fresh plasma</td>
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<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<tr>
<td>FRCs</td>
<td>Fragmented red cells</td>
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<tr>
<td>FT</td>
<td>Full thickness</td>
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<tr>
<td>GCS</td>
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<td>G-CSF</td>
<td>Granulocyte-colony stimulating factor</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage colony-stimulating factor</td>
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<td>HBSS+</td>
<td>Hank's Balanced Salt Solutions containing Mg$^{2+}$ and Ca$^{2+}$</td>
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<td>Human promyelocytic leukaemia cells</td>
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<tr>
<td>HMGB1</td>
<td>High mobility group box 1 protein</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IAP</td>
<td>Inhibitor of apoptosis protein</td>
</tr>
<tr>
<td>ICU</td>
<td>Intensive care unit</td>
</tr>
<tr>
<td>IG</td>
<td>Immature granulocyte</td>
</tr>
<tr>
<td>IGs</td>
<td>Immature granulocytes</td>
</tr>
<tr>
<td>IL-</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IL-4</td>
<td>Interleukin-4</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>IL-7</td>
<td>Interleukin-7</td>
</tr>
<tr>
<td>IL-8</td>
<td>Interleukin-8</td>
</tr>
<tr>
<td>IL-10</td>
<td>Interleukin-10</td>
</tr>
<tr>
<td>IL-13</td>
<td>Interleukin-13</td>
</tr>
<tr>
<td>ISS</td>
<td>Injury severity score</td>
</tr>
<tr>
<td>IQR</td>
<td>Interquartile range</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LTB4</td>
<td>Leukotriene B4</td>
</tr>
<tr>
<td>MDROs</td>
<td>Multi-drug resistant organisms</td>
</tr>
<tr>
<td>MERT</td>
<td>Medical Emergency Response Team</td>
</tr>
<tr>
<td>MFI</td>
<td>Median fluorescence intensity</td>
</tr>
<tr>
<td>MODS</td>
<td>Multiple organ dysfunction</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>MOF</td>
<td>Multiple organ failure</td>
</tr>
<tr>
<td>mtDAMPs</td>
<td>Mitochondrial derived damage associated molecular patterns</td>
</tr>
<tr>
<td>mtDNA</td>
<td>Mitochondrial deoxyribonucleic acid</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>fMLP</td>
<td>N-Formylmethionine-leucyl-phenylalanine</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate-oxidase</td>
</tr>
<tr>
<td>ncDNA</td>
<td>Nuclear deoxyribonucleic acid</td>
</tr>
<tr>
<td>NET(s)</td>
<td>Neutrophil extracellular trap(s)</td>
</tr>
<tr>
<td>NEUT GI</td>
<td>Neutrophil granularity index</td>
</tr>
<tr>
<td>NEUT RI</td>
<td>Neutrophil reactivity index</td>
</tr>
<tr>
<td>NISS</td>
<td>New injury severity score</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PAD4</td>
<td>Peptidylarginine deiminase 4</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PCT</td>
<td>Procalcitonin</td>
</tr>
<tr>
<td>PE</td>
<td>R-phycoerythrin</td>
</tr>
<tr>
<td>PFP</td>
<td>Platelet free plasma</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLT-F</td>
<td>Platelet fluorescence count</td>
</tr>
<tr>
<td>PLT-I</td>
<td>Platelet impedance count</td>
</tr>
<tr>
<td>PLT-O</td>
<td>Platelet optical count</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-phorbol myristate 13-acetate</td>
</tr>
<tr>
<td>PMX-DHP</td>
<td>Polymixin-B immobilized fibre cartridge</td>
</tr>
<tr>
<td>PT</td>
<td>Partial thickness</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>QC</td>
<td>Quality control</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>rBaux</td>
<td>Revised Baux score</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RvD2</td>
<td>Resolvin D2</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SIFTI</td>
<td>Scientific investigation of biological pathways following thermal injury</td>
</tr>
<tr>
<td>SIRS</td>
<td>Systemic inflammatory response syndrome</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic lupus erythematosus</td>
</tr>
<tr>
<td>SOFA</td>
<td>Sequential Organ Failure Assessment</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>sp</td>
<td>Species</td>
</tr>
<tr>
<td>STAT3</td>
<td>Signal transducer and activator of transcription 3</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris Buffered Saline</td>
</tr>
<tr>
<td>TBSA</td>
<td>Total body surface area</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris Buffered Saline containing 4% Tween-20</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TMB</td>
<td>Tetramethylbenzidine</td>
</tr>
<tr>
<td>UK</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>VDBP</td>
<td>Vitamin D binding protein</td>
</tr>
<tr>
<td>WBC</td>
<td>White blood cell</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction
Introduction

1.1 Trauma and thermal injury

There are approximately 5-8 million fatalities each year as a result of injuries and trauma (1, 2). As a group alone, fatalities account for more deaths than malaria, tuberculosis and HIV/AIDS. Trauma affects all age groups in developed and developing countries globally and associated mortalities account for around 10% of the deaths worldwide (1). Traumatic injury is an umbrella term which encompasses many forms of injury, including road traffic accidents, wars, falls and thermal injuries (1). For many years thermal injury and trauma were used interchangeably. However, investigation of thermal injury alone is now required.

Thermal injuries are a common and debilitating form of traumatic injury which is associated with considerable morbidity and mortality. They are among the most expensive forms of traumatic injury due to the consequent length of hospitalisation, rehabilitation and wound management (3). In 2015 it was reported that approximately 13,000 injuries which required hospital attention occur every year in England and Wales. Approximately 58% of these patients were admitted to hospital for further medical care. Throughout this thesis the term ‘thermal injury’ will refer to injuries caused by flame, contact and scald injuries, the most predominant causes in England and Wales (4).

Morbidity and mortality post thermal injury is heavily influenced by a plethora of elements. Firstly, severe thermal injuries which are larger in size and depth have an increased risk of nosocomial infection and thus an increased mortality rate (5). More recently, studies have shown that age has a considerable effect on the immune system (6-9) and thus ageing is a crucial confounding factor in the clinical
outcome. Elderly patients have a higher mortality rate, longer length of hospital stays and more complicated outcomes (10). This may be driven by the immune dysfunction and immunosenescence associated with ageing (7-9), or may also be affected by pre-existing medical and premorbid conditions which are common in elderly patients. Finally, patients with inhalation injuries have increased mortality rates despite increased understanding and advancements within respiratory care (11). A combination of these confounding factors in patients presenting with severe thermal injuries increases the associated mortality and morbidity rate.

1.2 Pathophysiology of thermal injury

The pathophysiology of burn is dependent upon the degree of initial injury and complications the patients incur. Severe thermal injury results in immediate local and systemic responses which are extremely complex and critical to outcomes post injury.

Jackson described 3 zones which make up the local response to burn injury (12). These include the zones of coagulation, stasis and hyperaemia (Figure 1.1). The zone of coagulation occurs at the point of maximal tissue damage and is comprised of necrotic tissue where there is irreversible tissue loss. This is surrounded by the zone of stasis which is characterised by decreased tissue perfusion. The tissue in this area can be rescued however secondary complications such as infection or ischemia may lead to further tissue necrosis. The zone of hyperaemia has increased tissue perfusion and without secondary complications, this will recover. The three zones of burn injury are subject to changes and loss of the zone of stasis will result in wound deepening and widening (12, 13).
Figure 1.1. Described model of burn zones according to adequate and inadequate resuscitation as described by Jackson. The burn wound consists of 3 zones which make up the local response to burn injury. These zones include a zone of coagulation, stasis and hyperaemia. Loss of the zone of stasis will result in wound deepening and widening of the wound. The figure is taken directly from ‘ABC of burns: pathophysiology and types of burns’ by Hettiaratchy and Dziewulski (12, 13).
1.3 Burn shock

Following thermal injury correct diagnosis, treatment and clinical practice are essential for positive outcomes. Smaller thermal injuries are commonly managed by outpatient care. However, clinical treatment of severe injuries is much more complicated and require hospitalisation, timely and accurate resuscitation, nutritional support, and early surgical management of burn wounds. All of which is directed at reducing morbidity and mortality (14).

Shock is a life-threatening condition characterised by reduced oxygen delivery and circulatory failure. Shock is commonly diagnosed by a combination of hypoperfusion and low or declining blood pressure (15). Burn shock is an umbrella term consisting of hypovolemic and septic shock which occurs following a severe thermal injury. Hypovolemic shock occurs immediately following severe thermal injury and without adequate fluid resuscitation it will lead to mortality. Septic shock is the major driver of delayed mortality associated with infection and is characterised by shock mediated by an infectious stimuli coupled with evident organ failure (15). The pathogenesis of burn shock is driven by the alteration in almost all components that control both fluid and protein loss from vascular space (16). Immediately following injury the microvasculature loses its vessel wall integrity which results in the loss of proteins to the interstitium (17) coupled with a decrease in the intra-vascular colloid osmotic pressure resulting in loss of fluid, electrolytes and further proteins from the vasculature system into the interstitium (17). Clinically this manifests itself as hypovolemia, haemoconcentration, oedema, reduced urine output and significant cardiovascular dysfunction (16). Timely and
appropriate treatment, namely fluid resuscitation, is required to prevent and manage immediate burn shock and prevent poor outcomes post injury.

1.4 Improvements in critical care management of patients with thermal injuries

Improvements in the immediate care of burn wounds have been made over the past four decades which have dramatically improved survival rates of patients. It is proposed that improvements in survival are a direct result of increased scientific understanding of burn injury and the immediate pathophysiology (18).

In 2014, Jackson and colleagues reported data of revised estimates of mortality from the Birmingham Burn Centre spanning from 2001 to 2010 and included 4577 patients. Although there was an increase in admissions, the overall mortality of the cohort decreased by approximately 3-fold compared to the previous decade. The authors concluded that improvements in outcome were multifactorial and included; improved prehospital care, rapid referral to burns institution, early burn wound excision and closure, and improved understanding and management of inhalation. Furthermore, the group proposes that further research into inhalation injury, fluid resuscitation, burn care in vulnerable populations (i.e. elderly patients), and skin substitutes are warranted if further improvements in outcome are to be met (19).

1.4.1 Fluid resuscitation

Fundamental to the care of patients with burn injuries is accurate and timely fluid resuscitation. Without intervention and fluid replacement in burns greater than 15-20% total body surface area (TBSA) burn shock will occur (20). A delay of just 2 hours of accurate fluid resuscitation results in a significant increase in adverse
outcomes (21). Therefore the primary aim of fluid resuscitation is to prevent the development of burn shock and to restore homeostasis during the immediate dysregulated cellular and hormonal response (22).

1.4.2 Inhalation injury

Advancements in the management of respiratory failure caused by inhalation injury and smoke inhalation have been fundamental in improving immediate outcome post thermal injury (23-25). The pathogenesis of respiratory failure is multifactorial and immediate airway management is essential (13, 26, 27). Inhalation injuries are further sub categorised in primary and secondary which differ by causes, progression and management. Primary inhalation injury is caused by direct damage to the respiratory system caused by the thermal stimulus which causes cellular damage, activation of inflammatory cells and oedema. This can cause blockage of the airways and consequently respiratory failure, a major clinical problem (28). Secondary injury to the respiratory system is initiated by the inflammatory response and is amplified by complications, including sepsis and multiple organ dysfunction (MODS) or multiple organ failure (MOF) (27, 29). Both of which require management and treatment to ensure a positive outcome (11, 13).

1.4.3 Burn wound care

Burn wounds provide a major source of inflammatory mediators which orchestrate the propagation and initiation of inflammation following thermal injury (30). Timely and correct management of burn wounds is essential to prevent an uncontrolled systemic inflammatory response, reduce infection risk, improve healing and
reduce the incidence of secondary complications (31, 32). Advancements in immediate cleaning, debridement and excision of wounds have been critical in preventing rapid colonisation of wounds, secondary complications and reducing mortality rates (20, 32).

Early excision and skin grafting generally occur between 24 hours and 7 days following injury to attenuate the inflammatory response, reduce rejection rate and reduce colonisation rate of wounds (20, 32). If wounds become colonised they are commonly treated by early debridement, wound excision and application of topical dressings. Novel topical dressings aim to promote wound healing, reduce scarring and identify/treat colonised wounds more efficiently (33, 34). Topical antimicrobial agents, which are commonly used, prevent graft loss and burn wound infection (32). Advancements in the understanding of the inflammatory response post thermal injury, reduced infection rates of wounds and novel topical agents have been pivotal to the improvements of burn wound care.

1.5 Systemic inflammatory response syndrome following thermal injury

Severe thermal injury results in loss of the natural defensive barrier and rapid onset of systemic inflammatory response syndrome (SIRS) (35). SIRS is an inflammatory response which can be initiated by a plethora of mechanisms including injury, surgery and ischemia. It clinically manifests as elevations of body temperature, heart rate, respiratory rate and abnormal white blood cell count or phenotypical changes (presence of band cells) (36). Whilst similar, sepsis is a SIRS response with a documented infection. Therefore, the difference between SIRS and sepsis is the stimuli responsible for its initiation. SIRS is initiated by a
sterile stimulus, e.g. trauma, and sepsis is mediated by an infectious stimulus, e.g. bacteria.

Many of the resultant consequences following thermal injury are mediated by the rapid release of pro-inflammatory mediators and immune suppression (37). This hyper-inflammatory state coupled with an increased propensity to infection often results in sepsis which is the major cause of mortality following burn injury (38). Systemic responses to burn injury include profound changes in the cardiovascular, respiratory, metabolic, immunological and haematological systems (37).

Classically, the SIRS and compensatory anti-inflammatory response syndrome (CARS) paradigm implies that CARS follows the SIRS response to restore homeostasis (37). However, Xiao and colleagues report a simultaneous SIRS and CARS following major trauma (37, 39). The SIRS response is characterised by increased circulating levels of pro-inflammatory cytokines and activation of innate immune cells (37) and the CARS response is characterised by an increase in circulating levels of anti-inflammatory cytokines and immunoparesis (37). Delayed recoveries in the SIRS and CARS response are associated with complicated outcome following major trauma due to the prolonged and dysregulated immune-inflammatory state (Figure 1.2) (37). Therefore, monitoring the amplitude and duration of the SIRS and CARS has the potential to aid in clinical decision making and may improve patient outcome following severe thermal injury.
Figure 1.2. Novel model of simultaneous SIRS and CARs following major trauma. (A) The traditional paradigm of immediate SIRS response followed by CARS following major injury. A second hit can lead to secondary complications and delayed amplification of the inflammatory response. (B) Model proposed by Xiao and colleagues showing an immediate and simultaneous SIRS and CARS response. The amplitude of response is associated with adverse clinical outcomes. Figure is taken directly from ‘A genomic storm in critically injured humans’ by Xiao et al (37).
1.6 Damage associated molecular patterns

Damage associated molecular patterns (DAMPs) are a collection of endogenous cytosolic, mitochondrial and nuclear derived proteins and DNA which are detected in high circulating concentrations following major trauma (40, 41). Elevation in circulating levels of DAMPs is associated with secondary complications, tissue damage and mortality (41-44). Namely, administration of mitochondrial DNA (mtDNA) to mice resulted in organ damage which was attributed to direct activation of neutrophils, subsequent neutrophil degranulation and local cytokine production (42, 43). Furthermore, in prospective observational cohort studies elevated levels of DAMPs are associated with MOF and mortality (41, 44). This highlights the potential interaction(s) between circulating levels of DAMPs and secondary complications following major trauma.

Exposure of monocytes to mitochondrial derived DAMPs (mtDAMPs) results in a tolerance state in which these cells are resistant to further endotoxin stimulation (45). It is proposed that this induced tolerance may underpin an increased propensity to infection. Although described in monocytes, it has yet to be examined if DAMPs induce this tolerance phenotype in neutrophils. Furthermore, it is yet to be established if this occurs following severe thermal injury and potential mechanistic role in the increased incidence of infection.

1.7 Nosocomial infections, sepsis and organ dysfunction following thermal injury

Mortality rates associated with severe thermal injuries continue to steadily decrease. This is in part caused by a multidisciplinary approach, improved burn wound management, advancements in fluid resuscitation and identification of
comorbid conditions e.g. inhalation injury (31). Although immediate mortality rates have improved, the incidence and associated mortality of secondary complications has increased (31). Upon detection of secondary complications rapid, accurate and timely diagnosis is required.

Sepsis is a life threatening condition characterised by an imbalance in both the immune and haemostatic systems. Sepsis is associated with cardiac dysfunction and acute respiratory distress syndrome which are synonymous with MOF (46). Although the accurate and timely diagnosis is crucial for determining patient outcomes, diagnosis remains difficult as criteria are insensitive and non-specific as they are masked by the ongoing SIRS (47). Therefore, further research into the pathophysiology, immune pathways and novel diagnosis criteria are required.

Whilst this is not a new concept, the prevalence of sepsis and its associated mortality remain high. A systematic review, which included 9 studies reporting survival data for thermal injuries, reported the prevalence of sepsis was between 8-42.5% in patients with burns. This was associated with a mortality rate (28-65%) that exceeded that of sepsis-related deaths for traumatic injury (2.4-19.6%) or critical care (21-53%) (38). In addition, the diagnosis of sepsis in burns patients represents a major challenge, as many of the classical diagnostic biomarkers of sepsis are masked by the ongoing SIRS that occurs following major burn injury (47). Understanding the mechanisms underlying the progression and increased propensity to sepsis is therefore critical if novel biomarkers for the accurate prediction and/or diagnosis of sepsis in patients with thermal injuries are to be found and novel therapeutic targets for its prevention and/or treatment identified.
Without intervention, sepsis can induce MOF which can be characterised by mild through to complete and irreversible damage to vital organs (48). Whilst MOF remains well defined the mechanisms responsible for its initiation and progression remain poorly understood. Of such, infection and immediate traumatic injury are attributed as initiators of MOF.

Disseminated intravascular coagulation (DIC) is characterised by abnormal and uncontrolled coagulation and is a common and contributing factor to MODS and MOF through thrombus formation and occlusion of blood vessels (49-52). DIC can be classified into acute and chronic depending upon stimulating factors, length and pathology (53-55). Trauma and thermal injury induce acute DIC which is a consumptive coagulopathy state characterised by excessive thrombin generation (56). DIC also causes the formation of schistocytes, due to high shear forces on red blood cells in occluded vessels (57), which appear to have biological functions in addition to being potential diagnostic markers of DIC (58). However, D-dimer, a soluble biomarker, is commonly used to diagnose DIC through evaluation of recent thrombus formation.

MOF remains the leading cause of delayed mortality following major trauma and is characterised by tissue hypoxia, tissue damage and organ dysfunction (59). In 2009, Nhu Nguyen reported that in 117 severely burn patients (≥40% TBSA) that the incidence of MODS was 45% and associated with severity of injury, sepsis, increased length of stay and importantly increased mortality rate (59). Although it is well recognised that damage to vital organs occurs during MODS/MOF and is associated with adverse outcome the mechanisms mediating this are unknown or poorly understood. Therefore, understanding the mechanisms driving the pathogenesis of MOF and organ damage has the potential to improve patient
outcome following thermal injury through early identification of patients at risk and novel clinical intervention.

1.8 Novel biomarkers of sepsis in thermally injured patients

Sepsis remains a significant healthcare problem in patients following thermal injury (38). Without timely diagnosis and treatment patients are at high risk of MOF and consequently mortality. The Surviving Sepsis Campaign is a joint collaboration of the Society of Critical Care Medicine and the European Society of Intensive Care Medicine committed to reducing mortality from severe sepsis and septic shock worldwide (60). It is advised that antibiotics should be administered to a patient within 1 hour of clinical diagnosis of sepsis and with each hour delay in the administration there is an associated significant increase in mortality (61-63).

A major limitation of post-burn sepsis research is the absence of an accurate definition of sepsis as many of the classically used diagnosis criteria are nonspecific and masked by the ongoing SIRS response (64). In 2007, a collective group of burns experts defined a clinical scoring criterion for the accurate diagnosis of sepsis in patients with thermal injuries (65). According to the American Burn Association (ABA), sepsis is diagnosed when three or more of the following criteria listed in Table 1.1 are satisfied in addition to the clinical response to antibiotics, positive blood or wound culture.

More recently a new sepsis diagnosis criterion was described in The Journal of the American Medical Association (66). This study aimed to replace previous diagnostic criteria with a new system termed the quick sequential organ failure assessment (qSOFA) score. Whilst this tool is an excellent bedside screening tool for identifying patients at risk of adverse outcomes it is non-specific in patients with
severe thermal injuries. This is due in part to the high incidence of intubation in patients with thermal injuries and the incorporation of the Glasgow coma scale (GCS) into the qSOFA score which is not part of the ABA scoring criteria. Thus, diagnosis of sepsis was made using the ABA scoring criteria for all analysis within this thesis.
<table>
<thead>
<tr>
<th>Finding</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>Fever &gt; 39°C, hypothermia (&lt; 36.5°C)</td>
</tr>
<tr>
<td>Heart Rate</td>
<td>Adults: Progressive tachycardia (&gt; 110 beats per min). Children: &gt; 2 standard deviations above age-specific norms (85% age-adjusted max heart rate)</td>
</tr>
<tr>
<td>Respiratory Rate</td>
<td>Adults: Progressive tachypnoea (&gt; 25 breaths per minute not ventilated or minute ventilation &gt; 12 L/min ventilated). Children: &gt; 2 standard deviations above age-specific norms (85% age-adjusted max heart rate)</td>
</tr>
<tr>
<td>Thrombocytopenia (only applicable &gt; 3 days post thermal injury)</td>
<td>Adults: Thrombocytopenia (&lt; 100,000/μl) Children: &gt; 2 standard deviations below age-specific norms</td>
</tr>
<tr>
<td>Hyperglycaemia (in the absence of pre-existing diabetes mellitus)</td>
<td>Untreated plasma glucose &gt; 200 mg/dl (11.1 mmol/l). Insulin resistance i) &gt; 7 units of insulin/h intravenous drip ii) significant resistance to insulin (&gt; 25% increase in insulin requirement over 24 h)</td>
</tr>
<tr>
<td>Feeding</td>
<td>Inability to continue enteral feedings &gt; 24 h i) Abdominal distension ii) Enteral feeding tolerance (residual &gt; 150ml/hour in children or two times feeding rate in adults iii) Diarrhoea (Adults: &gt; 2500 ml/day, Children &gt; 400 ml/day)</td>
</tr>
</tbody>
</table>

Table 1.1. The ABA Consensus conference 2007 criteria for sepsis diagnosis (65).
There is also the increasing problem of antibiotic resistance, a phenomenon in which bacteria can mutate making them more invulnerable to antibiotics. This resistance makes it harder to treat patients, increases medical costs, increases hospital length of stay and increases mortality (67). Van Langeveld et al investigated how multi-drug resistant organisms (MDROs) affect survival, hospital length of stay and secondary complications in patients with thermal injuries. Although the group found that MDROs had no effect on mortality they conclude that their findings suggest infections caused by MDROs are associated with a greater number of surgical procedures, longer duration of mechanical ventilation, more antibiotic days, and longer hospitalisation (68).

Therefore, novel and accurate biomarkers are required for earlier and accurate identification of patients at risk or who have developed sepsis following thermal injury. This may initiate a change in clinical practice and antibiotic stewardship which is required to overcome the growing burden of MDROs. There are a number of novel biomarkers which may be of clinical utility in patients with thermal injuries. However, many markers still lack sensitivity and specificity and therefore further research is required.

1.8.1 Procalcitonin

Procalcitonin (PCT) is a naturally occurring 116-amino acid prohormone of calcitonin produced in the thyroid, lungs and intestine (69). PCT is found in extremely low quantities in healthy individuals but challenges such as endotoxin insult cause a rapid upregulation of PCT production (70). Hence, the kinetics of PCT is favourable for the development of a biomarker of sepsis. A number of studies have reported the potential use of PCT in differentiating between
infectious and non-infectious systemic inflammation (71, 72). As such, PCT remains one of the most promising biomarkers in identifying infection/sepsis (73, 74).

Current studies aim to elucidate the potential role of PCT in antibiotic stewardship, patient stratification, diagnosis and prognostic utility following burn injury. In 2016, Cabral and colleagues published a meta-analysis of the current understanding of PCT as a biomarker in patients with thermal injuries. The meta-analysis included 14 studies published in both adults and children with thermal injuries (75). Pooled area under the receiver operator curve (AUROC) for PCT in diagnosis of sepsis was 0.83 (95% confidence intervals = 0.76, 0.90). However, substantial differences in AUROC values are reported for individual studies with values ranging from 0.55 – 0.98 (76, 77). Additionally, differences in the ‘cut off or threshold value’ for distinguishing sepsis exists. Many of these differences are attributed to the variances in methodology, heterogeneity between patient populations and timing of samples. Nevertheless, Cabral and colleagues conclude that PCT should be regarded as a strong diagnostic marker of sepsis in burns patients and further work is required to standardise PCT measurements (75).

One limitation of PCT is the reported elevation that occurs postoperatively in the absence of infectious stimuli (78). As patients with severe thermal injuries require surgical intervention, caution must be taken when interpreting PCT data in these patients. Furthermore, a number of studies have reported negative results for PCT in the diagnosis of sepsis following thermal injury (47, 76). Therefore controversy exists regarding PCT as a biomarker of sepsis in this patient population. Thus it is suggested that further studies are required to explore PCT diagnostic utility longitudinally compared to existing biomarkers of sepsis.
1.8.2 C-reactive protein

C-reactive protein (CRP) is a liver derived protein which can activate the classical complement pathway (79). CRP has been studied as a biomarker of infection, sepsis and mortality in a number of disease pathologies (80, 81). Of note, in a study of 43 patients admitted to intensive care unit (ICU) with burn injury, CRP did not correlate with severity of sepsis when PCT did (77). This further confirmed earlier work reporting the increased diagnostic utility of PCT compared to CRP in diagnosing sepsis (82). Furthermore, studies have questioned the ability of CRP to distinguish between inflammation caused by infectious and non-infectious stimuli (83, 84). As CRP is released in response to IL-6 (82), which is present in high quantities in patients with thermal injuries (85), it is thought this may cause the non-specific elevation in CRP and false positives reported in the context of infection. Hence, CRP should be regarded as a marker of inflammation in patients with thermal injuries rather than a potential biomarker of infection due to its non-specific nature.

1.8.3 Pro- and anti-inflammatory cytokines

Thermal injury results in the simultaneous release of pro and anti-inflammatory cytokines which are associated with severity of injury and secondary complications (86-88). As the exaggerated immune response is proposed to underlie the increased incidence of secondary complications post burn injury, groups have quantified levels of pro- and anti-inflammatory cytokines and correlated levels to clinical outcomes (89, 90).
In a study which included 28 children with severe burn injuries, elevated levels of a panel of cytokines showed positive discriminatory power to identify patients who were likely to succumb to their injuries. In this study, interleukin (IL)-4, IL-6, IL-7, IL-10, and IL-13 were abnormal within the first 7 days post injury in patients who did not survive their injuries when compared to patients who did survive. With abnormalities in IL-6, IL-7 and IL-10 displaying the highest predictive power for mortality (89). Furthermore, in a study of 468 paediatric burn patients, IL-8 serum levels were increased in patients who developed with MOF, succumbed to their injuries and, interestingly, sepsis (91). Hence, IL-8 may be a potential novel biomarker to monitor infection and septic episodes.

Recent studies have investigated if a combination of biomarkers offers greater diagnostic potential (92). For example, in a recent study of severely injured trauma patients, a combination of patient immune status coupled with measurement of IL-6 concentrations improved both the specificity and positive predictive value compared to the cytokine data alone (92). However, as cytokines are released in response to inflammation it has been questioned the specificity of cytokines in diagnosing sepsis following burn injury. Therefore, further validation of cytokines post burn injury is required.

1.8.4 Novel haematological parameters

Following thermal injury there is often an imbalance in the haemostatic parameters which potentially mediates life threatening thrombotic complications, higher incidence of MOF and increased susceptibility to sepsis. Although a thorough analysis of cellular kinetics has not been performed longitudinally, evaluation of
the influence thermal injury has on individual cellular kinetics has been studied (93-95).

Excessive stress on the bone marrow can result in emergency granulopoiesis that is characterised by the appearance of immature precursors of neutrophils, blood leucocytosis or neutrophilia. Of such, immature granulocytes (IGs) are a precursor of mature neutrophils and are elevated in septic patients (96). Automated systems now allow for the rapid quantification of IGs in human blood samples. Indeed, quantification of IGs in neonatal sepsis has shown positive diagnostic potential of this biomarker (97). More recently, Nierhaus et al investigated the diagnostic potential of IGs levels in 70 consecutive patients in ICU. Quantification of IGs could differentiate between sepsis and SIRS, within the first 48 hours after onset of SIRS, with a sensitivity of 89.2% and a specificity of 76.4%. Although IG count didn’t predict ICU mortality it exhibited a better discriminatory power than other inflammatory markers studied (i.e. IL-6, CRP and lipopolysaccharide binding protein) thus highlighting the potential predictive power of quantifying immature precursors of neutrophils in patients with suspected infections (96).

Of note is the interplay between the haemostatic system and inflammation in burn injuries. Interestingly, platelets are implicated in acute and chronic inflammation due to their ability to release inflammatory mediators and their interactions with inflammatory cells (98). Platelets are activated by a broad range of inflammatory stimuli and are now recognised as a bridge between innate immunity and haemostasis due to their direct interactions with pathogens and inflammatory cells and immune-modulatory effects (99).
Platelet levels following severe thermal injury have been evaluated as a potential prognostic marker for patients most at risk of complications (93). Indeed, platelet levels fall and remain lower in patients with poorer outcomes. Recently, it has been demonstrated that platelets can bind to and activate neutrophils, in turn, influencing their functions (100). Therefore, abnormalities in platelet number or function will affect host defence and response to the initial trauma and potential infection. However, it is not known how reliable this is as a prognostic marker given the inaccuracy of the most commonly used methodology (i.e. impedance analysis) in determining platelet counts (101, 102).

There are a number of sources which can induce error into traditional methods of platelet counting; via impedance or optical counting (101, 102). However, using a novel parameter to accurately measure platelet levels, platelet fluorescence (PLT-F) can eliminate this interference. This parameter utilises traditional fluorescence flow cytometry in which platelets are stained with the RNA binding dye oxazine which eliminates interference mediated by cellular debris approximately the same size as platelets. This is especially relevant in burn injury as red cell destruction occurs which in turn generates cellular fragments that can interfere with platelet counting (103, 104). As their name suggests fragmented red cells (FRCs) are products of red cell lysis or shearing present in a number of pathological conditions (105). However, in a healthy individual FRCs are completely absent or in extremely low quantities (106). Due to the heterogeneous nature of FRCs direct quantification has proven difficult.
1.8.5 Cell-free deoxyribonucleic acid

In recent years there have been a number of studies investigating the potential of quantifying circulating levels of cell-free deoxyribonucleic acid (cfDNA) in blood products from patients with various disease pathologies (107-109). Although quantification of cfDNA is non-specific, as it can be released from a number of sources, it has shown potential to predict poor outcome following trauma (110), thermal injury (111), cancer (112, 113) and critical illness (108).

Rhodes et al studied the significance of raised plasma cfDNA upon admission to the ICU and its relation with clinical outcome and severity of disease (108). After extraction, levels of plasma DNA was measured by polymerase chain reaction (PCR) and levels were compared between septic patients, non-septic patients and healthy volunteers. Plasma levels of cfDNA were significantly higher in septic patients and patients who died compared to healthy volunteers, non-septic or survivors. Thus, cfDNA may be a useful prognostic marker of sepsis and mortality in patients admitted to ICU (108). This was further confirmed in a larger study when Dwivedi et al demonstrated the potential prognostic utility of cfDNA levels in a cohort of 80 patients with severe sepsis in which levels of cfDNA, IL-6, thrombin, and protein C were measured and correlated with clinical outcome. AUROC analysis for cfDNA to predict ICU mortality was 0.97 (95% confidence intervals, 0.93, 1.00) and to predict hospital mortality 0.84 (95% confidence intervals, 0.75, 0.94). Importantly, cfDNA exhibited improved predictive power compared to IL-6, thrombin and protein C (109).

The recent development of fluorometric assays to measure cfDNA without a purification step has allowed for rapid and low cost quantification. Of note,
Shoham et al quantified levels of serum cfDNA using a rapid fluorometric assay in 14 serum samples taken within 6 hours of thermal injury. Levels of cfDNA were significantly raised post injury (1797 ng/mL ± 1523 ng/mL) compared to healthy controls (374 ng/mL ± 245 ng/mL). Importantly, levels were significantly higher in patients who died (3264 ng/ml ± 2215) compared to those who survived (1211 ng/ml ± 614). The group concluded that an admission cfDNA level equal to 1200 ng/ml represents a lethal level of admission cfDNA over which 50% of the patients died (111).

Quantification of plasma and serum cfDNA has therefore shown potential as a prognostic marker of outcome following trauma and thermal injury. Advancements in technologies available to quantify levels rapidly and accurately have furthered its application as a diagnostic and prognostic marker. However, cfDNA is non-specific and can originate from a number of sources (114, 115). Using more precise methods, such as PCR, the source of cfDNA can be categorized into source of origin e.g. nuclear DNA (ncDNA) or mtDNA. Furthermore, additional sources of elevated levels of cfDNA are from activated, apoptotic or necrotic tissues, cells or neutrophils.

1.9 Neutrophils

1.9.1 General background

Neutrophils are the most abundant circulating leukocyte comprising approximately 50-70% of the circulating leukocyte population in healthy individuals. They are short lived cells with an approximate half-life of 6-8 hours. Because of this they are produced rapidly and in large quantities each day (0.5 – 1 x 10^{11} cells per day)
Neutrophils are an essential component of innate immunity and are recruited rapidly to sites of infection in an attempt to tackle and clear invading pathogens.

Unsurprisingly, neutrophils are equipped with an arsenal of antimicrobial properties which function to control infection. Neutrophils can tackle invading pathogens via phagocytic uptake and elimination through exposure to intracellular free radicals, proteolytic enzymes and toxic molecules termed reactive oxygen species (ROS) (117). Also, neutrophils can release their nuclear material to form neutrophil extracellular traps (NETs) which can ensnare and immobilise pathogens. This was first described in 2004 and is now regarded as an emerging area of neutrophil biology and innate immunity (118). Although NETs are a defensive action of neutrophils, the direct killing ability of these structures is still highly debated (118, 119). In addition to pathogen uptake and immobilisation, neutrophils are involved in the fine regulation of inflammatory and immune response through the secretion of a large number of cytokines and chemokines (120-123). The essential defensive nature of neutrophils is highlighted in patients with congenital neutrophil disorders who have increased susceptibility to bacterial and fungal infections (124). An overview of traditional and novel neutrophil functions is depicted in Figure 1.3 (125).
Figure 1.3. Traditional and novel functions of neutrophils. Upon arrival to the site of infection and inflammation neutrophils can clear invading pathogens by a number of mechanisms, including phagocytosis, NET formation and the secretion of cytokines and chemokines. Once engulfed, neutrophils will generate toxic compounds called ROS to kill bacteria. Upon resolution, neutrophils undergo apoptosis and are cleared. Figure taken from ‘Diverse novel functions of neutrophils in immunity, inflammation, and beyond’ published by Attila Mócsai (125).
1.9.2 Chemotaxis and transmigration

It is essential for host defence that neutrophils can cross the endothelial barrier and migrate towards the local site of infection. Extravasation, or transmigration, is the process by which circulating neutrophils move into the infected tissue and surrounding area. This process is governed by a number of complex interactions between neutrophils and endothelium to ensure that transmigration remains controlled and effective. For transmigration to occur the neutrophils must first adhere to the vessel wall. Adherence occurs through transient selectin interactions on both the neutrophil and endothelium which are upregulated in inflammatory conditions (126). Neutrophils then move along the endothelium in processes termed ‘rolling and crawling’ before subsequently traversing the endothelial membrane (127, 128). Patients who have defective transmigration of neutrophils suffer from recurring infections (129).

For the elimination of invading microbes and pathogens, neutrophils must first migrate to the site of infection in a process termed chemotaxis. This process is the movement of neutrophils in the direction of a chemoattractant gradient and is a fundamental function of neutrophil biology and host defence. This directed movement is dependent upon the regulation of intracellular signalling pathways prompting rapid and accurate migration (130). In a healthy individual, neutrophils will rapidly migrate towards chemokines (e.g. IL-8), bacterial components (e.g. N-Formylmethionine-leucyl-phenylalanine (fMLP)) and complement proteins. In support of the tight regulation of chemotaxis, there appears to be a hierarchy of chemoattractant signals (131). The ability of neutrophils to sense chemoattractants is acquired during terminal differentiation in the bone marrow.
and is highly affected by both in vitro and in vivo stimuli (132). Thus, dysregulation of this process will result in abnormal neutrophil chemotaxis.

1.9.3 Phagocytosis

Upon trafficking to a pathogen, phagocytosis and elimination can occur. Phagocytosis is the engulfment of invading pathogens and is central to host defence. Neutrophils express a number of complement receptors (e.g. CD35) in addition to the Fc receptor CD16 which result in enhanced phagocytosis upon activation (133).

Once engulfed pathogens are localised within a phagosome and subjected to volatile components produced intracellularly by neutrophils (134). There are 2 independent mechanisms for killing engulfed pathogens. Firstly, neutrophils generate high levels of ROS through nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) oxidase complex (135). ROS species generated include hydroxyl radical and hydrogen peroxide which are unstable and toxic (136). Neutrophils from patients following traumatic injury have elevated baseline ROS production and enhanced production following secondary stimulation (137, 138). This is indicative of systemic priming of neutrophils most likely caused by the inflammatory environment induced immediately post injury. Secondly, neutrophils can kill pathogens via the delivery of granule products to the phagosome (139). One such enzyme derived from azurophilic granules is neutrophil elastase which is a serine proteinase and is known to directly digest bacteria (140). As these compounds, ROS and granular proteins are extremely toxic, dysregulation or abundant production is also implicated in host tissue damage which is a common feature post traumatic and thermal injury (134).
1.9.4 Apoptosis

Neutrophil apoptosis is a tightly regulated pathway which prevents tissue damage by inhibiting the release of toxic intracellular compounds of neutrophils. Recognition and clearance of dead neutrophils, via macrophages, is an essential step in the resolution of inflammation (141, 142). Apoptosis is controlled predominantly by a number of bcl-2 homologues within neutrophils. Of such, MCL-1 is a pro-survival protein which is produced and cleared rapidly and functions to prevent apoptosis and is regarded as a major orchestrator in neutrophil circulating life span (143, 144). In addition, neutrophils express pro-apoptotic bcl-2 homologues including, Bax (145), Bid (146) and Bim (147). Which all function to induce apoptosis and, unlike MCL-1, take a long time to be cleared upon generation. Severe traumatic injury results in a dysregulation of neutrophil apoptosis which is proposed to underlie the pro-inflammatory phenotype, increased incidence of infection and host tissue damage (148-151).

One mechanism behind dysregulated apoptosis is the elevated levels of pro-inflammatory cytokines and/or elevated levels of ROS, which can induce apoptosis via indirect activation of caspase 8 (152). Whilst it has been reported that neutrophil apoptosis is dysregulated following major trauma, the mechanism responsible and clinical implications remain unknown.

1.9.5 Anti-microbial actions

The importance of neutrophils in host defence is emphasised in disease pathologies in which fundamental neutrophil processes are dysfunctional. One such disease is chronic granulomatous disease (CGD) in which the individual is
incapable of producing ROS due to the absence of the NADPH oxidase complex and thus suffer from recurring infections throughout their life (153). Additionally, patients with Chediak-Higashi syndrome have a mutation in the LYST gene, which is responsible for a lysosomal trafficking regulator (154). Patients with this syndrome are susceptible to infections due to the inability to form functioning phagolysosomes. Furthermore, in a murine model of *Staphylococcus aureus* infection, there is a significant increase in mortality when mice are depleted of neutrophils (155). Hence neutrophils and their components are key orchestrators of host defence and essential for survival following bacterial challenge.

Although there is evidence showing that severe thermal injury results in altered neutrophil function. It is currently unclear if thermal injury results in a longitudinal disruption in neutrophil functions which may underlie the increased incidence of infection, tissue damage and secondary complications. Hence, longitudinal and extensive investigation of neutrophil function post thermal injury is required.

1.10 Neutrophil dysfunction post traumatic injury

Traumatic and thermal injury results in a profound immune dysregulation which is characterised by an immediate activation closely followed by a subsequent period of immune suppression (37). It has been proposed that this second period of dysregulation may underlie the increased incidence of nosocomial infections.

1.10.1 Maturity and phenotype of neutrophils post traumatic injury

Of note, trauma results in profound changes in maturity, survival, phenotype and function of neutrophils (156-161). As neutrophils are an essential component of innate immunity, functional dysregulation has been linked to host tissue damage
and increased propensity to infections. However, mechanisms responsible are poorly understood.

Maturation and production of neutrophils are controlled within the bone marrow by a complex panel of mediators and signals. The principal regulator of granulopoiesis is granulocyte-colony stimulating factor (G-CSF) which functions to commit progenitor cells to myeloid lineage (162), reduce maturation time (163), proliferation of granulocyte precursors and release of mature cells from the bone marrow. There are secondary signals of neutrophil production and release including; IL-6, IL-3 and granulocyte macrophage colony-stimulating factor (GM-CSF) (164-166). In states such as severe bacterial infection, the body can adjust to a state of ‘emergency granulopoiesis’ in which large quantities of neutrophils are released in part mediated by G-CSF (167). Gardner and colleagues reported in a mouse model of trauma that increased levels of G-CSF caused the release of high levels of mature neutrophils which protected mice against a lethal Klebsiella pneumoniae pulmonary challenge. Neutralisation of G-CSF reduced neutrophil release and reduced survival (168). Thus one might expect that G-CSF plays a predominantly positive role in host protection following major trauma.

However, excessive stress and stimulation of the bone marrow can result in emergency granulopoiesis that is characterised by the appearance of immature precursors of neutrophils, blood leucocytosis or neutrophilia (167). IGs are a precursor of mature neutrophils and are elevated in septic patients (96). Even though they are capable of mediating innate immune functions this is reduced compared to mature neutrophils and may underlie the increased susceptibility to infection following thermal injury (169). In a cohort of 83 severely injured patients, Cook et al reported that immediate and sustained elevation in G-CSF was
associated with the release of immature cells from the bone marrow and an increased risk of infection (157). Hence, the role of G-CSF in mediating survival following thermal injury remains unclear and is most probably multi-factorial and time dependent.

In 2011, Pillay and colleagues described a distinct change in the circulating neutrophil phenotype following endotoxin challenge or following severe injury (158). This group characterised neutrophil subsets by flow cytometry staining for CD16, a low affinity Fc receptor expressed on neutrophils, and CD62L, an adhesion molecule involved in leukocyte trafficking that is shed upon activation. Three hours after lipopolysaccharide (LPS) administration there is a dynamic change in neutrophil phenotype with the appearance of three subsets of neutrophils (Figure 1.4). Two of which were detectable in challenged participants and were absent from healthy individuals. These subsets were; CD16\textsuperscript{dim}/CD62L\textsuperscript{bright} and CD16\textsuperscript{bright}/CD62L\textsuperscript{dim} neutrophils.

Firstly, CD16\textsuperscript{dim}/CD62L\textsuperscript{bright} neutrophils were banded in their nuclear morphology and most likely released from the bone marrow prematurely in response to the LPS challenge. The second population of hyper-segmented CD16\textsuperscript{bright}/CD62L\textsuperscript{dim} neutrophils had increased maturation compared to normal blood neutrophils. Furthermore, each of the novel subsets had differing functions with CD16\textsuperscript{dim}/CD62L\textsuperscript{bright} neutrophils having reduced apoptosis compared to the CD16\textsuperscript{bright}/CD62L\textsuperscript{dim} population. Whereas the CD16\textsuperscript{bright}/CD62L\textsuperscript{dim} population exhibited increased ROS production compared to the CD16\textsuperscript{dim}/CD62L\textsuperscript{bright} population (158).
More recently the authors have shown that CD16\textsuperscript{bright}/CD62L\textsuperscript{dim} neutrophils are a distinct population of circulating neutrophils released in response to inflammation and not LPS challenge (170). Banded cells, CD16\textsuperscript{dim}/CD62L\textsuperscript{bright} phenotype, were released much quicker into the circulation following LPS challenge compared to CD16\textsuperscript{bright}/CD62L\textsuperscript{dim} cells. Furthermore, CD16\textsuperscript{bright}/CD62L\textsuperscript{dim} cells were different at the proteome level compared to band and/or hypersegmented cells. Whilst the source of these CD16\textsuperscript{bright}/CD62L\textsuperscript{dim} cells remains unknown, the group proposed that they are a unique population of cells released during inflammation and participate in the regulation of the immune system (170). Data like these highlight the importance of characterising neutrophil phenotype and its correlation with clinical outcome post thermal injury.
Figure 1.4. Administration of LPS results in distinct phenotypical changes in circulating neutrophils. (A) Flow cytometry of neutrophils, based on CD16 and CD62L staining, before administration of LPS. (B) Neutrophil populations 180 minutes following LPS administration. Three populations of neutrophils are observed based on CD16 and CD62L staining. Two populations are unique to LPS challenge and absent from healthy individuals. Figure taken directly from ‘A subset of neutrophils in human systemic inflammation inhibits T cell responses through Mac-1’ by Pillay et al. (158).
1.10.2 Functional dysregulation of neutrophils post traumatic injury

Chemotaxis describes the movement of a cell to a stimulus in a concentration dependent manner. Neutrophils utilise chemotaxis to migrate to sites of infection and tackle and clear invading pathogens and bacteria. Studies dating back to 1974 have identified an inherent defect in neutrophil migration following thermal injury (159-161). Until recently, technologies used to measure neutrophil migration have been limited, time-consuming and require a large volume of blood.

In 2010, Butler and colleagues described a novel microfluidic device that is simple to use, allows for precise and robust measurements of chemotaxis speed and persistence characteristics at single-cell resolution. Importantly, this device uses only a single drop of blood which is important to prevent anaemia in critically ill patients (171). This group has shown that thermal injury results in a significant reduction of directional migration speed within 24 hours of injury which reaches maximal reduction at 72-120 hours post injury when compared to healthy individuals. In this study one patient, with a 60% TBSA thermal injury, did not have reduced neutrophil migration. This patient was admitted with a documented infection unlike the rest of the cohort. The authors propose that the immediate inflammatory cascade post thermal injury results in suppression of chemotaxis which increases the propensity to infections and that the infection itself does not reduce chemotaxis (171). Hence, modulation of chemotaxis remains an early therapeutic target.

More recently the authors have described a novel phenotype of spontaneous neutrophil migration in septic patients following severe thermal injury. This phenotype was observed 1 – 2 days before the diagnosis of sepsis and was not
detectable in patients who did not develop an infection or healthy individuals (172). Therefore quantification of chemotaxis phenotypes may aid in early diagnosis of patients at risk of developing sepsis during their hospital stay. This could aid in the timing of antibiotic and clinical treatment in the aim of improving patient outcome post thermal injury (171).

Once a neutrophil has trafficked to the site of infection it functions to either phagocytose or generate NETs to ensnare the invading pathogen. Phagocytosis is a complex process that forms an essential component of innate immunity through the ingestion and elimination of pathogens. As such, a dysregulation in phagocytic function may precede the development of infections which are extremely common in patients with thermal injuries (38). Although this has been proposed, only a small number of studies have examined this relationship and none have studied the dynamic longitudinal changes in relation to clinical complications post thermal injury.

Sheng and colleagues studied neutrophil bactericidal activity in 36 patients with thermal injuries using a chemiluminescence assay. The group demonstrated that septic patients had lower neutrophil bactericidal function compared with healthy controls. Bactericidal activity was significantly lower in thermal injuries above 35% TBSA suggesting that severity of injury orchestrates the amplitude of dysregulation consistent with previous literature examining chemotaxis (171, 173). Alexander et al performed a prospective analysis of neutrophil function in 20 patients with severe thermal injuries (≥45% TBSA). The group reported a significant reduction in neutrophil bactericidal activity, in response to Staphylococcus aureus 502A, and chemotaxis in infected patients compared to non-infected patients. Thus suggesting a broader dysregulation of neutrophil
functions mediated by initial injury and subsequent sepsis not previously reported (171, 174).

ROS are essential anti-microbial compounds which function to directly kill and eliminate engulfed pathogens within a neutrophil. Production of ROS is controlled by NADPH oxidase complex which produces O2- and H2O2 upon activation which are toxic compounds. It has been reported that trauma results in an increase in ROS production (137). Conversely, multiple groups have shown that thermal injury results in significant reduction in the ability of neutrophils to generate ROS and a reduced phagolysosomal acidification (175-177).

Parment and colleagues in a prospective exploratory cohort study investigated neutrophil oxidative burst in response to Escherichia coli (E.Coli), phorbol 12-phorbol myristate 13-acetate (PMA), and fMLP. This study included 28 patients with thermal injuries with an average TBSA of 36%. Thermal injury resulted in a significant reduction in neutrophil oxidative burst, in response to all stimuli, from admission up to 3.5 months post injury compared to levels seen in healthy volunteers. Thus, thermal injury results in an immediate and sustained immunosuppression which appears to be different from general trauma (177).

Trauma and thermal injuries result in a significant dysregulation of a number of neutrophil functions. Although multiple groups have described the dynamic changes in neutrophil function no group has conclusively studied the longitudinal changes in neutrophil functions in relation to acute injury and subsequent infections following thermal injury.
1.11 Neutrophil extracellular traps

1.11.1 Structure and function

NETs, although first described in 2004, are still emerging as a potentially important area of neutrophil biology (118). NETosis (178) is a tightly regulated active cell death pathway facilitated by neutrophil activation resulting in the extrusion of DNA to the exterior of the cell decorated in granular proteins which can ensnare, trap or sometimes kill bacteria (118, 119, 179). Discovery of NETs sparked an entirely new area of neutrophil research and their association with innate immunity and haemostasis. Systemic markers of NETosis have been reported following major trauma, burn injury and during sepsis (180, 181).

1.11.2 Vital and suicidal NETosis

Traditionally NETs are generated by incubating neutrophils with chemical agonists, *e.g.* PMA, a known potent activator of protein kinase C (PKC). In 2013, this pathway of NET generation was termed ‘suicidal NETosis’ (178). This process results in complete loss of the nucleus and terminal cell death hence the name. Although NETosis was first described in 2004 there are earlier reports indicating that PMA could cause chromatin decondensation, nuclear swelling, release of nucleoplasm into the cytoplasm and membrane perforation (182). It wasn’t until 2004 that the process of NET release was discovered by Zychlinsky and colleagues (118). This initial description was advanced by further studies revealing that NETs are an important arm of innate immunity as they can bind to both gram-positive and gram-negative bacteria (183).
The mechanisms causing terminal NET production are still not fully understood. Many groups have reported that the generation of oxidants is an essential requirement for NETosis. However, signalling components downstream of oxidants responsible remain unknown. For instance, Fuchs et al reported that formation of NETs is dependent upon activation of NADPH oxidase. Additionally, the authors state that patients with CGD carry a mutation for NADPH oxidase and therefore cannot make NETs which may be responsible for recurring infections (184). Following activation of NADPH oxidase, ROS initiates the translocation of neutrophil elastase to the nucleus which results in chromatin decondensation and NETosis. Importantly, neutrophil elastase deficient mice do not form NETs in a pulmonary model of *Klebsiella pneumoniae* infection thus supporting the proposed mechanism of NADPH oxidase dependent NETosis. Hence, mobilisation of granular proteins following ROS production are fundamental in the regulation of chromatin density and structure (185). However, it has also been reported that biological stimuli, *e.g.* *Staphylococcus aureus*, can result in NADPH oxidase independent NET formation (186). Thus it is proposed that NETosis can occur through multiple signalling pathways which may be unique to individual stimuli.

A fundamental step in suicidal NET generation is the rupture of the cellular membrane and death of the neutrophil. Therefore one would propose that suicidal NETosis inhibits the ability of neutrophils to perform other traditional defensive actions, *e.g.* phagocytosis or chemotaxis, and may leave the host immunocompromised which would be fatal to patients following traumatic or thermal injury. Therefore, it is important to consider how neutrophils can generate NETs and still perform vital defensive actions. A number of possibilities exist including; 1) neutrophils generate NETs and survive the process; 2) neutrophils
are split into pools which perform different specialised functions depending upon orchestrating signals; 3) NET formation is the the last line of defence of the neutrophils when the host is overwhelmed.

A second pathway of NET generation has also been described and coined ‘vital NETosis’. The first major difference between the vital and suicidal pathway is the stimulus. Vital NETosis is mediated by naturally occurring stimuli, e.g. bacterial components and/or activated immune cells, and is therefore seen as the NETosis pathway occurring in vivo (178, 187). Moreover, vital NETosis occurs rapidly, within 30 - 45 minutes, whereas suicidal NETosis is much slower, within 3 - 4 hours post stimulation. Theoretically, this allows for rapid entrapment of invading pathogens and doesn’t compromise the host. The final difference between the two pathways is the mechanism of release. Vital NETosis is still in part mediated by activation of NADPH oxidase, mobilisation of granular proteins/enzymes and histone citrullination (178). However, vital NETosis also employs vesicular trafficking of DNA to the exterior of the cell. Vesicles co-localised with the cell membrane and delivered the nuclear material to the outside of the cell without membrane perforation (Figure 1.5 G, H and I) (186). This could represent a conserved mechanism to tightly control NET release and allow additional neutrophil functions to occur. In support of this hypothesis, Clark et al demonstrated that neutrophils which had released NETs were impermeable to SYTOX Green stain, suggesting that neutrophils can remain intact following NETosis (100). The differences between ‘vital and suicidal’ NETosis are depicted in Figure 1.5 (178).
Figure 1.5. Proposed novel mechanisms of NET release: suicidal and vital NETosis. (A-C) ‘Suicidal NETosis’ is initiated by chemical agonists, e.g. PMA, resulting in activation of PKC and translocation of granular proteins and eventual chromatin decondensation which ultimately leads to NETosis. This process requires approximately 180 minutes. (D-F) Bacteria and immune cells can cause rapid ‘vital NETosis’. Again this process requires the mobilisation of granular proteins for eventual NET release. (G-I) However vital NETosis can utilise vesical transport to expel DNA to the exterior of the cell without membrane rupture. The total figure was taken from (178) which took figures G-I from ‘A novel mechanism of rapid nuclear neutrophil extracellular trap formation in response to Staphylococcus aureus’ by Pilsczek et al (186).
Neutrophils may be split into specialised pools which perform actions depending upon complex orchestrating signals. In 2014, Branzk et al identified a novel microbe sensing mechanism that tailored neutrophil anti-microbial actions depending upon pathogen size. Neutrophils selectively released NETs in response to large microbes, namely Candida albicans hyphae and extracellular aggregates of Mycobacterium bovis, but did not respond to single bacteria or yeast which were phagocytosed instead. The 'decision' made by neutrophils to release a NET or undergo phagocytosis was controlled by competition for neutrophil elastase between the two processes. Dectin-1, a pattern recognition receptor involved in innate immunity, was the key signalling molecule in this decision process. If phagocytosis occurred via dectin-1, neutrophil elastase translocation was inhibited, thus preventing NET production. Conversely, knockout of dectin-1 led to excessive NETosis and tissue damage. Thus phagocytosis appears to act as a checkpoint to induce or prevent NET formation via dectin-1 and competition of neutrophil elastase (188). Therefore, NET formation may represents the last resort of neutrophil defence against invading pathogens to prevent complete loss of the circulating neutrophil pool and in turn immunocompromising and potentially damaging the host. This is of particular interest when studying patients with traumatic injuries as they are prone to nosocomial infections and sepsis (38). Hence, this pathogen sensing ability may aid patients by switching to a pro-phagocytosis phenotype, reducing the risk of the patient becoming immunocompromised.
1.11.3 Citrullinated histone H3 and reactive oxygen species generation

ROS are a heterogeneous collection of molecules which are highly reactive and formed in multiple cellular organelles as bi-products of redox reactions. NADPH oxidases are a group of membrane bound multiprotein enzymes that generate ROS and are expressed in large quantities in neutrophils (136). ROS play an important link between cellular activation and NET formation as discussed above (184). Although the full mechanisms behind ROS induced NET formation aren’t fully understood it is known that ROS can induce histone citrullination by activation of the enzyme peptidylarginine deiminase 4 (PAD4) which results in decondensation of nuclear chromatin (189). PAD4 is a nuclear enzyme which is highly abundant in human neutrophils and mediates the replacement of amino acid arginine with citrulline (190, 191). Citrullinated histone H3 (Cit H3) is expressed within NETs and is regarded as a specific marker of NET generation and has recently been detected in the bloodstream of critically ill patients (181). In addition to their role in the pathogenesis of inflammatory conditions, modified histones are potential biomarkers of sepsis (181, 192).

1.11.4 Histones, DNA and nucleosomes

Histones are a group of small positively charged proteins including; H1, H2A, H2B, H3 and H4. They are highly conserved and responsible for gene transcription (193, 194). Free histones are extremely cytotoxic due to their charged nature and administration results in rapid mortality in animal models (195-198). DNA is the fundamental orchestrator of cellular growth, repair, function and reproduction. DNA is released abundantly following severe tissue damage and
can activate a number of inflammatory cascades (199), because of this DNA is regarded as a DAMP. A DNA molecule consists of two polynucleotide chains with each nucleotide containing a 5-carbon sugar molecule attached to a phosphate group (194). These phosphate groups make DNA negatively charged which means that they can bind to histones and form nucleosome complexes (Figure 1.6).

Comprised of a nucleosome core, an octameric complex of histones and DNA, and linker DNA, nucleosomes are the principal component of chromatin (194, 200, 201). Nucleosomes have multiple functions including signalling as a centre for chromatin orchestrated processes and assembling higher-order structures which aid in compaction of genomic components (200). The presence of circulating nucleosomes is associated with NET release and cell death in patients with chronic myeloproliferative neoplasms and deep vein thrombosis (DVT) (202, 203). Nucleosomes have different cytotoxic and procoagulant properties compared to free histones and DNA (198, 204-206). Despite this, many studies investigating these components interchangeably use nucleosomes, histones and DNA to describe in vivo associations. Therefore, characterisation of the structure and properties of circulating nucleosomes, histones and DNA is required to conclusively understand their roles in inflammation, coagulation and tissue damage.
Figure 1.6. Nucleosome structure. Nucleosomes are comprised of a nucleosome core, an octameric complex of histones (H3-H4) and DNA, and linker DNA (H2A-H2B). Nucleosomes form through charge interactions with the overall charge being neutral. Figure adapted from ‘Histone exchange, chromatin structure and the regulation of transcription’ by Venkatesh and Workman (194)
1.11.5 Autophagy and neutrophil extracellular traps

Autophagy is a normal physiological response of the cell that maintains homeostasis by protein degradation and turnover of destroyed cell organelles to induce new cell formation (207). Recent evidence has demonstrated that NET formation is dependent upon both ROS generation and activation of autophagy (208). Additionally, multiple bacterial strains can activate autophagy, implicating its activation in neutrophil biology and host defence (209-212). To date, it is currently unknown if thermal injury and/or soluble factors released affect neutrophil function via alteration in autophagy signalling.

1.12 NETs and traumatic injury

Traumatic and thermal injury are characterised by an initial sterile injury and subsequent infectious stimuli. The generation of NETs is regarded as defensive action of neutrophils to invading stimuli in an attempt to ensnare and potentially trap bacteria. It has been demonstrated that NETs are present in septic individuals via the measurement of non-specific circulating markers of NET formation (213, 214). Furthermore, studies have concluded that NETs are produced during subsequent septic episodes following traumatic injury (181, 215, 216). Thus, NETs may form an orchestrating role in the defence against invading pathogens in patients with thermal injuries. However, it has yet to be conclusively established if NETs are generated following thermal injury.

Interestingly there is an emerging body of evidence to implicate NET formation and non-infectious stimuli (217-219). With elevations in circulating NET biomarkers reported post traumatic and thermal injury (215, 220). It is proposed that the severe tissue damage is enough to induce rapid NET formation within
minutes of injury through the release of inflammatory mediators. In a cohort of patients with burn injury, plasma cfDNA are increased compared to healthy individuals and thus suggests immediate NET formation (220). Although this study reports elevation in NET components it does not measure any specific markers of the process. Quantification of cfDNA alone is non-specific to NETosis, with plasma cfDNA originating from a number of sources including apoptotic, necrotic or NETosing cells and bacteria themselves (115). Therefore, to our knowledge, it has yet to be conclusively established whether NETosis is occurring in patients with burn injury.

1.13 In Vivo host defensive functions of neutrophil extracellular traps

Despite extensive studies of NETs, their direct in vivo interactions and effects are still being investigated with multiple groups reporting contradicting results (119, 221). Therefore, how NETs contribute to host defence post thermal injury is poorly understood and warrants further investigation.

Meng and colleagues provided evidence that NETs were responsible for in vivo killing of bacteria. Administration of deoxyribonuclease (DNAse) to mice in a cecal ligation puncture (CLP) model of sepsis resulted in reduced in vivo NET markers coupled with increased colony forming units of bacteria and an increased pro-inflammatory response (221). Hence, in this study NETs are directly responsible for killing bacteria and host protection. However, in 2012 Menegazzi challenged the current paradigm of NETs directly killing bacteria. The group reports that NETs ensnared bacteria but did not kill them as incubation of NETs with DNAse freed bacteria which were still alive (119). Therefore, NETs may have more of a physical bacteriostatic role in ensnaring bacteria and preventing dissemination rather than
a direct bactericidal activity. Though the potential implications this has in severe infections, e.g. sepsis following severe thermal injury, are currently unknown and seem rather redundant if neutrophil function is already reduced.

Furthermore, in a murine model of sepsis, NETs accumulate within various organs and interact with the vascular endothelium. Within the microvasculature NETs colocalised with inflammatory cells, including platelets, and may be contributing to thrombosis which is commonly observed post infection (222).

### 1.14 Excessive NETosis, thrombosis and host tissue damage

Advancements in the treatment of thermal injuries have improved initial survival following severe thermal injury. However, delayed mortality remains a significant health care issue with sepsis and MOF being the leading causes of mortality (4, 5). Even though MOF is well characterised the mechanisms mediating the pathogenesis are poorly understood. Therefore advancements in understanding the pathogenesis of MOF may improve patient outcome.

Although neutrophils are an essential component of innate immunity, recent evidence has shown that NETs can initiate a procoagulant phenotype, tissue and organ damage (195, 221, 223-225). Prolonged or uncontrolled NET generation results in damage to host tissues and significantly contributes to the pathogenesis of inflammatory conditions (195, 198, 226, 227). DNA and histones are key components of NETs, but elevation in circulating levels may also mediate organ damage through their cytotoxic nature (228). As such, NETs can provide a bridge between the innate immunity and haemostatic systems. For example, they can directly bind to and activate platelets along with various coagulation system components (100, 229). These interactions during excessive NETosis may then
promote thrombosis, DIC (52) and potentially mediate the pathogenesis of MOF following sepsis (230, 231).

Elevated levels of NET components have been implicated in thrombosis formation in a murine model of DVT induced stenosis in the inferior vena cava. 6 hours after stenosis, levels of plasma cfDNA were significantly elevated compared to sham-treated animals and neutrophils were visible in both the red (red blood cell rich) and white (platelet rich) thrombi. Moreover, Cit H3, a specific marker of NETs, colocalised with neutrophils in red thrombi. This data is consistent with a previous publication which identified nucleosomes in thrombi of baboons following induction of DVT (232). Infusion of DNase protected animals against flow restriction induced by DVT regardless of the length of stenosis. Furthermore, DNase treated mice had no visible thrombi suggesting DNase disrupts the mechanisms leading to thrombosis. The group hypothesised that ‘the anti-thrombotic effect of DNase is likely to be mediated by removal of NETs generated locally at the site of stenosis’ (233).

The procoagulant effect of NET components and cfDNA, is well documented (230, 234). However, the role of cfDNA on fibrinolysis is poorly understood. Fibrinolysis is the enzymatic breakdown of fibrin in blood clots predominantly by the enzyme plasmin. In 2015, Gould and colleagues investigated the effect cfDNA has on fibrinolysis in sepsis. The group quantified levels of cfDNA in plasma obtained from 400 patients with severe sepsis and clot lysis was measured in an isolated system and breakdown of formed clots was visualised using electron microscopy. Clots formed in plasma from septic patients had decreased permeability, decreased susceptibility to fibrinolysis and increased clot stability which could be reversed by the addition of DNase. It is proposed that this impairment of
fibrinolysis was caused by cfDNA forming a ternary complex between plasmin, cfDNA and fibrin that inhibits the activity of plasmin (235). Hence, this study highlights the multifaceted actions of NET components in the production and maintenance of thrombosis and potentially the pathogenesis of MOF following sepsis.

MOF is characterised by the formation of thrombi in the microvasculature resulting in tissue hypoxia, damage and ischemia. Boneschansker et al have demonstrated, using a microfluidic device replicating capillary plexuses, that NETs are capable of perturbing blood flow in capillary networks (Figure 1.7 A-B) (236). NETs generated from a very small number of neutrophils resulted in more than half of the microfluidic device being obstructed from red blood cell flow and this inhibition lasted for hours post NET formation despite the presence of naturally occurring DNAse in the blood. Administration of Turbo™ DNAse, a more potent analogue of naturally occurring DNAse 1, degraded NETs and restored blood flow within 30 minutes of infusion (Figure 1.7 B). Importantly, digestion products of NETs did not impair blood flow within the microfluidic device (236). Complete breakdown of the NET structures and removal of DNA from the channels was therefore sufficient to prevent this obstruction. This study highlighted a potential mechanism in which the physical properties of NETs may result in the pathogenesis of MOF by obstructing blood flow through capillary networks.

NETs and their components have the potential to not only induce thrombosis but can also propagate further detrimental effects by impairing fibrinolysis and obstructing blood flow. The formation of excessive or uncontrolled NETosis may result in tissue hypoxia, the onset of DIC, cause host tissue damage and potentially mediate the pathogenesis of MOF in patients with thermal injuries.
Importantly, digestion or breakdown of NETs via DNAse may be a therapeutic target to prevent NET mediated pathogenesis of MOF.
Figure 1.7. NETs form a physical barrier and impair blood flow through a microfluidic device. (A-B) Extracellular chromatin from NETs (yellow stain) extends deep into microfluidic devices and impairs blood flow (red stain). Incubation for 30 minutes with DNase results in degradation of NETs and restores blood flow through the device. The figure was taken directly from ‘Capillary Plexuses are Vulnerable to Neutrophil Extracellular Traps’ by Boneschansker et al (236).
1.15 Degradation of neutrophil extracellular traps

DNAse is the major extracellular endonuclease found in a number of bodily fluids and functions to breakdown chromatin and DNA which are released following cellular damage, cell death and NETosis. Levels of circulating cfDNA are elevated in a number of disease pathologies but the mechanisms behind uncontrolled elevation in this marker are poorly understood. Of note, elevated levels of plasma cfDNA are associated with and contribute to the progression of autoimmune diseases (237, 238). In a subpopulation of patients with systemic lupus erythematosus (SLE), DNAse activity was impaired by DNAse inhibitors or inhibitory antibodies (237). Although a reduction in DNAse activity and build-up of cfDNA has been shown to be associated with the development of autoimmune diseases, to date no group has investigated DNAse activity following thermal injury (237).

To date, only one study has investigated DNAse levels post traumatic injury. This study recruited 39 patients with traumatic injury and quantified levels of DNAse 1. Whilst there was a significant elevation in circulating DNA levels post-injury, only on the day of injury was a difference observed in DNAse 1 levels, with patients having significantly higher circulating DNAse 1 levels. At all other study time-points, DNAse 1 levels in patients were comparable to that for healthy controls (239). Thus, the accumulation of circulating NET markers may be due to a reduction in DNAse activity rather than quantity and may be one mechanism for the pathogenesis of MOF following thermal injury.
1.16 Actin

Actin is the most abundant protein in mammalian cells and is a fundamental protein for cell motility (240). Actin exists in a balance between monomeric and filamentous actin. Processes such as cell motility are dependent upon the rapid polymerisation of monomeric actin into filamentous actin (241, 242). However, this predisposition to polymerise is of detrimental consequences if actin is released into the circulation, as extracellular actin can cause direct damage to the microvasculature capillaries, impair clot lysis and activate platelets (240, 243, 244). Elevation in circulating actin is attributed to a saturation of the actin scavenging system.

Interestingly, DNAse activity is indirectly controlled by actin scavenging system (240, 245) as actin can bind to DNAse and inhibit its enzymatic activity (237, 245). Due to the extensive amount of tissue damage post severe thermal injury one might hypothesise that actin is released following severe thermal injury and results in the inhibition of DNAse activity and build-up of circulating cfDNA. Data reported by Lee et al support this hypothesis as elevated levels of actin have been detected in a cohort of septic patients (246). Therefore, DNAse activity may be inhibited in septic patients predisposing them to risk of actin toxicity, accumulation of circulating DNA, host tissue damage, a pro-thrombotic phenotype and MOF.

1.17 Actin Scavenging System

Control of the actin scavenging system is mediated by two key proteins; gelsolin (GSN) and vitamin D binding protein (VDBP) (240). Both proteins functions to clear monomeric and filamentous actin from the circulation through the liver. The actin scavenging system is summarised in Figure 1.8.
1.17.1 Gelsolin

Plasma GSN is found in high quantities in healthy individuals and can bind to both monomeric and filamentous actin (246). Levels of circulating GSN in healthy individuals range from 150 – 300 µg/ml and plasma GSN has a half-life of approximately 2 days (247). GSN is a 90 kDa cytoplasmic actin binding protein which also exists as a plasma variant. The plasma variant of GSN is larger, 93 kDa, and more positively charged compared to cytoplasmic GSN. Although both variants are similar in structure, their processing and eventual actions are independent (248).

GSN functions in collaboration with VDBP to rapidly depolymerise and reduce circulating levels of actin. GSN has three actin binding sites and functions to clear actin in two distinct processes (249-251). Firstly, GSN functions to prevent any further addition of monomeric actin to existing chains by binding to the barbed end of actin (250). Secondly, GSN binds to two distinct sites on the side of polymerised actin and severs the filaments resulting in depolymerisation (240, 251). As GSN binds to circulating actin it is not surprising that studies have reported a decrease in circulating levels of GSN in a number of disease pathologies connected with actin release (252-259). Furthermore, it has recently been reported that administration of GSN can reverse actin inhibition of lung macrophage binding and uptake of bacteria (260). In addition to being part of the actin scavenging system, GSN has direct interactions in modulating the immune response. Thus, reduced levels of GSN will have a multifaceted role in host response.

Huang et al reported in 95 patients with thermal injuries that plasma GSN levels were reduced and associated with mortality, development of sepsis and MODS
Furthermore, in a rat burn model (40% TBSA) plasma GSN levels decrease 12 hours post injury and remain lower for up to 6 days post injury. This was accompanied by increased pulmonary microvasculature permeability in burn treated animals. Importantly, administration of recombinant plasma GSN prior to burn injury protected against this pulmonary microvasculature dysfunction (261). Therefore plasma GSN may not only serve as a biomarker of poor outcome but could also be a potential therapeutic target to prevent secondary complications. However, the potential translation of GSN and its action in human burn injury have not been fully investigated.

1.17.2 Vitamin D binding protein

VDBP, also referred to as Gc-globulin, is a 58-kd alpha-2-globulin which is a key component of vitamin D transport (262). VDBP is found in abundant quantities in healthy individuals with levels comparable to GSN (263). Unlike GSN, VDBP binds to monomeric actin only allowing GSN to bind to polymeric actin (264). This relationship suggests an orchestrated response to enhance actin clearance in vivo by VDBP and GSN (265).

Binding of VDBP to actin prevents further polymerisation by clearing residual monomeric actin from the circulation. The clearance of actin-vitamin D binding protein complexes is rapid and is mediated through the liver primarily but this can also occur through the spleen and lung (266, 267). It is important to note that clearance of actin via GSN and VDBP results in consumption with a transient decrease in their circulating levels.
Levels of VDBP have also been suggested as a prognostic marker of outcome and organ damage following severe traumatic injury (268-271). Interestingly, it has been reported that trauma results in an eventual over production of VDBP (271). This implies a reactive response of the body to saturation or dysregulation of the actin scavenging system. However, this has not been studied longer than 1 week post trauma.

To date, no group has studied the relationship between VDBP, GSN and DNAse post thermal injury. As VDBP and GSN are responsible for actin clearance one might hypothesise that in thermal injury low plasma VDBP and GSN levels will be associated with dysregulated DNAse activity. Therefore dysregulation in the actin scavenging system may precede actin inhibition of DNAse activity and is potentially contributing to the complicated outcomes associated with MOF post thermal injury.
Figure 1.8. Actin scavenging system in a healthy individual. Following acute tissue injury there is the release of monomeric and polymerised actin into the circulation. VDBP rapidly binds to monomeric actin and clears it from the circulation, preventing further polymerisation. GSN clears polymerised actin by two mechanisms (249-251) in collaboration with VDBP. This rapid clearance of actin maintains circulating DNAse activity.
1.18 Targeting neutrophil extracellular traps

It is proposed that targeting the formation or breakdown of NETs in various diseases pathologies may have positive effects on disease progression (236, 237, 272, 273). One proposed mechanism for targeting NETs is the breakdown of pre-formed and excessive NETosis via the administration of DNAse to reduce thrombotic complications, host tissue damage and pro-inflammatory response during infection (230, 232, 235, 236, 274). However, administration of DNAse in a murine model of sepsis, induced by CLP, resulted in increases in the pro-inflammatory response, the colonisation of bacteria and mortality (221). This was attributed to increased bacterial dissemination caused by early and inappropriate degradation of NETs. This study highlights the potential importance of time dependent treatment as NETs should only be targeted when in excess and contributing to the pro-inflammatory and thrombotic phenotype.

NET production may also be inhibited via the inhibition of PAD4. In a murine model of sepsis, PAD4 deficient mice had comparable mortality to wild-type mice. Similarly, PAD4 knockout mice were partially protected against LPS induced shock, suggesting that knock-out of PAD4 and elimination of NETs may protect against the pro-inflammatory and toxic effects of NETs during sepsis. The authors conclude ‘preventing NET formation by PAD4 inhibition in inflammatory or thrombotic diseases is not likely to increase host vulnerability to bacterial infections’ (275). However, this model does not take into account pre-existing immunosuppression which is observed post burn injury (159-161, 173, 175-177). Moreover, in a murine model of infection, using necrotizing fasciitis, PAD4 knockout mice were more susceptible to infection than wild-type mice. The authors
attributed this susceptibility to the inability to form NETs in response to the infection \((276)\). Therefore full characterisation of NET formation kinetics, functions and the relationship with other neutrophil functions is required to understand if targeting NETs post thermal injury would provide any host protection.
1.19 Aims and hypothesis

Advancements in the initial care of burn wounds and immediate survival have increased dramatically over the past four decades (3, 18). However, sepsis and, consequently, MOF remain leading causes of delayed mortality following severe thermal injury (47). Although sepsis and MOF are well characterised, the mechanisms driving the increased incidence of infection and pathogenesis of MOF are poorly understood.

Evidence has implicated abnormal neutrophil function in the increased propensity to infection. However, this has not been studied longitudinally (159-161, 173, 175-177). The recent discovery of NETs has identified a novel paradigm in which neutrophil functions may be dysregulated post thermal injury and contributing to the pathogenesis of MOF (230, 232, 235, 236, 274). To date, no group has conclusively shown that NETs are produced following thermal injury and no group has examined the relationship between NETs, MOF and sepsis following severe thermal injury.

Accordingly, the aims of this thesis were to:

1. Determine the effect severe thermal injury has on neutrophil function and understand potential mechanisms mediating the dysfunction.
2. Investigate if thermal injury initiates NETosis and to investigate if NETosis is induced by sterile injury and/or infectious stimuli.
3. Determine if NET generation is associated with secondary complications, MOF and sepsis, and to investigate potential mechanisms and therapeutic targets.
4. Investigate if clearance of DNA was dysfunctional and associated with MOF.

5. Determine if reduced DNAse activity is a potential therapeutic target to reduce secondary complications.
Chapter 2

Materials and Methods
Materials and Methods

2.1 Scientific Investigation of Biological Pathways Following Thermal Injury Study

2.1.1 Ethical approval

Ethical approval for the study was granted by a UK NHS research ethics committee (Reference 12/EM/0432). Where possible, written informed consent was received from participants prior to their inclusion in the study. Due to the severe nature of the injuries being studied, the ethics committee approved the use of a legal consultee, either personal or nominated, if the patient was not initially able to consent for inclusion in the study themselves. When the patient was able, they were approached to give written consent to continue to participate in the study.

2.1.2 Study cohort

Scientific Investigation of the Biological Pathways Following Thermal Injury in Adults and Children (SIFTI) study is a multi-centre prospective observational cohort study. 150 patients were recruited in total to the study from January 2013 to October 2015. In addition, blood samples were collected from 10 healthy volunteers after obtaining written informed consent from the donors, who at the time of participation were in good health, free of immunological illness and significant co-morbidity, and were not taking any medication known to interfere with immunity. To be included in the SIFTI Trial the patient must meet the inclusion criteria summarised in Table 2.1. Further inclusion and exclusion criteria
are listed below Table 2.1. The study aimed to evaluate changes in the inflammatory, immune, endocrine, metabolic and coagulation responses to severe thermal injury in children, adults and the elderly. This study is the first multi-centre observational study of patients with thermal injuries to be conducted in the United Kingdom (UK) and Europe.

2.1.3 Blood sampling

Blood samples from patients and healthy individuals were collected at fixed intervals following injury (day 1 [< 24 hours post-injury], day 3 [+/- 1 day], day 7 [+/- 1 day], day 14 [+/- 3 days], day 21 [+/- 3 days], day 28 [+/- 3 days], month 2 [+/- 3 days], month 3 [+/- 7 days], month 6 [+/- 7 days] and month 12 [+/- 7 days]) as shown in Figure 2.1. Blood samples were collected into BD Vacutainers® (Becton Dickinson, UK) containing either ethylenediaminetetraacetic acid (EDTA), z-serum clotting activator or 1/10 volume of 3.2% trisodium citrate.

2.1.4 Preparation of platelet free plasma

Citrate anticoagulated blood was centrifuged at 2000 x g for 20 minutes at 4°C and the top 2/3rds of plasma carefully removed and placed into a new sterile 600 µl Eppendorf tube. Plasma was then centrifuged at 13,000 x g for 20 minutes at 4°C and the top 2/3rds of the platelet free plasma (PFP) removed and stored at -80°C ahead of further analysis.
2.1.5 Preparation of plasma

Heparin or citrate anticoagulated blood was centrifuged at 1500 x g for 8 minutes at 4°C and the top 2/3rds of plasma carefully removed and stored at -80°C ahead of analysis.

2.1.6 Preparation of serum

For serum, blood samples were collected into BD Vacutainers® containing z-serum clotting activator and allowed to clot for 30 minutes at room temperature (RT). Samples were centrifuged at 1500 x g for 10 minutes at RT. The top 2/3rds of serum was carefully removed and stored at -80°C before analysis.

2.1.7 Clinical diagnosis of sepsis and multiple organ failure

A diagnosis of sepsis was made when at least 3 of the sepsis trigger criteria agreed in 2007 by the ABA (65) were met along with either a positive bacterial culture or when a clinical response to antibiotics was observed. Sepsis criteria were assessed on a daily basis. The source of sepsis for each episode was made through the prospective recording of adverse events during the study. Pneumonia and ventilator associated pneumonia, urinary tract infection and central line associated blood stream infection were diagnosed according to the US Centres for Disease Control criteria. This method has been published (180).

The presence of MOF was assessed daily using the Denver post-injury MOF score and was defined as a score of >3 on two consecutive days with contribution from two organ systems (277). The APACHE II score (278) and SOFA score (279) were also evaluated for the first 24 hours of admission. The abbreviated burn
severity index (ABSI) (280) and the revised Baux score (rBaux) (281) were also calculated for each patient. Clinical methodology has been published (180, 282).
<table>
<thead>
<tr>
<th>Cohort</th>
<th>Age (years)</th>
<th>Burn size (TBSA %)</th>
<th>Burn depth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Child</td>
<td>&lt; 16</td>
<td>&lt; 10%</td>
<td>Full thickness</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(&gt;1%)</td>
</tr>
<tr>
<td>Child</td>
<td>&lt; 16</td>
<td>≥ 10%</td>
<td>Any</td>
</tr>
<tr>
<td>Adult</td>
<td>16 - 45</td>
<td>&lt; 15%</td>
<td>Full thickness</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(&gt;1%)</td>
</tr>
<tr>
<td>Adult</td>
<td>16 - 99</td>
<td>≥ 15%</td>
<td>Any</td>
</tr>
<tr>
<td>Elderly</td>
<td>65 +</td>
<td>&lt; 15%</td>
<td>Full thickness</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(&gt;1%)</td>
</tr>
</tbody>
</table>

**Inclusion criteria:** Patient of any age (0-99 years), arrival to the burn centre within 24 hours of injury (children and adults) and arrival to the burn centre within 7 days (elderly)

**Exclusion criteria:** Burn injury caused by chemical or deep electrical injury, associated multiple injuries ISS > 25, decision not to treat due to the severity of the injury or premorbid conditions (congestive heart failure, malignancy, patients receiving glucocorticoid treatments and/or multiple limb amputations).

**Table 2.1. Inclusion and exclusion criteria for patients recruited to the SIFTI trial.**
Figure 2.1. SIFTI Study blood sampling timeline. Blood samples were collected at fixed intervals following injury (day 1 [< 24 hours post-injury], day 3 [+/- 1 day], day 7 [+/- 1 day], day 14 [+/- 3 days], day 21 [+/- 3 days], day 28 [+/- 3 days], month 2 [+/- 3 days], month 3 [+/- 7 days], month 6 [+/- 7 days] and month 12 [+/- 7 days]). D = day and M = month.
2.2 Polytrauma patient group and study design

Data regarding recruitment, bleeding and ethics were provided by Professor Woolley as he was the senior investigator responsible for management of the polytrauma cohort. This information is also presented within his doctorate thesis. This was a prospective observational study which recruited trauma casualties (≥18 years of age) requiring full trauma team activation that presented to the Joint Force Role 3 Field Hospital in Camp Bastion, Afghanistan, between November 2011 and August 2013 with injuries caused by explosions. A full Trauma team activation occurs for any patients triaged pre-hospital as T1 (the most severe triage category) or meeting the activation criteria, Table 2.2. Due to the logistical constraints of performing research in a deployed military environment, and due to a lack of full time dedicated researchers, it was not possible to include all trauma patients. All patients met the inclusion and exclusion criteria described in Table 2.3.

By the end of the conflict in Afghanistan, casualties were evacuated from the battlefield either by the UK Medical Emergency Response Team (MERT) or via the USA forces PEDRO or Dustoff. Each of these evacuation platforms had different medical and military capabilities. Of note, the MERT and PEDRO could administer prehospital blood products to multiple casualties by the end of the conflict.

Clinical details, including injury severity score (ISS) and new injury severity score (NISS), were collected by the on-site investigator where possible or retrospectively analysed. Due to the nature of military trauma and sensitivity of data, some information was not available for analysis.
2.2.1 Study approval and consenting for patients injured in explosions

Due to the nature of major and massive haemorrhage after military trauma a formal ethics submission was not required by the Ministry of Defence Research Ethics Committee as the analysis was performed using leftover plasma (waste) which is a negligible amount in relation to the amount of clinical blood loss. The US ethical chain however granted ethical approval (log number M-10242). Informed consent was not required as this represented a minimal risk study.

2.2.2 Plasma preparation from patients injured in explosions

4.5 mls of blood was collected into tri-sodium citrate collection tubes (3.2%), with a final ratio of blood: anticoagulant 9:1. Blood was centrifuged at 3000 RPM for 20 minutes (Heraeus ® Megafuge 16 series®, Thermoscientific). The plasma was removed and frozen at -30°C. Samples were transported to the UK using boxes on cardice and stored until analysis at -80°C.
| Penetrating trauma | Gunshot or shrapnel wound  
| Blast injury (mine/IED/grenade)  
| Stab wound |
| Blunt Trauma | Motor vehicle crash with ejection  
| Motorcyclist or pedestrian hit by vehicle >30km/h  
| Fall >5 metres  
| Fatality in the same vehicle  
| Entrapment and/or crush injury  
| Inter-hospital trauma transfer meeting activation criteria |
| **AND** |
| Anatomy | Injury to two or more body regions  
| Fracture to two or more long bones  
| Spinal cord injury  
| Amputation of a limb  
| Penetrating injury to head, neck, torso, or proximal limb  
| Burns >15% BSA in adults **or** >10% in children **or** airway burns  
| Airway obstruction |
| **Or** |
| Physiology | Systolic blood pressure <90mmHg or pulse >120bpm (adults) •  
| Respiratory rate <10 or >30 per minute (adults); SpO2 <90%  
| Depressed level of consciousness or fitting  
| Deterioration in the Emergency Department  
| Age >70 years  
| Pregnancy >24 weeks with torso injury |

Table 2.2. Trauma team activation criteria according to Clinical Guidelines for Operations. Data within this table was provided by Professor Woolley as he was the senior investigator responsible for management of the polytrauma cohort. This information is also presented within his doctorate thesis.
<table>
<thead>
<tr>
<th>Inclusion Criteria</th>
<th>Exclusion Criteria</th>
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<tbody>
<tr>
<td>Full Trauma Team activation</td>
<td>Civilian casualties</td>
</tr>
<tr>
<td>Coalition Forces</td>
<td>Age &lt;18 yrs</td>
</tr>
<tr>
<td>Explosive Injury</td>
<td>Evidence of brain injury</td>
</tr>
<tr>
<td>Evidence of shock:</td>
<td>Died before start of surgery</td>
</tr>
<tr>
<td>Clinical suspicion associated with</td>
<td></td>
</tr>
<tr>
<td>any of the following criteria:</td>
<td></td>
</tr>
<tr>
<td>• ABD &gt;6</td>
<td></td>
</tr>
<tr>
<td>• SBP&lt;90mm Hg</td>
<td></td>
</tr>
<tr>
<td>• Pulse&gt;120bpm</td>
<td></td>
</tr>
<tr>
<td>• Depressed GCS</td>
<td></td>
</tr>
<tr>
<td>Clinical suspicion of requirement for blood</td>
<td>Evidence of brain injury</td>
</tr>
<tr>
<td>transfusion</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Enemy Forces</td>
</tr>
<tr>
<td></td>
<td>Pregnancy</td>
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</tbody>
</table>

Table 2.3. Inclusion and Exclusion Criteria for with severe injuries caused by explosions. Data within this table was provided by Professor Woolley as he was the senior investigator responsible for management of the polytrauma cohort. This information is also presented within his doctorate thesis.
2.3 Whole blood analysis using the Sysmex XN-1000 analyser

The Sysmex XN-1000 (Sysmex UK, UK) is a state of the art whole blood analyser that is capable of producing a full blood cell differential count from 88 µl of human blood. The analyser has a set of unique parameters including, IGs, red cell reticulocytes, accurate platelet counts and the immature platelet fraction. The instrument also produces three platelet counts: platelet impedance (PLT-I), platelet optical (PLT-O) and PLT-F count. The instrument was utilised to study the immaturity index and kinetics of various cell counts affected by thermal injury.

Cellular kinetics was determined in EDTA anticoagulated blood immediately following collection of sample. To ensure reproducibility the analyser was quality checked every day before analysis of clinical samples using an internal quality control (QC) (XN Check Reagent™, (Sysmex UK, UK)). The instrument was also enrolled into a national external QC scheme (UKNEQAS, UK). To determine normal ranges for each cellular parameter, EDTA anticoagulated blood from 40 healthy volunteers was analysed. Analysis of healthy volunteers was performed in collaboration with the Department of Pathology at the Queen Elizabeth Hospital in Birmingham (UK), by Chris Watson and Miss Sumiya Ahmed (Aston University, Birmingham, UK).

2.4 Measurement of neutrophil reactive oxygen species generation in whole blood

Analysis of ROS production was primarily performed by Dr Peter Hampson as part of the SIFTI study. The methodology has subsequently been published (180).
Neutrophil ROS production in response to 1.62 µM PMA stimulation was assessed using the commercially available PhagoBURST® kit according to manufacturer’s instructions (BD Biosciences, UK). 100 µl of heparinised blood was aliquoted in a fresh flow cytometry analysis tube, ensuring no blood was on the side of the tubes. To the ‘negative control’ tube, 20 µl of wash buffer was added. To the ‘test’ tube 20 µl PMA (1.62 µM) was added after gentle mixing. Samples were mixed gently and incubated at 37°C for 10 minutes. After incubation, 20 µl of substrate solution was added to each well and samples were incubated at 37°C for a further 10 minutes. The whole blood samples were lysed and fixed with 2 ml of RT 1 x lysing solution. Samples were vortexed and incubated for 20 mins at RT. Cells were pelleted by centrifugation (5 min, 250 x g, 2 - 8 °C) and the supernatant was discarded. 3 ml of wash solution was added to the tubes and samples were washed by centrifugation (5 min, 250 x g, 2 - 8 °C) and the supernatant was discarded. 200 µl of DNA staining solution was added to the tubes, mixed and incubated for 10 minutes on ice (protected from light). ROS production was measured within 30 minutes. 10,000 neutrophils, gated according to their forward scatter/sideward scatter properties, were analysed on an Accuri C6 flow cytometer and data evaluated using CFlow software. Data are presented as median fluorescence intensity (MFI) values, which reflect the enzymatic activity per cell (BD Biosciences, UK).

2.5 Isolation of neutrophils from whole blood

EDTA Anticoagulated blood was transferred to a sterile 50 ml Falcon tube (Thermo Fisher, USA) and 1 ml of 2% Dextran (Sigma-Aldrich, UK) was added for every 6 ml of blood. This was performed to cause sedimentation of the
erythrocytes so that the top layer, containing white blood cells (WBC), could be removed following 30 minutes of incubation at RT. After 30 minutes, the leukocyte-rich plasma was removed from the separated whole blood and was carefully layered onto a Percoll (Scientific Lab Supplies, UK) density gradient, which consisted of 2.5 ml of 80% Percoll and 5 ml 56% Percoll and centrifuged for 20 minutes at 218 x g with no brake at RT. Following centrifugation, the neutrophils that are located at the 56-80% Percoll interface were removed and placed into a sterile 50 ml Falcon tube and resuspended in RPMI-1640 media supplemented with 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Thermo Fisher, USA). Neutrophils were then centrifuged at 461 x g for 10 minutes at RT. Following centrifugation, the supernatant was discarded and the cells were resuspended in RPMI-1640 media and diluted to an appropriate final concentration. Cell count was determined using a Sysmex XN-1000 whole blood analyser (Section 2.3) with the aspiration sensor turned off. Following quantification, cellular concentration was adjusted for individual experiments.

Viability of neutrophil preparations was analysed by cytospin and differential staining using a commercially available Giemsa stain (Diff-Qik; Gentaur Europe, Belgium). Neutrophils preparations contained ≥ 98% of neutrophil in the total cell population.

2.6 Generation of neutrophil extracellular traps

To induce NET formation, 2 x 10^5 of isolated neutrophils (200 μl total volume) were seeded into wells of a clear flat bottomed 96 well plate (Sigma-Aldrich, UK). Neutrophils were then stimulated with 25 nM PMA or 100 ng/ml LPS (Both: Sigma-Aldrich, UK). Both PMA and LPS were diluted from stock solutions with RPMI-
1640 media supplemented with 2 mM L-glutamine which alone served as a buffer control:

- PMA stock solution (1.6 mM) was diluted 1:640 (dilution 1). Next, dilution 1 was further diluted 1:100 directly into the well containing 200 µl of isolated neutrophils. Final dilution was equal to 1:64000
- LPS stock solution (1 mg/ml) was diluted 1:100 (dilution 1). Next, dilution 1 was further diluted 1:100 directly into the well containing 200 µl of isolated neutrophils. Final dilution was equal to 1:10000

Neutrophils were incubated for 3 hours at 37°C and 5% CO₂ atmosphere with either PMA or LPS to induce NET formation. Following 3 hours, NETs were quantified by a fluorometric assay (Section 2.7).

2.7 Quantification of neutrophil extracellular traps

Following stimulation, the supernatant was removed from wells and placed into sterile 600 µl eppendorf tubes and centrifuged at 2200 x g for 10 minutes at 4°C to pellet cells and ‘non-NET’ material. Whilst it is not possible to guarantee that all NETs were removed, the level of DNA was comparable between individuals suggesting low variation between experiments. Furthermore, NET release was confirmed by fluorescence microscopy. Following centrifugation 100 µl of cell free supernatant was removed and placed into a black 96 well plate (Corning, USA). The supernatant was incubated with 1 µM SYTOX® Green Dye (Thermo Fisher, Life Technologies, UK) for 10 minutes at RT and in the dark. Fluorescence was measured using a BioTek® Synergy 2 fluorometric plate reader (NorthStar Scientific Ltd, UK) with excitation and emission set at 485 nm and 528 nm respectively. All samples were analysed in duplicate. As a control/calibration step,
cell free supernatant from unstimulated neutrophils and buffer controls were analysed in duplicate. For calibration of samples, a \(\lambda\)-DNA (ThermoFischer Scientific, UK) standard curve was utilised ranging from 1000 ng/ml down to 0 ng/ml. \(\lambda\)-DNA (stock concentration = 0.3 \(\mu\)g/\(\mu\)l) was diluted 1:20 in sterile phosphate buffered saline (PBS) (Sigma-Aldrich, UK). The top standard was then serially diluted 7 times and finally diluted 1:15 in the plate with 1 \(\mu\)M SYTOX® Green Dye.

2.8 Visualisation of neutrophil extracellular traps by fluorescent microscopy

This methodology was adapted from a previous publication (283) and has been previously described by Dr Jon Hazeldine within his doctorate thesis.

To visualise NET generation by fluorescence microscopy, 2 x 10\(^5\) isolated neutrophils, resuspended in 2 ml of RPMI media supplemented with 2 mM L-glutamine, 100 U/ml penicillin and 100 \(\mu\)g/ml streptomycin, were seeded onto 13 mm circular glass coverslips (VWR International, UK) and incubated for 30 minutes at 37\(^\circ\)C in a 5% CO\(_2\) atmosphere to allow cell adherence.

Post incubation, neutrophils were stimulated with 25 nM PMA or 100 ng/ml LPS for 3 hours (37\(^\circ\)C and 5% CO\(_2\) atmosphere). All agonists were prepared from stock solutions in RPMI media, which alone served as a buffer control. Following stimulation, cells were fixed with an additional 30 minute incubation with 4% paraformaldehyde (PFA) (Section 2.9) at 37\(^\circ\)C in a 5% CO\(_2\) atmosphere. Once fixed, slides were processed for fluorescence microscopy as detailed below:

- 5 minute wash in sterile PBS at RT (3 times)
- 1 minute wash with 0.1% Triton X-100 at RT
• 5 minute wash in sterile PBS at RT
• 5 minute incubation with 1 µM SYTOX® Green Dye at RT
• 5 minute wash in sterile PBS at RT

Slides were then mounted in fluoromount medium (Sigma-Aldrich, UK) and imaged using a LEICA DMI 6000 B microscope at X20 or X40 objective.

2.9 Paraformaldehyde preparation

To make 1 L of 4% PFA, 800 ml of 1 x PBS was heated to 60°C and stirred continuously. Next, 40 g of PFA powder (Sigma-Aldrich, UK) was added to the heated 1 x PBS and stirred. The pH of the mixture was adjusted with 1 N NaOH dropwise until the solution cleared. Once dissolved, the volume was adjusted to 1 L and pH was adjusted to pH 6.9. Finally, solution was filtered sterilised and stored at 4°C ahead of use in visualisation of NETs and degradation assays.

2.10 Human promyelocytic leukaemia cell culture

Human promyelocytic leukaemia cells (HL-60) (American Type Culture Collection (ATCC), USA) were cultured to produce a positive control, of Cit H3, for use on the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. HL-60 cells were cultured in RPMI-1642 media (Thermo Fisher, USA) supplemented with 10% (volume/volume (v/v)) heat-inactivated fetal calf serum (FCS; Sera Laboratories International, UK), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. HL-60 cells were cultured until there was a suitable number to differentiate into granulocyte cells.

To differentiate, cells were cultured for 3 days in RPMI-1642 media containing 1.25% dimethyl sulfoxide (DMSO). After 3 days, cells were re-suspended in
Locke's Solution and stimulated with 4 µM calcium ionophore (A23187, Sigma-Aldrich, UK) for 15 minutes at 37°C and 5% CO₂. Following stimulation, 1 x 10⁶ cells, determined by cell counts using a standard haemocytometer, were pelleted by centrifugation at 461 x g for 8 minutes at RT. Cells were then re-suspended in 1 mL of loading buffer ahead of SDS-PAGE analysis. HL-60 positive controls were further diluted 1:50 before use in western blot analysis.

2.11 K562 cell culture

K562 cells (ATCC number CCL-243) were cultured to create mtDAMPs (Section 2.21). Cells were purchased from the American Type Culture Collection (ATCC, USA) and were maintained in RPMI-1642 media supplemented with 10% (volume/volume (v/v)) heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin in 75 cm² cell culture flasks at 37°C in a humidified 5% CO₂ atmosphere. Cells were split 1 in 3 on the day preceding experimentation and washed once (220 x g, 8 minutes, RT) in PBS prior to use. Cultures of K562 cells were replaced on a monthly basis.

2.12 Western Blot Analysis

3 µl of patient plasma, 20 µl of in vitro preparations or 8 µl of molecular weight marker (New England Biolabs, UK) were diluted in hot sodium dodecyl sulphate (SDS) sample buffer (4% SDS (v/v), 0.1 M dithiothreitol, 20% glycerol (v/v), 0.0625 M Tris-HCL and 0.004% bromophenol blue (w/v)). Samples were then heated at 100°C for 10 minutes before being loaded onto a 5% acrylamide stacking gel/15% acrylamide running SDS-PAGE gel. Gels were run for 90 minutes at 33 mAmps. The gel was transferred to a polyvinylidene fluoride (PVDF) membrane (GE
Healthcare Life Sciences, UK) by wet transfer for 90 minutes (BioRad, UK). Following transfer the membrane was blocked, at RT for 60 minutes, with 25 ml 1 x Tris Buffered Saline (TBS) containing 4% Tween-20 (TBST), 5% milk (Marvel, UK), 2% bovine serum albumin (BSA) (Fisher Scientific, UK) and 276 µl of 35% hydrogen peroxide (to block the endogenous peroxide activity in the plasma samples). Blocking buffer was removed after 60 minutes and the membrane was washed 3 times (15 minute washes) with 1 x TBST.

2.12.1 Western blot protocol for Cit H3

Cit H3 in PFP was measured using SDS-PAGE and Western blotting, as described in Section 2.12. Following the final wash, primary antibody to Cit H3, diluted in 1 x TBST containing 2.5% BSA to a working concentration of 1 µg/ml, (ab5103, Abcam UK) was applied to the membrane overnight at 4°C with gentle shaking. Primary antibody was then removed and the membrane was washed three times (15 minutes each) with 1 x TBST. The secondary antibody (ECL™ Donkey Anti-Rabbit, Horseradish Peroxidase (HRP)-Linked Whole Antibody (GE Healthcare Life Sciences)) diluted 1 in 4000 in 1 x TBST containing 2% BSA, was then applied for 1 hour at RT. Following three washes of 15 minutes each with 1 x TBST, Amersham™ ECL Plus Western Blotting Detection System (GE Healthcare Life Sciences) was applied directly to the membrane for 3 minutes before it was developed using ChemiDoc™ Technology (BioRad, UK). Cit H3 is represented as a positive band at 17 kDa, calibrated using a loaded molecular weight marker.
2.12.2 Western blot protocol for detection of actin

Actin in PFP was measured using SDS-PAGE and Western blotting, as described in Section 2.12. Membranes were incubated with 1 µg/ml primary antibody anti-actin, N-terminal antibody produced in rabbit (A2103, Sigma-Aldrich, UK) overnight at 4°C. Primary antibody was then removed and the membrane was washed three times (15 minutes each) with 1 x TBST. Membranes were incubated with secondary antibody HRP-linked anti-rabbit IgG for 1 hour (GE Healthcare Life Sciences, USA) at RT. Following three washes of 15 minutes each with 1 x TBST, Amersham™ ECL Plus Western Blotting Detection System (GE Healthcare Life Sciences, USA) was applied directly to the membrane for 3 minutes before it was developed using ChemiDoc™ Technology. Actin is represented as a positive band at 42 kDa, calibrated using a loaded molecular weight marker.

2.13 Detection of cell-free DNA in plasma and serum samples using an in house fluorometric assay

PFP or serum samples were thawed at 37°C for 10 minutes before being gently mixed. Next, 10 µl of PFP or serum sample was added directly to a 96-well plate (Corning, USA) and incubated with 140 µl of cell-impermeable dye (1 µM) SYTOX® Green Dye for 10 minutes in the dark at RT, in order to stain cfDNA.

Fluorescence was measured using a BioTek® Synergy 2 fluorometric plate reader with excitation and emission set at 485 nm and 528 nm respectively. All samples were analysed in duplicate. As a control/calibration step, plasma from healthy volunteers was included on every plate and a buffer control was included. For calibration of samples, a λ-DNA standard curve was utilised ranging from 1000 ng/ml down to 0 ng/ml.
2.14 Detection of cell-free DNA in plasma and serum samples using a commercial CE marked assay

Levels of cfDNA were measured in plasma using a commercially available fluorometric assay (Trillium Diagnostics, USA) to compare to our in-house assay of cfDNA quantification. Plasma samples were thawed at 37°C for 10 minutes before being gently mixed ahead of analysis. Next, 10 µl of thawed PFP samples were incubated with 90 µl reagent A, which binds to cfDNA, at RT for 15 minutes. Fluorescence was measured using a BioTek® Synergy 2 fluorometric plate reader with excitation and emission set at 485 nm and 528 nm respectively. All samples were analysed in duplicate. As a control/calibration step, plasma from healthy volunteers was included on every plate and a buffer control was included. For calibration of samples, a λ-DNA standard curve was utilised ranging from 800 ng/ml down to 0 ng/ml.

2.15 Isolation of nuclear and mitochondrial DNA

This work was performed in collaboration with Dr Peter Hampson and Dr Jon Hazledine. The method has subsequently been published (180).

To isolate ncDNA or mtDNA from plasma or cellular preparations a Qiagen DNA isolation kit was used (Qiagen, Germany). Briefly, 200 µl of plasma or 200 µl cellular preparations were transferred into a sterile 1.5 ml Eppendorf tube which was centrifuged at 3000 x g for 10 minutes at 4°C. Following centrifugation, 150 µl was transferred to a new sterile Eppendorf containing 20 µl of Protease reagent. Next, 150 µl of Buffer AL was added to all samples and mixed by pulse vortexing for 15 seconds before being incubated for 10 minutes at 56°C. 150 µl of 100% ethanol was added to all samples and mixed by pulse vortexing for 15 seconds.
Following which, the mixture was carefully transferred to the QIAamp Mini spin column and centrifuged at 5500 x g for 1 minute at RT. The tube containing the filtrate was discarded and the QIAamp Mini spin column was transferred to a new 2 ml collection tube. Next, 500 µl of Buffer AW1 was added and the column was centrifuged at 5500 x g for 1 minute at RT. Again the QIAamp spin column was placed into a clean 2 ml collection tube and the tube containing the filtrate was removed. Next, 500 µl of Buffer AW2 was added to the QIAamp spin column and was centrifuged at 8000 x g for 3 minutes at RT. Following centrifugation, the QIAamp spin column was removed and centrifuged at 14500 x g for 1 minute to dry the column. Finally, 50 µl of Buffer AE was added to the QIAamp spin column and incubated for 5 minutes at RT before being centrifuged at 5500 x g for 1 minute. The liquid in the bottom of the tube contained the DNA which was quantified using the Nanodrop (Thermo Fisher, USA). DNA had a 260/280 ratio of 1.8 – 2 and was stored at -80°C ahead of use in PCR or DAMPs experiments.

2.16 Polymerase chain reaction for the quantification of plasma nuclear DNA and mitochondrial DNA levels

This work was performed in collaboration with Dr Peter Hampson and Dr Jon Hazledine. The method has subsequently been published (180).

To determine the source of the circulating cfDNA, PCR analysis was performed. DNA was isolated from 150 µl of PFP using a QIAamp DNA Blood Mini Kit (Qiagen, Germany) and eluted in 50 µl of nuclease-free water of which 5 µl was used in the PCR reaction. Plasma ncDNA was measured by PCR using the SYBR Green 480 Probes Master kit and analysed using a Light Cycler 480 (Roche, Switzerland).
Primer sets used to ncDNA was specific for the genes encoding β-globin:

- Forward 5'-GTGCACCTGACTCCTGAGGAGA-3' and reverse 5'-CCTTGATACCAACCTGCCAG-3'

Primer sets used to mtDNA was specific for the genes encoding cytochrome b:

- Forward 5'-GTGCACCTGACTCCTGAGGAGA-3' and reverse 5'-CCTTGATACCAACCTGCCAG-3'

Primers were synthesized by Eurofins MWG. Primer sequences have no significant homology with DNA found in any bacterial species published on BLAST. For concentration determination, a standard curve was created using purified ncDNA, isolated from K562 cells. Samples that produced no PCR products after 40 cycles were considered undetectable and the Ct number set to 40 for statistical purposes (180).

2.17 Quantification of DNAse activity in serum samples

The protocol was adapted from a previous publication (237). Neutrophils were isolated from whole EDTA anticoagulated blood samples, from healthy volunteers, by Percoll density gradient centrifugation (Section 2.5). 5 x 10⁴ neutrophils were seeded into each well of a 96 well flat bottom clear tissue culture plate. Neutrophils were stimulated with 25 nM PMA for 3 hours at 37°C in a 5% CO₂ atmosphere. Formed NETs were stored at 4°C for up to 24 hours ahead of degradation with serum, plasma or DNAse 1.

Following stimulation, neutrophils were incubated for 6 hours with 5% serum or plasma from healthy volunteers or patients at 37°C in a 5% CO₂ environment.
Serum was pre-diluted (1:4) in Hank’s Balanced Salt Solutions containing Mg	extsuperscript{2+} and Ca	extsuperscript{2+} (HBSS+) (Thermo Fisher, USA) before being diluted 1:5 directly into the NET containing wells. Following 6 hour incubation wells were fixed by adding 4% PFA for 30 minutes at 37°C in a 5% CO	extsubscript{2} environment. Wells were washed gently with sterile PBS to remove residual serum and non-fixed cellular debris. NETs were incubated with 1 µM SYTOX® Green Dye for 10 minutes at RT in the dark. Fluorescence was measured using a BioTek® Synergy 2 fluorometric plate reader with excitation and emission set at 485 nm and 528 nm respectively. For analysis of DNase activity in patient’s sera, NET degradation by the pooled-serum from 9 healthy donors was defined as 100% activity. A calibration serum from a healthy individual and a buffer control were included on each assay plate.

2.18 Visualisation of neutrophil extracellular traps degradation by fluorescent microscopy

To visualise NET degradation by fluorescence microscopy, 5 x 10\textsuperscript{4} isolated neutrophils, resuspended in 2 ml of RPMI media supplemented with 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin, were seeded onto 13 mm circular glass coverslips and incubated for 30 minutes at 37°C in a 5% CO\textsubscript{2} atmosphere to allow cell adherence.

Post incubation neutrophils were stimulated with 25 nM PMA for 3 hours (37°C and 5% CO\textsubscript{2} atmosphere). All agonists were prepared from stock solutions in RPMI media, which alone served as buffer controls. Formed NETs were stored at 4°C for up to 24 hours ahead of degradation with serum or plasma.

Neutrophils were then incubated for 6 hours with 10 U/ml rhDNase 1 (positive control), 5% serum from healthy volunteers or patients with thermal injuries.
rhDNAse 1 was chosen over other isoforms of DNAse as it is the predominant enzyme responsible for the degradation of circulating DNA. Serum was pre-diluted (1:4) in HBSS+ before being diluted 1:5 directly into the NET containing wells. Following stimulation cells were fixed with an additional 30 minute incubation with 4% PFA (37°C and 5% CO₂ atmosphere). Once fixed, slides were processed as detailed in Section 2.8.

**2.19 In Vitro inhibition of DNAse activity in serum samples**

NETs were generated as described in Section 2.17. Following generation NETs were stored at 4°C ahead of degradation with serum. To investigate the inhibition of DNAse activity, sera from healthy volunteers was incubated with either 1 µM, 2 µM or 5 µM actin from rabbit muscle (Sigma-Aldrich, UK) to cause inhibition of serum DNase activity. Actin was diluted from a stock (11.63 µM) in HBSS+ which also served as a buffer control. Actin was added directly into wells with serum making a total well volume of 250 µl and protocol was followed as previously described (Section 2.17).

**2.20 Enzyme linked immunosorbent assay**

All enzyme linked immunosorbent assays (ELISA) were performed as per manufacturer’s instructions.

**2.20.1 Quantification of deoxyribonuclease (DNAse 1) by ELISA**

As DNAse 1 is the predominant enzyme responsible for the degradation of circulating DNA levels were quantified in PFP using a human DNASE1/DNAse I ELISA Kit, catalogue number LS-F4463 (LifeSpan BioSciences, Inc). Briefly,
samples were thawed at 37°C, 5% CO₂ for 10 minutes before being gently mixed. Next, 10 µl of sample was added to 90 µl of Sample diluent (1:10 dilution) in a clear 96 well plate. Following initial dilution, 10 µl of diluted sample was added directly to assay plate which contained 90 µl of Sample diluent (1:10 dilution) making a final dilution of 1:100. Sample, standards and blanks were incubated for 1 hour at 37°C. Wells were then aspirated and 100 µl of a biotin-conjugated detection antibody (Detection reagent A) is then added which binds to captured antigen. The plate was then aspirated and washed three times with 300 µl of wash buffer to remove any unbound Detection reagent A. Following washing, 100 µl of an avidin-HRP (Detection reagent B) conjugate which binds to the biotin added to each well. Plate was incubated for 30 minutes at 30°C. The plate was then aspirated and washed five times with 300 µl of wash buffer to remove any unbound Detection reagent B. Following washing, 90 µl of tetramethylbenzidine (TMB) substrate solution was added to each well and incubated for 10 minutes at 37°C before adding 50 µl of a sulfuric acid stop solution to terminate the colour change. Optical density (OD) of each well is measured at a wavelength of 450 nm ± 2 nm. In all assays samples were analysed in singlet, standards (ranging from 5000 – 0 pg/ml) were analysed in duplicate and plasma from a known healthy volunteer was included to ensure reproducibility between plates. DNAse levels were extrapolated from standard curve values using GraphPad Prism® software (GraphPad Software Limited, USA). OD, for all assays, was measured using a BioTek® Synergy HT.
2.20.2 Quantification of vitamin D binding protein by ELISA

VDBP levels were quantified in serum samples using a VDBP ELISA kit (catalogue number K2314, ImmunoDiagnostik, Germany). This assay does not measure VDBP complexed with actin. All serum samples were diluted 1:40000 with SAMPLEBUF (sample dilution buffer). Firstly, 20 µl sample was added to 980 µl SAMPLEBUF and mixed well (1:50 dilution 1). Next, 20 µl of dilution 1 was added to 980 µl SAMPLEBUF and mixed well (1:50 dilution 2). Finally, 20 µl of dilution 2 was added to 300 µl SAMPLEBUF and mixed well (1:16 dilution 3). Final dilution of 1:40000. For analysis, 100 µl of dilution 3 was added to each well. Before analysis each well was washed 5 times with 250 µl of wash buffer. Following washing, 100 µl of prediluted sample was added to each well and incubated at RT for 1 hour with shaking. Following incubation contents of the well were discarded and washed 5 times with 250 µl of wash buffer to remove any unbound material. Next, 100 µl of peroxidase-labelled antibody was added to each well and incubated for 1 hour at RT with shaking. Again, wells were washed 5 times with 250 µl of wash buffer to remove unbound antibody. 100 µl of TMB solution was added to each well and incubated at RT for 10 minutes. Colour change was stopped by the addition of 100 µl of stop solution which was mixed briefly. Absorption was determined immediately with an ELISA reader at 450 nm against 620 nm (or 690 nm) as a reference. VDBP levels were extrapolated from standard curve values using GraphPad Prism® software.

2.20.3 Quantification of human gelsolin by ELISA

GSN levels in serum, from patients with thermal injuries, and plasma samples, patients with injuries caused by explosions, were quantified using LSBioTM
Human GSN/Gelsolin ELISA Kit, catalogue number LS-F5675 (LifeSpan BioSciences Inc, UK). Briefly, samples were thawed at 37°C, 5% CO₂ for 10 minutes before being gently mixed. Next, 1 µl of sample was added to 49 µl of Sample diluent (1:50 dilution) in a clear 96 well plate. Next, 1 µl of diluted sample was further diluted in 99 µl of assay buffer and mixed gently (1:100 dilution). Following dilution, 10 µl of diluted sample was added directly to assay plates which contained 90 µl of Sample diluent (1:10 dilution) making a final dilution of 1:50000. Sample, standards and blanks were incubated for 2 hours at 37°C. Wells were then aspirated and 100 µl of a biotin-conjugated detection antibody (Detection reagent A) was then added which binds to captured antigen for 1 hour at 37°C. The plate was then aspirated and washed three times with 300 µl of wash buffer to remove any unbound Detection reagent A. Following washing, 100 µl of an avidin-HRP (Detection reagent B) conjugate which binds to the biotin was added to each well and plate was incubated for 60 minutes at 37°C. The plate was then aspirated and washed five times with 350 µl of wash buffer to remove any unbound Detection reagent B. Following washing, 90 µl of TMB substrate solution was added to each well and incubated for 20 minutes at 37°C before adding 50 µl of a sulphuric acid stop solution to terminate the colour change. OD of each well was measured at a wavelength of 450 nm ± 2 nm. In all assays samples were analysed in singlet, standards (ranging from 4000 – 0 pg/ml) were analysed in duplicate and plasma from a known healthy volunteer was included to ensure reproducibility between plates. GSN levels were extrapolated from standard curve values using GraphPad Prism® software. OD, for all assays, was measured using a BioTek® Synergy HT.
2.21 Isolation of nuclei and mitochondria from K562 cells

Dr Jon Hazeldine isolated nuclei and mitochondria from K562 cells ahead of use during in vitro experiments.

Firstly, RIPA lysis buffer (Thermo Fisher, USA) was diluted 1:10 in distilled water containing protease inhibitory cocktail to make 1 x RIPA buffer. Cells were transferred into a sterile 1.5 ml Eppendorf tube and pelleted by centrifugation at 1500 x g for 2 minutes at RT. Post centrifugation, the supernatant was removed and cellular pellet was re-suspended in 1 ml 1 x RIPA lysis buffer. Eppendorf was left on ice for 10 minutes before the solution was transferred to a tight fitting homogeniser using a Pasteur pipette and 120 strokes were performed to lyse cells. Next, the solution was transferred to a fresh sterile 1.5 ml Eppendorf tube using a Pasteur pipette and centrifuged at 800 x g for 10 minutes at 4°C to pellet the nucleus. Post spin, the supernatant was carefully removed and placed into a new sterile 1.5 ml Eppendorf tube using a Pasteur pipette and centrifuged at 3000 x g for 30 minutes at 4°C to pellet the mitochondria. Both the nuclear and mitochondria pellet were resuspended in 200 µl of sterile PBS. mtDNA and ncDNA were then isolated using a QIAamp DNA isolation kit (Section 2.15).

2.22 mtDAMPs effects on neutrophil function and phenotype

All experiments were performed in collaboration with Dr Jon Hazeldine (Institute of Inflammation and Ageing, University of Birmingham, UK). Dr Hazeline performed western blot analysis and assessment of neutrophil activation by surface phenotyping using flow cytometry. All other measurements and experiments were performed in collaboration between Dr Jon Hazeldine and Robert J Dinsdale.
2.22.1 Inhibition of neutrophil extracellular traps formation with isolated mtDAMPs

Neutrophils were isolated from fresh blood by Percoll density gradient (Section 2.5). Next, 2 x 10^5 were treated for 15 minutes, at 37°C and 5% CO₂, with 40 or 100 µg/ml mtDAMPs or vehicle control. Following incubation neutrophils (2 x 10^5) were then stimulated for 3 hours with 25 nM PMA. Post-treatment, supernatants were carefully collected and centrifuged at 2200 x g for 10 minutes at RT, after which the DNA content of cell-free supernatants was analysed using the SYTOX™ Green dye assay. Background fluorescence values acquired from SYTOX™ Green staining of mtDAMPs in the absence of neutrophils were subtracted from test readings. NET production is expressed as a fold increase compared to unstimulated neutrophils alone.

For fluorescent microscopy, 2 x 10^5 neutrophils were seeded onto glass coverslips and stimulated with 40 or 100 µg/ml mtDAMPs, 40 µg/ml mtDNA or vehicle control for 15 minutes at 37°C in a humidified 5% CO₂ atmosphere. Post treatment, samples were stimulated with 25 nM PMA for 3 hours (37°C, 5% CO₂), after which samples were fixed for 30 minutes with 4% PFA (37°C, 5% CO₂) and permeabilized with 0.1% Triton X-100. NETs were stained with 1 µg/ml of a rabbit polyclonal anti-histone H3 (citrulline R2, R8 and R17) antibody for 90 minutes, washed three times with PBS and stained for 90 minutes with 10 µg/ml of a goat anti-rabbit ALEXA Fluro-555 conjugated antibody. Following three washes in PBS, cells were stained with 1 µM SYTOX Green dye. Once stained, slides were mounted in fluoromount medium and visualized using a LEICA DMI 6000 B microscope at x20 objective.
2.22.2 Neutrophil transmigration following stimulation with mtDAMPs

Neutrophils were isolated from whole blood by Percoll density gradient as previously described (Section 2.5). Freshly isolated neutrophils (1 x 10^7/ml) in HEPES buffer containing 1 mM Ca^{2+} were incubated for 30 minutes in a 37°C water bath with 3 µg/ml calcein-acetoxymethyl ester (calcein-AM, Fisher Scientific UK, UK). Post incubation, neutrophils were treated for 15 minutes (37°C, 5% CO_2) with 40 or 100 µg/ml mtDAMPs or vehicle control, after which cells were pelleted by centrifugation at 1500 x g for 2 minutes at RT, supernatants removed and neutrophils re-suspended to a concentration of 1 x 10^7/ml in HEPES buffer containing 1 mM Ca^{2+}. A total of 100 µl, containing 1 x 10^6 neutrophils, were then dispensed into the upper chambers of cell culture inserts (polycarbonate membrane, 3 µM pores, Corning, USA) that had been pre-loaded into wells of a 24-well flat bottomed plate (BD Biosciences, UK) that contained pre-warmed phenol red free RPMI-1640 media supplemented with GPS, 10% heat inactivated-FCS and 1 ng/ml leukotriene B4 (LTB4; R&D Systems, UK).

Following a 90-minute incubation at 37°C in the dark, cell culture inserts were removed from plates without any attempt to dislodge adherent neutrophils and plates read immediately for calcein fluorescence using a BioTek Synergy 2 fluorometric plate reader with excitation and emission set at 485 nm and 528 nm respectively. Fluorescent intensities were converted into neutrophil numbers via the use of a standard curve that was generated from calcein-AM loaded neutrophils that had been incubated alongside the test samples in the conditions described above. The number of neutrophils measured in media in which no chemokine was added was subtracted from the numbers calculated for wells that
contained 1 ng/ml LTB4 in order to determine specific chemokine-mediated migration.

### 2.22.3 Assessment of neutrophil phenotype following stimulation with mtDAMPs

Freshly isolated neutrophils (1 x 10⁵) were stimulated with either 40 or 100 µg/ml of mtDAMPs or vehicle control for 15 minutes at 37°C in a humidified 5% CO₂ atmosphere. Post treatment, samples were placed on ice and stained for 20 minutes with the following mouse anti-human monoclonal antibodies or their concentration-matched isotype controls: 2 µg/ml fluorescein isothiocyanate (FITC)-labelled CD62L (clone DREG56; eBioscience, UK), 1 µg/ml CXCR1-FITC (clone eBIO8F1-1-4; eBioscience, UK), 0.5 µg/ml R-phycoerythrin (PE)-labelled CXCR2-PE (clone eBio5E8-C7-F10; eBioscience, UK), 2.5 µg/ml allophycocyanin (APC)-labelled CD11b (clone ICRF44, BioLegend, UK) or 2 µg/ml CD16-APC (Clone 3G8; BD Biosciences, UK). Post incubation, cells were pelleted (250 x g, 5 minutes, 4°C), supernatants discarded and neutrophils washed once in PBS containing 1% BSA. Following resuspension in PBS, samples were transferred to polypropylene FACS tubes for flow cytometric analysis, which was performed on an Accuri-C6™ bench top cytometer. 10,000 neutrophils, gated according to their forward scatter/sideward scatter properties were acquired for analysis, where receptor expression was measured as MFI values.

### 2.22.4 Activation of ERK1/2 following stimulation with mtDAMPs

To study mitogen activated protein kinase (MAPK) activation following mtDAMP stimulation, freshly isolated neutrophils (1 x 10⁶ in RPMI + GPS + FCS media) were treated for 0, 2, 5, 10, 15 or 30 minutes (37°C, 5% CO₂) with 100 µg/ml
mtDAMPs or vehicle control, after which cells were pelleted (1500 x g, 2 minutes, 4°C) and supernatants removed.

Cell lysates prepared by the addition of SDS sample buffer and boiled for 10 minutes (Section 2.12). Lysates were separated on 12% SDS-PAGE and membranes probed with pERK1/2 (Cell Signalling Technology, USA) and ERK1/2 antibodies overnight at 4°C (diluted 1:1000). Primary antibody was then removed and the membrane was washed three times (15 minutes each) with 1 x TBST. Membranes were incubated with secondary antibody goat anti-rabbit secondary antibody conjugated to HRP (diluted 1:4000 in TBST; GE Healthcare, UK) at RT. Following three washes of 15 minutes each with 1 x TBST, Amersham™ ECL Plus Western Blotting Detection System was applied directly to the membrane for 3 minutes before it was developed using ChemiDoc™ Technology.

2.22.5 Reactive oxygen species generation following stimulation with mtDAMPs

100 µl aliquots of heparinised whole blood were treated for 15 minutes (37°C, 5% CO₂) with 40 or 100 µg/ml mtDAMPs or vehicle control. Post-treatment, neutrophil ROS production in response to 1.62 µM PMA stimulation was assessed using the commercially available PhagoBURST® kit according to manufacturer’s instructions (Section 2.4). 10,000 neutrophils, gated according to their forward scatter/sideward scatter properties, were analysed on an Accuri C6 flow cytometer and data evaluated using CFlow software. Data are presented as MFI values, which reflect the enzymatic activity per cell.
2.23 Statistical analysis

Data were analysed using GraphPad Prism® (GraphPad Software, Inc), SPSS (IBM) and R version 3.0.1 (http://www.r-project.org) together with the Ime4, effects, rms and pROC packages. All data within this thesis was checked for normality using the Kolmogorov-Smirnov test.

2.23.1 Analysis of in vitro experiments

If data was normally distributed the following statistical tests were performed; Unpaired t test was performed to analyse two independent groups. A paired t test was performed to analyse groups from paired samples. Not normally distributed paired groups were analysed by a Wilcoxon matched pairs signed rank test. If data was normally distributed and there was more than one matched group within the analysis a one-way analysis of variance (ANOVA) followed by the Bonferroni post hoc test was used. If data was not normally distributed and there was more than one matched group within the analysis a one-way analysis of variance (ANOVA) followed by the Friedman’s test followed by Dunn's Multiple Comparison Test post hoc test was used.

2.23.2 Analysis of data generated from patients with thermal injuries

When comparing to healthy individuals the level of statistical significance was adjusted manually for all analysis due to data not being equally distributed across all longitudinal time points. The threshold for significance was set at \( p < 0.005 \), unless otherwise stated within figure legend, as there are 10 longitudinal time points measured within this analysis. If data was normally distributed data was
analysed using an unpaired t test. If data was not normally distributed data was analysed using a Mann Whitney test. When analysis was performed on patient groups, e.g. sepsis and no sepsis, the threshold for significance was not adjusted (p < 0.05). If data was normally distributed data was analysed using an unpaired t test. If data was not normally distributed data was analysed using a Mann Whitney test. Categorical variables were compared using a Chi-squared test.

2.23.3 Logistic regression analyses of potential biomarkers of sepsis

Logistic regression analyses were conducted to examine the relationships between biomarkers at pre-specified sample times (e.g. day 7) and the presence of sepsis. Discriminatory power was assessed through the area under the receiver operator characteristic curve (AUROC). Longitudinal analyses were performed using linear mixed-effects models. Sample day was included in these models as a restricted cubic spline to allow for a flexible non-linear relationship between time and the response variable. Analysis was performed using the statistical software packages SPSS (IBM) and R version 3.0.1 (http://www.r-project.org) together with the Ime4, effects, rms and pROC packages. Analysis was performed by Dr Jonathon Bishop (University of Birmingham, UK).

2.23.4 Analysis of data generated from patients with polytrauma

As data was equally distributed amongst analysis groups the threshold for significance did not need to be adjusted manually (p < 0.05). When comparing to healthy controls, a one-way ANOVA followed by Bonferroni’s post hoc test was performed when data was normally distributed. If data was not normally distributed a one-way ANOVA followed by Dunn’s Multiple Comparison Test was performed.
When analysis was performed between patient cohorts (blood products and no blood products) the threshold for significance was not adjusted ($p < 0.05$). If data was normally distributed an unpaired t test was performed. If data was not normally distributed a Mann Whitney test was performed. Categorical variables were compared using a Chi-squared test.
Chapter 3

Neutrophil extracellular trap release following thermal injury
3.1 Introduction

Neutrophils are essential effector cells of the innate immune system and are equipped with a number of antimicrobial functions. In 2004, Brinkmann and colleagues defined a novel anti-microbial process in which neutrophils release their nuclear material to the outside of the cell in a ‘net-like’ structure which is designed to ensnare and trap bacteria (118, 119). This process is termed NET release or NETosis (178).

The majority of research within the field of NETosis has focussed around their role during infection and sepsis. This is in part due to the specific functions of NETs in orchestrating the response to infection and due to their components being potential biomarkers (181, 192). Sepsis is a life threatening condition characterised by an imbalance in both the immune and haemostatic systems. It often results in cardiac dysfunction and acute respiratory distress syndrome, which are associated with MOF (46). Although accurate and early diagnosis of sepsis is crucial for determining patient outcomes, this is challenging as the criteria used are insensitive and nonspecific (47).

A number of studies have concluded that NET production occurs in septic patients following traumatic injury (181, 215, 216). Furthermore, it has also been suggested that NETs can also be produced in response to sterile thermal and traumatic injury (111, 181, 215). However, many of the studies use quantification of plasma cfDNA alone as a marker of in vivo NETosis. This method is non-specific to NETosis as plasma cfDNA can originate from a number of sources including bacteria themselves (115). Therefore, to our knowledge, it has yet to be conclusively established whether NETosis occurs in patients following burn injury.
There is emerging evidence that neutrophil function is dysregulated following severe thermal injury (156, 159-161, 173, 174, 177). It has been proposed that this dysregulation of neutrophil function may underlie the increased incidence of nosocomial infections. Parment and colleagues have reported a reduced ability of neutrophils to generate ROS, an essential precursor of NETosis, following thermal injury (177). Although precursors of NET formation are reduced no group has investigated the effect severe thermal injury has on NET formation. Furthermore, if dysfunction exists no group has fully investigated the potential mechanisms responsible. Potential causes of neutrophil dysfunction include non-functioning immature neutrophils and circulating soluble mediators (40, 41, 45, 169).

3.1.1 Aims

The aims of this chapter were:

- Conclusively investigate NET formation following severe thermal injury by the measurement of specific circulating biomarkers.
- Investigate the association and predictive power of circulating NET components and secondary complications, e.g. sepsis.
- Investigate the ability of neutrophils to form NETs ex vivo to understand if thermal injury results in impaired neutrophil function.
- Investigate potential mechanisms responsible for the dysregulation of neutrophil function.
3.2 Results

3.2.1 Study cohort

All analysis was performed on adult patients (≥ 18 years of age) with burns ≥ 15% TBSA. The consort diagram for this analysis is included (Figure 3.1). Analysis was restricted to burns ≥ 15% TBSA to minimise the confounding bias of age and severity of injury in analysis. Furthermore, this has reduced the span and confounding bias of fluid resuscitation. Importantly, all patients were admitted within 24 hours of their injuries and all received standardised burn resuscitation protocols as per Parkland’s formula and as such have received equivalent fluid resuscitation: average = 5.4 mls/kg/%TBSA (Standard deviation = 2.1) (284, 285).

In total there are 63 patients included in this analysis. The median age of the cohort was 39 years of age (range 16 - 88) and the median TBSA was 30% (range 15 - 95%). Patients were further subcategorised into septic and non-septic according to the ABA sepsis scoring criteria (65). Of note, 6 patients died within 7 days of injury from non-septic causes and were removed from the analysis, as it was not possible to determine whether these patients would have developed sepsis. Septic patients had significantly larger and more severe injuries (p < 0.001) and consequently a higher mortality rate (p = 0.002). Full patient demographics are reported in Table 3.1.

The incidence of sepsis was 61.4 % and the median time to first septic episode was 4 days (Interquartile range (IQR): 4, 8). The major source of sepsis in this cohort was pulmonary infection, accounting for 69%, and the most common colonising organism was Bacillus sp, accounting for 20%. Full details of colonising organisms and source can be found in Table 3.2.
Figure 3.1. CONSORT diagram showing the sub-classification and breakdown of numbers of study subjects. Data for surface area of burn is presented as median value (minimum-maximum value).
<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Healthy Controls (n = 10)</th>
<th>All Patients (n = 63)</th>
<th>Sepsis (n = 35)</th>
<th>No Sepsis (n = 22)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>58 (22-96)</td>
<td>39 (16-88)</td>
<td>41 (16-88)</td>
<td>31 (18-77)</td>
<td>ns</td>
</tr>
<tr>
<td>Gender (M:F)</td>
<td>6:4</td>
<td>42:21</td>
<td>23:12</td>
<td>14:8</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>%TBSA (min-max)</td>
<td>30 (15-95)</td>
<td>45 (16-95)</td>
<td>22 (15-31)</td>
<td>&lt; 0.0001</td>
<td></td>
</tr>
<tr>
<td>ABSI (min-max)</td>
<td>8 (4-14)</td>
<td>10 (4-14)</td>
<td>6 (4-8)</td>
<td>&lt; 0.0001</td>
<td></td>
</tr>
<tr>
<td>Survived (Y:N)</td>
<td>43:20</td>
<td>21:14</td>
<td>22:0</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>MOF (Y:N)</td>
<td>33:30</td>
<td>19:16</td>
<td>1:21</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.1. Patient Demographics (Chapter 3). Continuous data are quoted as median values with minimum to maximum values. Sepsis and no sepsis patient variables were compared using either a Mann-Whitney (continuous variable) or Chi-squared test (categorical variables).
<table>
<thead>
<tr>
<th>Source, n (%)</th>
<th>Pulmonary Infection</th>
<th>Wound Infection</th>
<th>Central line associated blood stream infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>24 (69)</td>
<td>9 (26)</td>
<td>1 (3)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Organism, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus sp.</td>
</tr>
<tr>
<td>Staphylococcus sp.</td>
</tr>
<tr>
<td>Enterobacter sp.</td>
</tr>
<tr>
<td>Candida sp.</td>
</tr>
<tr>
<td>E. Coli</td>
</tr>
<tr>
<td>Haemophilus influenzae</td>
</tr>
<tr>
<td>Acinetobacter sp.</td>
</tr>
<tr>
<td>Klebsiella sp.</td>
</tr>
<tr>
<td>Pseudomonas sp.</td>
</tr>
<tr>
<td>Proteus mirabilis</td>
</tr>
<tr>
<td>Pantoea sp.</td>
</tr>
<tr>
<td>Serratia marcescens</td>
</tr>
</tbody>
</table>

**Table 3.2. Information on first septic episode.** Continuous data are quoted as median values with interquartile range. Data has been published within ‘Neutrophil Dysfunction, Immature Granulocytes, and Cell-free DNA are Early Biomarkers of Sepsis in Burn-injured Patients: A Prospective Observational Cohort Study’ by Hampson and Dinsdale *et al* (180).
3.2.2 Assessment of neutrophil extracellular trap release in healthy controls

Before investigating if thermal injury affects NET generation it was necessary to establish a number of techniques to measure NETs *in vitro*. To measure NET production in healthy controls two methods were established; quantification of cfDNA released during NETosis and visualisation of released NET structures by fluorescence microscopy, as previously described (283).

Stimulation of neutrophils isolated from healthy controls with 25 nM PMA results in a significant release of cfDNA compared to unstimulated neutrophils (Figure 3.2). Stimulation of neutrophils with 100 ng/ml LPS did not cause an increase in cfDNA compared to unstimulated neutrophils (Figure 3.2).

Analysis of NET formation by fluorescence microscopy showed that neutrophils isolated from healthy volunteers stimulated with 25 nM PMA are positive for both DNA (green stain) and Cit H3 (blue stain). Interestingly, Cit H3 is decorated across NETs but is more abundantly expressed within the remaining decondensed nuclear structure. All neutrophils in stimulated images also have decondensed nuclei which is a hallmark characteristic of NET formation. In contrast, unstimulated neutrophils retain their natural polymorphic nuclear morphology and do not release NETs following isolation (Figure 3.3). Stimulation of isolated neutrophils with LPS results in minor NET release and is characterised by few decondensed nuclei and a low number of neutrophils releasing DNA strands. Therefore, DNA and Cit H3 are components of NET formation and PMA is an appropriate *in vitro* stimulus to induce maximal NET formation.
Figure 3.2. Stimulation of neutrophils with 25 nM PMA results in robust NET formation. Ex vivo NET generation in response to 25 nM PMA or 100 ng/ml LPS by neutrophils isolated from healthy individuals. Data at each time point were compared with healthy control (HC) values using a repeated measures ANOVA and a Bonferroni post hoc test. *P < 0.05, **P < 0.01, ***P < 0.001. n = 5 for all experiments. Box and whisker plots represent minimum to maximum value and mean for each group.
Figure 3.3. Visualisation of ex vivo NET generation in response to 25 nM PMA or 100 ng/ml LPS by neutrophils isolated from a healthy control by fluorescence microscopy. DNA was stained by 1 μM SYTOX Green Dye (green stain) and Cit H3 was stained using 1 μg/ml AB5103 in addition to secondary antibody ALEXA Fluor 555 (blue stain). Slides were mounted in fluoromount medium and visualized using a LEICA DMI 6000 B microscope at X20 objective. Data shown is representative of 5 individual experiments.
3.2.3 Detection of circulating cell-free DNA in healthy controls

To measure circulating levels of ‘NET markers’ an assay to quantify levels of plasma cfDNA was established from a previous publication (286). A λ-DNA standard curve was included in each assay which ranged from 1000 ng/ml – 0 ng/ml. λ-DNA standard curves were linear, reproducible and accurate. Importantly, the inter-assay and intra-assay coefficients of variation for the assays were 5.3% and 5.1%, respectively.

cfDNA could be detected in plasma from healthy volunteers but in low quantities (133.9 ng/ml) and comparable to that of previously published literature (109). Notably, cfDNA levels have been quantified in both plasma and serum (109, 111). Therefore to validate the assay, levels of cfDNA were measured in matched plasma and serum samples from healthy individuals. Levels of cfDNA were significantly higher in matched serum samples (188.5 ng/ml) when compared to plasma samples (133.9 ng/ml) (Figure 3.4).

3.2.4 Analysis of plasma cell-free DNA levels following thermal injury

It has previously been reported that levels of cfDNA are associated with poor outcome following major trauma and sepsis (109, 287). Therefore levels of cfDNA were analysed in patients with severe thermal injury.

Levels of cfDNA in plasma taken within 24 hours following injury (Figure 3.5A), were not elevated (DNA = 153.46 ng/ml) compared to healthy control samples (161.04 ng/ml). This was unexpected as it has been previously published that thermal injury results in an initial increase in admission serum cfDNA levels (111). To try and explain the difference between the two studies, levels of cfDNA were also quantified in serum and compared to the matched plasma samples.
Admission levels of cfDNA were significantly higher in matched patient serum samples (439.38 ng/ml) compared to patient plasma samples (168.95 ng/ml) (Figure 3.6 A). Importantly the net change in cfDNA concentration between serum and plasma samples also correlated positively with WBC count ($p = 0.0036$, Spearman $R = 0.6824$, Figure 3.6 B).

Levels of plasma cfDNA were significantly elevated at days 7 and 14 post-burn (Figure 3.5 A). Longitudinal analysis showed that plasma cfDNA levels were elevated in septic patients compared to non-septic patients from 7 days up to 2 months post injury (Figure 3.5 B). In non-septic patients, there was a minimal increase in plasma cfDNA, being similar to levels found in healthy volunteers.

In order to establish that our in house assay was comparable to that of a commercially available CE marked kit plasma samples were tested in both assays simultaneously. Importantly levels of cfDNA in our assay correlated with a commercially available kit ($p < 0.0001$, Spearman $R = 0.78$, Figure 3.7).
Figure 3.4. Levels of cfDNA are significantly higher in serum compared to plasma samples from healthy individuals. Levels of cfDNA were quantified in plasma and serum samples from healthy individuals (n = 12). Data was compared by a Wilcoxon matched pairs signed rank test. ***P < 0.001. Data is presented as before and after to depict changes within individual donors.
Figure 3.5. cfDNA is elevated post thermal injury and elevated in septic patients. (A) Plasma cfDNA levels (log scale) across time following burn injury (n = 50). Data at each time point were compared with healthy control (HC) (n = 10) values using a Mann-Whitney test; *P < 0.005. (B) Levels of cfDNA compared between septic and non-septic patients. Data was analysed using a Mann-Whitney test; *P < 0.05. Box and whisker plots represent minimum to maximum value and median for each group. HC = healthy control, D = day, M = month.
Figure 3.6. Admission levels of cfDNA are higher in matched serum samples and correlate with WBC. (A) Levels of cfDNA in admission plasma and matched serum samples (n = 16). Data were compared using paired t-test; ***P < 0.001. Data is presented as before and after to depict changes within individual donors. (B) Correlation of WBC and change in DNA concentration between matched admission plasma and serum samples (n = 14). Data was analysed using Spearman’s rank.
Figure 3.7. Measurement of cfDNA by in house assay correlated positively with a commercially available kit. Correlation of plasma cfDNA levels using in house assay compared to Trillium Diagnostics kit to detect extracellular DNA (n = 32). Data was analysed using a Spearman’s rank (n = 32).
3.2.5 Diagnostic use of cell-free DNA in septic patients

In order to investigate the potential diagnostic utility of plasma cfDNA during sepsis, we performed statistical regression analysis on the longitudinal kinetics for septic and non-septic patients (Table 3.3). There was a clear significant difference in plasma cfDNA trajectories following thermal injury between septic and non-septic patients (Figure 3.5 B). On days 1 and 3 post injury the model gave reasonable discriminatory power between septic and non-septic patients with a c-statistic of 0.691 (95% CI (0.542, 0.840)) and 0.640 (95% CI (0.479, 0.802)) respectively. Discriminatory power of the model increased with time with a c-statistic of 0.775 (95% CI (0.604, 0.947)) on day 7 post injury. However, the maximal difference between the two patients groups was observed at day 14 following injury. Levels of plasma cfDNA at day 14 following thermal injury provide good discriminatory power of whether an individual was in the septic group or not with a c-statistic of 0.883 (95% CI (0.767, 1.000)).

3.2.6 Longitudinal analysis of nuclear and mitochondrial DNA

In order to investigate the potential source(s) of circulating cfDNA, quantitative PCR analysis was performed to determine the concentration of ncDNA and mtDNA. Analysis of plasma levels of mtDNA revealed no significant elevation following thermal injury compared to healthy volunteers (Figure 3.8 A). However, there was a significant elevation of ncDNA between day 1 and day 28 post-injury (Figure 3.8 B). Plasma ncDNA levels had comparable kinetics to cfDNA, with levels peaking at day 7 and 14, and there was a strong positive correlation between the two measurements (r= 0.763, p < 0.001). However, there was a significant elevation of ncDNA at day 1-3 post-injury unlike plasma cfDNA. This is
most likely due to the higher sensitivity of the PCR assay compared to the fluorometric assay (lower limit of detection; 0.1 ng/ml vs. 60 ng/ml).
<table>
<thead>
<tr>
<th>Variable</th>
<th>Number of Patients</th>
<th>Number of Septic Patients</th>
<th>AUROC (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cfDNA</td>
<td>50</td>
<td>19</td>
<td>0.691 (0.542, 0.840)</td>
</tr>
<tr>
<td>Day 3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cfDNA</td>
<td>51</td>
<td>19</td>
<td>0.640 (0.479, 0.802)</td>
</tr>
<tr>
<td>Day 7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cfDNA</td>
<td>35</td>
<td>12</td>
<td>0.775 (0.604, 0.947)</td>
</tr>
<tr>
<td>Day 14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cfDNA</td>
<td>48</td>
<td>18</td>
<td>0.883 (0.767, 1.000)</td>
</tr>
</tbody>
</table>

Table 3.3. Discriminatory power of cfDNA for predicting sepsis at different time points was assessed through AUROC and 95% confidence intervals. Data is presented as c-statistic and 95% confidence intervals on each day tested.
Figure 3.8. Circulating DNA is predominantly of nuclear origin. (A) mtDNA levels (log scale) and (B) ncDNA levels (log scale) across time following burn injury (n = 50). Data at each time point were compared with healthy control (HC) (n = 10) values using a Mann-Whitney test; *P < 0.005. Box and whisker plots represent minimum to maximum value and median for each group. HC = healthy control, D = day, M = month.
3.2.7 Neutrophil extracellular trap formation following thermal injury

Having found evidence of elevated levels of plasma cfDNA in septic patients, we investigated whether NETs could be a source of cfDNA. To confirm NETosis, plasma samples were analysed for the presence of Cit H3, which is a characteristic feature of NET generation (276).

A western blot protocol was set up and validated along with a positive control for Cit H3 generated through HL-60 cells. Interestingly, following differentiation with DMSO, HL-60 cells retained their rounded morphology and do not exhibit a ‘granulocyte like’ nuclear morphology. Western blot analysis of calcium ionophore stimulated HL-60 cells shows a concentration dependent decrease in signal with higher dilutions. As the 1:50 dilution of HL-60 gave a robust signal without saturating the blot this was used as a positive control for future measurements (Figure 3.9 A).

Cit H3 was clearly detectable in the plasma of septic patients (Figure 3.9 B-C). The results obtained for two representative septic patients are shown in Figure 3.9 B-C (representative of n = 9). The data presented here is for a patient with a 66% TBSA who was first diagnosed with sepsis on day 7 (Figure 3.9 B) and a patient with a 71% TBSA who was first diagnosed as septic on day 9 (Figure 3.9 C). Cit H3 was clearly detected in the plasma and coincided with the peak in cfDNA levels. Although this analysis included patients with severe thermal injuries there was only trace levels of Cit H3 found in the initial sample for one patient. However, Cit H3 was not detectable in any plasma sample obtained from non-septic patients or healthy controls (n = 10).
Figure 3.9. Longitudinal analysis of NET formation. In all experiments, Cit H3 was detected in the plasma of septic patients as a 17kDa band by Western Blotting. (A) HL-60 positive control diluted 1:20, 1:50, 1:100, 1:150. (B-C) Two representative septic patients following thermal injury (data is representative of 9). Data presented includes longitudinal plasma cfDNA measurements (log scale) and corresponding Cit H3 blots. A positive control (generated in HL-60 cells) was included on all blots. HC = healthy control, D = day, MWM = molecular weight marker.
3.2.8 Ex vivo NETosis

There is an increasing amount of evidence that thermal injury results in suppression or dysregulation of neutrophil functions (156, 173, 174). However, to date, no group has investigated if thermal injury also disrupts NET formation. Therefore, to investigate whether thermal injury affected NET production by circulating neutrophils, cfDNA release by isolated neutrophils stimulated with 25nM PMA or 100 ng/ml LPS was quantified by fluorescent microscopy and fluorometry.

Firstly, to investigate if neutrophils from patients with thermal injuries were primed and in turn released higher levels of NETs, neutrophils were stimulated with 100 ng/ml LPS. In healthy individuals, neutrophils stimulated with LPS do not result in a significant increase in NET release compared to unstimulated neutrophils (Figure 3.2). Interestingly, we found that neutrophils from burn injury patients did not release more NETs when stimulated with 100 ng/ml LPS compared to healthy controls at all time points tested (Figure 3.10 A).

To confirm maximal NET generation, neutrophils were stimulated with PMA for 3 hours. Interestingly, neutrophils from burn injury patients released significantly lower levels of DNA compared to neutrophils isolated from healthy controls on days 3 and 7 post injury (Figure 3.10 B). Fluorescence microscopy imaging confirmed that neutrophils isolated from burn injury patients are partially resistant to NETosis induced by 25nM PMA, with the majority of the neutrophils from burn injury patients retaining their natural cell and nuclear morphology following stimulation (Figure 3.10 C). However, in healthy controls nearly all cells release NETs and have decondensed nuclei.
Figure 3.10. *Ex vivo* NET production in response to a biological stimulus, LPS, and a chemical stimulus, PMA. (A) *Ex vivo* NET generation in response to 100 ng/ml LPS across time following burn injury (n = 24). (B) *Ex vivo* NET generation in response to 25 nM PMA across time following burn injury (n = 24). Data at each time point were compared with healthy control (HC) values by unpaired t-test *P < 0.008. *Ex vivo* NET production in response to LPS was only measured for up to 21 days post injury due to the availability and number of neutrophils isolated. Data is presented as box and whisker plots and represents mean, minimum and maximum value (C) *Ex vivo* NET generation in response to PMA by neutrophils isolated from a healthy control or a burn patient. Slides were mounted in fluoromount medium and visualized using a LEICA DMI 6000 B microscope at X20 objective. Green stain = DNA. HC = healthy control, D = day.
3.2.9 Reactive oxygen species generation of neutrophils following thermal injury

Analysis of ROS generation was performed in collaboration with Dr Peter Hampson as part of the SIFTI Trial (University of Birmingham, UK). Data has been published (180).

Of note, we have shown that there is a significant reduction in the ability of neutrophils to phagocytose and ex vivo NETosis following thermal injury compared to healthy individuals (180). Thus, we investigated if neutrophils were dysfunctional in ROS production, suggesting a broader inhibition of neutrophil antibacterial functions. Compared to the levels measured in healthy volunteers, neutrophil ROS production was significantly reduced in patients with severe thermal injuries (Figure 3.11 A). This reduction ensued 3 days following injury and was lowest at 7 days post injury. The reduction in ROS formation persisted for up to a month following thermal injury (Figure 3.11 A).

3.2.10 Neutrophil function is reduced to a greater degree in septic patients

Analysis of ROS production in relation to sepsis status was performed to understand if dysregulated ROS production was responsible for the increased incidence of sepsis post thermal injury. ROS was reduced to a similar degree over the first 7 days following injury in both patient cohorts. However, ROS production was significantly lower in septic patients from day 14 – month 2 compared to non-septic individuals (Figure 3.11 B).
Figure 3.11. Thermal injury results in a reduction in ROS production which is more pronounced in patients who develop sepsis. (A) Neutrophil ROS production longitudinally (n = 63). Data at each time point were compared with healthy control (HC) values using a Mann-Whitney test; *p < 0.005. (B) ROS production at each time point was compared between septic and non-septic patients using a Mann-Whitney test (n = 57). *p < 0.05. Box and whisker plots represent the median, minimum and maximum values. HC = healthy control, D = day, M = month.
3.2.11 White blood cell kinetics

As neutrophil function is reduced following thermal injury we wanted to investigate if this was associated with a change in kinetics or phenotype of neutrophils. Thermal injury results in a significant elevation in the circulating levels of WBC compared to healthy individuals (Figure 3.12 A). WBC were elevated on day 1 post-injury and again from day 14 to day 28 (Fig 3.12 A). Levels of WBC on day 1 post-injury correlated positively with percentage TBSA (p < 0.001, r = 0.55, n = 39).

Levels of circulating neutrophils were also significantly elevated compared to the control cohort on day 1 and again from day 7 to month 2 post injury (Fig 3.13 A). Levels of circulating neutrophils on day 1 positively correlated with percentage TBSA (p < 0.001, r = 0.55, n = 39). Importantly, levels of WBC and neutrophils only differed between septic and non-septic patients at day 1 and day 28 following injury. Thus suggesting that alteration in the whole number is not fully responsible for the reduction in neutrophil function post thermal injury (Figure 3.12 B Figure 3.13 B).

Levels of IGs were quantified to investigate if a change in neutrophil phenotype was partly responsible for the reduction in neutrophil function. Levels of IGs were significantly elevated on day 1 post injury compared to healthy individuals (Figure 3.14 A). There was also a secondary increase in IGs from 7 days to 2 months following injury compared to healthy individuals (Fig 3.14 A). Levels of IGs were significantly higher in septic patients compared to non-septic patients on days 1, 7, day 21 - 2 months following injury (Figure 3.14 B). Morphological analysis of isolated neutrophils confirmed the presence of immature cells with the appearance of the classical banded nuclear morphology (Figure 3.14 C, black arrows). The
image shown is from a patient with 45% TBSA thermal injury with neutrophils isolated on day 7 post injury during a known septic episode.
Figure 3.12. Circulating levels of WBC are elevated post thermal injury. (A) WBC across time (n = 39) compared to healthy controls (n = 40) using a Mann-Whitney test *p < 0.005. (B) WBC count at each time points was compared between septic and non-septic patients using a Mann-Whitney test (n = 39) *p < 0.05. Box and whisker plots represent median, minimum and maximum values. HC = healthy control, D = day, M = month.
Figure 3.13. Circulating levels of neutrophils are elevated post thermal injury
(A) Neutrophil count across time (n = 39) compared to healthy controls (n = 40) using a Mann-Whitney test *p < 0.005. (B) Neutrophil count at each time point was compared between septic and non-septic patients using a Mann-Whitney test (n = 39) *p < 0.05. Box and whisker plots represent median, minimum and maximum values. HC = healthy control, D = day, M = month.
Figure 3.14. Thermal injury results in the release of IGs which are associated with sepsis. (A) IGs across time (n = 39). Differences in kinetics were compared to data from control cohort (n = 40) using a Mann-Whitney test; *p < 0.005. (B) IG count at each time points was compared between septic and non-septic patients using a Mann-Whitney test (n = 39). *p < 0.05. (C) Giemsa stain of peripheral blood neutrophils on day 7 post burn injury. Arrows identify cells with immature banded nuclear morphology. Box and whisker plots represent median, minimum and maximum values. HC = healthy control, D = day, M = month, IGs = immature granulocytes.
3.2.12 Release of immature granulocytes post-burn injury is associated with reduced reactive oxygen species generation

This data was performed in collaboration with Dr Hampson as part of the SIFTI Trial. Flow cytometric analysis of neutrophils following severe injury revealed a subset of neutrophils that exhibited greater forward-scatter and reduced sideward-scatter properties when compared to the ‘normal’ neutrophils (Figure 3.15 A). The second population of neutrophils was termed a ‘dual population’. Of note, this dual population of neutrophils produced lower amount of ROS compared to ‘normal neutrophils’ (Figure 3.15 A).

The ‘dual population’ of neutrophils were significantly elevated from day 3 – day 28 post injury compared to healthy individuals (Figure 3.15 B). The kinetics of release were comparable to IG release, emphasised by the significant correlation ($r = 0.65$, $p < 0.0001$) between the percentage of ‘dual population’ neutrophils and percentage of IGs (Figure 3.15 C).

3.2.13 Immature granulocyte percentage is associated with reduced ex vivo neutrophil extracellular trap generation

The ability of the ‘dual population’ to generate NETs could not be investigated directly. However, we used IGs percentage as a surrogate marker as there was a significant correlation between the percentage of neutrophils that fell within the ‘dual population’ and IG frequency (Figure 3.15 C). As the appearance of IGs is in part responsible for the reduction in ROS generation, the ability to undergo ex vivo NETosis was correlated with IG percentage. Ex vivo NET production correlated negatively with IG percentage ($n = 56$, $r = -0.3517$, $p = 0.0079$, Figure 3.16).
Figure 3.15. Burn injury leads to the release of a dual population of non-functioning neutrophils into the circulation. (A) Presence of a neutrophil ‘dual population’ following burn injury which display reduced ROS production. (B) The percentage of cells within the ‘dual population’ as analysed by flow cytometry longitudinally compared to healthy individuals (n = 33). Data was compared to healthy controls (n = 15) by Mann Whitney test *p < 0.005. Box and whisker plots represent median, minimum and maximum values. (C) Correlation of percentage ‘dual population’ and percentage of circulating IGs (n = 33), as determined by the Sysmex XN-1000 analyser. Data was analysed using a Spearman’s rank (n = 33). HC = healthy control, D = day, M = month, FSC = forward scatter, SSC = sideward scatter, IGs = immature granulocytes.
Figure 3.16. IGs are a potential contributor to the reduced ex vivo NETosis observed following thermal injury. Correlation of DNA release from isolated neutrophils following 3 hours of stimulation with 25 nM PMA with IG percentage. Data was analysed by Spearman's rank (n = 56).
3.2.14 Thermal injury results in a change in extended neutrophil parameters

Following severe thermal injury the circulating pool of neutrophils is heterogeneous and measurement of IGs alone does not fully investigate the total changes in granulocyte phenotype. Therefore, a more comprehensive analysis of neutrophil phenotype and maturity was performed. These include neutrophil granularity index (NEUT GI), neutrophil reactive intensity (NEUT RI), neutrophil fluorescence intensity and the width of dispersion (NEUT WY), neutrophil complexity and width of dispersion (NEUT WX) and neutrophil cell size and the width of dispersion (NEUT WZ).

NEUT WY was significantly elevated on day 1 to day 21 post-injury compared to levels in healthy individuals (Figure 3.17 A). NEUT WY was significantly higher in septic patients compared to non-septic patients from day 3 – day 21 post injury (Fig 3.17 B). In contrast, NEUT RI was significantly elevated from day 3 to month 2 post injury compared to healthy individuals (Fig 3.18 A). NEUT RI was significantly elevated in the septic patients from day 3 to day 28 post injury compared to the non-septic patients (Figure 3.18 B). Unlike with NEUT WY and NEUT RI, NEUT WX was not significantly different from the control cohort at any time point. NEUT WZ was only significantly elevated at day 14 (Figure 3.19 A-B).

3.2.15 Extended neutrophil parameters correlate with reduced neutrophil function

Levels of ex vivo NET production were correlated with NEUT WY and NEUT RI to investigate if a change in neutrophil phenotype was partly responsible for the reduced neutrophil function. Levels of DNA release negatively correlated with
NEUT WY (R = -0.4494, p = 0.0036, n = 41, Figure 3.20 A) and NEUT-RI (R = -0.7368, p < 0.0001, n = 41, Figure 3.20 B).
Figure 3.17. Burn injury results in increased NEUT WY. (A) NEUT WY across time (n = 39) compared to healthy volunteers. Differences in kinetics were compared to data from control cohort (n = 40) using a Mann-Whitney test *p < 0.005. (B) NEUT WY at each time points was compared between septic and non-septic patients using a Mann-Whitney test (n = 39) *p < 0.05. Box and whisker plots represent median, minimum and maximum values. HC = healthy control, D = day, M = month.
Figure 3.18. Burn injury results in increased NEUT RI. (A) NEUT RI across time (n = 39) compared to healthy volunteers. Differences in kinetics were compared to data from control cohort (n = 40) using a Mann-Whitney test; *p < 0.005. (B) NEUT RI at each time points was compared between septic and non-septic patients using a Mann-Whitney test (n = 39). *p < 0.05. Box and whisker plots represent median, minimum and maximum values. HC = healthy control, D = day, M = month.
Figure 3.19. Burn injury does not result in dynamic changes in NEUT WX and NEUT WZ. (A) NEUT WX (n = 39) and (B) Vector sum of NEUT WX and NEUT WY (NEUT WZ) across time (n = 39). Differences in kinetics were compared to data from control cohort (n = 40) using a Mann-Whitney test; *p < 0.005. Box and whisker plots represent median, minimum and maximum values. HC = healthy control, D = day, M = month.
Figure 3.20. Changes in neutrophil nucleic acid content potentially contribute to reduced ex vivo NETosis observed following thermal injury. (A) Correlation of ex vivo NETosis and NEUT WY (n = 39). (B) Correlation of ex vivo NETosis and NEUT RI (n = 39). Data was analysed by Spearman’s rank.
3.2.16 mtDAMPs activate neutrophils

This work was performed by Dr Jon Hazeldine (University of Birmingham, UK) as part of a collaboration and joint first authorship manuscript (in preparation).

An additional mechanism for reduced neutrophil function post thermal injury is direct immune suppression by mtDAMPs. To confirm previous observations that mtDAMPs could induce neutrophil activation (43), we investigated the surface expression of CD62L, CD11b, CXCR1 and CRX2 on human neutrophils following 15-minute stimulation with 40 or 100 µg/ml mtDAMPs.

Treatment of neutrophils with both 40 and 100 µg/ml of mtDAMPs resulted in a significant reduction in surface density of CXCR1, CXCR2 and CD62L, compared to control neutrophils stimulated with vehicle buffer (Figure 3.21 A-C). Neutrophils treated with 40 and 100 µg/ml mtDAMPs had significantly higher levels of CD11b surface expression compared to control neutrophils (Figure 3.21 D). Activation of neutrophils was further confirmed by western blotting for MAPK ERK1/2. Neutrophils stimulated for up to 30 minutes with 100 µg/ml mtDAMPs had detectable phosphorylation of ERK1/2 within 2 minutes of stimulation (Figure 3.21 E). Total EKR1/2 was included as a loading control for all experiments (Figure 3.21 E).
Figure 3.21. **mtDAMPs induce neutrophil activation.** Neutrophils were stimulated with 40 or 100 µg/ml mtDAMPs for 15 minutes. (A) Surface CXCR1 expression on neutrophils following pre-treatment with mtDAMPs. (B) Surface expression of CXRC2 expression on neutrophils pre-treated with mtDAMPs. (C) Surface CD62L expression on neutrophils following pre-treatment with mtDAMPs. (D) Surface CD11b expression on neutrophils following pre-treatment with mtDAMPs. Data is presented as MedFI and was compared to neutrophils pre-treated with buffer control via paired t test. *p < 0.05, **p < 0.01 and ***p < 0.001. Data is presented as mean +/- SEM (E) Western blotting for MAPK ERK1/2 and phosphorylated MAPK ERK1/2 following pre-treatment with 100 µg/ml mtDAMPs.
3.2.17 mtDAMPs pre-treatment results in impaired neutrophil extracellular trap generation upon secondary stimulation

To investigate if mtDAMPs were in part responsible for the reduced ex vivo NET generation, neutrophils were pre-treated with mtDAMPs and then stimulated with PMA to induce maximal NETosis. Neutrophils which had been pre-treated with 40 or 100 µg/ml mtDAMPs produced significantly fewer NETs compared to neutrophils pre-treated with vehicle control (Figure 3.22 A). This inhibition of NET formation was confirmed by fluorescence microscopy with neutrophils pre-treated with mtDAMPs displaying lower NET release. Of note, nuclei of pre-treated neutrophils do decondense suggesting initiation of NETosis process but an inability to release the decondensed nuclear material (Figure 3.22 B-C). Importantly, mtDAMPs alone do not induce decondensation of nucleus without secondary stimulation with PMA (Figure 3.22 C)

mtDAMPs are a collection of endogenous cytosolic, mitochondrial and nuclear derived proteins and DNA detected in high circulating concentrations following major trauma (40, 41). The two major components include fMLP and mtDNA.

Neutrophils were pre-treated with mtDNA in order to investigate what component of the mtDAMPs was causing the inhibition of NET production. Only 40 µg/ml could be used in this experiment due to the availability of mtDNA. Neutrophils pre-treated with 40 µg/ml mtDNA had no inhibition of NET formation relative to control neutrophils pre-treated with vehicle control when visualised by fluorescence microscopy (Figure 3.23).
3.2.18 Effect of mtDAMP pre-treatment on PMA-induced neutrophil reactive oxygen species production and citrullination of histone H3

Two processes central to the formation of NETs are the generation of ROS and the citrullination of histone H3 (189-191). The effect of mtDAMPs on ROS production was examined to investigate if this was the mechanism responsible for the reduced NET formation. Pre-treatment of neutrophils with 40 µg/ml of mtDAMPs did not affect ROS production following secondary stimulation. Pre-treatment of neutrophils with 100 µg/ml of mtDAMPs resulted in an increase in ROS production relative to neutrophils pre-treated with buffer control (Figure 3.24 A).

The activation of intracellular NADPH and the formation of ROS is a prerequisite for the citrullination of histone H3, a specific marker of NETosis (189-191). As we had found no inhibition of ROS production we investigated if Cit H3 production was affected. Cit H3 was localised in the nucleus and dispersed on the NETs of vehicle control neutrophils stimulated with PMA (Figure 3.24 B). Furthermore, Cit H3 was detectable in the nucleus of PMA stimulated neutrophils pre-treated with 40 and 100 µg/ml mtDAMPs (Figure 3.24 B). This is consistent with the appearance of the decondensed nuclear structure. Although visible, Cit H3 appeared to be expressed in lower quantities in PMA stimulated neutrophils pre-treated with 100 µg/ml compared to 40 µg/ml mtDAMPs (Figure 3.24 B).
Figure 3.22. Pre-treatment of neutrophils with 40 or 100 µg/ml mtDAMPs inhibits NET formation. (A) Fold increase in DNA release following pre-treatment with 40 or 100 µg/ml mtDAMPs and secondary stimulation with PMA. Data was compared to unstimulated neutrophils via paired t test (**p < 0.01). Data is presented as mean +/- SEM (B) Fluorescence microscopy displaying neutrophils pre-treated with, either, buffer control or 40 or 100 µg/ml mtDAMPs followed by secondary stimulation with PMA. Images at 20x (C) Fluorescence microscopy displaying neutrophils pre-treated with, either, buffer control or 40 or 100 µg/ml mtDAMPs followed by secondary stimulation with. Images are taken at 63x magnification. (B-C) Data is representative of 3 independent experiments.
Figure 3.23. Pre-treatment of neutrophils with 40 µg/ml mtDNA does not inhibit NET formation. Fluorescence microscopy displaying neutrophils pre-treated with, either, buffer control or 40 µg/ml mtDNA followed by second stimulation with 25 nM PMA. Images are taken at 20x magnification and data is representative of 3 independent experiments.
Figure 3.24. Pre-treatment of neutrophils with 100 µg/ml mtDAMPs augments ROS production but reduces citrullination of histone H3. (A) Quantification of ROS production in whole blood following pre-treatment of neutrophils with, either, 40 or 100 µg/ml mtDAMPs and a secondary stimulation of 25 nM PMA. Data was compared to neutrophils pre-treated with buffer control via paired t-test (**p < 0.01). Data is presented as mean +/- SEM. (B) Fluorescence microscopy displaying neutrophils pre-treated with, either, buffer control, 40 or 100 µg/ml mtDAMPs followed by secondary stimulation with 25nM PMA. Green stain is for DNA stained by SYTOX Green Dye. Blue stain is for Cit H3. All images are taken at 20x magnification. Data is representative of n = 3.
3.2.19 Exposure to mtDAMPs suppresses neutrophil migration to LTB4

Suppression or dysregulation in various neutrophil functions have been reported following thermal injury including chemotaxis and migration (159-161, 171, 172). We aimed to investigate if mtDAMPs could cause a broad inhibition of neutrophil function. Therefore, neutrophils were pre-treated with mtDAMPs and their ability to transmigrate to the arachidonic acid-derived fatty acid LTB4, a chemotactic signal, was investigated. Neutrophils pre-treated with both 40 and 100 µg/ml of mtDAMPs had a significant reduction in their migratory activity towards LTB4 compared to vehicle control treated neutrophils (Figure 3.25).
Figure 3.25. Pre-treatment with 40 and 100 µg/ml mtDAMPs reduced migratory activity of neutrophils towards the arachidonic acid-derived fatty acid LTB4. Neutrophils were pre-treated with 40 or 100 µg/ml mtDAMPs and the amount of neutrophils which transmigrated towards to LTB4 was quantified. Data was analysed via repeated measures ANOVA and Bonferroni test compared to vehicle control neutrophils (Unt). ***p < 0.001
3.3 Discussion

When first described, NETs sparked a change in the traditional paradigm of neutrophil biology (118). NETs are comprised of DNA, granular proteins and modified histones and function to ensnare, trap and potentially kill invading pathogens (118, 119). Multiple groups have used quantification of DNA and histones as a method for quantifying NET release in vivo and in vitro (288). Here we report, consistent with previous literature, neutrophils stimulated in vitro with PMA results in maximal NET formation which can be characterised by increased release of DNA from neutrophils. One limitation of studying DNA release as a single entity of NET release is the lack of specificity as DNA can originate from a number of sources including apoptosis and necrosis (115). Therefore, specific markers of NETosis, e.g. Cit H3 which decorates the remaining decondensed nuclear structure and extruded NET structures, need to be investigated to confirm NETosis (289). In this thesis, it has been shown that NETs are decorated with Cit H3, consistent with previous literature (181, 190, 233).

*In vitro* NETosis is further limited by the stimulus used. The use of PMA is commonly criticised because it lacks biological relevance as a stimulus. Therefore, additional stimuli including IL-8, fMLP and LPS have been shown to induce NET formation in vitro following initial priming of neutrophils (9). Here we report that LPS alone does not induce NETosis in isolated neutrophils from healthy individuals. Therefore, stimulation of neutrophils isolated from patients with thermal injuries with LPS allows the investigation of priming status of neutrophils and their propensity to undergo NETosis.

NETosis is a conserved and active cell death pathway which forms a fundamental part of innate immunity. NETs are released from neutrophils in an attempt to
ensnare, trap and potentially kill invading bacteria (118, 119). Previous groups have shown that NETs are implicated in the response to sterile traumatic injury, consequent infection and sepsis in various disease pathologies (111, 290). It has been proposed that NETosis can be induced rapidly by a number of soluble mediators released following tissue damage (9). Moreover, Branzk and colleagues demonstrated that neutrophils were able to adapt and tailor their host defensive functions based upon the pathogenic challenge. If the challenge is too large in size to be engulfed by phagocytosis, then a pro-NETosis phenotype was adopted (188). In our patient cohort the most colonising bacteria was *Bacillus sp*. Interestingly, when neutrophils are incubated with *Mycobacterium bovis* bacillus Calmette-Guérin they formed NETs in preference to phagocytosis (188). Therefore, given the nature and sources of infections in patients with thermal injuries it is not unreasonable to hypothesise that patients will have a pro-NETosis phenotype.

Elevation in circulating cfDNA post thermal injury has been attributed to *in vivo* NET formation (111, 215, 290). However, this method alone is non-specific and circulating DNA can originate from a number of sources including cellular damage, apoptotic cells, necrosing cells, NETosis and bacteria themselves (115). A specific marker of NET formation is the presence of Cit H3 which is facilitated by the activation of the enzyme PAD4. This mediates the decondensation of nuclear chromatin and extrusion of NETs to the outside of the cell decorated in Cit H3 (189).

Hirose and colleagues detected the presence of Cit H3 in the blood stream of critically ill patients following traumatic injury (181) thus confirming NET formation. Here, we expand upon this and demonstrate that Cit H3 is detectable in the
plasma of patients following thermal injury during sepsis and coinciding with elevated levels of cfDNA. Thus, we propose that NETosis is a contributing factor to the increase in cfDNA detectable in septic patients. Importantly, Cit H3 was only detectable in trace quantities immediately following injury in one patient. Hence, the initial sterile injury does not induce NET formation following thermal injury. Data presented in this thesis would suggest that NETs are a defensive action of neutrophils to control and eliminate infection post thermal injury rather than a response initiated by the sterile injury.

In contrast, Hazeldine et al report NET formation within 60 minutes of sterile traumatic injury. This occurs in the absence of an infectious stimulus and appears to be mediated by a different mechanism of NET generation following thermal injury. Thus, there appears to be a difference between burn and general traumatic injury in the generation of NETs (291). However, variations in results may be attributed to differences and difficulties in methodologies used to study NET formation. Namely, current Western blotting assays, for Cit H3, are limited due to its semi-quantitative nature and trace quantities of Cit H3 may be below the limit of detection in plasma samples of patients with thermal injuries. Advancements in ELISA based technologies may aid in the sensitive quantification of Cit H3 and further its validation as a biomarker of sepsis, NETosis and secondary complications (292). Nevertheless, measurements of Cit H3 in conjunction with cfDNA have provided important information on the kinetics and timing of NETosis following thermal injury.

NETosis was long regarded as process localised to tissues at sites of infection and injury (293-295). However, recent evidence has implicated NETosis as a process which can occur within the circulation, resulting in formation and
propagation of thrombosis in a mouse model of DVT (233). Hence, it is plausible that thermal injury results in both NET formation within the tissues and circulation. This is one potential explanation for the detection of NET components in plasma following thermal injury.

NETosis can be initiated in vivo by a plethora of mechanisms. One of which is neutrophils directly interacting with circulating bacteria and the formation of NETs during sepsis (225). Furthermore, neutrophils can also bind to inflammatory cells which initiate and amplify neutrophil response and function. In addition to their central role in haemostasis, platelets are key players in host defence against infection in a process termed ‘immunothrombosis’ (296, 297). Of particular interest is ‘vital NETosis’ governed by the interaction between activated platelets and neutrophils (178). In our cohort of patients, Montague et al report activation of platelets, by measurement of circulating sGPVI, at the later time points correlating with NET production (Montague et al, manuscript submitted). Thus, it is reasonable to hypothesise that activated platelets are also in part responsible for the NETosis in patients following thermal injury.

In addition to their procoagulant activity, it has been reported that heme-related molecules can induce rapid NETosis in vitro (58). In a murine model of sickle cell anaemia, heme causes NET release and is associated with increased mortality (298). Thermal injury results in the production of FRCs (282), which will potentially carry heme-related molecules, which could bind to and activate neutrophils to induce NETosis. Future studies should focus on the mechanism(s) driving NETosis post thermal injury to better understand their role during in vivo responses.
Severe sepsis and septic shock remain a global health care challenge with a systematic review, published in 2012, reporting sepsis as the major cause of mortality in burn injury patients (38). Accurate and early diagnosis of sepsis still remains challenging with many of the diagnostic criteria being non-specific in patients with burns. This is due to many of the classically used diagnosis criteria being masked by the ongoing SIRS which occurs immediately following severe thermal injury. The Surviving Sepsis Campaign published guidelines, in 2012, which recommends administration of antibiotics within 60 minutes of diagnosis of sepsis (299). Furthermore, a delay in diagnosis of sepsis is associated with significantly increased mortality (300). In 2016, the Surviving Sepsis Campaign stressed the importance of antibiotic stewardship aided by further testing to ensure infection and in turn reduce overuse of antibiotics (301). This is in an effort to overcome the growing problem of antibiotic resistance. Blood cultures remain the gold standard for diagnosis of sepsis even though many problems including specificity and time required to process have been reported (302, 303). Hence, the identification and development of novel and accurate biomarkers of sepsis is imperative.

Recent literature has shown a potential role for cfDNA in the diagnosis of sepsis (109, 304-306). Quantification of cfDNA in serum samples, taken within 6 hours of injury, from patients with burns correlated with injury severity and outcome (111). Surprisingly, we report no elevation in plasma cfDNA within 24 hours of injury compared to levels detected in healthy individuals. There are a number of possible explanations for these discordant observations. Firstly, a difference in the timing of the first sample could explain the results. In our analysis, the median time to first blood sample following burn injury was 9 hours. As it has previously been shown
that cfDNA is cleared within 30 minutes of appearance in circulation this may result in clearance and reduced levels of cfDNA (307-309). Another key difference between the two studies is in the preparation of blood samples. In our analysis cfDNA was quantified in plasma samples were as Shoham and colleagues measured cfDNA in serum (111). Analysis of matched plasma and serum samples shows that levels of cfDNA are significantly higher in admission serum samples compared to matched plasma samples from burn injury patients. Furthermore, the change in DNA between matched samples positively correlated with WBC count. In addition, levels of cfDNA were significantly higher in serum samples compared to matched plasma samples from healthy volunteers. Interestingly, the ‘net’ change in DNA was much lower in healthy individuals compared to the ‘net’ change observed in patients. As the ‘net’ change of DNA correlates with WBC, we propose that this increase is mediated by the significant increase in WBC count in patients following thermal injury compared to healthy individuals. Therefore, the false elevation in serum could be a consequence of in vitro clotting, cell death or non-specific binding of SYTOX™ Green dye to any residual WBC. This is not only an important standardization issue but is also in agreement with previously published data (310).

It has been suggested that plasma cfDNA is a potential novel biomarker of sepsis. Consistent with previous publications (109, 220), longitudinal analysis of cfDNA levels in our cohort of patients revealed a potential diagnostic use for this biomarker. Levels of cfDNA are significantly higher in septic individuals and AUROC analysis showed good discriminatory at days 7 and day 14 post injury. Quantification of cfDNA by fluorometric analysis has a number of advantages. Firstly, the process is rapid and requires no isolation of DNA. Unlike Cit H3,
plasma cfDNA can be quantified which allows for evaluation of the marker in multiple clinical settings. Finally, the assay is relatively cheap, requires a small volume of blood and is rapid to perform. In all, this makes the assay applicable as a point of care test to quickly identify patients with infection. Conversely, there are a number of limitations of the assay and cfDNA as a biomarker. It is possible that DNA binding dyes such as SYTOX Green™ could bind non-specifically to other charged molecules in the plasma samples causing false elevation. Additionally, cfDNA lacks specificity as it can originate from a number of sources (115). Hence, it is important to consider the source of circulating DNA.

Using a murine cecal ligation model of sepsis it has been reported that the major source of elevated cfDNA during sepsis is host tissue damage and not NETs (311). Thus, the elevation in cfDNA reported in our cohort could be caused by an accumulation of DNA from initial tissue damage and consequential tissue damage that occurs during sepsis and MOF. Hamaguchi and colleagues went on to show that depletion of neutrophils only had a minor effect upon cfDNA levels confirming an insignificant role of neutrophils in mediating the elevation in cfDNA (311). Thus, the contribution of NETs in the elevation of cfDNA may be minor following thermal injury but still may explain some of the later elevation in cfDNA reported within this thesis.

Circulating DNA can also be of bacterial origin (312, 313). Here, PCR analysis was performed to determine the source of cfDNA. Analysis shows that the predominant source of circulating DNA is nuclear in origin and not mitochondrial following thermal injury. Importantly, the primers used shared no homology with any bacterial species and therefore the ncDNA must, therefore, be host derived; either from host tissue damage, apoptosis, necrosis or NETosis. However, we
cannot exclude the possibility that bacterial DNA is a contributing factor to the total cfDNA measured.

ncDNA levels were significantly elevated within 24 hours of injury relative to healthy individuals which was contradictory to the cfDNA results. The significant elevation of ncDNA at day 1 – 3 following injury is in part due to the higher sensitivity of the PCR assay. As the lower limit of detection of the PCR assay was 0.1 ng/ml and the lower limit of detection for the fluorometric based assay was 60 ng/ml. Still, ncDNA followed comparable kinetics to total cfDNA and they correlated strongly with each other (r = 0.763).

mtDNA is derived from the mitochondria of cells and can act as a DAMP (314). Elevated levels of mtDNA are associated with secondary complications following traumatic injury (314-317). This is attributed to their direct interactions with immune cells and damage to host tissue due to their cytotoxic nature (314, 317, 318). Itagaki and colleagues reported an association between mtDNA levels and NET formation following traumatic injury. Consistent with their previous findings, trauma patients had a significant elevation in mtDNA compared to healthy individuals and incubation of isolated neutrophils with mtDNA resulted in the induction of NET release (315). This may in part be attributable to DAMP induced activation of neutrophils, however the exact mechanism remains unknown (319). Conversely, in our analysis we report no significant elevation in mtDNA concentrations in patients with severe thermal injuries compared to healthy individuals. It is currently unknown if this is a contradictory finding perhaps due to the different mechanism(s) of injury. However, it could be in part explained by differences in timing of blood sampling resulting in clearance of mtDNA or pre-analytical variables by which centrifugation has removed the larger microvesicles
containing mitochondria and hence mtDNA (320). Finally, higher levels of mtDNA are detected in elderly trauma patients compared to young trauma patients (315). As the mean age of our cohort was 39 years this may result in the lower levels of mtDNA detected. As we have few elderly patients with burns ≥ 15% TBSA it was not possible to investigate this hypothesis within our cohort. Thus, further research is required to investigate mtDNA concentrations following thermal injury in relation to outcome, age and secondary complications.

Itagaki and colleagues report preliminary evidence showing a reduced ability of neutrophils from elderly trauma patients to form NETs ex vivo relative to age matched controls and young trauma patients (315). Given the high incidence of infections post burn injury and apparent reduced neutrophil function it has led to the investigation of this relationship (38, 172, 174, 321).

As early as 1985, Arturson et al reported, in a cohort of 50 patients with thermal injuries, a reduction in chemotaxis, phagocytosis and impaired killing of bacteria (175). As recently as 2014, a spontaneous migratory phenotype for neutrophils isolated from patients with severe thermal injuries was observed. This migratory phenotype was present 1 - 2 days prior to clinical diagnosis of sepsis and was not present in healthy or non-septic individuals (172). Whilst these studies documented a dysregulation in neutrophil function following severe thermal injury, they have only studied this in the early days post injury. Thus, the longitudinal neutrophil response has not been thoroughly examined. Whilst the ability of neutrophils to generate NETs post trauma has been investigated no group has investigated the effect thermal injury has on this NET production (315).
Traumatic injury results in the systemic priming of neutrophils which has been attributed to the enormous release of soluble mediators (322, 323). Zhang et al reported a link between initial traumatic injury and consequent immune cell activation and inflammation (42). Further research has shown that adenosine triphosphate (ATP) and N-formyl peptides can stimulate ROS production, degranulation and cytokine secretion in neutrophils (43, 324, 325). Due to the elevated levels of NET components in the circulation we had hypothesised that neutrophils from patients following thermal injury would be primed and in turn would overproduce NETs rapidly with ex vivo stimulation. To test this hypothesis, neutrophils isolated from patients with burn injury were stimulated with LPS alone to investigate their priming status and ability to form NETs ex vivo (9, 293). However, neutrophils from patients following thermal injury did not generate NETs when stimulated with 100 ng/ml LPS. This would suggest that neutrophils are not primed or have a reduced ability to form NETs following thermal injury.

We expanded upon this finding and report that neutrophils stimulated with 25 nM PMA release significantly lower amounts of NETs on day 3 – 7 post thermal injury relative to healthy individuals. This was further confirmed by fluorescent microscopy in which minimal release of DNA was observed from neutrophils isolated from patients. These findings are consistent with previous literature which reports a reduction in maximal NET generation in patients with traumatic injuries (326). In a number of patients it was clear that neutrophils also retained their natural morphology following stimulation without significant decondensation of nuclei. As PMA is not receptor dependent this indicates a clear defect in later signalling cascades. Thus, there appears to be a reduction in the ability to form NETs following thermal injury which may leave patients at risk of nosocomial
infections. One limitation of this data is the absence of a second marker of NET formation, e.g. neutrophil elastase, in our fluorescence microscopy based assay. However, within this analysis we use an isolated in vitro system to induce maximal NET formation. Thus, DNA released from neutrophils following stimulation with PMA can be regarded as a surrogate marker of NET formation. Therefore, we would propose that the lack of a more comprehensive staining panel does not affect our conclusions.

Initially a reduction in ex vivo NETosis appears to contradict our in vivo data. However, there are a number of possible explanations for NET markers detected in vivo and reduced ex vivo NETosis. One possible explanation is that functional neutrophils have migrated and are generating NETs in the damaged tissues and thus leaving non-functional neutrophils in the circulation. Secondly, there may be a dysregulation in the breakdown and clearance of NETs allowing for the build-up of NET components. One limitation of our current data is the inability to investigate the relationship between ex vivo NETosis and susceptibility to infection post thermal injury due to limited sample size. Thus, further studies are required to fully understand the clinical relevance of this finding post thermal injury and to investigate if reduced ex vivo NETosis is associated with an increased incidence of infection and a potential therapeutic target.

Whilst a reduction in NET formation has been shown post traumatic injury (315), to our knowledge no groups have studied potential mechanisms for this dysregulation in the same patient cohort. ROS are a heterogeneous collection of molecules which form an important link between cellular activation and NET formation. Production of ROS is controlled by NADPH oxidase complex which
produces $O_2^-$ and $H_2O_2$ upon activation. ROS induced decondensation of nuclear chromatin precedes NETosis and is facilitated by the activity of PAD4 (189-191).

There are contradicting data regarding ROS production following traumatic injury. Namely, it has been shown that baseline ROS production is increased and may be contributing to host tissue damage (137, 138). Conversely, Parment and colleagues report a reduction in oxidative capacity for up to 3.5 months post thermal injury (177). In agreement with Parment and colleagues, we report a significant reduction in ROS formation following thermal injury relative to healthy individuals. Furthermore, in our analysis septic patients had significantly lower levels of ROS production than non-septic patients. This highlights the immediate and sustained immunosuppression induced by thermal injury in septic patients. Given that ROS production is regarded as a prerequisite of NETosis, this may be a potential mechanism by which neutrophils have a reduced ability to generate NETs ex vivo. However, ROS independent mechanisms of NET formation must also be considered. Hence, given the limited sample size, further research is required to fully study in vivo reduced ROS formation and ex vivo NETosis following thermal injury.

Independent of NETosis, ROS also forms a key component of host defence against engulfed pathogens (134). Neutrophils are essential effector cells of innate immunity and function to clear invading pathogens. Dysfunction or dysregulation of neutrophil functions may leave the host immunocompromised as highlighted in CGD patients who are incapable of forming ROS due to the absence of NADPH (153). Moreover, phagocytosis is significantly reduced following thermal injury and is more pronounced in septic individuals (180). This may be contributing to the increased incidence of infection as septic patients with thermal injuries have
reduced ROS formation and phagocytosis following thermal injury. In addition, it has recently been suggested that pathogens can use evasion strategies to avoid killing by neutrophils. Laskay et al suggest an evasion strategy, by Leishmania, that allows pathogens to use granulocytes as host cells and replicate. As ROS is a critical effector of intracellular killing and reduced post thermal injury, this process may be occurring. Therefore, pathogens may be using neutrophils as a ‘Trojan horse’ to aid in their dissemination due to the inability to kill engulfed pathogens (327).

Given that NETosis can also be mediated through ROS independent mechanisms it is important to consider additional mechanisms by which dysregulation in NET formation may occur (186). IGs are released prematurely from the bone marrow in response to stress and infection (96). Although they can still phagocytose and migrate, it is reduced compared to mature neutrophils (169). In addition, immature cells are termed ‘band cells’ due to their classical banded morphology and increased nuclear content. Here we show that neutrophils which are banded in morphology are present in isolated neutrophil preparations from patients. Analysis of neutrophils following severe thermal injury also revealed a ‘dual population’ of neutrophils which have distinct forward and sideward scatter properties comparable to data published by Pillay et al in 2011 (158). The kinetics of the ‘dual population’ of neutrophils correlated with the appearance of IGs and in our analysis this ‘dual population’ of neutrophils was defective in generating ROS and phagocytosis (180). IG frequency correlated with reduced ex vivo NETosis and therefore immature cells may be contributing to the reduced neutrophil function post burn.
It is important to consider the heterogeneous nature of the circulating population of neutrophils. Quantification of IGs alone is therefore limited as it does not account for phenotypical changes in the total neutrophil population. We performed a more comprehensive analysis of neutrophil phenotype by quantifying five additional novel parameters; NEUT WY, NEUT WX, NEUT WZ, NEUT GI and NEUT RI. Here we report a significant increase in NEUT WY and NEUT RI post thermal injury. The elevation is potentially caused by the cumulative release of IGs and promyelocytes, myelocytes or metamyelocytes. Moreover, the presence of IGs and extended measurements of neutrophil phenotype correlated with reduced ex vivo NETosis. Thus, changes in neutrophil maturity and morphology could be in part responsible for the reduced neutrophil function observed following severe thermal injury.

Quantification of circulating IGs is able to discriminate between septic and non-septic patients with a sensitivity of 89.2% and a specificity of 79.4% (96). Here we show that in addition to their potential mechanistic role in sepsis, IGs have a potential diagnostic use as they are significantly higher in septic patients compared to non-septic individuals. Further investigation and formal statistical analysis on the kinetics of IGs release in relation to infection is required to fully understand the diagnostic potential of this marker.

Recently it has been reported that quantification of extended neutrophil parameters could predict an increase in circulating IGs and discriminate between IG release from infectious or non-infectious causes (328). Therefore a combination of IG number and extended neutrophil parameters may provide a novel panel of biomarkers to discriminate between sterile injury and infection following burn. In a recent study by Luo et al, NEUT X, NEUT Y and NEUT Z could identify septic
patients (329). In our analysis only NEUT WY and NEUT RI, measurements of nucleic acid content, demonstrated any significant and dynamic changes in thermal injury patients compared to control cohort. The differences may be mediated by methodology as, NEUT-Y, NEUTX and NEUT-Z do not account for width of dispersion unlike NEUT-WY. Stiel et al have reported that an increase in nucleic acid content had a strong discriminatory power for diagnosing sepsis with sensitivity equal to 90.91% and specificity equal to 80.60% (330). In agreement, septic individuals had higher NEUT WY and NEUT RI values compared to non-septic patients. Quantification of immature neutrophils and measurement of nucleic acid content may be a potential novel biomarker of sepsis following thermal injury. Furthermore, measurement of the extended neutrophil parameters of maturity and phenotype may have better potential compared to quantifying IGs alone.

Although quantification of novel biomarkers is showing promise in the early diagnosis of sepsis in patients with thermal injuries, understanding the mechanisms responsible for the immunosuppression offers the opportunity to explore novel therapeutic interventions. However, current mechanisms responsible for immunosuppression are poorly understood. One potential source of immediate immunosuppression is the release of mtDAMPs. 

mtDAMPs are comprised of a heterogeneous mixture of molecules including; mtDNA, cytochrome C, N-formyl peptides and ATP (331). However, fMLP and mtDNA are regarded as the two major effectors on neutrophils. This was demonstrated in 2015 by Hazeldine et al who report neutrophil activation through N-Formyl peptides within mtDAMPs (43).
Once released into the circulation from damaged tissues and cells, mtDAMPs can activate and induce a state of tolerance in immune cells (42, 45, 332). In 2014, Fernández-Ruiz et al defined endotoxin tolerance in monocytes induced by circulating mtDAMPs. Notably, this phenomenon of tolerance was associated with an increased infection rate (45). In a more recent study, the authors report that patients with ischemic stroke display this endotoxin tolerance phenotype and it is association with elevated circulating mtDNA. Furthermore, monocytes from healthy individuals developed this endotoxin tolerance phenotype when cultured with serum from patients with ischemic stroke or elevated levels of mtDNA (332). Whilst this refractory state has been described in monocytes it is possible that circulating mtDAMPs induce a comparable phenotype in neutrophils following thermal injury and maybe responsible for the reported dysfunction.

Following severe traumatic injury mtDAMPs, including N-formyl peptides and mtDNA, are released into the circulation (42, 333). Whilst here we report no significant elevation in mtDNA following thermal injury the difference may be caused by a number of factors including pre-analytical variables, time to blood sampling and mechanism of injury. Furthermore, we have been unable to measure circulating levels of fMLP due to lack of sample availability. Therefore, further study is warranted to fully investigate mtDAMP kinetics post thermal injury and their association with neutrophil function. Of note, in septic patients or patients with DIC, elevated levels of circulating high mobility group box 1 protein (HMGB1) have been observed (334, 335). Thus, one would hypothesise that fMLP is released in response to consequent host tissue damage and infection following thermal injury in a similar manner to HMGB1 (42, 333).
In 2015, Hazeldine et al. reported activation of human neutrophils following stimulation with 40 or 100 µg/ml mtDAMPs. The authors showed for the first time that activation of neutrophils by mtDAMPs was dependent upon signalling through the MAPK p38 and ERK 1/2. Selective inhibition of the formyl peptide receptor-1 inhibited this activation and the group concluded that N-formyl peptides are the main constituents driving mtDAMP-induced neutrophil activation (43). Consistent with their findings we report that mtDAMPs result in activation of neutrophils characterised by CD62L shedding and phosphorylation of ERK 1/2 (43). Here we expand upon this observation by investigating CXCR1 and CXCR2 surface expression on neutrophils following 15 minutes incubation with mtDAMPs. Following activation of neutrophils, CXCR1 and CXCR2 are internalised resulting in a decrease in their surface expression (336, 337). Here, we report that stimulation with mtDAMPs results in a rapid decrease in both CXCR1 and CXCR2 surface expression. The decrease in CXCR2 is more pronounced than CXCR1 which may be explained by CXCR2 being rapidly internalised following activation compared to CXCR1 (337).

Various endogenous and exogenous inhibitors of NET release have been reported (338-341). Although dysregulation in NET formation has been reported following major trauma (180, 315), the mechanisms driving this dysregulation are poorly understood. Furthermore, the role mtDAMPs and inhibition NET formation has yet to be fully explored. Pre-treatment of neutrophils with either 40 or 100 µg/ml of mtDAMPs results in a significant reduction in NET formation following secondary stimulation with PMA. To investigate the component responsible for inhibition of in vitro NET formation neutrophils were pre-treated with mtDNA. Consistent with previous findings we report no inhibition of NET formation when neutrophils are
pre-treated with mtDNA (315). Hence, an additional component of mtDAMPs that is not mtDNA is potentially responsible for the inhibition of PMA induced NET formation. Thus data presented within this thesis does not contradict previous observations (315).

Neutrophils pre-treated with mtDAMPs and then stimulated with PMA had decondensed chromatin and nuclear structure and did not retain their natural polymorphic nuclear structure. This is indicative of dysregulation of NET release rather than recognition of stimulus. Since PMA activity is not dependent upon receptor activation it appears that inability to release NETs is mediated by dysregulation in a downstream cascade. This is different to our ex vivo data in which neutrophils do not decondense following stimulation with PMA. However, this difference may be explained by cumulative dysregulation in our ex vivo data caused by a change in phenotype and abnormal ROS production. Importantly, this is not accounted for in our in vitro analysis of mtDAMPs functions.

Formation of NETs is generally NADPH oxidase-dependent with many activators of ROS being reported as inducers of NETosis (189-191, 342). In addition, mice with NADPH oxidase deficient neutrophils are not able to form NETs (342). To investigate if a dysregulation in ROS production was responsible for inhibition of NETosis, levels of ROS were quantified following pre-treatment with mtDAMPs and secondary stimulation with PMA. Of note, Hazeldine et al have previously reported that stimulation of neutrophils with mtDAMPs results in a significant increase in ROS production from resting neutrophils (43). Here we expand upon this and show that pre-treatment of neutrophils with mtDAMPs and a secondary stimulation with PMA results in a significant increase in ROS production compared to vehicle control neutrophils. ROS induced decondensation of nuclear chromatin
precedes NETosis and is facilitated by the activity of enzyme PAD4 (189-191, 343). Here we report, although reduced, neutrophils still have Cit H3 detectable and a decondensed nuclear structure. As ROS formation can mediate citrullination of histones by PAD4 and eventual chromatin decondensation this may explain our results (135, 189, 208, 343).

It is important to consider the limitations of these experiments. As we are measuring ROS production in whole blood we cannot eliminate that mtDAMPs are indirectly increasing neutrophil ROS production via activation and interaction with other inflammatory cells. However, our data suggest that a reduction in ROS is not causing inhibition of NETosis following pre-treatment with mtDAMPs.

Multiple groups, including ourselves, have reported a reduction in neutrophil ROS production following severe thermal injury (175-177). Here we report that pre-treatment of neutrophils with mtDAMPs increases ROS production following secondary stimulation. Therefore, mtDAMPs are probably not responsible for the in vivo reduction in ROS production reported in this thesis. As discussed earlier, this reduction in ROS formation is most likely caused by a change in neutrophil maturity, phenotype or an unknown soluble mediator.

Ramijsen et al report that the formation of NETs is dependent upon both ROS generation and autophagy (208). Autophagy is an essential cellular mechanism for cellular homeostasis and is regulated by activation of the mammalian target of rapamycin (mTOR). Pharmacological inhibition of mTOR accelerated NETosis following stimulation with fMLP and is therefore a negative regulator of autophagy. Inhibition of autophagy by wortmannin abolishes citrullination of histones induced by co-treatment of neutrophils with rapamycin and fMLP (344). We hypothesise
that mtDAMPs may be activating mTOR which inhibits autophagy and in turn prevents NET production. Therefore, one may hypothesise that the reduction in Cit H3 staining observed is in part caused by mtDAMP inhibition of autophagy. However, this is only a hypothesis and further study is required to fully understand the effect mtDAMPs have on autophagy and consequently NET formation.

For neutrophils to function in tissues they must migrate towards the site of infection before phagocytosing or generating NETs against the pathogen. One hypothesis speculates if the dysfunctional pool of circulating neutrophils consists of cells which are unable to migrate to sites of injury and in turn leaving the patient immunocompromised. Hence this may manifest as the clear dysregulation in migratory phenotype of neutrophils observed following severe thermal injury (159-161, 171, 172). In addition, studies have reported impaired migration, of neutrophils isolated from trauma patients, towards IL-8 and LTB4 (326, 345). In this thesis, pre-treatment of neutrophils with mtDAMPs causes a reduction in neutrophils ability to migrate towards LTB4. Suggesting that mtDAMPs may be a contributory factor to the dysregulated chemotaxis and migration reported following severe thermal and traumatic injury.

We propose that the reduced migration of neutrophils is mediated by both mtDNA and fMLP. Firstly, Li et al have reported using an ex vivo model that pre-treatment of neutrophils with 10 nM fMLP resulted in reduced transmigration in response to both 1 and 10 nM LTB4 (326). This was earlier shown by Campbell et al who reported decreased migration towards IL-8 following pre-treatment with 10 nM fMLP (346). Furthermore, mtDNA contains CpG DNA repeats as mitochondria evolved from bacteria (347, 348). Incubation of neutrophils with CpG DNA significantly impairs migration towards LTB4 which occurred simultaneously to a
reduction in BLT1, a high affinity receptor for LTB4 (349). In addition, the authors show a reduction in migration towards IL-8 following pre-treatment with CpG DNA. Again, this reduction occurred simultaneously to a reduction in the IL-8 specific receptor, CXCR1 (349). In our studies, pre-treatment of neutrophils with mtDAMPs results in a significant reduction in both CXCR1 and CXCR2. Therefore, whilst we have yet to investigate an ability to migrate towards IL-8, it is possible that mtDAMPs may result in disrupted transmigration towards this chemokine. Finally, reduced transmigration towards LTB4 may be caused by internalisation of BLT1 receptors following treatment with mtDAMPs.

In conclusion, this data supports the hypothesis that mtDAMPs may induce a state of neutrophil dysregulation and may be responsible, in part, for the reduced ex vivo NETosis and dysregulated migration reported following thermal injury (159, 161, 171, 172). These results may provide a novel area of research linking the initial traumatic injury, subsequent suppression of neutrophil function and increased incidence of nosocomial infections. Following further studies, this may identify therapeutic targets to manipulate and correct the reduced neutrophil function following thermal injury. However, it is important to consider that all of these experiments are performed in isolation without any potential cellular interactions or additional soluble mediators. However, we propose that the reduced neutrophil function is in part mediated by the initial and rapid release of mtDAMPs.

In summary, data presented in this chapter show NETs are generated following thermal injury. Consistent with previous literature (181, 350, 351), NETs are generated during the infectious phase and are most likely forming a key part of host defence against invading pathogens. Shoham et al have previously reported
an increase in serum cfDNA and NET markers immediately post thermal injury (111). However, our data suggests that this was caused by an artefactual increase in cfDNA induced by blood clotting in the preparation of serum. Thus, there is a need for standardisation of cfDNA and NET marker measurements in clinical samples.

Furthermore, data presented in this chapter reports a dysfunction in the ability of neutrophils to form NETs following thermal injury. Whilst preliminary evidence has reported this dysfunction following trauma (315), to our knowledge it has yet to be reported following thermal injury. As shown in Figure 3.26, the reduced ability to generate NETs may be attributed to three main causes. Firstly, septic patients have significant changes in the maturity phenotype of circulating neutrophils which are known to be dysfunctional (169). mtDAMPs, and other soluble mediators, which are released immediately following thermal injury are potential contributors of the reduced ability to generate NETs. This provides a link between the initial traumatic injury, subsequent suppression on innate immunity and provides an explanation for the increased incidence of nosocomial infections following thermal injury. Whilst still a hypothesis it is possible that functioning neutrophils have migrated to sites of injury within the tissues, in turn leaving behind non-functioning circulating neutrophils. In conclusion, this research reports both novel biomarkers of sepsis and potential mechanisms of reduced neutrophil function following thermal injury which may be therapeutic targets.
Figure 3.26. Mechanisms by which neutrophil dysfunction, including reduced NET formation, may occur following severe thermal injury. Following severe thermal injury there is a heterogeneous pool of circulating dysfunctional neutrophils which may underlie the increased infection rate. Dysfunction may occur by a change in neutrophil phenotype, tolerance induced by prior stimulation with soluble mediators (e.g. mtDAMPs) or due to functioning neutrophils migrating to the tissues leaving behind non-functioning circulating neutrophils.
Chapter 4

Reduced DNAse activity in burns patients is associated with compromise to the blood-based actin scavenging system and increased risk of multiple organ failure
4.1 Introduction

Sepsis is a life threatening condition characterised by an imbalance in both the immune and haemostatic systems, which results in cardiac dysfunction and acute respiratory distress syndrome. Without intervention, sepsis can lead to MOF, which is a leading cause of delayed mortality following major trauma and is characterised by tissue hypoxia, tissue damage and organ dysfunction (59). Although it is well recognised that damage to vital organs occurs during MOF, the mechanisms mediating this damage and progression remain poorly understood (352). Understanding the pathogenesis of MOF and organ damage has the potential to improve patient outcome following major injury including burns.

Immunothrombosis is a recently described phenomenon in which thrombosis forms a vital part of host protection against invading pathogens (296). This process is supported by innate immune cells and forms a matrix upon which recognition and elimination of pathogens can occur. Of note, neutrophils have a multifaceted role in coagulation and are implicated in thrombosis, tissue damage and various disease pathologies (229, 230, 237, 353). One proposed mechanism by which this occurs is through the generation of NETs which are comprised of DNA, histones, granule-derived peptides and enzymes and have been shown both in vitro and in vivo to ensnare, trap and in some instances directly eliminate pathogens (119).

Recent studies have shown that NETs and their components can initiate a procoagulant phenotype and cause tissue and organ damage (195, 223, 224). NET induced thrombi have decreased permeability, decreased susceptibility to fibrinolysis and increased clot stability (235). Build-up of circulating NET components may occur due to overproduction of NETs, due to systemic priming,
or a reduced ability to degrade and breakdown NETs. As described in an earlier chapter (3.2.8 Ex vivo NETosis, Figure 3.10), neutrophils isolated from burn-injured patients are dysfunctional in producing NETs ex vivo compared to healthy individuals. Therefore, we hypothesise that the accumulation of NET components following injury is likely caused by impairment in clearance mechanisms.

DNAse 1 is the major extracellular endonuclease found in blood and functions to break down chromatin and DNA that are released following cellular damage, cell death and NETosis. Reduction in DNAse activity is associated with the pathogenesis of autoimmune disease through physical inhibition of the enzyme by circulating antibodies (237). DNAse activity is also indirectly regulated by the actin scavenging system that prevents the formation of DNAse and actin complexes. The system is comprised of two key proteins; GSN and VDBP (237, 240, 245, 246). Whilst it has been shown that reduced levels of GSN are associated with mortality, development of sepsis and MOF (254). No group has studied dynamic changes in GSN and VDBP levels in relation to DNAse activity and build-up of circulating toxic NET components following severe thermal injury.

4.1.1 Aims

The aims of this chapter were:

- Investigate if clearance of DNA was dysfunctional following thermal injury.
- Investigate if a reduction in DNAse activity was associated with secondary complications namely, sepsis and MOF.
- Investigate if reduced DNAse activity is a potential therapeutic target to reduce secondary complications.
4.2 Results

4.2.1 Study cohort

Consistent with Chapter 3, all analysis was performed on adult patients (≥ 18 years of age) with burns ≥ 15% TBSA. The consort diagram for this analysis is included (Figure 4.1). Analysis was restricted to burns ≥ 15% TBSA to minimise the confounding bias of age and severity of injury in analysis. Furthermore, this has reduced the span and confounding bias of fluid resuscitation (284, 285).

Patient and healthy controls demographics are shown in Table 4.1. A total of 64 adult burns patients were included in the study, with a mean age of 43 years (range 16 - 88) and mean burn size of 35% TBSA (range 15 - 95). In addition, 19 healthy controls were included in this analysis with a mean age of 44.5 years (range 20 - 95.9). The incidence of sepsis was 59%, with 38 patients experiencing one or more episodes during their hospital stay. The median time to first septic episode in this cohort was 5 days post-injury (IQR 4 - 7). The incidence of MOF in the cohort was 37.5% (24/64), with the median time to first episode 4 days post-injury (IQR 2 - 11). A significantly greater injury severity, incidence of sepsis and mortality rate was recorded in those patients who developed MOF (p < 0.05). All patients received standardised burn resuscitation protocols as per Parkland Formula and as such received equivalent fluid resuscitation (average = 5.4 mls/kg/%TBSA, standard deviation = 2.1).

Different from Chapter 3, all patients were included in the analysis within this chapter irrelevant of time to mortality as no attempt to evaluate the diagnostic or prognostic accuracy of biomarkers using AUROC was made.
### Table 4.1. Patient demographics (Chapter 4).

MOF and no-MOF variables were analysed by Mann-Whitney (continuous variables) or Chi-squared test (categorical variables). Abbreviations: ABSI, %TBSA = percentage TBSA.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Healthy Controls (n = 18)</th>
<th>All Patients (n = 64)</th>
<th>MOF (n = 24)</th>
<th>No MOF (n = 40)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>44.5 (20-95.9)</td>
<td>43 (16-88)</td>
<td>44 (24-77)</td>
<td>42 (16-88)</td>
<td>ns</td>
</tr>
<tr>
<td>Gender (M:F)</td>
<td>9:9</td>
<td>43:21</td>
<td>12:12</td>
<td>31:9</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>%TBSA (min-max)</td>
<td>35 (15-95)</td>
<td>48 (17-95)</td>
<td>27 (15-58)</td>
<td>&lt; 0.0001</td>
<td></td>
</tr>
<tr>
<td>ABSI (min-max)</td>
<td>8 (4-14)</td>
<td>10 (6-14)</td>
<td>7 (4-11)</td>
<td>&lt; 0.0001</td>
<td></td>
</tr>
<tr>
<td>Survived (Y:N)</td>
<td>44:20</td>
<td>10:14</td>
<td>34:6</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>Sepsis (Y:N)</td>
<td>38:26</td>
<td>22:2</td>
<td>16:24</td>
<td>&lt; 0.0001</td>
<td></td>
</tr>
</tbody>
</table>
Figure 4.1. CONSORT diagram showing allocation and disposition of study subjects.
4.2.2 Nuclear DNA levels are elevated in patients with multiple organ failure

Firstly, it was essential to understand if an elevation in NET markers was associated with the development of MOF or sepsis. Analysis of ncDNA was chosen due to the lower limit of detection compared to cfDNA alone. Therefore, changes within 24 hours following thermal injury and its relation to MOF and sepsis could be investigated.

Longitudinal changes in the kinetics of ncDNA were compared between patients who did or did not develop MOF. Levels of ncDNA were significantly elevated on day 1–day 3 post-burn in patients who developed MOF compared to those who did not (Figure 4.2 A). This was followed by a secondary significant elevation in ncDNA between day 14 and month 2 in patients with MOF. ncDNA levels returned to values comparable to that of healthy controls 3 months post-injury (Figure 4.2 A).

4.2.3 Nuclear DNA levels are elevated in patients who develop sepsis

Longitudinal changes in the kinetics of ncDNA were compared between patients who did or did not develop sepsis. ncDNA was significantly elevated on day 14–month 2 post-burn in patients who developed sepsis compared to those who did not (Figure 4.2 B). This was followed by a secondary significant elevation in ncDNA at month 12 in patients who had developed sepsis which is most probably caused by small sample number at this time point within the analysis.

Elevation in ncDNA at later time points coincides with NET generation as determined by the presence of Cit H3. The representative western blot (Figure 4.3) is for a 55 year old male patient who had a 65% TBSA burn who survived their injury but developed both sepsis and MOF. Cit H3 is detectable at both day
21 and day 28 post thermal injury coinciding with infection and elevation in ncDNA post thermal injury. Therefore, it is highly likely that NETs are contributing to the increase in ncDNA at the later time points post injury (Figure 4.3). Of note, levels of ncDNA within 24 hours following thermal injury significantly correlated with TBSA burn, ABSI and rBaux score (R = 0.42, 0.31 and 0.37 respectively, Figure 4.4 A-C).
Figure 4.2. Patients with MOF and sepsis have higher levels of circulating ncDNA. (A) ncDNA across time (n = 64) differences in kinetics were compared between patients who did and did not develop MOF using a Mann-Whitney test; *p < 0.05. (B) ncDNA across time (n = 64) differences in kinetics were compared between patients who did and did not develop sepsis using a Mann-Whitney test; *p < 0.05. Both panels are presented on a log scale. Box and whisker plots represent median, minimum and maximum values. HC = healthy control, D = day, M = month.
Figure 4.3. Cit H3 coincides with elevations in ncDNA following thermal injury. ncDNA (log scale) and Cit H3 western for a representative patient: 55 year old male patient who had a 65% TBSA burn who survived their injury but developed both sepsis and MOF. Positive control = Calcium ionophore stimulated HL-60 cells (Section 2.10), HC = healthy control, D = day.
Figure 4.4. Levels of ncDNA correlate with severity and size of injury. (A) Correlation of ncDNA and TBSA burn (%) (n = 64) (B) Correlation of ncDNA and ABSI (n = 64). (C) Correlation of ncDNA and rBaux score (n = 64). All measurements were taken within 24 hours of injury. Data was compared by Spearman’s rank.
4.2.4 Patients with thermal injury have reduced DNAse activity

To investigate if the accumulation of ncDNA was associated with reduced clearance, DNAse activity was quantified in serum samples. There was a significant reduction in DNAse activity, from day 1 – day 28 post-injury compared to healthy individuals (Figure 4.5). Interestingly, DNAse activity on day of injury did not correlate with size of thermal injury (TBSA %), severity of injury (ABSI) or rBaux (Figure 4.6 A-C).

Reduced DNAse activity was confirmed by fluorescence microscopy with NETs still visible in slides treated with patient serum (Figure 4.7). Conversely, all healthy control serum samples digested NETs completely (Figure 4.7). Data presented is from 5 independent experiments and is representative of 10 experiments. DNAse activity significantly and inversely correlated with ncDNA levels across all time points (n = 64, r = -0.3574, p < 0.0001).

4.2.5 DNAse activity is lower in patients who develop multiple organ failure or sepsis

To understand if a reduced DNAse activity was associated with MOF, by facilitating the accumulation of ncDNA, patients were split into those who did and those who did not develop MOF. Importantly, patients who developed MOF had significantly lower DNAse activity at day 28 and month 2 post injury compared to patients who did not develop MOF (Figure 4.8).

Patients were further subcategorised according to their sepsis status to investigate if infection had any effect on DNAse activity. Patients who developed sepsis had significantly lower DNAse activity at day 21 to month 2 post injury compared to patients who did not develop sepsis (Figure 4.9).
4.2.6 DNAse-1 antigen levels are elevated following thermal injury

As we have reported a sustained elevation in NET markers in the presence of an inability to produce NETs \textit{in vivo} we investigated if clearance was impaired. Levels of DNAse 1 were quantified to understand if the reduction in DNAse activity from day 1 – day 28 post injury was mediated by a reduction in circulating DNAse antigen. Levels were quantified in 24 patients who had reduced DNAse activity (below 50% compared to healthy controls) within 24 hours of injury. Interestingly, there was no significant difference in DNAse antigen levels from day 1 – day 3 post injury. However, there was a significant increase in DNAse antigen levels from day 7 – day 14 and on day 28 post injury compared to levels measured in healthy volunteers (Figure 4.10).
**Figure 4.5.** Thermal injury results in reduced DNAse activity. DNAse activity following thermal injury (n = 64). Differences in kinetics were compared to data from control cohort (n = 10) using a Mann-Whitney test; *p < 0.005. Box and whisker plots represent median, minimum and maximum values. HC = healthy control, D = day, M = month.
Figure 4.6. DNAse activity within 24 hours of injury does not correlate with severity and size of injury. (A) Correlation of DNAse activity and TBSA burn (%) (n = 64) (B) Correlation of DNAse activity and ABSI (n = 64). (C) Correlation of DNAse activity and rBaux (n = 64). All measurements were taken within 24 hours of injury. Data was compared by Spearman’s rank.
Figure 4.7. Thermal injury results in reduced DNase activity visualised by fluorescence microscopy. Fluorescence microscopy imaging of NET degradation of preformed NETs. DNA is stained green with SYTOX™ Green Dye. A buffer control (PMA control) was included in every experiment to show maximal NET generation without degradation. NETs treated with serum from patients (Thermal Injury Patients), healthy individuals (Healthy Control) or 10 units/ml DNAs 1 (DNase 1 Positive Control). Data presented here is for 5 independent experiments and is representative of 10 independent experiments. All images were taken at 20x magnification.
Figure 4.8. Patients with MOF exhibited lower DNase activity. Comparison of DNase activity (n = 64) between patients with (n = 24) and without MOF (n = 40); *p < 0.05. Data was compared between patient groups at time points using a Mann-Whitney test. Box and whisker plots represent median, minimum and maximum values. HC = healthy control, D = day, M = month.
Figure 4.9. Patients who developed sepsis have lower DNAse activity. Comparison of DNAse activity (n = 64) between patients diagnosed with (n = 38) and without sepsis (n = 26); *p < 0.05. Data was compared between patient groups at time points using a Mann-Whitney test. Box and whisker plots represent median, minimum and maximum values. HC = healthy control, D = day, M = month.
Figure 4.10. Thermal injury significantly increases DNAse 1 antigen levels. DNAse 1 antigen levels following thermal injury (n = 24). DNAse 1 antigen compared to healthy controls (n = 10). Differences in kinetics were compared to data from control cohort (n = 10) using an unpaired t test (*p < 0.008). Box and whisker plots represent mean, minimum and maximum values. HC = healthy control, D = day.
4.2.7 Actin is released following thermal injury

Actin, an inhibitor of DNAse activity, was measured in plasma to determine the potential mechanism behind reduced in vivo DNAse activity following thermal injury. Sterile injury results in the immediate release of cellular actin which was detected in 8 out of 9 patient samples taken within 24 hours of injury (Figure 4.11 A). This data is representative of 20 individual patients in which actin is present in 16 out of 20. Actin was not detected in plasma from 2 healthy controls, this data is representative of n = 5 in which no actin was detectable.

Actin was measured longitudinally in 6 patients to understand the kinetics of actin release. Importantly, actin was detectable up to day 28 post injury in patients. Western blot data presented is for three patients: Patient 10 had a 66% TBSA burn who developed MOF. Actin was detected within 24 hours and up to 3 days following thermal injury. Interestingly, actin was also detectable at later time points, day 20 – day 23. Patient 11 had a 53.5% burn TBSA who developed sepsis. A weak actin band was detected on days 19 and day 28 post injury. Patient 12 had a 65% TBSA burn who developed sepsis. Actin is detected only on day 1 following injury. Western blot analysis for 4 patients shows that actin is detectable at day 21 following injury in Patient 11 and 12 whilst being absent on day 14 and day 28 in patient 13 and 14 (Figure 4.11 B)

4.2.8 Actin inhibits DNAse activity in vitro

Healthy control serum was incubated with increasing concentrations of actin in order to understand the potential in vivo mechanism of reduced DNAse activity. Incubation with 1, 2 or 5 µM actin resulted in a significant decrease in DNAse activity compared to buffer control (Figure 4.12).
Figure 4.11. Thermal injury causes the release of circulating actin. (A) Western blot of actin in plasma samples from 9 patients (P 1 - 9) taken within 24 hours of injury. Actin was not detected in plasma from healthy controls (HC) n = 2. (B) Western blot for Patient 10 with a 66% TBSA who developed MOF. Patient 11 had a 53.5% burn TBSA who developed sepsis. Patient 12 had a 65% TBSA burn who developed sepsis. Western blot analysis for 4 individual patients (P13-16). HC = healthy control, D = day.
Figure 4.12. Actin inhibits DNAse activity in vitro. Actin inhibition of DNAse activity in serum by 1, 2 or 5 µM actin. Differences in kinetics were compared to data from control serum (n = 8) using a Friedman's test followed by Dunn's Multiple Comparison Test; *p < 0.05. Box and whisker plots represent median, minimum and maximum values. Pooled serum from 9 healthy volunteers was used to calibrate 100% degradation.
**4.2.9 Thermal injury results in a decrease in circulating vitamin d binding protein and gelsolin levels**

Levels of VDBP were quantified by Dr Khaled Alitarrah (University of Birmingham, UK) as part of the SIFTI Trial. All data was analysed by Robert J Dinsdale.

The actin scavenging system is controlled by two proteins; VDBP and GSN. Levels of VDBP were quantified in patients with burns ≥ 20% TBSA (n = 50) to establish the effect thermal injury has on the actin scavenging system.

Thermal injury resulted in a rapid and significant reduction in VDBP from day 1 - day 3 post injury compared to levels in healthy controls (Figure 4.13 A). There was a significant increase in VDBP levels at month 3 post thermal injury relative to healthy individuals (Figure 4.13 A).

Levels of VDBP weakly correlated with DNAse activity across all time points (r = 0.15, p = 0.013). However, there was no significant difference in VDBP kinetics between patients with and without MOF (Figure 4.13 B).

**4.2.10 Thermal injury results in a decrease in circulating gelsolin levels**

Levels of GSN were quantified to fully investigate the effect severe thermal injury has on the actin scavenging system. Levels of GSN were measured in all 64 patients.

Thermal injury resulted in a significant reduction in GSN from day 1 - day 14 post injury (n = 64) compared to levels in healthy control (Figure 4.14 A). Additionally, kinetics of GSN was comparable between patients with and without MOF (Figure 4.14 B). Levels of GSN also weakly correlated with DNAse activity across all time points (r = 0.1331, p = 0.0058). There were no significant differences in circulating
VDBP and GSN levels between septic and non-septic patients following thermal injury (Figure 4.15 A-B)

4.2.11 *In vitro* actin inhibition of DNAse activity can be corrected by gelsolin

As we have shown reduced DNAse activity in patients who developed MOF and an association between serum GSN levels and DNAse activity, we next investigated if administration of GSN could potentially reverse the *in vitro* inhibition of DNAse activity caused by raised actin levels. Incubation of actin spiked serum (5 µM) with 100 µg/ml GSN resulted in a significant increase in DNAse activity comparable with that of actin free serum (Figure 4.16).
Figure 4.13. Levels of VDBP are reduced following thermal injury. (A) VDBP across time (n = 50). Differences in kinetics were compared to data from 10 healthy volunteers using a Mann-Whitney test; *p < 0.005. (B) VDBP across time (n = 50) between patients with (n = 18) and without MOF (n = 32) using a Mann-Whitney test; *p < 0.05. Data is presented as a box and whisker plots and represents median, minimum and maximum values. HC = healthy control, D = day, M = month.
Figure 4.14. Levels of GSN are reduced following thermal injury. (A) GSN levels across time (n = 64) compared to healthy controls (n = 10). Data was analysed by Mann-Whitney test compared to healthy controls *p < 0.005. (B) GSN levels across time comparing those who did (n = 24) and those who did not (n = 40) develop MOF. Differences in kinetics were compared between patient groups using a Mann-Whitney test; *p < 0.05. Data is presented as a box and whisker plots and represents median, minimum and maximum values. HC = healthy control, D = day, M = month.
Figure 4.15. GSN and VDBP levels are not significantly different between septic and non-septic patients. (A) GSN levels across time according to sepsis status (n = 64). Differences in kinetics were compared between patient groups using a Mann-Whitney test; *p < 0.05. (B) VDBP levels across time according to sepsis status (n = 50). Differences in kinetics were compared between patient groups using a Mann-Whitney test; *p < 0.05. Data is presented as a box and whisker plots and represents median, minimum and maximum values. HC = healthy control, D = day, M = month.
Figure 4.16. GSN recovers actin inhibition of DNAse activity *in vitro*. DNAse activity following incubation of actin spiked serum with vehicle control or 100 µg/ml GSN (n = 8). Data was analysed using a paired t test (*p < 0.05). Data is presented as before and after to display changes within independent experiments.
4.2.12 Patient demographics for patients with severe injuries caused by explosions

To investigate if blood products, namely fresh frozen plasma (FFP), can potentially increase DNAse activity by correcting the blood based actin scavenging system, levels of GSN, VDBP and DNAse activity were quantified in plasma from patients following severe traumatic injury caused by explosions. This cohort was split into patients who had (n = 6) or had not received FFP (n = 6) prior to hospital admission and blood sampling. There was no significant difference in ISS, NISS and time to admission following injury between the 2 groups. Full patient demographics can be found in Table 4.2. On average patients received 3 units of blood products before admission to hospital. Due to the nature of this work and the cohort it was not possible to obtain clinical data on outcomes or secondary complications.

4.2.13 Fresh frozen plasma increases gelsolin levels and DNAse activity following severe injury caused by explosion but has no effect on vitamin d binding protein levels

All patients in this analysis were matched for clinical scores of severity and admission times. In the total cohort (n = 12), DNAse activity and VDBP levels were comparable between healthy controls and patients with severe injuries caused by explosions (Figure 4.17 A, C). However, GSN levels were significantly lower in patients with severe injuries caused by explosions when compared to healthy individuals (Figure 4.17 B).

Patients who did not receive blood products before hospital admission had significantly lower DNAse activity and plasma GSN levels compared to healthy controls (Figure 4.18 A-B). However, there was no difference when comparing
plasma VDBP levels (Figure 4.18 C). DNAse activity, GSN and VDBP were comparable between patients who received blood products and healthy controls (Figure 4.18 A-C).

Administration of blood products and FFP before admission to hospital significantly increased DNAse activity compared to patients who had not received blood products upon admission to hospital (Figure 4.19 A). Furthermore, administration of blood products and FFP before admission to hospital significantly increased circulating GSN levels compared to patients who had not received blood products and FFP (Figure 4.19 B). Finally, administration of blood products and FFP did not affect circulating levels of VDBP in both patient groups (Figure 4.19 C).
Table 4.2. Patient demographics for patients with severe injuries caused by explosions (Chapter 4). FFP post or pre admission variables were analysed by Mann-Whitney (continuous variables) or Chi-squared test (categorical variables).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No FFP pre admission (n = 6)</th>
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</tr>
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<td>27 (17-59)</td>
<td>22 (16-42)</td>
<td>ns</td>
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<tr>
<td>NISS (min-max)</td>
<td>36 (18-75)</td>
<td>35 (16-66)</td>
<td>ns</td>
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<tr>
<td>Minutes to admission (min-max)</td>
<td>75 (30-135)</td>
<td>83 (50-130)</td>
<td>ns</td>
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</tbody>
</table>
Figure 4.17. Severe injury caused by explosion caused a significant reduction in circulating GSN levels compared to healthy controls. (A) Plasma DNAse activity in healthy individuals (n = 10) and patients with severe injuries caused by explosions (n = 12). (B) Circulating plasma GSN levels in healthy individuals (n = 10) and patients with severe injuries caused by explosions (n = 12). (C) Circulating plasma VDBP levels in healthy individuals (n = 10) and patients with severe injuries caused by explosions (n = 12). All data was compared by Mann-Whitney test *p < 0.05. Data is presented as a box and whisker plots and represents median, minimum and maximum values. HC = Healthy control.
Figure 4.18. Patients who do not receive blood products before admission to hospital have significantly lower DNAse and GSN levels compared to healthy individuals. (A) Plasma DNAse activity in healthy individuals (n = 10) and patients who did and did not receive blood products before admission (n = 6). (B) Circulating plasma GSN levels in healthy individuals (n = 10) and patients who did and did not receive blood products before admission (n = 6). (C) Circulating plasma VDBP levels in healthy individuals (n = 10) and patients who did and did not receive blood products before admission (n = 6). All data was compared by one way ANOVA and Dunn's multiple comparison test *p < 0.05. Data is presented as a box and whisker plots and represents median, minimum and maximum values. HC = Healthy control.
Figure 4.19. Blood products increase circulating GSN levels and protects against inhibition of DNAse activity. (A) Comparison of DNAse levels at hospital admission between patients with (n = 6) or without (n = 6) prior blood product administration. (B) GSN levels at hospital admission between patients with (n = 6) or without (n = 6) prior blood product infusion at time of blood sampling. (C) Comparison of VDBP activity levels at hospital admission from patients with (n = 6) or without (n = 6) prior blood product infusion at time of blood sampling. For panel A and C data was analysed by unpaired t-test. For panel B data was analysed by Mann-Whitney test. *p < 0.05. For panel A and C, data is presented as a box and whisker plots and represents mean, minimum and maximum values. For panel B, data is presented as a box and whisker plots and represents median, minimum and maximum values. HC = Healthy control.
4.3 Discussion

Circulating levels of cfDNA have been described as a novel biomarker of secondary complications and mortality with studies reporting positive prognostic and diagnostic utility (109, 180, 220, 304-306). In a cohort of 67 patients with severe sepsis, quantification of ncDNA showed positive prognostic utility for predicting 24 hours mortality in patients who presented to the emergency department (354).

Circulating DNA can arise from a number of sources including tissue damage, apoptosis, necrosis and NETosis (114). In our analysis levels of ncDNA within 24 hours of injury correlated with measurements of burn size and severity. Therefore, we propose that the initial elevation in ncDNA is originating from tissue damage caused by the burn injury. Due to the cytotoxic nature of DNA (355, 356), the initial elevation in circulating ncDNA may be contributing to the immediate host tissue and organ damage which occurs in patients with burn injuries. Consistent with previous literature (354), ncDNA levels are significantly higher in septic patients compared to non-septic individuals. We propose that tissue damage, surgery and NETosis may also be contributing to this secondary increase. Interestingly, levels of ncDNA are significantly higher at 12 months following injury in patients who developed sepsis compared to those who didn’t. This elevation is either caused by lower sample numbers in the analysis for 12 month samples, a reactive response by the body in which DNA cannot be cleared or further release from tissue remodelling and scar formation.

NETs and histones form key components of host defence and innate immunity (118, 355, 357). Engelmann and Massberg described an innate immune response
in which coagulation is activated in an attempt to ensnare, recognise and remove bacteria (296, 358, 359). This process is termed immunothrombosis and is in part mediated by the production of NETs (296). NETs are capable of providing both a stimulus to and scaffold for thrombus formation. Uncontrolled or excessive NET generation and immunothrombosis can result in increased risk of thrombosis, host tissue damage and the accumulation of pathogenic components, including DNA, in the microvasculature (226).

Fuchs and colleagues demonstrated that NETs perfused with blood caused recruitment of red cells, increased fibrin deposition, platelet adhesion and activation. Importantly, this could be prevented by incubation with DNase which promotes NET breakdown (232). In a murine model of DVT, Cit H3 was found in close proximity to thrombi. Furthermore, DNA and histones form components of the scaffold required for the pathogenesis of DVT (233). In addition to promoting thrombosis, elevated levels of NET components are cytotoxic and can result in host tissue damage. For example, incubation of activated endothelial cells with NETs results in cell damage which, again, can be prevented by degrading NETs with DNase (360).

Here, we report that ncDNA was elevated from day 1 to day 3 and again at day 14 to 2 months post-injury in patients who developed MOF compared to those who did not. Here we report an accumulation of circulating ncDNA for up to 28 days following injury. We hypothesised that clearance of host and NET derived DNA was dysregulated. Multiple groups have shown that degradation of NETs with DNase can protect against tissue damage, thrombosis and procoagulant interactions (232, 360). Furthermore, elevated levels of circulating DNA are also associated with and contribute to the progression of autoimmune diseases,
notably SLE (237, 238). This build-up of circulating DNA can be explained in part to the impaired DNAse activity caused by the presence of ‘DNAse inhibitors’ or inhibitory antibodies in these patients (237).

As we report a build-up of circulating DNA and reduced ex vivo NET formation we next investigated DNAse activity in patient samples. Here we report a reduction in DNAse activity from day 1 – day 28 post injury in the total cohort. Of note, there was large variation in DNAse activity within the whole cohort at all time points. Furthermore, levels of DNAse activity did not correlate with injury severity. This may be explained by timing of sample, clinical intervention or heterogeneity within the patient cohort. Importantly, DNAse activity was lower in patients who developed MOF or sepsis compared to those who did not develop either. Both groups had comparable reduction in DNAse activity for the first 14 days following thermal injury. Therefore, one might hypothesise that the initial injury causes the immediate reduction in activity reported in both groups and the later reduction may be caused by further tissue damage or surgical procedures. Within this cohort the average amount of individual septic episodes was 2.5 episodes per patient. The average time to first episode was 5 days following injury (range 3 – 70 days) and the time to last episode was 23 days following injury (range 3 – 130 days). Furthermore, whilst most patients underwent surgery within the first 5 days for debridement and immediate treatment of burn wounds, patients did have further surgical procedures if required, e.g. skin graft rejection. Thus, the variation within DNAse activity at the later time points following thermal injury may be explained by the ongoing tissue damage from surgical procedures and sporadic infectious episodes.
It is important to consider the mechanism by which DNAse activity inhibition occurs. Here, we report that levels of total DNAse antigen are increased post thermal injury, which is consistent with existing literature (239). Therefore, the apparent reduction in DNAse activity is indicative of inhibition of enzymatic activity. In this thesis, only levels of DNAse 1 antigen were quantified as this is the predominant enzyme responsible for the degradation of circulating DNA. However, there are different isoforms of DNAse, including DNAse-γ, therefore we cannot exclude the possibility that the reduction in DNAse activity may be mediated in part by reduced DNAse-γ antigen levels. Thus, further study of DNAse isoforms and their contribution to reduced DNAse activity following injury is required.

A naturally occurring inhibitor of DNAse activity is actin, which is an abundant protein in mammalian cells (240). Actin exists in a balance between monomeric and filamentous actin, which is essential for cellular function (241, 242). However, this predisposition of actin to rapidly polymerise is extremely dangerous if it occurs in the circulation. Actin is recognised as a DAMP due to its rapid release, immunostimulatory actions and conserved structure (361). Polymerised actin, F-actin, binds to the DNGR-1 receptor (CLEC9A) and primes cytotoxic T-cells against dead cell antigens (362-364). In addition to its DAMP properties, extracellular actin can cause direct damage to the microvasculature, impair clot lysis and activate platelets (240, 243, 244).

Actin can bind to DNAse forming a stoichiometric 1:1 complex which inhibits enzymatic activity (245, 365). In this analysis circulating actin is detected in patients immediately following and for up to 28 days post-injury. The initial release of actin most likely originates from the extensive tissue damage occurring following severe thermal injury. This will not only inhibit DNAse activity but may
also be responsible for the immediate stimulation of the immune system and host tissue damage that occurs following thermal injury due to its DAMP properties and cytotoxic nature (240, 243, 244, 361-364).

Here we show that actin is detectable for up to 28 days post injury. In Patient 10 (Figure 4.11 B) actin is cleared at day 10 and is then detectable at day 20 - day 23. Therefore, this secondary appearance of actin is not from the initial tissue damage and may be caused by further host tissue damage during surgery, infection or MOF. Patient 10 has been chosen as a representative of this study due to quality of blot. However, this appearance of actin at the later time points is present in multiple patients. This is consistent with previous literature reporting elevated levels of actin in septic individuals (246). This prolonged appearance of actin may be in part responsible for the prolonged inhibition of DNAse activity and may also be responsible for host tissue damage and a pro-thrombotic phenotype associated with actin toxicity. One limitation of these data is the inability to quantify circulating actin by an ELISA based method. Thus, within this analysis we make no attempt to relate circulating levels of actin with DNAse activity, nor do we suggest there is an increase in circulating levels at different time points tested. Further studies are warranted to investigate the relationship between exact actin concentrations and secondary complications in order to study the dynamic kinetic changes and interactions. Furthermore, performing western blot analysis on plasma is extremely difficult and challenging due to the increased concentration of high molecular weight proteins which distort gels and cause high background signal. This has affected the quality of western blot produced and prevents further analysis of blots by densitometry. Therefore, novel and more accurate
methodologies are required to quantify levels of actin in blood products from patients.

Many of the detrimental effects of circulating levels of actin are attributed to saturation of the actin scavenging system. Control of the actin scavenging system is mediated by two key proteins; GSN and VDBP (240). GSN is normally found in high quantities in healthy individuals and can bind to both monomeric and filamentous actin (246, 247). GSN functions to clear circulating actin via 2 distinct processes; preventing further polymerisation or severing existing filamentous actin (240, 249-251). It is therefore not surprising that studies have reported a decrease in circulating levels of GSN in a number of disease pathologies connected with actin release (252-259). Of note, Huang et al reported in 95 patients with thermal injuries that plasma GSN levels were reduced and associated with mortality, development of sepsis and MODS (254). In a rat burn model, plasma GSN levels decreased within 12 hours to 6 days post-injury. This was accompanied by increased pulmonary microvasculature permeability, which was corrected by administration of recombinant plasma GSN prior to burn injury (261). Moreover, administration of GSN can also reverse actin inhibition of lung macrophage binding and uptake of bacteria (260). As GSN functions may extend beyond simply actin scavenging, GSN levels may not only serve as a biomarker of poor outcomes but could also be a potential therapeutic target to reduce or prevent secondary complications.

VDBP is an abundant circulating protein that is a key component of vitamin D transport (262, 263). Unlike GSN, VDBP binds to monomeric actin only (264, 265). Binding of VDBP to actin prevents further polymerisation by rapidly clearing residual monomeric actin from the circulation (266, 267). Levels of VDBP have
been suggested as good prognostic markers of outcome and organ damage following severe trauma (268-271).

Following severe thermal injury there is an immediate and transient decrease in both VDBP, up to 3 days post injury, and GSN, up to 14 days post injury, compared to levels quantified in healthy volunteers. Thus, severe thermal injury results in dysregulation of the actin scavenging system which will facilitate the accumulation of monomeric and polymerised circulating actin which can then cause host damage and inhibit DNAse activity (240, 243, 244, 361-364). Of note, patients with thermal injuries have significantly higher levels of VDBP at 3 months following injury compared to healthy individuals. This overproduction is consistent with previous publications (271) and suggests a reactive response mediated potentially by the injury, recovery, medications or diet.

The decrease in both VDBP and GSN may be caused by a number of mechanisms which include; loss of protein through endothelium dysfunction, dilution during fluid resuscitation or saturation through elevated levels of actin. Severe thermal injury results in systemic endothelial dysfunction and capillary leak (366). Like albumin, it is possible that VDBP and GSN may be lost by this mechanism. However, it is reported that endothelial dysfunction and capillary leak are only present for up to 5 hours post-injury (367). Therefore, this would not explain the decrease seen at 48 hours post-injury in this analysis as capillary leak and endothelial dysfunction will have been corrected. It is also possible that fluid resuscitation may cause the immediate decrease in both GSN and VDBP. All patients received a standardised burn resuscitation protocol as per the Parkland Formula thus this would not fully explain the difference in the later kinetics of GSN and VDBP. Of note, the VDBP ELISA does not bind to VDBP complexed with
actin. Hence, this may explain the initial reduction in VDBP reported within this thesis.

In addition to their mechanistic functions, GSN and VDBP have been proposed as biomarkers of poor outcomes following trauma and burns (254, 268-271). In our cohort, there are no differences in VDBP and GSN levels or kinetics between patients who did and did not develop MOF or sepsis. This is surprising and contradictory to existing literature; however, there are a number of potential explanations. The significant reduction in VDBP and GSN occurred before the onset of sepsis and MOF in this cohort. Therefore, the decrease may solely be caused by immediate actin release following injury due to the severe nature of the injuries included within this analysis. Furthermore, differences may not have been seen due to the severity of injuries included within this analysis, low numbers of patients or differences in timing of sample between studies.

From this analysis, we have developed a hypothesis model depicting how patients are predisposed to the pathogenesis of MOF following severe thermal injury (Figure 4.20). We hypothesise that following severe thermal injury there is the initial release of both monomeric and polymeric actin which results in the rapid consumption of circulating GSN and VDBP, potentially due to extensive tissue damage and actin release. This reduction facilitates the rapid accumulation of circulating actin and binding to DNAse and inhibition of enzymatic activity. All patients are then predisposed to the development of MOF following severe thermal injury but that a second stimulus is required to elevate DNA and reveal this susceptibility. This could arise through repeated surgery with resultant tissue damage or through NETosis in septic patients. This is supported by the growing amount of evidence reporting the involvement of NETs in immunothrombosis (273,
This phenotype will facilitate the build-up of circulating DNA and NET components which further the pathogenesis of MOF by promoting impaired fibrinolysis, thrombotic complications, tissue damage and occlusion of the microvasculature (195, 224, 235, 236). This hypothesis model provides a novel mechanistic link between the initial traumatic injury and subsequent infection in potentially mediating the pathogenesis of MOF.
Figure 4.20. Hypothesis model: The link between severe thermal injury and pathogenesis of MOF through disruption of the actin scavenging system. Following severe thermal injury polymerised and monomeric actin is released which immediately reduces VDBP and GSN levels. The polymerised actin can then bind to DNAse and inhibit its activity. Following infection, NETs are released and accumulate due to reduced degradation. This increases the pathogenesis of MOF.
In 2011, Cohen and colleagues demonstrated the therapeutic potential of GSN in a rat model of sepsis caused by double puncture of the cecum. Administration of 1 mg/ml recombinant human GSN, only once, resulted in significantly improved survival and reduced tissue damage compared to sham animals (369). The group concluded that administration of GSN is a potential therapy to reduce the severity of illness caused by infection and sepsis. They propose that GSN may have an effect on expression and regulation of pro-inflammatory cytokines. However, we would suggest that GSN is having a multifaceted role by regulating cytokine release (369), inflammatory cell function (260) and readdressing the overwhelmed actin scavenging system. We show that administration of GSN in vitro can restore DNase activity in actin spiked serum, providing further evidence that GSN is a potential therapy. However, this experiment is performed in vitro with no contribution from VDBP or any other cellular interactions. Therefore, further experiments are required to investigate the potential of GSN and VDBP combined in vivo.

To investigate this possibility we performed a preliminary retrospective analysis in samples from military patients following severe injury caused by explosions receiving FFP prior to first blood sampling and hospital admission. Severe trauma caused by explosions is becoming an increasingly common form of injury due to conflicts and terrorism. Injuries can be classified into primary to quaternary injuries depending upon severity and mechanism of blast injury (370). Burn injuries are also common among patients with injuries caused by explosions and therefore clinical treatment is comparable.

Patients with severe injury caused by explosions who are hemodynamically unstable receive packed red blood cells and FFP in a 1:1 ratio immediately
following their injuries. By definition, FFP contains high levels of GSN and VDBP which may explain some of the known therapeutic potential in the context of traumatic injury by boosting the depleted levels of the actin scavenging proteins (371). In our preliminary analysis, we have split our cohort into two groups who did or did not receive blood products before admission to hospital. The decision to give blood products was based upon resources available during transportation from the battlefield and not injury severity or mechanism of injury. Importantly, subgroups within this analysis are matched for ISS, NISS and time to admission following injury. Theoretically, the only difference between the two groups is the infusion of blood products.

Here we provide preliminary data showing that early administration of blood products significantly increases GSN levels immediately post severe trauma caused by explosion. This increase in GSN was also accompanied by a significant increase in DNAse activity. However, VDBP levels were not affected. This data would suggest that an early increase in circulating GSN is able to improve DNAse activity rapidly following injury independently of VDBP. In 2005, Chhabra et al showed that the N-terminal fragment of GSN could bind to and disrupt actin-DNAse complexes, in turn, restoring enzymatic activity (372). Therefore, this may be a potential mechanism by which GSN can rapidly restore or enhance DNAse activity independently of VDBP. However, it is important to note that blood products will contain many other soluble factors which may enhance DNAse activity, including DNAse 1 itself. Therefore, the restoration of DNAse activity may not be solely attributed to the increases in GSN. Thus, further studies are required to investigate all components within blood products to fully understand if they are contributing any therapeutic benefit.
Although not common, patients recruited within our primary analysis did receive blood products but only when clinically required. In total 21 patients received at least 1 unit (220 ml) FFP following injury which may be an important confounder in this study. This may also contribute to the variation and lack of difference between patients who did and did not develop MOF. The median time to first unit received was 3 days post injury (range 1 – 57 days post injury) with 4 patients received FFP during the first 24 hours post injury. However, as not all patients received FFP, coupled with the sporadic nature and lack of sustained administration we cannot study any differences between patients who did and did not receive FFP following thermal injury.

These data provide preliminary evidence that early administration of FFP to patients with severe injuries may offer a simple way of boosting the depletion of actin scavenging system and therefore improve DNAse activity. Larger studies are therefore required to fully investigate the therapeutic potential of both FFP and GSN alone post major trauma and severe thermal injury. Given the extensive literature and ongoing debate on the utility of FFP in trauma and resuscitation it may be more applicable to utilise GSN in isolation to scavenge excess actin (373-375). GSN is unlikely to be immunogenic due to the high concentrations found in healthy individuals. Therefore, we propose that administration of FFP (containing GSN) or GSN alone immediately to patients with severe thermal injuries may be a potential safe therapeutic which addresses dysregulation of the actin scavenging system. In turn, reducing the accumulation of DNA and NET components and potentially protecting against the pathogenesis of MOF.

In summary, this chapter presents data of several novel findings and a model of post-injury complications in which DNAse activity is reduced following thermal
injury, driven most likely by raised circulating actin and acute reductions in the actin scavengers GSN and VDBP. The reduced DNAse activity may be contributing to the pathogenesis of MOF by mediating the sustained elevation of circulating DNA from the injury and secondary complications. In addition, two potential therapeutic agents, FFP and GSN, been identified which have the potential to restore DNAse activity and thus protect against host tissue damage associated with MOF.
Chapter 5

General Discussion
General Discussion

5.1 Limitations

Despite showing for the first time the association between and potential mechanisms responsible for dysregulated NET release following severe thermal injury, there are a number of limitations which must be considered.

This study was designed to be exploratory and hypothesis generating in nature and not confirmatory. Hence we cannot estimate a specific outcome or obtain a pre-determined level of precision from our results. Data generated should be regarded as a pilot to obtain data to inform a potential larger confirmatory study.

Furthermore, in this thesis, clinical samples were obtained and analysed at fixed time points. Whilst this remains a strength of the current study it limits the ability to study the full kinetics of many in vivo markers and their relation to secondary complications or clinical treatments. A second study has been established in which blood samples are taken daily over the first 14 days which will allow us to investigate and characterise the daily kinetics of neutrophil function, NET release and diagnostic utility of described biomarkers.

Whilst we have shown the protective role of immediate administration of blood products in patients with severe injuries caused by explosions it remains extremely preliminary data in a small population of patients. Due to the nature of injuries and sensitivity of data it has not been possible to correlate increases in DNAse activity and GSN with improved outcome in this patient cohort. In addition, in this analysis only GSN, VDBP and DNAse activity have been quantified and investigated. Blood products contain many soluble mediators, including DNAse, and therefore
the protection against reduced DNAse activity cannot be solely attributed to increases in GSN levels. Therefore, further characterisation of blood products or the use of GSN and/or VDBP in isolation is required.

5.2 Future Work

Severe thermal injuries constitute a major form of traumatic injury (1). Advancements in the initial care of patients with burns have dramatically improved immediate survival (18, 19, 31). However, delayed mortality associated with sepsis and MOF remain a significant health care problem (38, 47). Although sepsis and MOF are well characterised the mechanisms mediating the increased susceptibility to infection and the pathogenesis of MOF are poorly understood. This thesis has investigated and identified a number of potential mechanisms, therapeutic targets and biomarkers which have the potential to improve patient outcome following severe thermal injury.

Infection and sepsis remain a major clinical burden in patients with severe thermal injuries. Whilst this has been reported, the mechanisms responsible for the increased susceptibility and incidence are poorly understood. Data from this thesis reports a reduction in ex vivo NET production and intracellular ROS formation which may underlie the inability to clear pathogens and be in part responsible for the increased incidence of infections (Figure 5.1 A). The exact mechanism by which this occurs is yet to be described but possible factors include; abnormal neutrophil maturity phenotype or release of soluble mediators (e.g. mtDAMPs) (Figure 5.1 B). Thus, understanding the mechanisms responsible for reduced neutrophil function may aid in both the early identification of at risk patients and
development of novel therapeutics which reduce infection and secondary complication rates following severe thermal injury.

Furthermore, data within this thesis identifies a major biological mechanism driving MOF after major trauma, namely the compromise to the actin scavenging system which leads to reduced DNAse activity and a build-up of cfDNA. NETs are generated during sepsis in patients with thermal injuries. The exact mechanism of NETosis reamins to be identified, however, potential mechanisms include direct interactions with bacteria or inflammatory cells (Figure 5.1 C). Included in this thesis is a model of post-injury complications in which DNAse activity is reduced following thermal injury caused by actin release from host tissue damage and dysregulation of the actin scavenging system (Figure 5.1 D). Our data provide a novel mechanistic link between the initial traumatic injury and subsequent infection in potentially mediating the pathogenesis of MOF (Figure 4.20). Although our study is based in burns patients it is likely to have relevance to all major trauma and to support this, pilot observational trial data from patients with severe injuries caused by explosions suggests that DNAse activity can be restored by the prehospital administration of FFP. MOF is a leading cause of mortality following severe thermal injury and data within this thesis identifies several potential novel therapies to overcome the suppression of DNAse activity and improve outcomes after trauma.

Future work investigating potential therapeutics to modulate abnormal neutrophil function and the actin scavenging system is described below in priority order.
Figure 5.1. Hypothesis model: Linking initial injury to secondary complications following severe thermal injury. (A) Severe thermal injury significantly reduces, both, *ex vivo* NET formation and ROS generation. (B) This reduction is potentially mediated by a phenotypical change in the circulating neutrophil pool, soluble mediators *e.g.* mtDAMPs or functional neutrophils have potentially migrated to the tissues leaving a non-functioning pool of circulating neutrophils. (C) During infection, NETs are generated following thermal injury. Potential mechanisms include direct induction through bacteria or through neutrophil interactions with inflammatory cells. (D) Thermal injury results in dysregulation of the actin scavenging system which facilitates the build-up of circulating DNA, from initial injury and NETs, which are potentially responsible for the pathogenesis of MOF. Panel D has been presented earlier in this thesis (Figure 4.20).
5.2.1 *In vivo* characterisation of NETosis

Levels of cfDNA are associated with cellular damage and secondary complications in a number of disease pathologies (108, 376). However, it is important to consider the nature and structure of circulating DNA as it is released, complexed with histones, in nucleosomes following NETosis. Whilst this is known, the broad effects of free histones, free DNA and nucleosomes are described interchangeably whereas there is a clear difference in their biological activity (377).

Free histones are cationic, highly conserved between species and orchestrate gene transcription. Intravenous administration of free histones results in rapid mortality of animals which can be prevented using an anti-histone antibody (198). Histones can also mediate cytotoxic damage to endothelial and epithelial cells via activation of TLRs (197). Whilst the mechanism by which histones are cytotoxic remains unclear, studies have proposed that their cationic nature facilitates the direct binding of histones to cells resulting in perforation, cell death and damage caused by calcium influx (195, 196).

One potential therapeutic to reduce histone cytotoxicity is the administration of negatively charged molecules such as heparins (378, 379). The non-anticoagulant form of unfractionated heparin, for example, has been trialled as a novel therapeutic in sepsis (378). Whilst this study did not report an improvement in 28 day mortality, Wildhagen and colleagues report a beneficial effect of this novel therapy in mediating the inhibition of histone cytotoxicity (378). During sepsis there is an abnormal consumption coagulopathy and thus an increased risk of bleeding. Hence, administration of a non-anticoagulated form of heparin may have promising effects on reducing tissue damage mediated by histones during sepsis without potentiating bleeding.
DNA is regarded as a DAMP due to its rapid release and stimulation of immune processes (199). The origin of DNA, nuclear, mitochondrial or bacterial, affects its biological activity. In 2015, Bhagirath and colleagues isolated DNA from septic patients and determined the different function of nuclear, mitochondrial and bacterial DNA on coagulation and inflammation. All three sources of DNA were capable of inducing thrombin formation, by an intrinsic dependent pathway mechanism, and activation of platelets by platelet integrin αIIbβ3. Although, all three have similar procoagulant properties, their functions in initiating inflammation differ. Whilst, ncDNA and mtDNA prolonged in vitro neutrophil life, bacterial DNA did not. However, only bacterial DNA promoted in vitro secretion of IL-6 (206).

Due to their opposing charges, DNA and histones will bind together and form a nucleosome complex with an overall neutral charge. Like free histones and DNA, nucleosomes can activate neutrophils (380). However, nucleosomes have different cytotoxic properties compared to free histones and DNA. Unlike free histones, administration of nucleosomes does not result in a significant increase in mortality (204, 205). This may be explained by the inability of nucleosomes, unlike histones, to cause direct damage or cell death to cultured endothelial cells. However, subsequent physical or enzymatic degradation of nucleosomes then resulted in damage to endothelial cells (381). Thus components of nucleosomes, histones and DNA, are then responsible for the cellular damage and this effect is nullified when components are complexed. In this thesis, we have investigated three potential sources of DNA; nuclear, mitochondrial, and NET derived. However, the status by which they exist, free or complexed, has not been fully investigated. Thus, further study is required to investigate the relationship between
free histones, DNA and nucleosome complexes with secondary complications and host tissue damage.

In 2013, Abrams et al reported the potential therapeutic benefit of CRP in neutralising in vivo and in vitro histone induced endothelial damage, enhanced coagulation and increased vascular permeability (381). Supported by earlier work (382, 383), CRP is able to compete with phospholipid-containing liposomes and form CRP-histone complexes, confirmed using immunofluorescence staining and a gel overlay assay. The formation of CRP-histone complexes reduced integration of histones into the cellular wall of endothelial cells and in turn cellular damage. The exact mechanism of binding between CRP and histones remains to be identified, however, the authors propose that the positively charged histones are likely binding the negatively charged central pore of the CRP pentamer (381). Interestingly, it has yet to be established if CRP-histone complexes have increased clearance. Thus, Abrams and colleagues demonstrated, for the first time, that CRP is a conserved mechanism which protects the body against histone toxicity in the acute phase following trauma and illness (381). Hence, it may be possible to use CRP, or a therapeutic mimicking CRPs mechanism of action, as an intervention to neutralise the cytotoxic effect of circulating histones following severe thermal injury.

As described above cfDNA can originate from a number of sources (114). In 2016, Lehmann-Werman and colleagues described a novel technique by which the tissue specific origin of cfDNA could be elucidated (384). It is likely that following thermal injury circulating DNA is heterogeneous in origin given the severe nature of injury, surgical procedures, infection, host tissue damage and NET release. Despite the shared homology in nucleotide sequences, DNA also has unique
methylation patterns depending upon the cell or tissue of origin (385). Thus, it is possible to infer cell death within specific organs and its contribution to the circulating cfDNA. As proof of principle, Lehmann-Werman and colleagues detected pancreatic β-cell DNA in patients with diagnosed type-1 diabetes and islet-graft recipients, neuronal or glial DNA in patients with traumatic brain injury or cardiac arrest and exocrine pancreas DNA in pancreatic cancer or pancreatitis patients (384). Given the exploratory nature of this study, however, they were unable to conclusively evaluate the diagnostic or prognostic utility of methylation patterns. However, investigation of methylation patterns in patients following thermal injury may have the potential to aid in the accurate diagnosis of specific organ dysfunction and responses to clinical treatment.

5.2.2 DNAse isoforms and their functions

In 1997, Rodriguez and colleagues described three members of a DNAse 1 family. All three displayed similarity in their nucleotide, amino acid sequences (386) and biochemical properties, dependency on Ca$^{2+}$ and Mg$^{2+}$ (387). DNAse 1 is the major endonuclease responsible for the breakdown of circulating nucleic acids. However, in recent years groups have begun investigating the physiological roles of DNAse-γ (386-388). Like our analysis, many assays established to measure DNAse activity, therefore, quantify total DNAse. Furthermore, most groups focus upon the role of DNAse 1 alone and thus do not investigate the role of DNAse-γ (237, 353, 389).

As it functions internucleosomally and degrades nuclear chromatin, DNAse-γ was originally reported to be involved in apoptotic DNA fragmentation (390-392). However, cells transfected with DNAse-γ can secrete this enzyme thus suggesting
a mechanism by which exocytosis can occur (393). Consequently, DNase-γ, potentially from monocytes (394), may contribute to total circulating DNase activity.

DNase 1 and –γ function synergistically to clear total circulating DNA through distinct substrate consumption and degradation. DNase 1 has a higher affinity and accelerated clearance of free DNA compared to DNase-γ which is more efficient at degrading chromatin (387, 395).

Recent studies report a clear link between genetic mutations of DNase-γ and development multiple autoimmune diseases (396-398). Highlighted in DNase-γ mice which develop an SLE phenotype associated with the presence of autoantigens against chromatin (399). Although DNase-γ has been related to autoimmunity, there have there have been no investigations into its function and potential role in secondary complications following trauma or severe burn. Thus, further work could investigate the combined and individual functions of DNase-1 and –γ activity in the degradation of chromatin and DNA release following severe thermal injury. This may reveal additional mechanistic information and/or novel therapeutics to reduce secondary complications driven by elevated circulating DNA or chromatin.

5.2.3 Targeting the build-up of toxic and pro-thrombotic DNA following thermal injury

Following thermal injury, there is the disruption of the actin scavenging system which predisposes patients to the accumulation of tissue derived and NET derived DNA (Figure 4.20). This can then facilitate further host tissue damage, obstruct blood flow and induce thrombosis (233, 236, 360). Modulation of this system by
targeting DNAse, GSN or VDBP has the potential to protect against this pro-thrombotic and host cytotoxic phenotype.

5.2.4 DNAse as a therapy

DNAse is the major extracellular endonuclease found in a number of bodily fluids and is responsible for the breakdown and clearance of circulating chromatin and DNA. Severe thermal injury results in the immediate release of DNA which is followed by a secondary release during infection. Both populations of DNA are implicated in the generation of thrombosis, host tissue damage, endothelial damage and secondary complications in trauma patients and animal models of thrombosis (232, 233, 356, 400). Thus, targeting total DNA accumulation by administration of DNAse may provide therapeutic benefit.

Using a murine model of DVT, extracellular chromatin formed a scaffold upon which venous thrombosis could occur and was involved in the pathogenesis of the DVT. Administration of DNase 1 was capable of protecting animals from DVT (233). Furthermore, PAD4 deficient mice form fewer thrombi after inferior vena cava stenosis compared to wild-type animals (401). Thus confirming the role of NET derived DNA in the propagation of venous thrombus formation. In addition to their procoagulant activity, DNA, derived from NETs, can also cause direct damage to endothelial cells (360). Interestingly, coculture of neutrophils with activated endothelial cells results in NETosis which caused direct cellular damage thus it appears to be self-propagating. This could also be abrogated by the degradation of NETs using DNAse (360). These data provide evidence that administration of DNAse to target host derived and NET derived DNA may have potential to alleviate host tissue damage and reduce the pro-thrombotic
phenotype. Importantly, administration of DNAse 1 is a licenced therapeutic in patients with cystic fibrosis and used to reduce the viscosity of sputum through direct degradation of NET derived DNA.

It is also very important to consider the potential complications associated with DNAse. Administration of DNAse in a murine model of sepsis resulted in an increased pro-inflammatory response along with increased colonisation of bacteria and mortality (221). This was further confirmed by Mai et al who highlighted the importance of timing when targeting NETs. Early administration of DNAse resulted in increased mortality, whereas later administration reduced tissue damage and decreased circulating DNA levels resulting in improved survival (402). These data provide evidence that DNA should only be broken down when in excess and contributing an enhanced pro-inflammatory and thrombotic phenotype. Given the clinical complexity and heterogeneous nature of patients following severe burns this may be challenging. Furthermore, as actin is released following severe thermal injury this may inhibit any administered DNAse, rendering the therapy potentially non-effective. Hence, a more targeted approach towards NETs or modulation of the actin scavenging system may be more applicable.

5.2.5 Inhibition of PAD4

NETosis can also be prevented by inhibition of PAD4. Indeed, PAD4 knockout mice are totally protected against venous thrombosis formation and tissue damage following liver injury (225, 275). However, these studies do not take into account the pre-existing immunosuppression which occurs following thermal injury. Therefore, elimination of NETs completely could leave, an already immunocompromised patient, susceptible to further infection. Moreover, PAD4
deficient mice are more susceptible to necrotizing fasciitis infection than wild-type mice and this susceptibility is attributed to the inability to form NETs (276).

5.2.6 Modulation of actin scavenging system

The actin scavenging system is comprised of two key proteins; VDBP and GSN. Following severe thermal injury, there are reductions in circulating levels of both proteins which predispose patients to reduce DNAse activity and the build-up of circulating DNA (Figure 4.13 - 4.14). As both VDBP and GSN are extremely abundant within the blood they are unlikely to be toxic if used as direct therapeutics.

One potential mechanism to replenish depleted VDBP and GSN is through administration of blood products, namely FFP. Although not known, replenishment of the actin scavenging system may play a role in a number of the reported therapeutic benefits of using blood products following major trauma (371). In this thesis, administration of FFP during transportation from site of injury to hospital in major trauma victims improved DNAse activity in addition to increasing circulating GSN levels but without increasing circulating VDBP levels (Figure 4.19). Whilst this data suggests a role of GSN in recovery of DNAse activity any improvement by FFP will be multifactorial and caused by a number of soluble factors which may enhance DNAse activity, including DNAse 1 itself. Therefore, the restoration of DNAse activity cannot be solely attributed to the increase in GSN. Thus, further studies are required to investigate whether other components within blood products are contributing.

Whilst VDBP remains a potential therapy, to date, no group has investigated the effect of administration of VDBP on outcome following trauma or burns. Despite
this, large quantities of protein can be produced from plasma fractions (403). Following thermal injury GSN and VDBP both become saturated, most likely caused by the release of monomeric and polymeric actin. Administration of GSN and VDBP as a combination therapy may therefore aid in the rapid recovery of the actin scavenging system.

There is growing evidence for the use of GSN in the treatment of infection, trauma and burns (261, 404-406). In 2007, Lee and colleagues reported a reduction in mortality and a dynamic change in cytokine release in GSN depleted mice that were given 20 mg/mL recombinant human plasma GSN after challenge with endotoxin. Although the mechanism responsible remains unclear, improvements in outcome were associated with depolymerisation of actin in these animals (404). Interestingly, levels of total circulating actin did not decrease which suggests that GSN can modulate the activity of actin independent of VDBP which is consistent with previous literature (407, 408).

A clinical trial has been established to assess the pharmacokinetics and safety of recombinant plasma GSN given to patients admitted to an ICU, including patients with trauma, infection and burns. The hypothesis of the study states that administration of GSN will increase circulating GSN levels and decrease the incidence of secondary complications, e.g. MOF or death (Trial Number: NCT00671307). Results for this study are not currently available, however, a second study may be warranted to focus upon patients admitted to an ICU with a dysregulated actin scavenging system.

Whilst the beneficial effects of GSN alone have been described (261, 404-406), the cumulative benefit of GSN with VDBP or DNAse have not. Given the
saturation and dysregulation of the actin scavenging system, one might hypothesise that a combination therapy may be more appropriate. Furthermore, it may be possible to produce novel therapies which contain the active N-terminal fragment of GSN which is responsible for its ability to restore DNAse activity independently of VDBP (372). Therefore, one might propose the development of a novel compound which mimics the N-terminal fragment of GSN for its use in a clinical trial investigating early and sustained administration of GSN and its effects on DNAse activity and outcome following severe thermal injury. Of note, GSN function also extends beyond control of actin homeostasis and as a potential use as a biomarker of poor outcome as it also exhibits various anti-inflammatory activities (260, 408). Thus, understanding the mechanism(s) by which GSN provides benefit is fundamental to its development as a therapy.

5.2.7 Targeting reduced neutrophil function following thermal injury

The association between severe thermal injury and abnormal neutrophil function has been well reported (156, 159-161, 173, 174, 177). Furthermore, this reduction or dysregulation in neutrophil function may underlie the increased incidence of nosocomial infections reported within these patient cohorts. In this thesis, it has been shown that measurements of abnormal neutrophil function are potential biomarkers of sepsis, in addition to being novel therapeutic targets.

5.2.8 Granulocyte colony-stimulating factor and neutrophil maturity

Traumatic injury induces marked alterations in haematopoiesis which is characterised by a repolarisation towards a myeloid lineage (409, 410). Production and maturation of neutrophils is multifaceted with the principle regulator of granulopoiesis being G-CSF (162, 163) which causes activation of signal
transducer and activator of transcription 3 (STAT3), via MEK-1/-2 and ERK-1/-2 (411, 412).

Severe thermal injury results in the release of IGs/neutrophils which are associated with sepsis status and reduced neutrophil function (Chapter 3, Figure 3.14, 3.17 and 3.18). One limitation of the work presented in this thesis is the lack of mechanistic insight responsible for the release of IGs following thermal injury. This release may be induced by emergency granulopoiesis caused by excessive stress from immediate injury and consequent infectious episodes. In a study which included 83 severely injured patients, sustained elevation in G-CSF was associated with the release of immature cells and increased risk of infection (157). In addition, Ertel and colleagues report an inhibition of neutrophil apoptosis caused by local G-CSF which propagates damage caused by neutrophils at sites of injury (413). Therefore, one might propose that inhibition of G-CSF can potentially reduce circulating IGs and protect against, neutrophil mediated, immunosuppression.

Conversely, administration of G-CSF has positive effects in mice (168, 414). Namely, an increase in neutrophil chemotaxis in animals who received recombinant G-CSF following burn injury which was attributed to a combination of myeloid expansion and direct enhancement of neutrophil function (414). Gardner et al report a key role for G-CSF/STAT3 axis in providing protection against post-traumatic infection which was consistent with human gene analysis. Paradoxically, thermal injury resulted in protection from a lethal Klebsiella pneumoniae pulmonary challenge. Inhibition of G-CSF reduced STAT3 activation and diminished this protective response by preventing myeloid differentiation and neutrophil release (168). Thus, caution must be advised when targeting G-CSF
following severe thermal injury as it potentially exhibits multiple roles in host protection and innate immunity (157, 168, 413, 414). Therefore a more targeted approach modulating neutrophil maturation may be more applicable.

5.2.9 Resolvins

Acute inflammation is necessary for the host to respond to initial tissue damage or invading pathogens and is required to maintain host protection and homeostasis. However, if inflammation is prolonged or uncontrolled it can lead to host tissue damage and secondary complications (415). Resolution of inflammation was long characterised as an eventual reduction of pro-inflammatory mediators by a passive process. As reviewed by Serhan and colleagues, resolution of inflammation is now considered an active and responsive process controlled by the release of a number of negative regulators (416). Resolvins, protectins and lipoxins are three examples of anti-inflammatory mediators which induce resolution and containment of acute inflammation (417, 418).

Resolvins are named after their role in the resolution of inflammation and produced from eicosapentaenoic or docosahexaenoic acid, the most abundant omega-3 polyunsaturated fatty acids (417, 419). Administration of resolvin D2 (RvD2) can enhance both neutrophil phagocytosis and killing of engulfed E.Coli (420). In a cecal ligation and puncture model of sepsis, treatment with RvD2 causes a reduction in leukocyte-endothelial interactions, cytokine production, and a reduction in local and systemic bacterial burden (420). Furthermore, Kurihara et al reported a restoration of neutrophil chemotaxis following burn injury when animals were administered RvD2 which was attributed to the direct action of RvD2 on neutrophils (421). Whilst it is known that resolvin D1 can act through ALX/FPR2
and GPR32 G-protein coupled receptors (422), the receptors responsible for RvD2 actions are currently unknown. Hence, further characterisation of RvD2 and its receptors is essential to understand the mechanisms by which immunoregulation and enhancement of neutrophil function occur. Nevertheless, RvD2 may be a potential therapeutic to reduce both the uncontrolled inflammation and reduced neutrophil function which occurs following severe thermal injury.

5.2.10 Haemoperfusion therapy

Direct haemoperfusion therapy can filter cells or soluble mediators from the blood of patients. This results in reduced uncontrolled activation of the immune system and a reduction in host tissue damage (423, 424). Whilst direct haemoperfusion therapy is a recognised treatment for septic shock in Japan, only parts of Western Europe currently use this technology (425). Direct haemoperfusion therapy using a polymixin-B immobilized fibre cartridge (PMX-DHP) primarily works by removal of endotoxin from the blood. However, recent evidence has shown a broader mechanistic role for PMX-DHP in the removal of activated neutrophils which can cause endothelial damage (424). Whilst the mechanism responsible for selective removal of activated cells remains unclear the authors propose that it is caused by a direct interaction between activated neutrophils and polymixin-B located on the fibres of the filter (424). Thus, depletion of activated neutrophils by PMX-DHP may help to control inflammation in patients with septic shock.

More recently, Lee and colleagues developed a microfibre mesh decorated in polyethyleneimine and polyamidoamine dendrimers which can capture DAMPs, including extracellular DNA. In vitro immobilisation of DAMPs located within blood from trauma patients results in reduced activation of toll-like receptor (TLR) -2, 3, 4
and 9 compared to unfiltered serum in a monocyte derived reporter cell line. Furthermore, immobilisation of DAMPs in hearts transplanted in vivo abrogated thrombus formation (423). In this thesis, DAMPs cause dysregulation of neutrophil function in vitro which mimics our in vivo findings in patients with severe thermal injuries (Figure 3.21 - 3.25). Hence, immobilisation of DAMPs using the microfibre mesh decorated in polythyleneimine and polyamidoamine dendrimers has the potential to protect against dysregulation of neutrophil function following thermal injury and reduce thrombotic complications associated with elevated DNA and histones. However, direct haemoperfusion therapy using polythyleneimine and polyamidoamine dendrimers remains experimental with limited translation to clinical practice due to difficulty in integrating these microfibre meshes into available technology.
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