

**STEROIDS AND IMMUNITY FROM INJURY
THROUGH TO REHABILITATION
(SIR Study)**

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Abstract

There are over one million deaths from road traffic collisions. In Afghanistan, there have been 2005 UK battle injuries over 10 years. Advances in military trauma care have improved survival, resulting in more severely injured individuals entering the trauma care pathway. Improved understanding of immunoendocrine changes after severe trauma may facilitate novel interventions to improve outcomes. We prospectively recruited 102 severely injured patients at the QEH Birmingham; 52 military and 50 civilian patients with a mean Injury Severity Score of 27.2 ± 13.9 . Blood and 24-hr urine were collected at baseline (injury < 24h) and at regular intervals from while in hospital and at 3, 4, and 6 months. Results demonstrated a reduced neutrophil function following a surge of DAMPs and cytokines that were released into the circulation. Both DHEA and DHEAS were significantly down-regulated ($p < 0.0001$). Serum testosterone was initially completely suppressed ($p < 0.0001$) but normalised after week 4. Protein and muscle loss followed a U-shaped curve; catabolism began to recovery 4-6 weeks following injury. In conclusion, the acute response to severe injury comprises increased glucocorticoid activation and down-regulation of adrenal and gonadal androgens. Delineation of whether the endocrine changes are beneficial or adverse will determine the potential for future intervention studies.

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List of Abbreviations

AAS	Anabolic androgenic steroids
ACF	Auto-correlation factor
ACTH	Adrenocorticotrophic hormone
ADH	Anti-diuretic hormone
AIC	Akaike's Information Criterion
ANOVA	Analysis of variance
APACHEII	Acute physiology and chronic health evaluation II
ARDS	Acute respiratory distress syndrome
ARDS CTN	Acute respiratory distress syndrome clinical trials network
ATLS	Adult trauma life support
ATP	Adenosine tri-phosphate
BALTI-2	Beta-Agonist Lung Injury Trial-2
BMP	Bone matrix protein
BSA	Bovine serum albumin
CARS	Counter Anti-inflammatory Response Syndrome
CBG	Cortisol-binding globulin
CCAST	Critical Care Air Support Team
CDBI/INF	Draft Additional Protocol to the Council of Europe Convention on Human Rights and Biomedicine on Biomedical Research
CD	Cluster of differentiation
CLR	C-type lectin Receptors
CNS	Central nervous system
COPD	Chronic obstructive pulmonary disease
CORD	Chronic obstructive respiratory disease
CORTICUS	The Corticosteroid Therapy of Septic Shock
CRASH	Corticosteroid randomisation after significant head injury

CRASH-2	Clinical Randomisation of an Antifibrinolytic in Significant Haemorrhage
CRF	Corticotropin-releasing factor
CRH	Corticotropin-releasing hormone
CRP	C-Reactive protein
CSF	CSF Cerebrospinal
CTIMP	Clinical Trial of an Investigational Medicinal Product
CT	Computer Topography
CYB11B	11 β -hydroxylase
CYP11A1	Cytochrome P450 11 hydroxylase
CYP17A1	Cytochrome P450 17 hydroxylase
CYP21A2	Cytochrome P450 21 hydroxylase
CYP19A1	P450 aromatase dehydrogenase
DAMP	Damage associated molecular pattern
DASA	Defence Analytical Services and Advice
DCR	Damage control resuscitation
DCS	Damage control surgery
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone sulphate
DHT	Dihydrotestosterone
DMRC	Defence Medical Rehabilitation Unit
DMS	Defence Medical Service
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
EPC	Endothelial progenitor cell
ESC	Embryonic stem cell
FACS	Fluorescence-activated cell sorting

FAST	Focused Assessment with Sonography for Trauma
FFP	Fresh Frozen Plasma
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
FSH	Follicle stimulating hormone
GSW	Gun shot wound
HLADR	Human leukocyte antigen DR (isoform)
HMG	High-Mobility Group
HOSPEX	Military Hospital Exercise
HPA	Hypothalamic–pituitary–adrenal
HRP	Horseadish peroxidase
HSC	hematopoietic stem cell
HSD	Hydroxysteroid dehydrogenase
HSS	High strength silica
ICF	The International Classification of Function, Disability and Health
ICU	Intensive care unit
IED	Improvised explosive device
IFN	Interferon
IGF	Insulin growth factor
IGFBP	Insulin growth factor binding protein
IQR	Interquartile range
ISS	Injury severity score
LOS	Length of stay
LPS	lipopolysaccharide
MAPK	Mitogen-activated protein kinases
MCA	Mental Capacity Act
MDSC	Myeloid derived suppressor cells

MERT	Medical Emergency Response Team
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MIP	Macrophage Inflammatory Proteins
MOD	Multi-organ dysfunction
MODS	Multi-organ dysfunction syndrome
MOF	Multi-organ failure
MOST	Military <i>Operational Surgical Training</i> course
MPO	Myeloperoxidase
MRSA	Methicillin resistant staphylococcus aureus
MSC	Mesenchymal stem cell
MTBE	Tert-butyl-methyl-ether
mtDNA	Mitochondrial derived DNA
NADPH	Nicotinamide adenine dinucleotide phosphate
NCEPOD	The National Confidential Enquiry into Patient Outcome and Death
NET	Neutrophil extracellular traps
NHS	National Health Service
NICE	National Institute for Health and Care Excellence
NIHR	National Institute for Health Research
NISS	New injury severity score
NLR	Node-Like Receptors
NMR	Nuclear magnetic resonance
NOD	Nucleotide-binding oligomerization-domain protein
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	polymerase chain reaction

PDGF	Platelet derived growth factor
PICS	Persistent inflammation, immunosuppression, and catabolism syndrome
PIS	Patient information sheet
PKA	Protein kinase A
PLS-DA	Partial least squares discriminant analysis
PMA	Phorbol-12-myristate-13-acetate
PMN	Polymorphonuclear
POISE	Peri Operative ISchemic Evaluation Trial
PROWESS	Recombinant Human Activated Protein C Worldwide Evaluation in Severe Sepsis
PRR	Pattern recognition receptor
QEHB	Queen Elizabeth Hospital Birmingham
RAGE	Receptor for advanced glycation end products
RBC	Red blood cell
RCT	Randomised controlled trial
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RTC	Road Traffic Collusion
SAPS II	Simplified Acute Physiology Score II
SGCNS	Surgeon General's Casulaty Nutrician Study
SIR	Steroids from Injury Through to Rehabilitation
SIRS	Systemic Inflammatory Response Syndrome
SOD	Superoxide dismutase
SOFA	Sequential Organ Failure Assessment
SPSS	Statistical Package for the Social Sciences
SRD5A	5 α -reductase isoenzymes
SRMRC	Surgical Reconstruction and Microbiology Research Centre

SSC	Side scatter
STS	Steroid sulfatase
SULT2A1	Sulfo-transferase
TBI	Traumatic brain injury
TBSA	Total body surface area
TGF- β	Transforming growth factor
TLR	Toll-like receptor
TMB	Tetramethyl benzidine
TNF	Tumour necrosis factor
TNP	Topical negative pressure
TRISS	Trauma and Injury Severity <i>Score</i>
TSH	Thyroid stimulationg hormone
TXA	Tranexamic acid
UHB	University Hospital Birmingham
USA	United States of America
UUE	Urinary Urea Excretion
WMA	World Medical Association

Chapter 1: Introduction

1.0 Trauma – the size of the Problem.

Trauma is the leading cause of the death in the first four decades of life (1), with approximately 20,000 severely injured people each year in England alone, resulting in 5400 deaths. Another 28,000 injuries not classed as severe should be treated in the same way (2). In 2011, worldwide there were 1.21 million deaths from Road Traffic Accidents (3). In addition and of relevance to this thesis, in Afghanistan between Jan 2001 to Jan 2014 there were 447 deaths of UK military personnel. Of the 2173 battle injuries there were 610 seriously or very seriously injured (4).

1.1 Military Trauma Care System

Lorne Blackborne summarises in three papers the approach that the UK/US military medical services have adopted in the treatment of injured personnel (5-7). In the Defence Medical Services (DMS), improvement to First Aid and Field Medic Training and the introduction of the Medical Emergency Response Team (MERT) have meant timely delivery of an alive patient to the Field Hospital in Camp Bastion. Improvements in early survival from military major trauma have been achieved using blood component therapy (8,9) for resuscitation at the scene and during transfer by the MERT, and the early implementation of CRASH-2 (10) evidence. Reducing time to definitive injury diagnosis and treatment has been achieved through improved collective training and availability of Focused Assessment with Sonography for Trauma (FAST), CT scanner and an on-site Radiologist. Following accurate diagnosis, Damage Control Resuscitation and Surgery (DCR & DCS) is performed, and the casualty is then better-prepared to return to the Birmingham Level 1 Trauma Centre using Critical Care Air Support Team (CCAST)(11) within 24-48h of injury.

Appreciating the value of high performance teams in delivering health care, the DMS provides high-fidelity simulation training before deployment to conflict areas on military Hospital Exercise (HOSPEX) (12) and Military Operational Surgical Training (MOST) course(13).

Improvements in care have resulted in more survivors with an Injury Severity Score (ISS) which would previously have predicted death (14). This means that a greater proportion of traumatised patients are surviving severe catastrophic injuries(8,9), but must endure a protracted time to healing(10), long intensive care unit (ICU) admission, Multi-Organ Dysfunction/Failure(12), and repeated episodes of infection(13) that may compromise their immune system long term (15). However, the majority of patients recover and leave ICU to be nursed on a military themed ward before care is transferred to the Defence Medical Rehabilitation Centre (DMRC) Headley Court in Surrey.

1.2 NHS Trauma Care System.

The NCEPOD document, 'Trauma: Who Cares' drove the development and implementation of Level 1 Trauma Centres in the UK (11). It highlighted that in 60% of cases patients received a standard of care that was less than good because of clinical and organisational failings. The solution was to bypass District General Hospitals and establish high-volume consultant-led centres which had all the necessary specialties to treat the severely injured 'under one roof'. The subsequent establishment of 22 UK Trauma Centres in 2012 (14) was estimated to save 600 lives a year in England through better organised services and timely transfer of patients to

a centre that can deal with severe injuries. NHS England reported audit work carried out by Manchester University looking at how the Trauma Networks have been performing since their inception (16). They found 20% improvement in odds of surviving and quicker transfer of patients in to the MTCs.

1.3 Injury Severity Scoring

Outcomes of trauma since Imhotep, around the 27th century BC(572). Homer, around 850BC, was able to determine the risk of mortality from a casualties injuries(573). Modern trauma injury scoring was done by De Haven's in an attempt to objectively measure injury following light aircraft crashes(574) but most modern systems use the Abbreviated Injury Scale (AIS) which was developed to analyse fatalities following RTC.

Scoring systems can be separated into 3 groups:

1. Anatomical.
2. Physiological.
3. Combination of Anatomical and Physiological.

The Anatomical group can also be subdivided into consensus or data derived(575). The consensus derived scores are based on AIS codes (such as ISS/NISS), whereas the data-derived are survival probabilities calculated for each injury code from a large trauma database (such as ICISS). Alternative categorisations can be done by their application: scoring of injury severity for outcome prediction (such as ISS), scoring of injury distribution (AIS), or following a clinical course (APACHE)(574).

1.3.1 Abbreviated Injury Scale (AIS)

The Committee on Medical Aspects of Automotive Safety developed a tool first published in 1971 to rank injury severity in Road Traffic Collisions. The score is used to indicate the relative risk of threat to life in an average person who sustains the coded injury as his or her only injury. The AIS injuries are ranked on a scale of 1 to 6; minor, moderate, serious, severe, critical, and fatal(576).

Initially only for blunt injuries but now including all injuries, the AIS 2005 Update 2008 is the latest. AIS on its own only retrospectively describes a 'relative' risk to life and it is unable to predict mortality, complications or direct aid treatment(577). AIS only describes one injury in each body region, requires coders to be trained to apply AIS codes, it is resource intensive, an unfortunate 'side-effect' is that the updates have led to discrepancies in codes across different versions(578-9).

1.3.2 Injury Severity Score (ISS)

Soon after the AIS was published, Baker *et al.* (580) developed the Injury Severity Score (ISS) in order to improve on the AIS by accounting for multiple injuries in a single patient. The ISS uses the AIS 1-6 score from three different body regions out of six (which can be head & neck, face, chest, abdomen, extremity, external). These three scores are squared and then added together to give the ISS which can be between 1 -75 ($ISS = a^2 + b^2 + c^2$). ISS scores above 15 are considered to be major/polytrauma(581)The ISS was reported by its own developers to be a good predictor of mortality in a group of 2128 people involved in Road Traffic

Collisions(580). Still used regularly to assess trauma it failed to account for another AIS in the same region that may well represent the injury severity more closely.

1.3.3 New Injury Severity Score (NISS)

In 1995, Professor Baker proposed a modification to the protocol of calculating the ISS(581). The altered protocol allowed the use of the three worst injuries regardless of body region and a New Injury Severity Score was created. Comparisons across two independent trauma populations of 3,136 and 3,449 patients indicated that NISS had improved predictive power over ISS as NISS was able to better separate survivors from non-survivors. NISS was also better at the classification of penetrating injuries. However, at the time of the NISS publication the authors were already mindful that NISS was still limited by the dependence upon on AIS derived anatomical information and excluding the possibilities of physiological information(582).

Comparisons between ISS and NISS by Tohira *et al.* have indicated that including the mechanism of injury influenced the outcome prediction. They confirmed that ISS is better at predicting outcomes of blunt trauma patients whereas NISS is better at predicting outcomes of penetrating trauma patients(583).

The contemporary role of trauma scoring systems is now multifaceted. From early beginning predicting outcome from trauma, they have evolved to measure the performance of trauma systems, aid in comparing other parallel trauma systems, guiding research and directing clinical governance(584). Trauma scoring systems are used to compare therapeutic methods, and as a pre- or inter- hospital triage tool(574).
Are they accurate, reliable and specific?

1.3.4 Triage Index and Revised Trauma Score (RTS)

The Trauma Score was developed in 1980 as a result of consensus physician peer review(585). The Trauma Score evolved from the Triage Index, which consists of 5 variables. The variables are respiratory expansion, capillary refill, eye opening, verbal response, and motor response(15).

The trauma score added in physiological factors of systolic blood pressure, respiratory rate and the Glasgow Coma Scale to make a useful predictor of outcome for patients with blunt or penetrating injuries (585). The Trauma Score was later modified as only the variables of systolic blood pressure, respiratory rate and the Glasgow Coma Scale had any predictive value(577) to become the Revised Trauma Score (RTS) (587)It was found that the capillary refill and respiratory expansion were difficult to assess, especially at night, and that retractive respiratory expansion was always difficult to observe. RTS is a more reliable predictor of survival and death than the Trauma Score(585) and can account for the severe isolated head injuries due to the GCS weighting. When scoring; the greater the degree of physiological abnormality, the lower the score.

Revised Trauma Score = $(0.7326 \times \text{SBP}) + (0.2908 \times \text{RR}) + (0.9368 \text{ GCS})$

The revised trauma score gives a total score of 0-7.8408, which is then rounded to the nearest whole number and referenced to a graph which plots the survival probability against the score. RTS is limited because it is not easy to calculate in the pre-hospital setting and the inherent bias for the intubated and ventilated patient. As a purely physiological score it is unable to integrate severe injuries with optimal resuscitation.

1.3.5 TRISS

TRISS was developed in the 1980's(586) in order to compare trauma outcomes(6) by combining physiological and anatomical variables. TRISS has also been used for other purposes which include quantifying the severity of injury of a particular population, identifying cases for peer review and comparing the death/survival rates of different hospitals(588). Calculating the TRISS probability of survival requires knowledge of the patient's age, mechanism of injury, ISS, and the Revised Trauma Score (RTS). The regression coefficients are derived from the Major Trauma Outcome Study (MTOS) database and differ depending on whether the mechanism is blunt or penetrating(585).

TRISS is the main tool from which inter-hospital trauma care is compared i.e. The Trauma Audit & Research Network (TARN) in the United Kingdom (UK).

There have been criticisms of TRISS that the values derived from the RTS are very dependent upon pre hospital care given. Gabbe *et al.* is critical of the reliance upon physiological values(588). However, Champion observed that anatomical scoring alone has a lower correlation with outcome than in combination with physiologic states(585). Supported by Chadwa *et al.* who argues that outcomes based purely on anatomical injury description alone is incomplete and not enough to help predict outcome(574).

Other limitations of TRISS include the knowledge that it misclassifies severely injured patients with an ISS >20, those with severe head injuries(26) and it inherits the limitations of ISS; namely that it is unable to account for multiple severe injuries in one body region.

1.3.6 The American College of Surgeons Committee of Trauma (ASCOT).

In an attempt to take into account the influence that observed physiology had upon patients' outcome the American College of Surgeons Committee of Trauma created a predictive measure of outcome ASCOT. Intended as a refinement of TRISS(6), ASCOT incorporates AIS injury descriptions, age, and particularly physiological data into a single score that is a predictive measure of outcome. ASCOT is perceived as having better outcome prediction than TRISS(589), and particularly for penetrating trauma. It involves complex calculations for small improvements in predictive power(577, 585). There is also evidence to indicate that the ASCOT and TRISS scoring systems have no significant difference in predictive power(582).

1.3.7 The International Classification of Disease-9 based Injury Severity Score (ICISS)

Osler *et al.* in 1996(582) looked at alternatives to the AIS consensus driven scoring. They had access to a database of which scored the same patients using both the AIS and ICD9 criteria. This served as an environment ripe for comparison of the two methods of injury scoring. All ICD 9 injuries were allocated a survival probability from a database of 300,000 patients spanning 5 years which served to produce the probability of survival from particular injuries which were described as survival risk ratios (SRRs). This new protocol was called ICISS. It is essentially the product of all the injuries sustained

$$CISS = (SRR)_{injury1} \times (SRR)_{injury2} \times (SRR)_{injury3} \times (SRR)_{injury4} \dots$$

The ICISS was judged to be an improvement because all injuries are taken into account, and all injuries are more accurately modelled. The authors were, however, mindful that there were limitations in the application of the ICISS model. Many of the limitations are actually related to the calculation of 'survival probabilities'. The 'survival probabilities' according to each injury are dependent upon the location. Another limitation is that the 'survival probability' of trivial injuries is over estimated in some cases as the trivial injury may occur in patient who has sustained other more severe injuries and then die. The reality of multiple injuries is complex, and they can have synergistic effects. By modelling multiple injuries as having an independent effect is simplistic. A final limitation is that the 'survival probabilities' require an extremely large database to draw upon their calculated value as it was observed that at least half of the ICD9 Codes occurred less than 30 times in a database of over half a million injuries in 300,00 patients. For the ICISS to be truly representative, it requires modelling of the multitude of different interactions that multi-trauma creates. It has been confirmed that ICISS is 'unstable' partly due to its dependence upon data sources from which the relevant SRRs are derived. As much as the ICISS sought to move away from the 'subjective determinations' that form the AIS codes, the ICISS performed much worse when the ICD10 descriptors were used instead of those from ICD9(583). Due to these differences it is unlikely that ICISS would be implemented as the injury severity score of choice(588).

1.3.8 Other non-trauma scoring systems

With a patients being admitted to ICU a significant proportion of trauma patients will have an ICU based scoring systems applied to them. But the scoring systems used in

conventional ICU do not necessary fully embrace the nature of the trauma patient and this is particularly true of the military ICU(577). There is however the Sequential Organ Failure Assessment (SOFA) which has shown reliability in trauma patients and will be discussed later.

Physiological values form the basis for outcome scoring systems in Intensive Care Units (ICU). Examples include the Acute Physiology and Chronic Health Evaluation (APACHE)(590), the simplified acute physiology score (SAPS)(591) and the Sequential Organ Failure Assessment (SOFA) score (592).

1.3.9 The Acute Physiology and Chronic Health Evaluation (APACHE) Scoring Systems

The Acute Physiology and Chronic Health Evaluation (APACHE) system was developed on the postulation that the severity of illness in groups of patients could be measured by quantifying the degree of abnormality in the physiology of ITU patients(593). The APACHE score is composed of two components: the Chronic Health Evaluation (CHE), which describes a patients co-morbidities and the Acute Physiology Score (APS), describing the physiological values in the initial hours after ITU admission. The allocation of scoring values was through consensus review rather than through databases.

The APACHE was then revised in 1985, by reducing the number of physiological variables from 32 to 12, to become the APACHE II. The physiological variables recorded the worst value during the first 24 hours of a patient's admission to the ICU(590).

The APACHE II score is calculated from the sum of the:

Acute Physiology Score + Age Points + Chronic Health Points

The APACHE II score can give values from 0 -71 with higher scores indicating an increased risk of hospital death.

The Acute Physiology Score is calculated by taking the sum of the 12 variables, including a Chronic Health Score.

APACHE II has since become the most frequently used predictor of general mortality on ITU. The APACHE II scoring system is known to have been validated as a predictor of mortality(594) in diverse patient groups(6). But only 5% of study population in the APACHE II score were multi-trauma patients with a mortality of only 8%(590). Therefore the applicability of APACHE II for trauma patients on ITU has been questioned. APACHE III was created in 1991 to try and address the issues of scoring for trauma but there are still doubts about its scoring as it hasn't be convincingly validated in trauma patients(574).

1.3.10 The implementation of scoring systems

The scoring systems most frequently encountered within the injury literature are ISS, NISS, TRISS and ICISS, rather than ASCOT or the anatomic profile score(APS)(583). Part of the reasons ISS has remained 'the standard' is because of their ease of use; validation in multiple populations, high correlation with mortality, length of stay, and cost(595).

The Scoring system used in the UK by The Trauma Audit & Research Network (TARN) is the TRISS model. It uses the data captured to provide a means of systematically auditing the standards of care for the severely injured(596). It is also the most commonly used scoring system tool to compare hospital performance, despite the significant amount of criticism detailing the limitations of TRISS(588).

Trauma scoring have relied upon adjustments to anatomy weighting, physiology and statistical modelling but with very little adoption.

There is a move to revise of the ICD to address issues associated with describing multiple pathologies and scoring the severity of injury (48). However, even with this progress the revision of the ICD to improve scoring of injury severity. Current scoring systems will be limited at its source as an anatomical scoring system which is unable to differentiate between the responses of theoretically two individuals with exactly the same anatomical injury.

A system is needed that captures in the injury, how the tissues have responded immediately and over time and the capacity is for regeneration. Ageing, genetic make-up are an important co-factors, these will be realised in the production and reponse to DAMPs; cellular and cytokine response may anticipate how patients respond. When investigating the role of inflammatory mediators in relation to outcome prediction It has been noted that the level of circulating Il-6 and IL-8 are not dependant to the ISS score (597). By factoring in age, biochemical markers, co-morbidities and mechanism of injury variables it may be easier predict outcome.

With the mechanism of injury Russell *et al.* stressed that one has to take into consideration blast as a new mechanism which combines both blunt and penetrative injuries(584). The mechanism of injury has also been shown to predict outcome in paediatric patients(595)and after blunt trauma in adults(598). Haider *et al.* predicted outcome in paediatric patients from mechanism of injury is particularly strong as it covers a group of 35,097 patients. They were explicit in stating that force and energy transfer were major factors affecting patient outcomes and may be major contributors to observed differences in outcome.

Combining the anatomy, physiology, age and co-morbidities are important in the general classification of injury severity against survival probability(585). There is evidence that the addition of age and physiological variables to Injury Scoring systems improves the predictive accuracy in all injury severity scores. What the addition of age and physiology does do is to reduce the variation in discrimination between different scoring systems(598). These suggested variables are relatively simple to obtain and will better help demarcate the outcome of those with the same ISS and similar physiological values but different endogenous reaction to trauma.

Current trauma scoring systems have not been able to adjust to the advances in medical care that are now unable to accurately predict outcomes as demonstrated by Penn-Barwell *et al.* in a study of survival during the advances in military trauma care over the last 10 years(599). The importance of how a patient responds to medical treatment over time may also be relevant. A single scoring system has not become acceptable to all users or applicable to all trauma populations. The 'holy grail' in

trauma scoring is to develop a system that is prospective, pragmatic, reliable and reproducible for all groups of patients and all injuries.

1.4 Clinical Course following Severe Trauma

These improvements in military and civilian trauma outcomes achieved through early resuscitation in the field, and better integrated specialist care (17) are counterbalanced over the following few weeks by an acute systemic inflammatory response syndrome (SIRS) with immuno-incompetence leading to infection, multi-organ dysfunction/failure (MOD/MOF) and late deaths (18). Cytokine production and cell-derived responses include neutrophil priming and changes in the innate and adaptive cellular responses that are linked to outcomes (19). The mechanisms driving the non-infective inflammatory response were poorly understood until relatively recently when pioneering work by Carl Hauser revealed that the release of Damage Associated Molecular Patterns (DAMPs) by injured cells precipitates systemic inflammation (20). Mitochondrial DNA has recently emerged as a potential initiator from necrosed and damaged cells (21). Importantly, if this is proportional to the extent of injury then this may provide a more accurate measure of tissue damage and severity. Infection, poor wound healing and the inability to tolerate long procedures restrict surgical reconstruction following severe injury(22,23). A catabolic state predominates and patients lose lean muscle(24,25). Eventual survival is associated with emergence from this state of chronic inflammation, the patient gains lean mass, organ systems recover and their wounds heal(26-29). Trauma patients will continue to gain weight after leaving hospital to continue their prolonged rehabilitation as an outpatient (30).

Injury has mental as well as physical consequences. At least a quarter of patients have psychological morbidity at one year after injury (31). The effects of anxiety and depression on the effectiveness of rehabilitation has not been demonstrated in severe trauma but the early identification and management of depression in a geriatric group improves rehabilitation outcomes following hip fracture (32). Psychological stress has been shown to slow wound healing (33).

The complexity of the trauma pathway is reflected in the variety of outcome measures in addition to the key outcome of survival. Secondary measures, such as 'Ventilatory Days' and 'Length of Stay' (LOS) are often also reported. These measures are unfortunately influenced by organisational variables such as bed availability, discharge destination, and the individual clinician treating the patient. They are also affected by the nature of their injuries: where loss of tissue involves a functional deficit this may disproportionately affect how long they stay. A chest injury may keep a patient ventilated longer than one with a limb amputation and a hand injury may prevent discharge until devices that need to be fitted at home to aid 'activities of daily living' are done.

This introductory chapter will outline important components of the immune-endocrine response to trauma, the main focus of the thesis. In reviewing the literature the narrative will outline previous attempts to ameliorate the inflammatory and endocrine response and highlight areas which have still to translate to the bed-side. The review will also consider variables impacting upon the response to injury and patient outcomes, most notably the role of age and to a lesser extent gender.

1.5 The Acute Inflammatory Response to Injury

Wound healing has been recognised as important to wellbeing from around the time of Hammurabi ('The Healer') in 2150BC. Advances in knowledge and practice have developed into the surgical and medical art of caring for the injured patient. Hippocrates recognised that "Healing was a matter of time, but it is also sometimes a matter of opportunity."

The aim of repair is to regain tissue integrity and homeostasis and this is achieved by three broad phases after injury; haemostasis and inflammation, proliferation, and remodelling (34,35). It is by no accident that haemostasis and inflammation are considered together. At the time of tissue injury exposure and presence of tissue factor initiates coagulation (36,37). The expression of tissue factor (TF) on mural and sub-endothelial cells complexes with factor VII/VIIa initiating factors IX and X. Factor Xa converts prothrombin to thrombin enough to activate VIIa and Va cofactors which activate IX and X to mediate the conversion of prothrombin to thrombin. Concurrently platelets, activated by TF, adhere to the damaged tissue and release mediators (e.g. serotonin, adenosine diphosphate and thromboxane A₂). The release of adhesive proteins (fibrinogen, fibronectin and von Willebrand factor) generate thrombin inducing further platelet aggregation and so a blood clot is formed and bleeding stops (38). The production of thrombin is regulated to keep the clot localised with key factors involved being Protein C-thrombomodulin, heparin anti-thrombin and the tissue factor pathway inhibitor (39).

Most theories on inflammation focus on a system of reactions between host and non-host, immediate and later responses and the innate and adaptive immunity. In the

case of inflammation in response to injury, sterile inflammation, the question is how do pathogenic and sterile tissue damage result in such a similar response?

In its simplest form, inflammation is the response to the interruption of tissue homeostasis. The damaged tissue initiates a process that recruits plasma proteins, antibodies and leukocytes from the blood to the site of injury facilitated by increased vascular permeability and blood flow (40). Bacterial infections have provided the earliest insights into triggering the innate immune inflammatory response through receptors like Toll-Like Receptors and NOD (nucleotide-binding oligomerization-domain protein)-like receptors expressed on innate immune cells, which recognise non-self molecules on pathogens, termed Pathogen Associated Molecular Patterns (PAMPs) (41). PAMPs include cell wall components such as lipopolysaccharide and peptidoglycans as well as nucleic acids to detect viruses (42). The initial infection stimulates local macrophages and mast cells to produce a variety of inflammatory mediators such as chemokines, cytokines, vaso-active amines, eicosanoids and proteolytic cascade products (43). Recruited neutrophils are able to extravasate between activated endothelial cells in response to chemokines such as IL-8. On reaching the infected or damaged area neutrophils become activated on contact with microbes or through cytokines and other molecules released from damaged tissues. The neutrophil's main function is to kill pathogens through the generation of reactive oxygen species (ROS) and release of cytotoxic granules after phagocytosis of the microbe, a process which can also cause bystander damage to local tissues (44,45). More recently it has been shown that neutrophils can kill extracellularly by releasing their nuclear material to generate a net of DNA also containing proteases to trap and kill bacteria, a process termed netosis (46).

The complement system also aids removal of foreign material by opsonising microbes or tissue fragments to aid recognition by phagocytic cells. Comprising more than 30 soluble and bound proteins it can be activated by three cascade pathways; 'classical', 'lectin' and 'alternative' (47). Activation causes the generation of anaphylotoxin, opsonisation, lytic membrane attack and amplification of the presence of foreign material as part of the innate immune system (48). Complement Receptor 1 (CD35) is found on leucocytes and has been found to be upregulated in sepsis and trauma (49).

The innate immune system not only recognises PAMPs but also signals that injury has occurred by recognising 'Alarmins' or DAMPS.

Apoptosis is the process of programmed cell death. The sequence leads to characteristic cell shrinkage and nuclear fragmentation that releases characteristic cytokines(50). It can occur in response to a stress signal from glucocorticoids, heat, radiation, viral or hypoxia to mention a few(51). Traumatic injury to the cell on the other hand is in response to acute cellular injury is chaotic and cell contents are spilt with out being packaged for recycling like they are in apoptosis. The release of cell contents triggers an inflammatory response(52).

1.5.1 Damage Associated Molecular Patterns (DAMPs)

DAMPs are described as molecules released from damaged cells that initiate the sterile inflammatory pathways (53). This concept was born out of work done identifying PAMPs, a group of molecules that are recognised by the host as non-self

and whose actions are initiated through Pattern Recognition Receptors (PRRs)(54). An attempt to classify DAMPs to fulfil 4 criteria was suggested by Kono and Rock (55):

1. A DAMP should be active as a highly purified molecule.
2. The biological effect should not be due to contamination with microbial molecules. Caution is particularly warranted if the putative DAMP is found to work through receptors for PAMPs such as toll-like receptors (TLRs).
3. The DAMP should be active at concentrations present in pathophysiological situations.
4. Selective elimination or inactivation of the DAMP should ideally inhibit the biological activity of dead cells in in-vitro or in-vivo assays.

Important molecules that act as DAMPS (Figure 1) are mitochondrial DNA (mtDNA), N-formyl peptides, HMGB1, Heat Shock Protein, uric acid, galectins, thioredoxin, adenosine and many more (56).

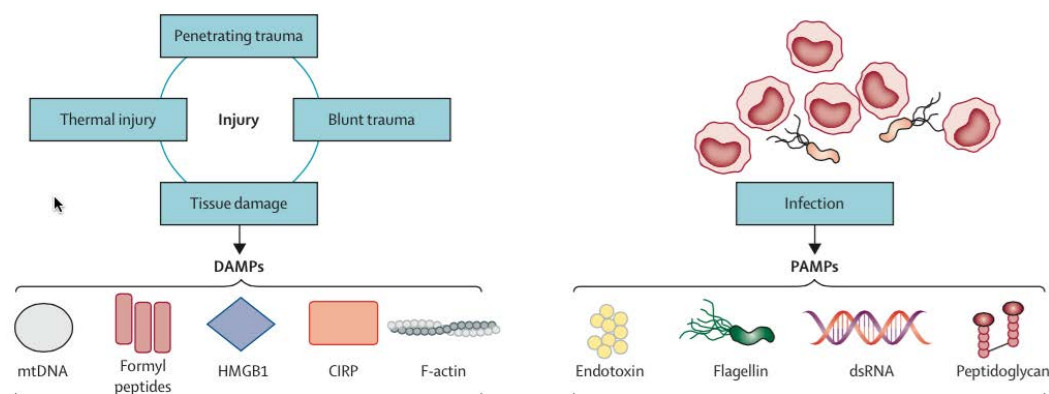


Figure 1.1. Damage Associated Molecular Patterns (DAMPs) and Pathogen Associated Molecular Patterns (PAMPs) from a review by Lord *et al*(48).

1.5.1.1 Mitochondrial DAMPS.

Mitochondria have recently emerged as a source of DAMPs involved in the sterile inflammatory response. Initiation may come from the release of organelles and molecules from the cytoplasm of damaged cells, namely molecules associated with the mitochondria, such as mtDNA and mitochondrial formyl peptides (57). Collectively this source of DAMPS is known as mitochondrial DAMPs (MTDs). The 'endosymbiont theory' of the origin of the mitochondrion, states that both chloroplasts and mitochondria came from the inclusion of alphaproteobacteria more than 1 billion years ago. An alternative explanation is presented in a review by Michael Gray that the organelle may have appeared simultaneously at the beginning of the eukaryotic cell containing it (58). Mitochondria retain many of the biochemical and morphological features of bacteria: a double membrane; unique membrane lipids; no histones; replication independent of the nucleus; circular DNA and the ability to form N-formyl peptides. Either way the striking similarities between the initiation of the inflammatory response by bacteria and by mitochondrial DAMPs is difficult to ignore.

Elevated plasma DNA levels were reported by Lo *et al.* in trauma patients without the ability to explain its origin (59). Investigators have attempted to quantify the amount of mtDNA and mitochondrial DAMPs (MTDs) in relation to the inflammatory response. Zhang *et al.* measured the mtDNA in 15 trauma patients before resuscitation with no open wounds or gastrointestinal injuries, and demonstrated increased activation of neutrophils with increasing concentrations of mtDNA (60). The levels peak on day 1 after trauma but are sustained after severe sepsis (52). Acting through formyl peptide receptors and TLR9, N-Formyl peptides and mtDNA respectively have been shown to

have a direct effect on arteries to cause shock seen in sepsis and severe trauma(59,61). Up to now there has been no evidence to link the severity of injury with the level of MTDs.

1.5.1.2 High Mobility Protein Group Box 1 (HMGB1).

The High-Mobility Group (HMG) is a group of non-histone chromosomal proteins, present in almost all cells with a nucleus, that are involved in the regulation of DNA-dependent processes including transcription, replication, recombination and DNA repair. Divided into three large families (A, B and C), HMGB1 is the most important in the context of inflammation (31). In 2001, Wang *et al.* (62) showed how HMGB1 stimulates an inflammatory response when released from necrotic cells but not after apoptosis(63). HMGB1 is also secreted from dendritic cells, macrophages and monocytes in response to IL1b, TNFa and LPS (31). This appears to encourage migration from the nucleus and the acetylation of HMGB1 before being transferred into secretory vesicles and thus *secreted* during the inflammatory response. When cells become necrotic the HMGB1 loosely bound to chromatin diffuses out of the cell. This does not occur in granulocytes(64).

Whether actively secreted or diffused out of necrotic cells, HMGB1 is able to activate Pattern Recognition Receptors (PRRs) to alert immune cells to the presence of damage or microbes. It is known to bind with the surface cell receptor for advanced glycation end products (RAGE) when released into the circulation (65). RAGE is present at low levels but is upregulated when there is an accumulation of ligands, where it activates NF-kB and MAPK (66). TLR2 and TLR4 are also implicated in the

mediation of various cellular responses including the release of proinflammatory cytokines TNF- α , IL-1 β , IL-1 α , IL-6 and MIP (67).

As a cytokine, HMGB1 may well be dependent on the environment as hypoxic surroundings during acute inflammation results in structural changes due to sulphide bonds forming during redox modulation. The amount of oxidation is also significant, as the production ROS is a common event and where HMGB1 is oxidised appears to be significant in its ability to signal a response(68). A regulated change in the intra and extra cellular redox reactions plays a vital role. When released by necrotic cells it appears to be oxidised although the exact mechanism is not elucidated. For the activation of TLR4, HMGB1 needs a cysteine at position 106, which if oxidised by ROS during apoptosis, renders it unable to stimulate the immediate pro-inflammatory response from macrophages (69).

1.5.1.3 Other DAMPS

Molecules implicated in acting as DAMPs or Alarmins can be differentiated as endogenous or exogenous, protein or non-protein or those released by necrotic or apoptotic cells. For example uric acid and ATP are non-protein DAMPS released from apoptotic cells (70). A newly emerging DAMP is S100B whose level is raised in severe injury and is seen as a marker of endothelial dysfunction associated with the up regulation of IL-6 and IL-8 (71).

1.5.2 Pattern Recognition Receptors (PRRs).

Early understanding of PRRs came from plants (72). In 1979, the first PRRs to be recognised were trans-membrane scavenger receptors that bind the lipopolysaccharide (LPS) of endotoxins, Low Density Lipids (LDLs), and other polynucleotides (73). In 1985 in a completely different field, Christiane Nusslein-Volhard (74) observed 'weird' looking *Drosophila* fruit flies developing as a result of 'Toll' genes, in her embryogenesis work. It would be ten years later in another lab, that Toll genes were also found to encode for immune peptides (74,75). A breakthrough in the search for human analogues came in a report by Charles Janeway's group, this would eventually be recognised as TLR-4 which binds LPS (76). Other examples of PRRs are C-type lectin Receptors (CLR) that are membrane bound, Node-Like Receptors (NLR) in the cytoplasm of immune cells and Mannose-binding Lectins (MBL) that do not remain in the cells that produce them (77).

'Danger Theories' first coined by Matzinger present a model of immune recognition that indicates danger to cells but without necessarily being foreign in origin (78). Proponents believe that the initiation of the immune system depends on the way the cells die. Endogenous signalling molecules, also called alarmins, are produced by damaged cells, whereas PAMPs are exogenous molecules, but both have hydrophobic portions that are able to engage PRRs. Grouped together these molecular patterns demonstrate the close relationship between trauma and a pathogen-induced response(79).

1.5.3 Toll-Like Receptors

The TLR group are one of the most ancient, conserved parts of the immune system. Located both internally and on the cell surface, TLRs appear on a wide variety of immune and non-immune cells ((80)). Many TLRs have a wide range of ligands that initiate an inflammatory response (81) in response to PAMPs, DAMPs and environmental stress. Further to link coagulation and innate immunity, TLRs have been demonstrated on platelets and can initiate an inflammatory response in this context (82). TLRs can be separated into two main groups; those that appear on the cell surface and interact with membrane components of microbes such as LPS, Lipids and Lipoproteins, and those located internally and expressed on the surface of vesicles such as endosomes, endoplasmic reticulum and endolysosomes where they recognise microbial nucleic acids. TLR2, TLR4 and TLR9 are particularly important in trauma, haemorrhage, reperfusion injury (83,84) and sepsis(85).

Receptor	Ligand(s)	Source	Types of Cell
TLR 1	Multiple triacyl lipopeptides	Bacteria	Monocytes/macrophages
TLR 2	Multiple glycolipids, Lipopeptides, lipoproteins	Bacteria	Some dendritic cells B lymphocytes Monocytes/macrophages Mast cells
	lipoteichoic acid	Gram-positive bacteria	
	HSP70	Host cells	
	Beta-glucan	Fungi	
TLR 3	Double-stranded RNA	viruses	
TLR 4	Lipopolysaccharide	Gram-negative bacteria	Dendritic cells B lymphocytes monocytes/macrophages Myeloid dendritic cells[28]
	Several heat shock proteins	Bacteria and host cells	
	Fibrinogen, heparin sulfate & hyaluronic acid fragments	Host cells	
	Nickel		
	Opioid drugs		
TLR 5	Flagellin	Bacteria	Mast cells
TLR 6	Multiple diacyl lipopeptides	Mycoplasma	B lymphocytes
TLR 7	Imidazoquinoline, loxoribine	Small synthetic compounds	Intestinal epithelium
	single-stranded RNA	RNA viruses	
TLR 8	small synthetic; single-stranded RNA		
TLR 9	Unmethylated CpG Oligodeoxynucleotide DNA	Bacteria, DNA viruses, Host cells	
TLR 10	unknown		
TLR 11	Profilin	Toxoplasma gondii	monocyte/macrophages
TLR 12	Profilin	Toxoplasma gondii	a subset of dendritic cells
TLR 13	bacterial ribosomal RNA	Virus, bacteria	Intestinal epithelium

Table 1.1: Toll-Like Receptor Ligand Source and Type of Cell. Adapted from various sources (86-88)

Abundantly expressed in peripheral blood leucocytes, TLR2 recognises foreign materials such as LPS, Heat Shock Protein and various other ligands that are mainly bacterial in origin. Although much work has focused on its role in sepsis recent work has identified its function as a DAMP. Injury appears to enhance the response of TLR2 to stimuli independent of TLR4 and without an increasing surface expression of this PRR (54,89). While the production of cytokines and the attraction of immune cells dominates in the early inflammatory response, TLR2 and TLR4 are also implicated in a later suppression of cytokine production through stimulation of T-regs who have also been shown to have TLR2 receptors(90). This may be important in the resolution phase of the inflammatory response.

Found on the cell surface, TLR4 has often been linked to the prolonged inflammatory response and the so-called second hit phenomenon responsible for late infections and multi-organ failure in trauma patients (86). TLR4 stimulation induces production of proinflammatory cytokines such as IL-6, TNF α and IL-1 β (91,92). Like TLR2 it has been implicated in adaptive immunity. There is functional expression of TLR4 on T cells (93). After injury an exaggerated response to stimulation is maintained after injury as seen through late splenocyte dysfunction mediated through TLR4 but not through TLR2 (94).

As a member of the TLR family, TLR9 has a fundamental role in recognising PAMPs and activation the innate immune response. The differences here are that it recognises mitochondrial associated molecules specifically, which are thought to be bacterial in origin. The receptor is expressed internally on endoplasmic reticulum, endosomes, lysosomes and endolysosomes that are involved in bacterial killing but can be

stimulated on contact with foreign DNA. TLR9 recognises unmethylated 2-deoxyribo(cytidine-phosphateguanosine) (CpG) DNA molecules that occur frequently in bacteria and viruses but are rare in mammals (95). It is likely that activation also occurs after stimulation with mitochondrial DAMPs from injured cells during the initial response to trauma. TLR9 related stimulation on DCs appears to induce activation of parts of the adaptive response(90).

The emergence of mitochondrial derived DAMPs as key to the inflammatory response after injury will focus attention on the potential role of TLR antagonists in dampening the inflammatory response.

1.6 Genomic response

The individuals response to severe injury results in a change to many systems. The genomic response reveals how cells adjust to their environment and the alerted threat. A study by Xiao et al.(600) directly contradicts the models proposed for a counter anti-inflammatory response syndrome(CARS) that follows a pro-inflammatory response syndrome. Xiao et al. highlight that they were unable to find a single gene or cluster that changed uniquely but a common inflammatory response that revealed the activation of a vast number of genes that encode for inflammatory mediators. They also were able to observe the simultaneous suppression of genes involved in antigen presentations, apoptosis and T cell receptor function and NK cell function.

Cuenca et al. report the development of a genomic score that can predict outcome in trauma patients. Predicting a complicated outcome with more MOD/F and inflections based on the micro-array determination of circulating leukocyte transcriptome

(601). Desai et al. showed a down-regulation of MHC-II genes are associated with poorer outcomes after injury, highlighting the NK-kB pathway known to be downstream of TLR(602).

Other gene pathways may also be important, such as the lipid mediator pathways described by Orr et al. In their study of 96 trauma patients they showed that complicated recoveries had a dysregulation in lipid mediator signaling(603). They also highlight the use of resolvins in animal studies of burn injury demonstrating improved neutrophil function and survival(604).

Suggestions that age influences outcome following severe injury is not questioned. This is reflected in a study by Vanzant et al. comparing the over and under 55years of age. They showed a higher slightly later genomic response from the neutrophil transcriptome in those with a complicated recovery in trauma patients over 55 years of age(605).

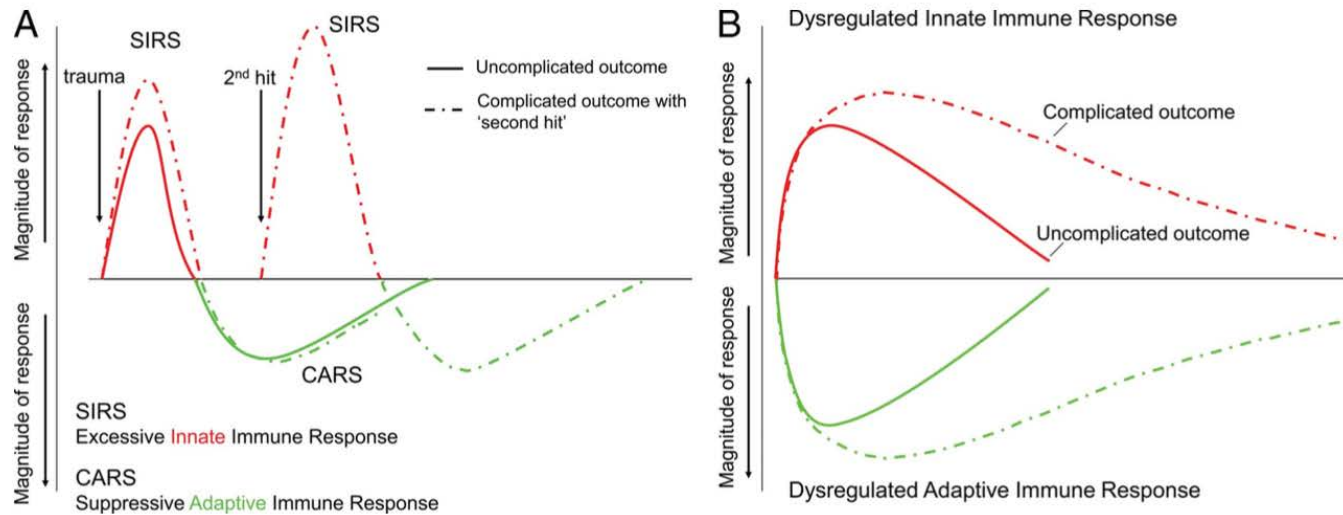


Figure 1.2. A genomic storm: Refining the immune, inflammatory paradigm in trauma. (A) The current paradigm explains complications of severe injury as a result of excessive proinflammatory responses (SIRS) followed temporally by compensatory antiinflammatory responses (CARS) and suppression of adaptive immunity. A second-hit phenomenon results from sequential insults, which leads to more severe, recurrent SIRS and organ dysfunction. (B) The proposed new paradigm involves simultaneous and rapid induction of innate (both proand antiinflammatory genes) and suppression of adaptive immunity genes. Complicated recoveries are delayed, resulting in a prolonged, dysregulated immune–inflammatory state. Taken directly from a paper by Xiao et al(600).

1.6.1 The Early Cellular Inflammatory response

1.6.1.1 Neutrophils

Neutrophils are a lead effector of innate immunity, targeting foreign microbes and cellular debris, they are released into the blood in large numbers where they remain for over 5 days (96). Chemotaxis and activation can be induced by chemokines and N-formyl peptides via G protein coupled receptors on the cell surface.

Chemokines, cytokines and immune mediators signal the site of danger to both monocytes and neutrophils that marginate to the area of inflammation. On adhering to the endothelial wall, selectins, a group of adhesion molecules, slow the cell allowing the neutrophil to leave the circulation and migrate towards the site of infection or tissue injury (82,97). This margination can be achieved either through or between the endothelial cells(98).

Neutrophils express a large number of PRRs; TLRs, NLR and C-type Lectins. Numerous cytokines, chemokines and angiogenic factors can be expressed by the neutrophil and its role as a central mediator of inflammation is well established. As well as producing cytokines, neutrophils can directly cross-talk with DCs, NK Cells and T and B Lymphocytes' described eloquently in a review by Mantovani *et al* (94).

1.6.1.2 Neutrophil response to Injury.

Neutrophils play an important role in the early response following injury. It is likely that mitochondrial derived and other DAMPS recruit neutrophils, activate them, and along

with other stimuli generate ROS (99). Within 10 minutes neutrophils migrate to the site of injury and reach peak levels in the peripheral circulation within 24 hours. Neutrophils are sequestered in to the injured tissue, 'primed', immediately phagocytosing and killing any microbes present and promoting inflammation to effect wound healing (100). Neutrophils thus co-ordinate the inflammatory response directed at sterilising injured tissue and directing macrophages towards the site. Apoptotic neutrophils are engulfed by macrophages, termed efferocytosis (101). The maintenance of the inflammatory response is controlled through IL-23 which induces IL-17 production from Th17 cells, which in turn induces chemokine production to attract further neutrophils (102,103). G-CSF is also increased during infection or after trauma and acts on bone marrow progenitor cells to cause increased differentiation of progenitor cells to neutrophils (104)

Expression of CD11b has been proposed as being directly related to base deficit and subsequent multi-organ failure with neutrophils as the cause (105,106). Decreased phagocytosis has been shown in sepsis and trauma possibly related to reduced CD16 (FcγRIII) expression, which is required for phagocytosis mediated by antibody bound to microbes or damaged tissue (107-109).

1.6.1.3 Phagocytosis and microbial killing.

Phagocytosis is a combination of repulsive protrusion between cytoskeleton and plasma membrane, and the active membrane flattening using myosin units (110). This process can internalise opsonised and non-opsonised material, though the latter is very inefficient. In neutrophils the principle receptors for opsonisation are Fc receptors and a group of β 2 integrins (CD11b/CD18) that bind complement C3b and C3bi(111).

FcγRIIA (CD32) and FcγβRIIIb (CD16) are the main Fc receptors (112). Granulocytes contain many different types of granules which vary depending on the location, age and role of the neutrophil (113) and a large array of secretory vesicles are present to support an ongoing inflammatory reaction by producing cytokines and chemokines such as IL-8 (114). Granules can be primary, secondary or tertiary and contain anti-microbial molecules. The granules are membrane bound; primary (azurophilic) granules are associated with CD63, secondary (specific) granules are associated with CD66b and tertiary granules with vesicle-associated membrane protein-2(115,116). Antigens within the membrane of the granules can be detected when the granule fuses with the cell membrane as exocytosis occurs(117,118).

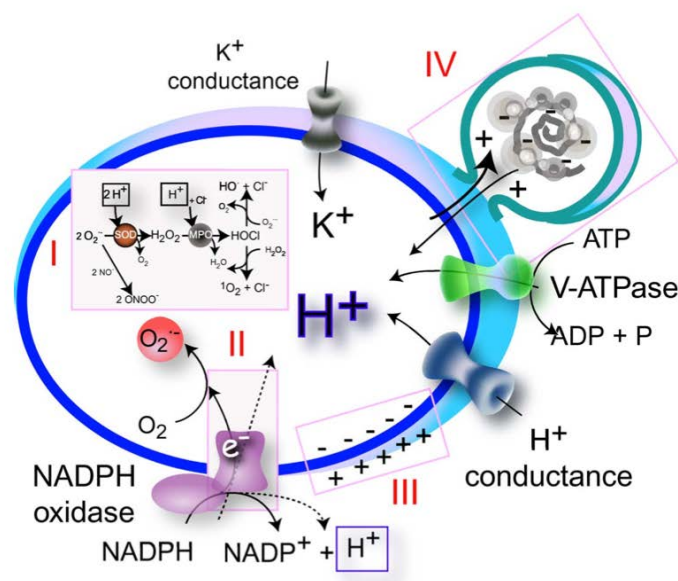


Figure 1.3. Oxidant generation and ionic homeostasis of the phagosome. (I) Production of superoxide anions results in consumption of protons during dismutation, leading to formation of other reactive oxygen species via superoxide dismutase (SOD) and myeloperoxidase (MPO). (II) The NADPH oxidase has been postulated to function as a proton channel. (III) The oxidation of NADPH and transport of electrons into the phagosome generates a membrane potential across the phagosomal membrane, which promotes proton influx, counteracting cytosolic acidification. (IV) Cations translocated in response to the electrical potential change (H^+ and/or K^+) enhance granule secretion by increasing the ionic strength of the phagosomal lumen. Taken directly from a review by Lee *et al*(56).

ROS are produced by the NADPH oxidase, a multi-protein enzyme complex, which is inactive in the resting neutrophil((28,106). Interaction of the neutrophil with pathogens and their subsequent envelopment of the phagosome will cause activation of the NADPH complex to produce ROS (119-121).

Recently neutrophils have been found to use their DNA to tackle pathogens by the release of neutrophil extracellular traps (NETs)(122). Fibres of neutrophil DNA bind to bacteria and kill them using cytotoxic mediators on the DNA (111). The production of NETs appears to be part of an apoptotic process that is more readily stimulated by IL-8, LPS, bacteria, fungi or activated platelets than single DAMPs (Figure 1.4)(123).

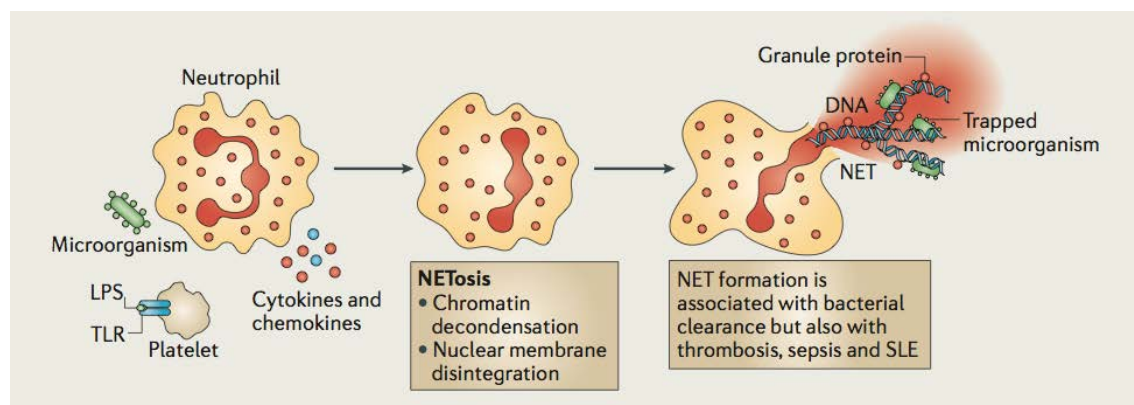


Figure 1.4. Neutrophil Extracellular Traps. Adapted from a review by Mantovani et al(104).

The key role for neutrophils in wound healing is sterilisation and the attraction of macrophages to help clear debris and remove apoptotic cells (124). If activated tissue neutrophils do not encounter any pathogens, as would occur at a sterile site, they release their cytotoxic granules(125) which can cause damage to healthy tissues. CD11b, part of the Membrane-activated complex 1 (Mac-1; CD11b/CD18) is a β_2 integrin implicated in neutrophil-mediated tissue injury(126). This is seen as harmful

but in the early stages the breakdown of collagen can aid neutrophil-pathogen contact and may help to collapse lymphatic structures, so slowing the spread of a bacteria attack(56). Ultimately it is important to slow and stop neutrophil recruitment to resolve the inflammation and ensure wound healing is complete.

The neutrophil's other main contribution to wound healing is the attraction of macrophages at 24-48 hours post injury. Neutrophils orchestrate events, which include minimising their accumulation, activation, and promote their own apoptosis. (46). The neutrophil is thus replaced by macrophages as the dominant cell type. Production of pro-inflammatory cytokines continues from macrophages along with the release of Platelet-Derived Growth Factor (PDGF) which attracts fibroblasts as inflammation resolves and epidermal cell proliferation begins(127).

1.6.1.4 Neutrophil Phenotype

The circulating neutrophil phenotype was originally thought not to vary, however it is now clear that there can be different types of neutrophil in health and during injury or disease(128-130).

Subsets are emerging whose primary role may not be pathogen killing and may be more immune regulatory in nature. Unique populations of apparently immature neutrophils that only express a limited amount of CD16 on their surface, so called CD16 dim cells, are seen in sepsis and trauma (106). They appear to have a reduced ability to phagocytose and generate ROS. These cells have a longer lifespan which may have an advantage with heavily contaminated wounds or septic episodes(131). It may be that these neutrophils have other primary functions other than bacterial killing.

CD35 is upregulated during degranulation on most circulating cells including neutrophils(132). CD35 binds C3b(133). It has also been found that the cleavage of CD35 occurs when neutrophils come into contact with strong chemotactic agents such as fMLP (134). Activation of the cell when in contact with complement in particular C3b/C4b that is involved in opsonising bacteria and erythrocytes (135) that has led some to using CD35 to attempt differential bacterial from viral infection with some success(136). CD35 or Complement Receptor 1 (CR1) is a known potent inhibitor of complement.(137).

Another phenotype has been described, that of Myeloid-Derived Suppressor cells (MDSC) and there is still substantial debate as to whether these are the same or distinct from immature neutrophils (Figure 4) (125).

1.6.1.5 Myeloid-Derived Suppressor cells (MDSC).

A new subtype of granulocytes/neutrophils and monocytes known as Myeloid-Derived Suppressor Cells (gMDSC or mMDSC) has now been described which dampen the pro-inflammatory response. MDSC are upregulated during exaggerated immune responses when they migrate to the lymphoid tissue such as lymph nodes and spleen. There are studies showing increased populations of MDSC in the spleens of murine trauma and burns models that are CD11b⁺/Gr-1⁺(138). These appear to be a subset of 'left shifted' immature neutrophils that are released in response to on-going pro-inflammatory stimulus. There is some debate about whether MDSC consistently have an immature morphology(139). Neutrophil derived gMDSC in humans are CD11b⁺/CD33⁺/CD14⁻/HLADR⁻/CD15⁺. There is variability in receptor expression and morphology for gMDSC in the literature, which probably represents the presence of

multiple subsets (140). What is undisputed is that MDSC are immunosuppressive and also contribute to the suppression of the adaptive immune response (128-130).

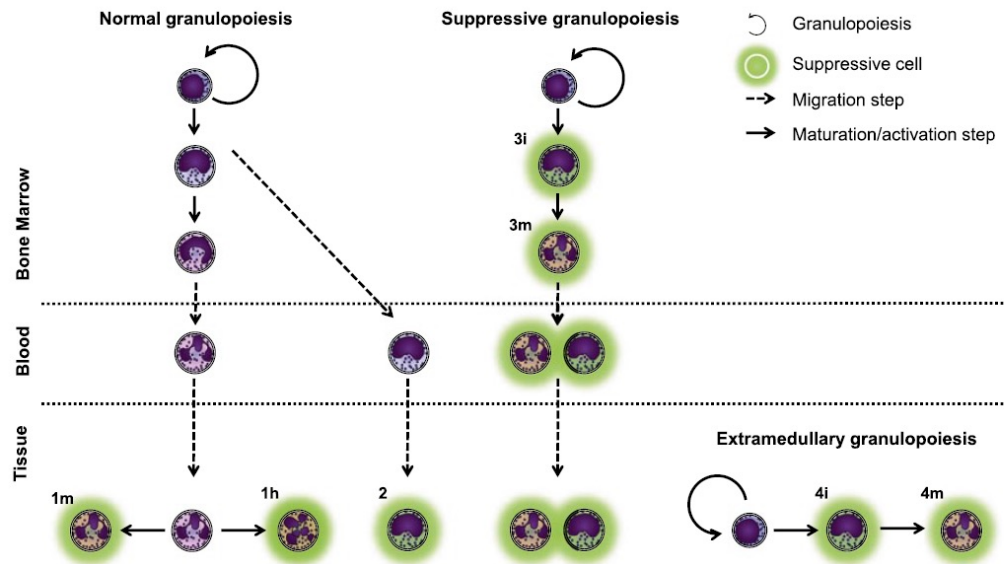


Figure 1.5. Overview of neutrophil subtypes. Figure taken from a review article by Pillay *et al*(141). gMDSC are thought to arise from mature (1) or immature neutrophils in the tissues in response to cytokines. The is also thought to be those that arise in the marrow; a granulopoiesis that only produces gMDSC that can either be mature(3m) or immature(3i). Also, it appears that they may be a subset that undergoes extra medullary granulopoiesis to produce MDSCs.

1.7 Cytokine response in Trauma.

The cytokine response to trauma is complex with distinct pro- and anti-inflammatory components that change over time. After injury there is an initial rise in both pro and anti-inflammatory cytokines, modified by resuscitation, DCS and definitive reconstruction (142,143). In contrast as ischaemia is corrected, sedation and effective analgesia will dampen the production of cytokines (144,145). There are additional peaks to the inflammatory course outlined in (146). Infection, MOD/F, poor analgesia (147) and the multiple surgical insults of debridement and definitive reconstruction give additional stimulus to an already overburden immune system (148). The temporal influences on the inflammatory response can be represented by some of the major cytokines involved in the immune response to injury and these are now discussed.

Interleukin 1 β (IL-1 β)

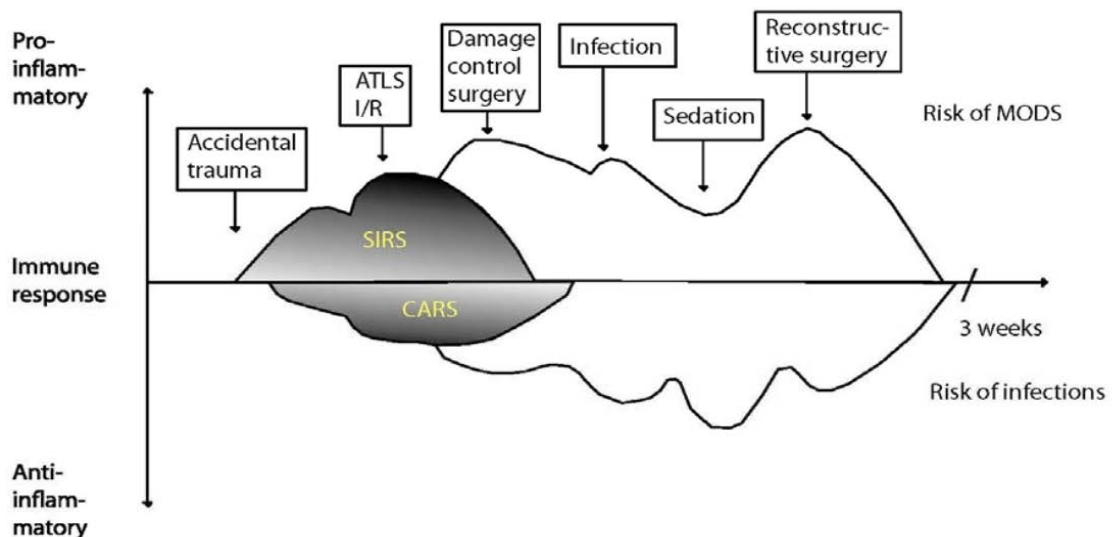


Figure 1.6. The pro- and anti inflammatory response to accidental trauma modified from a review by Brøchner *et al.* (149).

IL-1 β is a lymphocyte attracting cytokine that is produced by activated macrophages. As a proprotein, IL-1 β is converted and activated by caspase1 (33,150). IL-1 β is a mediator of the inflammatory response that is involved in proliferation, differentiation and apoptosis of cells (151). It has also been found to induce pain hypersensitivity in the central nervous system by the induction of cyclooxygenase-2 (PTGS2/COX2) (152).

Interleukin 4 (IL-4)

IL-4 has many roles, including proliferation of activated B-cell and T-cells and the differentiation of B cells into plasma cells (153). IL-4 influences the nature of the immune response by the production of Th1 cells, macrophages, IFN- γ , and dendritic cell derived IL-12 and increasing MHC class II production (154) as well as inducing B-cell class switching to produce IgE(15).

Interleukin-6 (IL-6)

IL-6 is a pro-inflammatory cytokine that is present in the circulation very early in any inflammatory response (155). Released from T cells, neutrophils and macrophages, IL-6 can cross the blood-brain barrier and is known to change the temperature set point in the hypothalamus inducing fever (151). IL-6 also promotes B cell production and suppresses T Regs differentiation (156). IL-6 is interesting as it can also be anti-inflammatory, inducing macrophages to release the powerful endogenous immunosuppressant prostaglandin E2(157). Later in the response IL-6 also attenuate TNF- α and IL-1b activity by promoting release of IL-1b receptor antagonist (IL1RA) and soluble TNF receptors (154).

Interleukin-8 (IL-8)

IL-8 is a potent chemokine produced by macrophages and epithelial cells it selectively attracts neutrophils to the site of injury (158). All cells with TLRs can secrete IL-8. It also induces the synthesis of neutrophil derived platelet activating factor and its levels rise dramatically in acute inflammatory conditions particularly sepsis and trauma (159).

Interleukin-10 (IL-10)

IL-10 is an anti-inflammatory cytokine that has multiple immunomodulatory functions and is produced by several regulatory immune cells including T regs and B regs (160,161). It downregulates the expression of Th1 cytokines, MHC antigens and co-stimulatory molecules on macrophages and can inhibit the proliferation of activated T cells (162)IL-10 also promotes B-cell survival and enhances their proliferation as well as antibody production. Early IL-10 has been shown to correlate with severity of injury(163). It has been linked to multi-organ failure in trauma (164), though this may be indirect through its immune suppressive actions(165,166). .

Interleukin-13 (IL-13)

Closely related to IL-4, IL-13 is a mediator of the Th2 response and physiological aspects of the allergic response (167). Like IL-4 it induces IgE class switching and secretion from B cells (168).

Interleukin-17 (IL-17)

IL-17 is produced by T-helper cells in response to IL-23 and has a strong role in adaptive immunity and maintenance of the inflammatory response(169). For example,

it has been associated with Type 2 allergic response and is implicated in the development of asthma and cystic fibrosis(170). As stated earlier it is involved in the activation and recruitment of neutrophils through inducing cytokines and chemokines such as IL-6, IL-8, GM-CSF, IL-1 β and TNF α (171).

Granulocyte-macrophage colony-stimulating factor (GM-CSF)

GM-CSF is produced by macrophages, T cells, mast cells, fibroblasts and endothelial cells and stimulates stem cells to produce granulocytes and monocytes(172). GM-CSF can also act to prolong the active lifespan of neutrophils and macrophages at sites of inflammation(173). Neutrophil MHC-II expression is also upregulated increasing the activation of T cells in response to antigens (174).

1.8 Inflammaging.

The reduced ability of the older adult to recover from injury or fight infection is multifactorial; reduced mucosal barriers, changes in the mechanics of the GU tract, comorbidities, age-related changes in immunity, nutrition, medication and genetic differences to list a few(175,176). The changes in immune system with age have been coined inflammaging; slightly elevated levels of pro-inflammatory cytokines, acute phase proteins and a reduction in anti-inflammatory cytokines are common(177). The inflammatory response that is mounted by older adults to alarmins, PAMPs and DAMPS appears subdued(178-180). Differences are seen in both the innate and the adaptive response. The innate cellular response of neutrophil function demonstrate reduced chemotaxis, phagocytosis(181), ROS production(182) and NET formation (183). Differences in monocytes and macrophages are not demonstrated by a fall in

numbers but in their reduced ability to produce ROS and phagocytose (184). Dendritic cells show reduced migration and impaired cytokine production (185). There are also reductions in the effectiveness of natural killers cells. The age –related changes in innate cellular immunity are summarised in Table 1.2.

Studies have shown increases in TNF α , CRP and IL-6 in older adults that could be described as a chronic inflammation(186,187). Immunescence has been used to describe the older persons increased susceptibility to a mired of infections in later life(188). Longitudinal studies are now attempting to explain how these defects in the immune system in older adults may be repaired (189).

	Composition	Phenotype	Function
Monocytes/ Macrophages	No change in circulating frequency and/or absolute number. Increased percentage of CD14 ⁺ 16 ⁺⁺ non-classical monocytes. Reduced percentage of CD14 ⁺ 16 ⁻ classical monocytes.	Reduction in CD62L, TLR1/4 expression. No change in TLR2 expression. Increase in CD11b, TLR5 expression.	Decreased monocyte phagocytosis. Decreased ROS production by LPS-challenged monocytes. Increase in TLR4-driven TNF- α production. Decreased production of IL-6 and TNF- α following TLR1/2 stimulation.
Dendritic cells (DCs)	Reduction in Langerhans cell density. Decreased/unchanged frequency of pDCs. Decreased/unchanged frequency of mDCs.	Comparable MHC II, CD11c, CD123 expression on mDCs/pDCs. Comparable TLR7/9 expression on pDCs. Increased percentage of CD83 ⁺ , CD86 ⁺ PBDCs.	Reduction in phagocytosis by MDDCs. Decrease in migration. Impaired TNF- α , IL-6, IL-12 and type I and III interferon production by pDCs.
Neutrophils	No change in circulating numbers.	No change in CD11a, CD11b expression	Reduced chemotaxis in vitro. Reduced phagocytosis. Impaired NET formation. Increased/decreased ROS formation. Impaired receptor recruitment into lipid rafts.
Natural killer (NK) cells	Increase in CD56 ^{DIM} frequency and/or number. Reduction in CD56 ^{BRIGHT} frequency and/or number. Increased CD56 ^{DIM} :CD56 ^{BRIGHT}	Comparable NKG2D, CD2, CD16 expression. Decreased CD94, KLRG1, NKp46 expression.	Reduction in NKCC at the single cell level. Impaired perforin release. Maintenance of ADCC. Reduced cytokine/chemokine output.

Table 1.2. Changes in innate age-related immunity. Taken directly from a review by hazeldine *et al* (190).

1.9 Endocrine and metabolic response to Injury

Early enlightenment into the metabolic response to surgery and injury was made by Cuthbertson in 1932 and then built on by Moore who described classical 'ebb and flow' physiology relating muscle loss and altered nitrogen balance (191). A decrease in tissue perfusion after injury causes a reduction in metabolism, that is followed by increases in metabolic rate and a hyperdynamic circulation after 3-5 days. Their work highlighted the need for adequate nutritional support and rehabilitation (192). Although much of the evidence extrapolated from animal models and observations appears exaggerated(193), the pattern of recovery is seen in the modern survivor from severe injury.

In practice, the so called 'ebb' phase is short and within a few hours the flow phase is seen. Increased metabolic turnover and activation of the innate immune system overwhelms the victim of trauma.

The endocrine, metabolic and inflammatory response to tissue injury is coined the 'stress response.' Historically this has been easiest to study this after elective surgery where a controlled traumatic injury is inflicted at a set time and the response thereafter can be studied (194,195). However, the stress response from surgery has become more difficult to study as modern regional and general anaesthetic techniques dampen the neuroendocrine response to tissue damage.

1.9.1 The Initial Endocrine Response.

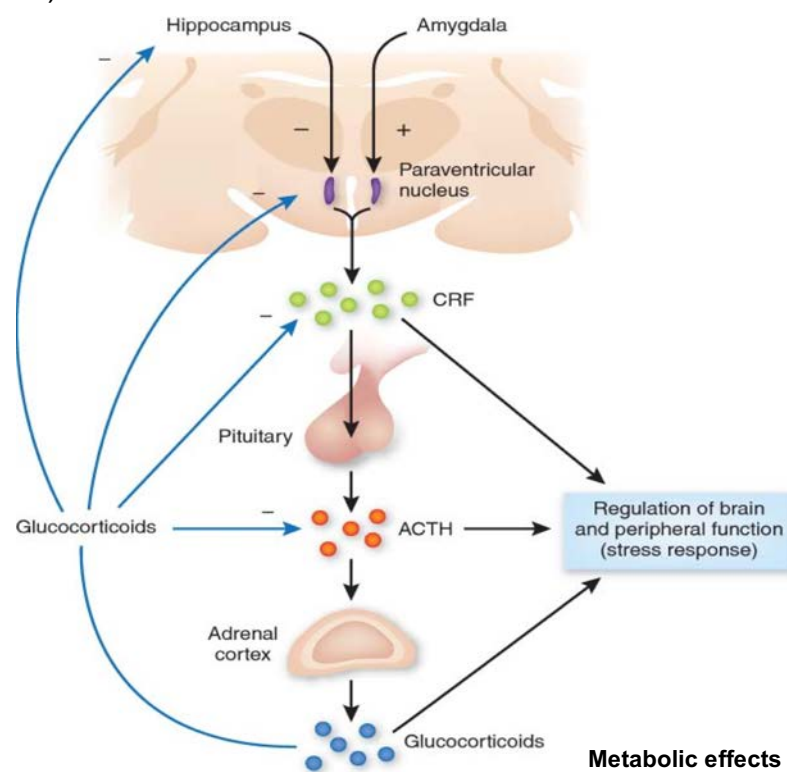
The major endocrine response to injury is to produce catecholamines and corticosteroids. These groups of molecules produce a short-term and long-term stress

response that has evolved to protect the individual from life threatening injury. The ability to enhance the fight or flight reaction is realised through a number of physiological changes.

Afferent somatic and autonomic nerves from the area of injury stimulate the sympathetic nervous system and the hypothalamic-pituitary axis (196). The modes of stimuli are diverse; pain, fear, hypovolaemia, tissue damage and/or immunological activation (197). The hypothalamus responds by firing sympathetic fibres to the adrenal medulla causing catecholamines to be immediately released into the circulation. The hypothalamus also releases corticotropin-releasing hormone (CRH) which acts on the anterior pituitary to release adrenocorticotrophic hormone (ACTH) that stimulates the Adrenal cortex to produce mineralocorticoids and glucocorticoids. The pathway and actions of these hormones is described in Figure 1.6(198,199).

The dominant catecholamines, adrenaline and noradrenaline, are released from the adrenal medulla by the action of ACTH from presynaptic nerve terminals as a result of hypothalamic stimulation. The resulting adrenergic response yields a marked tachycardia and hypertension. Hepatic, pancreatic and renal organ systems respond. An early survival response is to 'retain water' as renin is converted from angiotensin I to angiotensin II stimulating aldosterone from the adrenal cortex to reabsorb more sodium. The pancreas releases glucagon that stimulates the liver to breakdown glycogen releasing glucose and lactate into the circulation. Free fatty acids are mobilised from lipid stores and insulin acts on fat and carbohydrate metabolism (200-202)

The immune system is also affected and neutrophils are mobilised from the marrow in response to increased catecholamines and cortisol (203), though systemically cortisol suppresses neutrophil function but also keeps numbers high by inhibiting their apoptosis. The immune system also stimulates an adrenergic response and pro-inflammatory cytokines act directly on the hypothalamus and there is thus extensive cross-talk between the immune and endocrine systems during the stress response (196). Cytokines stimulate the production of prostaglandin E2 directly to stimulate the hypothalamic–pituitary–adrenal (HPA) axis or the HPA axis detects lower levels in the periphery via sensory vagal afferent fibres to inform the CNS of local inflammation(197).



Immune effects of Cortisol

- Up-regulations of anti-inflammatory proteins
- Down regulation of pro-inflammatory proteins
- Apoptosis of lymphocytes
- Inhibition of neutrophil function

Metabolic effects of Cortisol

- Gluconeogenesis in liver
- Mobilisation of amino acids
- Lipolysis
- Proteolysis

Figure 1.7. The HPA axis is under the excitatory control of the amygdala and inhibitory control of the hippocampus. Modified from an article by Steve Hyman(198).

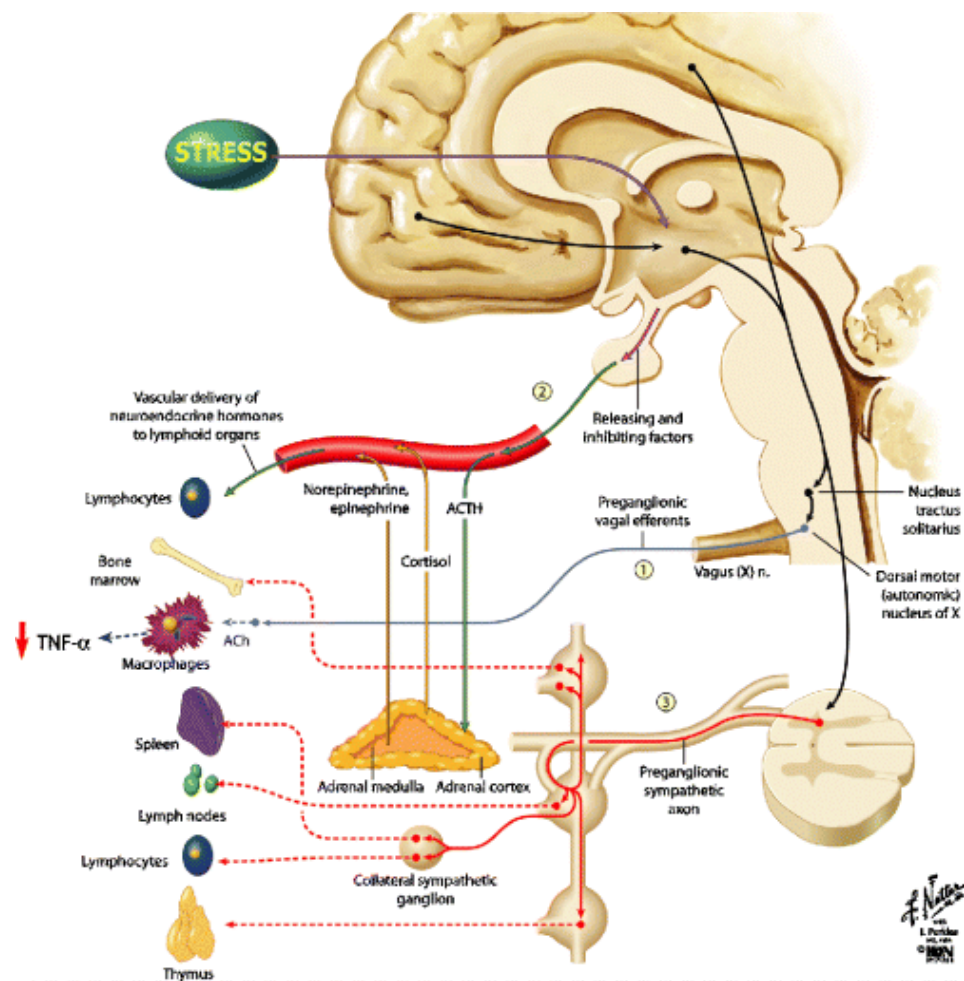


Figure 1.8: Examples of efferent nerve pathways that modulate immune function modified from a review by *Blalock et al (204)*.

- (1) Vagal acetylcholine acts on macrophages to blunt proinflammatory cytokine synthesis.
- (2) Hormones from the hypothalamic-pituitary-adrenal axis modulate lymphocyte function.
- (3) Sympathetic outflow can regulate the function of immune tissues and their cells.

The HPA axis is classically hormonally controlled with stimulation and the levels of hormones being controlled by a negative feedback loop. Managed by hypothalamic and inhibiting factors, the anterior pituitary receives its control from the hypothalamic-

hypophyseal portal system; whereas the posterior pituitary receives direct neural control from the hypothalamus. The anterior pituitary hormones (ACTH) and growth hormone (GH) are released in response to hypothalamic releasing factors, corticotrophin releasing hormone (CRF) and somatotropin. When injured, ACTH stimulates cortisol production within a few minutes (205). Thyroid function also surges initially (206) but is quickly suppressed with 48 hours of injury (207). Other hormones such as luteinizing hormone (LH) and follicle stimulating hormone (FSH) are not thought to significantly change in response to injury(203).

1.9.2 Cortisol and the Cortisol:Cortisone Ratio.

Cortisol baseline levels are 400 nmol/l which can increase to >1500 nmol/l within 4-6 hours of injury and feedback mechanisms fail to lower ACTH and cortisol concentrations in trauma(202). The magnitude of the cortisol response is thought to be proportionate to the size of insult and it is unresponsive to cortical steroid administration(203), although Offner *et al.* found that cortisol levels did not correlate with the ISS (208). Other commentators have found it difficult to agree on absolute values and what constitutes a normal cortisol response during critical illness (209). Cortisol enhances muscle breakdown to provide substrates for repair (207), with gluconeogenic precursors and amino acids mobilised for protein synthesis in the liver and from lipolysis. Cortisol also has a mineralocorticoid effect to retain sodium and water and lose potassium. In contrast, cortisol's anti-inflammatory effects are to decrease cytokines, leukotrienes and prostaglandins (203).

Some cortisol is bound to cortisol-binding globulin (CBG) and albumin and when these are low in trauma and critical illness this might lead to under estimation of the free

cortisol available. Molenaar *et al.* examined this in a critical care population and concluded that as previously reported 25% of the total cortisol is protein bound (202). Others dispute this and argue that CBG should be used to calculate free cortisol when estimations of adrenal function are being made (210).

The concentration of active cortisol in the target tissues is important. This balance between metabolism and production of cortisol is regulated by the action of various enzymes the most important of which is 11 β -hydroxysteroid dehydrogenase(11 β -HSD) as shown in Figure 8. The activity of this enzyme is represented by the Cortisol:Cortisone ratio (F:E) (211).

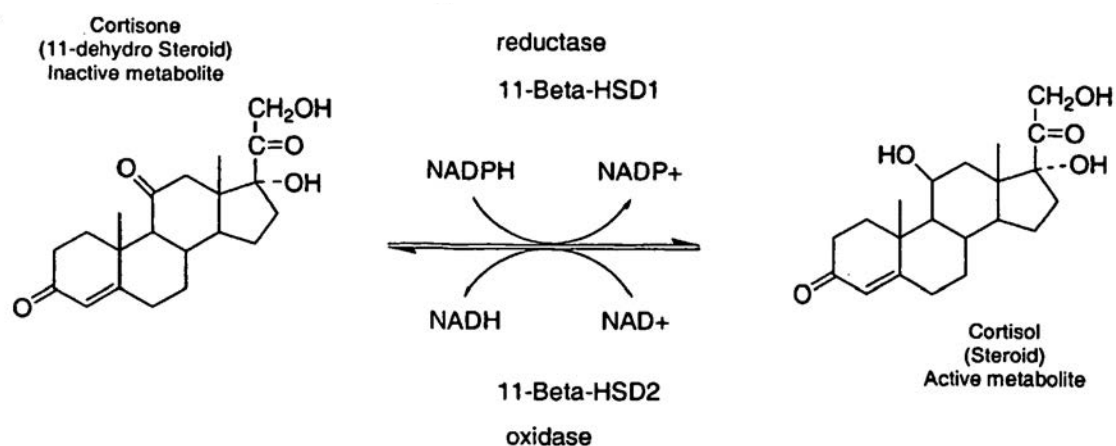


Figure 1.9. Cortisol is stored as Cortisone and mobilised through 11 β -HSD1.

Taken from a patent(212).

1.9.3 Other hormones.

Increases in growth hormone after trauma have metabolic and anabolic effects. The metabolic effects are mild including glycogenolysis and lipolysis. There is an inhibition of glucose uptake and utilisation by cells (213). Growth hormone appears to slow the catabolic effects of the glucocorticoids. The related Insulin-like Growth Factor 1(IGF1)

stimulates growth and proliferation of cells and slows protein catabolism, but there appears to be a paradoxical reduction in IGF1 in relation to growth hormone in trauma and sepsis (214). Prolactin is increased with injury in proportion to the severity of injury (215). The posterior pituitary hormone ADH is another important hormone that acts as a vasopressor and enhances release of ACTH important after injury and haemorrhage (216,217).

Insulin is secreted in response to hyperglycaemia promoting glucose utilisation and glycogen synthesis. This reduces lipolysis and muscle protein breakdown. Trauma causes inhibition of beta-cells in the pancreas by the α 2-adrenergic inhibitory effects of catecholamines (218). The initial response is followed by insulin resistance, the development of which increases mortality and the control of which has improved survival in trauma and sepsis (212).

Thyroxine (T4) and tri-iodothyronine (T3) are secreted by the thyroid in response to thyroid stimulating hormone (TSH). T3 is more active than T4 and they are both highly bound to albumin, thyroxine pre-albumin and thyroid-binding globulin. They increase metabolic rate and heat production in many organ systems resulting in increased oxygen consumption. In trauma concentrations of thyroid hormones appear to be inversely proportional to catecholamine concentrations which return to normal. Initially levels are low after injury and rise over the next two weeks the exact kinetics of this are poorly understood and more research is needed with longer follow-up(219).

1.9.4 Metabolic response to injury

The metabolic response appears to prime the immune system as well as mobilise building blocks for repair from carbohydrate, lipid and protein sources.

Carbohydrate metabolism is driven by catecholamines and cortisol to promote glycogenolysis and gluconeogenesis. Insulin secretion is initially inhibited and subsequently insulin resistance can develop. Prolonged high levels of glucose are associated with increases in mortality (220), impairing wound healing, increasing infections and ischaemic damage to nervous and myocardial tissue.

Inhibition of protein anabolism and promotion of catabolism is driven by cortisol and cytokine production(221). The amount of protein lost is proportional to the amount of surgical stress(222). Skeletal muscle is mainly utilised but there is some visceral muscle protein which is catabolised to release essential amino acids(223). Newly formed proteins in the liver are acute phase proteins and albumin production is reduced which will affect the oncotic pressure of the blood and the extracellular volume(224). This protein loss can be estimated by measuring nitrogen excretion in 24 hour urine(225,226). Attempts at overcoming catabolism by providing additional substrate have been disappointing and will be discussed later (see section 1.10.1.5).

Lipolysis and ketone production is driven by catecholamine, cortisol and glucagon secretion in combination with insulin deficiency(227). Triglycerides are metabolised into free fatty acid and glycerol(228). High glucagon and low insulin promote oxidation of FFAs to acyl CoA that is converted in ketones by the liver(229). The common ketones β -hydroxybutyrate, acetoacetate and acetone are water soluble and are used as fuels(230). The ability to predict the duration and severity of catabolism is a

desirable outcome measure that will allow patients to be stratified as well as allow investigators opportunity to try and reduce it. One strategy is to analyse the pattern of metabolic products commonly known as metabolomics.

1.9.5 Metabolomics

The physiological changes following severe injury yield a pattern of metabolic products that are likely to have a unique signature. Metabolomics is the study of the pattern of metabolic products using mass-spectrometry or Nuclear Magnetic Resonance (NMR) to analyse the spectra and quantities of specific metabolites(231). Complex statistics such as partial least squares discriminant analysis (PLS-DA) can be used to separate individuals even within a specific disease states and has been used to predict their likely response to therapies for example Rheumatoid Arthritis or Type II diabetes(232). This approach has also been used to study metabolic profiles in the critical ill and of particular relevance is the differentiation of septic and sterile SIRS(227).

1.9.6 Androgens and the response to injury

The role of testosterone and oestrogen in injury is not clear. Ninety-five percent of testosterone is produced by the Leydig Cell of the gonad in males, it is responsible for anabolism and secondary sexual characteristics. In women small amounts are synthesised by the thecal cells of the ovaries as well as the zona reticularis of the adrenal cortex in both sexes. Most testosterone is bound to sex hormone binding globulin and regulated by the HPA axis. When low, gonadotrophin-releasing hormone (GnRH) is released by the hypothalamus to stimulate the pituitary to produce LH and FSH controlled through a negative feedback loop (228). Primary and secondary

hypogonadism are reported with changes in LH and FSH in critically ill patients (229). Testosterone levels were found to be reduced by half in men who had myocardial infarction, traumatic brain injury, or elective surgery within 24 hours (230). FSH and LH levels were lower or unchanged. Central hypogonadism occurs with acute illness in both genders. Hypogonadism has been shown after traumatic brain injury (TBI), critical illness and burns (231). The mechanisms of hypogonadism are unclear; low FSH and changes in the pulsatile nature of LH are thought to contribute (232). A direct action on the Leydig cells by TNF α and IL-1 as well as their proposed inhibition of the hypothalamus and pituitary also contribute (233).

While the ovaries and the adrenal glands (in both sexes) produce very little testosterone, they secrete weaker androgens; in particular, dehydroepiandrosterone (DHEA) and androstenedione. These are known to be important in women as they undergo peripheral conversion to the more potent androgens, testosterone and 5 α -dihydrotestosterone (DHT). Androstenediol is a weaker steroid that binds to oestrogen receptors (234). Dehydroepiandrosterone (DHEA) is a C19 steroid that is synthesised in the zona reticularis of the adrenal gland. It is secreted in response to ACTH and circulates predominantly in its sulphated form DHEAS. This abundant early sex hormone serves as a metabolic intermediate in the production of androgens namely testosterone and oestrogen. The role of DHEA metabolites and intermediates in stimulating androgen receptors is now being better understood (235), though the role of this steroid after severe injury is still to be evaluated. The production and metabolism of the androgens is shown in Figure 1.10.

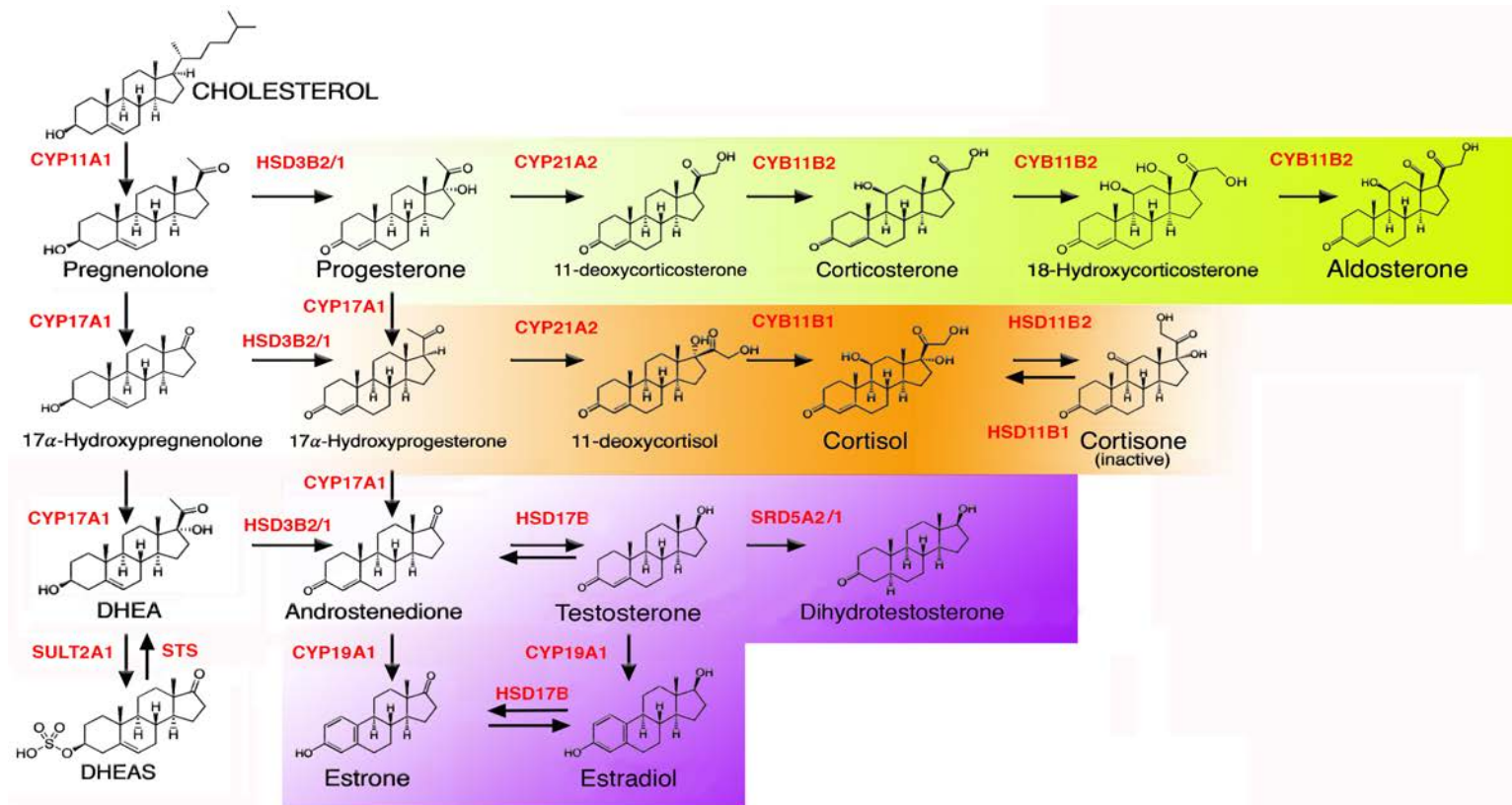


Figure 1.10. The steroidogenesis pathway. CYP11A1, Cytochrome P450sc. HSD3B, 3-hydroxysteroid dehydrogenase isoenzymes. CYB11B, 11 beta-hydroxylase. CYP21A2, Cytochrome P450 21 hydroxylase. CYB11B2 Cytochrome P450 17 hydroxylase. HSD11B, 11 β -hydroxysteroid dehydrogenase. CYP17A1, Cytochrome P450 17 hydroxylase. HSD3B, 3 β -hydroxysteroid dehydrogenase isoenzymes. SRD5A, 5 α -reductase isoenzymes. STS, steroid sulfatase. SULT2A1, sulfo-transferase. CYP19A1, P450 aromatase dehydrogenase HSD17B, 17 β -hydroxysteroid dehydrogenase isoenzymes.

1.10 Ameliorating the immune-endocrine response to severe injury.

1.10.1 Implications of current treatment regimes.

Modifying the clinical course of prolonged SIRS, MOD/F, multiple infections and the severe catabolic state that results in significant morbidity may not be possible with a single modality. This review will now turn to look at treatments that modify the immune-endocrine response following injury before examining novel treatment ideas.

1.10.1.1 Haemostasis

The doctrinal shift in the early use of tourniquets by the military has led to this practice being more frequently adopted by the NHS (236). The use of novel haemostatic agents has been met with mixed reviews(237). The Medical Emergency Response Team (MERT) was based out of a Chinook helicopter in Afghanistan and had a pre-hospital care doctor on board (238). Blood products could be given in transit to the hospital and the administration of tranexamic acid (TXA) was routinely given. Early adoption of the evidence from the CRASH2 study of the benefits of early TXA will have saved lives (239), particularly when the study showed that TXA was best given within 1 hour when a reduction in mortality of over 30% was seen (OR 0.68) for bleeding deaths (240,241). TXA inhibits plasmin and reduces clot breakdown. No increase in thrombosis was seen in CRASH2 and in a subsequent retrospective cohort study the increases in thrombosis observed were not associated with an increase in mortality (242).

1.10.1.2 Massive Transfusion.

The changes in practice have seen a much greater use in blood component therapy. Previous resuscitation protocols advocated large volumes of crystalloid born out of ATLS guidelines. Fluid overloaded coagulopathic patients resulted from this iatrogenic practice that has had a significant effect of mortality and morbidity (243). In 2005 the use of component therapy based on the needs of the patient were advocated (244). Warnings came from the literature about the use of RBCs and the development of MOF, infections and transfusion related lung injury (245). The literature has consistently shown the adverse effects of transfusion (246). A paradigm shift occurred to use blood components to maintain perfusion without the use of crystalloid or colloid as a plasma expander (10). In a 'best evidence review' despite a survivor bias, the authors suggest a clear survival advantage for the early use of fresh frozen plasma, packed red blood cells and platelets in the ratio of 1:1:1(247). The practice of component therapy was adopted despite the appreciation of the immunosuppressive effects that appear to come from donor leukocytes, stored cytokines and other cell debris acting as DAMPS (248).

1.10.1.3 Damage Control Resuscitation and Surgery.

The term 'Damage Control', coined by Stone(249) came from the Navy and can be traced back to Navy doctrine where sacrifices are made to keep the ship afloat (250-252). The concept in surgery was to do enough to save the patient's life but not try to attempt a definitive procedure at the risk to the trauma patient's life and should be carried out after the correction of clotting and other metabolic disturbances i.e. acidosis. Damage control orthopaedics was performed in selected patients and their

then fractures were treated with external fixators and the definitive fixation was performed 24-48 hours (253-255). Not everyone benefits from a damage control approach (256). Although commonly practiced in battlefield conditions and in other areas of trauma, delayed primary closure is not strongly supported in the literature (257).

Damage control resuscitation is carried out concurrently as surgeons work to 'turn off the tap (258).' The proactive correction of acidosis, coagulopathy and hypothermia with component therapy (259) can negate the need to carry out a limited surgical procedure in a well-resuscitated patient. Decisions can then be made to the subsequent pathway of damage control or definitive surgery depending on the status of the patient. Damage control resuscitation may well decrease inflammation as well as correcting coagulopathy (260). Evidence from controlled surgical studies demonstrates that maximum stress response occurs at the end of surgery with good intraoperative anaesthesia (261).

1.10.1.4 Wound Closure.

Good evidence on reduction of the metabolic demands following wound closure came from the burns field. Janzekovic's landmark paper (262) took some time to be adopted. Herndon *et al.* and Wolf *et al.* have driven a wealth of literature supporting the practice (263,264). Their work showed a 40% reduction in metabolic rate in large burns compared to those not grafted for a week. Less bacterial colonisation, sepsis and pain left the authors in not doubt (8,265). While burn wounds are different to trauma, once the burn eschar has been removed, the burn patient shows many similarities to the trauma patient.

Current practice is to serially debride wounds and apply topical negative pressure (TNP) dressings until wounds look ready to close. Low levels of evidence support the use of TNP in a traumatic setting (266) and there are no RCTs. Whether the clinicians can extrapolate the improved healing times into reduced metabolic load over time is controversial. Moreover TNP and early wound closure treatment benefits only lend themselves to severe injuries that have large open wounds.

1.10.1.5 Immunonutrition.

Malnutrition involves all aspects of the immune system. Changes in lymphocyte subsets (decreased CD3+, CD4+ and CD8+) in malnutrition and increases in cortisol and a Th1 to Th2 cytokine shift are observed (267). Enteral nutrition has consistently shown superiority over parenteral nutrition as a means of providing calories to the critically ill (268). The timing of feeding is also important. A meta-analysis by Diog *et al.*, identified 6 RCTs which while small and poorly powered showed a significant reduction in mortality and pneumonia (OR=0.34, p=0.02) when enteral feeding was started within 24hours (269). Most commentators believe a translocation of gut flora is increased by overgrowth, immune-function and/or changes in permeability of the gastrointestinal mucosa (270-272). Another consideration to gut integrity and the immunological consequences of critical illness is gut ischaemia and any reperfusion injury. Feeding may well promote perfusion and minimise this immunological insult (273).

While early enteral feeding does limit some of the proteolysis seen after severe trauma, it doesn't reverse it. Glutamine as a supplement has received notoriety in improving outcomes when used in feeds. An RCT by Hall *et al.* did not show improved mortality

and only trends towards less ICU stay and hospital stay (274). Other studies have shown lower infection rates or less deaths attributed to infection (275). L-arginine as a supplement has also been shown to improve wound healing in a trauma and chronic wound models (276,277). As a substrate for NO production L-arginine restores blood flow and reduces pro-inflammatory cytokines (278). Micronutrient supplementation in the critical ill adult was reviewed in a meta-analysis by Visser *et al*; significant reductions in infections, LOS in ICU or otherwise were not seen. Subgroup analysis did show reductions in mortality but they reported that timing, duration and dosing are key factors (279-281). Pre-operatively giving nutritional immune-enhancing supplements was demonstrated in an RCT by Tepaske *et al*. showing less infections, reduction in sensitivity reaction and lower IL-6 compared with controls(282). A meta-analysis and review of immunonutrition for high risk surgical patients by Marik *et al*. showed a reduction in the risk of infection (OR 0.40), wound complications (OR 0.60) and a reduction of length of stay by over 3 days(283). A review of the field was conducted by Reddell *et al*, they highlight the use of anti-oxidants and/or their co-factors that should be optimised in the trauma patient in ICU (284,285).

1.10.1.6 Intensive care.

The search for a magic bullet in treating critically ill patients has largely focused on the treatment of sepsis. Early, goal-directed therapy and protective lung-ventilation saw early wins with Rivers *et al*. (286) and the ARDS clinical trials network (CTN) (287) reporting benefits. There was early adoption of the use of Activated protein C following the PROWESS trial (288), but questions were asked about the methodology and subsequent trials have failed to show benefits with significant bleeding seen in the

treatment group (289) . Glucocorticoids have been used in sepsis, with or without evidence of adrenal dysfunction. Of the 22 RCTs, there are two large trials in the use of glucocorticoids in the treatment of sepsis with conflicting results; Annane *et al.* (290) showed a reduced 28-day mortality (n=300, 55 vs 61%) where as The Corticosteroid Therapy of Septic Shock (CORTICUS) Trial showed no benefit at 28 day mortality (n=500, 35 vs 32%) with an increase in new sepsis and septic shock in the corticosteroid group. Two meta-analysis of 12 trials supported treatment with corticosteroids as decreasing mortality (38vs44%) (291). Further analysis shows no difference in mortality, no harm and an earlier reversal of shock (292). The surviving sepsis campaign provided a large observational database that also showed no benefit (293). Despite numerous trials the treatment of sepsis has not seen any significant novel drug treatment that has changed outcomes above the use of broad-spectrum antibiotics. However, a recent examination of the impact of beta-blockade in late-stage sepsis has suggested a potential survival advantage which needs to be confirmed in a large scale study(294).

1.11 Novel Treatments to ameliorate of the immune-endocrine response to trauma.

Head injuries were treated with corticosteroids for 30 years before a large multicentre trial involving 10,000 patients proved that the chance of dying was increased by 3% if you were given corticosteroids (295). This highlights the difficulty in trying to target elements of the immune system that often have more than one downstream effect.

1.11.1 Corticosteroids

Adrenal deficient or adrenal suppressed patients fail to respond to the stress response and often need supplementation during/after surgery. The exact optimal regime to mimic the stress response following surgery is still to be evaluated (296,297). The diagnosis of adrenal suppression is much debated. In the management of sepsis and trauma it is clear that single morning cortisol levels are not helpful and even the use of the short or long synacthin tests at times are misleading (298). There are limited data for corticosteroid use in trauma patients. In an observational study α 1-adrenoceptor stimulation was shown to be more responsive after corticosteroid administration (299,300). While not supported by ARDS Clinical Trials Network for ARDS (301), the use of corticosteroids in trauma has been shown to reduce hospital acquired pneumonia with shorter ventilatory days in one study but failed to demonstrate differences in overall mortality (302).

The translation of animal modelled treatments into the clinical setting has had many failures as exemplified by the anti-TNF treatment in septic shock trial (303). Anti-inflammatory cytokine targeting such as transforming growth factor (TGF- β) or IL-10 to reduce the inflammatory response haven't seen their way to clinical trials yet (304). Gene therapy using adenoviral vectors has the potential to treat trauma and sepsis victims; potential targets such as the transcription factors NF-kB, AP-1 and IL-10 due to their role in the regulation of pro-inflammatory genes may be an option (218,305). Limiting the bystander damage caused by the inflammatory response in the lung in acute lung injury using a RNA interference technique is also being explored (306).

Oxidative stress during the initial phases of injury has been implicated in causing tissue damage and increasing tissue leak. In a burn model anti-oxidants have been used to counter this phenomenon. *N*-acetyl-cysteine is one treatment that has experimentally reduced apoptosis in many clinical scenarios such as burns and nerve damage (307). Vitamin C or ascorbic acid reduces the fluid resuscitation by mopping up free-radicals, reducing vascular permeability and lowering concomitant respiratory complications (308). Although there are studies examining the beneficial effects of vitamin C in other inflammatory conditions such as pneumonia, the strength of the evidence to date is not sufficient to recommend its use in trauma (309).

1.11.2 Beta-adrenergic receptors.

In a landmark study by Poldermans *et al.* a 91% relative risk reduction was seen in a RCT involving 112 non-cardiac surgery patients that was stopped early (310). In 2011 Dr Poldermans was dismissed when the integrity of his data was found to be lacking (314,315). A later meta-analysis which excluded the discredited Poldermans DECEASE trials has raised new questions about the risk benefit ratios that have been established to guide the use of peri-operative beta-blockade (316,317). This new analysis established a 27% increase in all-cause mortality.

The use of beta-agonists to control the development of acute respiratory distress syndrome was trialled by Perkins *et al.* Animal and human studies showed reduced neutrophil sequestration and resolution of pulmonary oedema (318). The use of a systemic beta-agonist was stopped early due to deaths in the treatment arm; the study was not going to show efficacy (319).

Beta-blockade has been advocated in trauma (320). A number of studies in burns have supported blocking the action of the 10-fold increase in circulating catecholamines to reduce obligatory thermogenesis, tachycardia, cardiac load and resting energy expenditure (321). Beta-adrenergic antagonists particularly propranolol have been shown to reduce muscle wasting and decrease healing times (322)(323). Friese *et al.* (324,325) in 2008 during a pseudo-randomised trial of trauma victims over 55 years of age found lower levels of IL-6 in their patients receiving beta-blockers. They found decreased healing times and shorter hospital stay in the beta-blockade group and this was particularly significant in the group taking beta-blockers pre-hospitalisation. The only other significant study found a significant increased survival in patients with head injury and a trend towards those without(326).

1.11.3 Anabolic Hormones.

1.11.3.1 Insulin.

Hyperglycaemia is associated with poor outcome in critical care. An RCT from a single centre published data on intensive glucose control by insulin infusion with a relative reduction in mortality of 42% (330). The trial was stopped early and the results became the basis of practice guidelines to keep glucose below 8.4mmol/L. Many found it difficult to replicate these results as the risk of hypoglycaemia in this vulnerable group was high and this was later confirmed by a meta-analysis (331). Supported by later reviews some commentators' believe tighter glucose control may be possible with improved technology. When David Klonoff reviewed the 5 meta-analyses involving

tight glucose control, the benefits to mortality and morbidity are not consistent and is often blurred due to the heterogeneity of the patient groups (327).

When attention is turned to insulin's role as an anabolic hormone the benefits of using insulin to control catabolism may be important. Insulin is thought to inhibit proteolysis and this was confirmed in a study by Whyte *et al* (332). In this small study involving 25 treated patients plus controls, the authors showed that modest glucose control with insulin infusion between 7-9 mmol/L was enough to reduce proteolysis compared with matched controls. Reduction in proteolysis did not appear to be dose dependent and lower cortisol levels were observed in the treatment group. In a severe injury group the use of low levels of insulin infusion may be valuable.

1.11.3.2 Growth Hormone

Utilising the anabolic actions of growth hormone (GH) was an attractive proposal particularly to slow proteolysis in hypercatabolism (333). Trials revealed a positive nitrogen balance and better utilisation of nutrition (334,335) GH was used safely in burns adults and children, where it improved survival, as well as increased muscle growth and decreased wound healing time (336). The results of two RCTs in critical ill patients contradicted this and not only showed increases in mortality but also showed prolonged ventilator times, and hospital stay (337). The observed increase in hyperglycaemia in the GH infusion group and the subsequent increases in MOF and sepsis have been attributed to the worse outcomes. The mechanisms are complex and changes in the pulsatile nature of GH secretion and GH resistance are blamed.

Insulin-like growth factor 1 (IGF-1) has been shown to produce less hyperglycaemia, but when IGF-1 combined with a binding protein -3 (IGFBP-3) it has shown more promise in experimental work (338). IGFBP-3 has a much longer half-life and avoids renal clearance from the circulation.

1.11.3.3 Oxandrolone – a testosterone analogue.

Anabolic androgenic steroids (AAS) act via a single androgenic receptor in various tissues. The potency of AAS relates to their binding affinity to the androgenic receptor. Anabolic steroids have both androgenic and anabolic effects. They can act directly or indirectly. AAS stimulate protein synthesis in skeletal muscle. Testosterone has been shown to reduce muscle catabolism in thermal injury (339). The androgenic side effects of testosterone as well as potential liver toxicity drove the development of testosterone analogues.

Oxandrolone is a C17 α -alkylated steroid but it has 6.6 times the anabolic effects of methyltestosterone but a tenth of the androgenic properties. Oxandrolone is readily metabolised by 5 α -reductase in androgenic tissue, its metabolite then has little effects on the androgenic receptor (340). Oxandrolone has been shown to be very effective at halving protein breakdown and doubling protein synthesis. Evidence from seven prospective randomised studies, 2 of which were double blinded, all showed improved outcomes based on decreased healing times, shorter hospital stay and less muscle wasting in burn injury (341). There have been 2 studies testing oxandrolone in a trauma population. Gervasio *et al.* (342,343) in 2000 showed no improvement in a prospective, randomised, double-blind, placebo-controlled study. Sixty-two patients were given 20 mg OD of oxandrolone for 28 days. Their main positive finding was

increased muscle protein but they failed to reach any other conclusions. In another prospective, randomised, double-blind, placebo-controlled trial by Bulger *et al.* (344) in 2004 studied a more selective group of intensive care patients that remained on ICU for more than 7 days. The forty patients involved showed no difference in outcomes but more ventilatory days and a greater re-intubation rate in the oxandrolone group. Five patients in the control group died compared to one patient in the treatment group and when the mortalities were removed and the analysis repeated this was not significant. The number of ICU days, length of hospital stay and incidence of ARDS was also not significant.

Oxandrolone has the potential to produce the unwanted effects of androgenic steroid hormones. Transient elevations of transaminase levels, as well as reductions in high-density lipoprotein levels have been the most common effects seen in trials (345-347). Other side-effects noted are raised liver enzymes and cholestatic jaundice, the more severe complications of hyperplasia, adenomas and hepatocellular carcinoma are rare. These have occurred in high doses, with prolonged use, when other anabolic agents have also been used and/or in the treatment of aplastic anaemia and Fanconi's anaemia (348). The adenomas and carcinomas have regressed on withdrawal of the drug. These effects have not been seen in any of the studies involving burn patients (349). In nearly 1000 patients androgenic effects were seen in 14 patients; facial hair growth, acne, alopecia, increased libido and clitoromegaly (346,350-355).

Oxandrolone is the standard of care in most burns centres for patients with over 30%TBSA. Burn injury produces an extreme inflammatory response that reduces after the burn excision (356). This has driven targeted approaches using beta-

blockade and anabolic steroids to ameliorate hypermetabolism and catabolism which is now in common use for severe burns (357). However, it is not known whether the perceived benefits of oxandrolone and propranolol in burns patients can be replicated in trauma (358,359). The difficulties of researching this area are the heterogeneous nature of the patients and their injuries which hampers the interpretation of trials in this area.

1.12 Severe Injury in Older Adults

The way older people respond to burns and trauma and their subsequent inflammatory response affects their survival and LOS(360). These differences are relative unexplored following severe trauma but evidence is emerging in some settings. Investigators consistently describe poor survival and increased morbidity following injury(361).

There are anatomical and physiological differences that reflect worse outcomes in the older trauma patient. These are pulmonary, cardiac, renal hepatic, gastrointestinal, musculoskeletal and neurological as well as the immunological differences already discussed (362). Examples such as reduced chest wall compliance and lung volumes that impedes ventilation or the reduced muscle bulk that slows rehabilitation demonstrate how vulnerability the older individual is to severe injury. At all stages of the clinical pathway, geriatric trauma patients show differences in their response (363).

The response to trauma in the elderly follows an expected dampening of the immune response that is seen in other inflammatory conditions and is linked to poorer outcomes with late infections and more MOD/F (364). Vester *et al.* showed how pro-inflammatory

cytokines such as IL-6 were and IFN- γ concentrations were lower in older patients compared to young after orthopaedic trauma(365). With altered coagulation, for example, older patients are often more severely injured than their mechanism suggests(366). The elderly trauma patient is typically under triaged; tachycardia is reduced or absent despite significant bleeding (367,368). Age is an independent risk factor for mortality in head trauma; dura is more adherent and intracranial vessels are easily damaged (369,370). Even a seemingly mild traumatic injury can cause devastating injury to brittle bones and poorly supported soft tissues and demonstrated by pre-tibial lacerations and hip-fracture (371-373).

The combination of complex co-morbidities, lack of physiological reserve and the poor expectation among treating surgeons has lead to poor outcomes. It is unclear that claims of reluctance to manage elderly trauma are manifested in poor outcomes (374,375). What has been demonstrated though, is that an aggressive approach to diagnosis and treatment of elderly trauma can improve mortality (363,375).

1.13 Sexual Differences in Immunity – Implications for Trauma

Animal studies have consistently shown a survival advantage for proestrous females in trauma (376). The evidence demonstrates the wide effects of sex hormones on the recovering injured female (377). Studies involving a murine trauma-haemorrhage model, ovariectomised female rats had improved cardiac and hepatobiliary function when given 17 β –estradiol at the beginning of resuscitation compared to controls (273,378,379). Others have shown that proestrus females are protected against lung injury by maintenance of lung myeloperoxidase and also hepatic function that is likely

to be mediated through a haem oxygenase-1 pathway (380-382). These effects are most apparent at maximal levels of oestrogen (383).

In humans a survival advantage for females is generally seen in trauma but this has not been universal as the studies have mainly been retrospective. Offner *et al.* in 1999 prospectively looked at 545 trauma patients and found males suffer more infections and greater MOF but LOS and mortality were not affected(384). Magnotti *et al.* conducted a retrospective review of 36,000 blunt trauma patients and showed an increase morbidity, ventilator associated pneumonia and bacteraemia but no survival advantage for women (385). In fact the evidence from numerous studies has shown either no difference or a survival advantage for females with most also demonstrating less morbidity for younger females(386-389). In addition, the type of injury may also influence the gender differences following injury as some studies have shown better survival in males for those sustaining thermal injury(389).

1.14 Resolution and Recovery

As the injured patient survives and progresses to recovery there is reduction in inflammation, catabolism and a reparative phase predominates. The hypermetabolic state under neural, hormonal and immunological control via catecholamines, glucagon and cortisol stimulates glucose production in the liver, induces muscle to release amino acids and production of glycerol and free fatty acids from fatty tissue.

It is unknown why the production of substrates for repair is not better utilised, lean muscle loss is as much as 30-40% after trauma and this directly impedes rehabilitation(390). Much of the protein is lost as nitrogen in the urine and

intake/absorption is never able to keep up. The time at which this occurs is thought to be related to the size of the injury, the host response and affected by complications such as infection and surgical reconstruction(202).

The hypermetabolic state eases and catabolism is replaced by anabolism(37). The exact nature of this switch is still to be fully understood. The adrenergic response of stress hormones wanes and a recovery of anabolic hormones dominate. Wound closure, recovery of organ dysfunction and physiotherapy resistive exercise strategies all contribute to a reduction in the metabolic response (388)

The strategies to improve recovery have lagged behind efforts to improve survival in severe trauma. Most of the work comes from either burn injury, TBI or post intensive care illnesses (391). The strategies to ameliorate the endocrine response in burns have been multifactorial and already discussed. It appears that these may be applicable to our severe injury population. Most practitioners are attempting to start rehabilitation much earlier in the patient pathway following injury (392). Research in this area is lacking. The NHS through NICE has acknowledged the lack of resource in this area (393,394). Rehabilitation has consistently been evaluated as lacking for major trauma (395). Part of the problem is the lack of robust outcome measures to test the benefits of interventions. The most popular Patient Reported Health Outcome Measures in Trauma in a review by Hoffman *et al.* reports that the most cited measure SF-36 only covers 6% of The International Classification of Function, Disability and Health (ICF) domains (202).

With difficulties in stratifying the trauma population and measuring robust outcomes it is no wonder that randomised controlled trials in trauma are lacking and difficult to setup.

1.15 Summary, hypothesis and Thesis Aims

The improvements in survival after major trauma have been affected by changes in early management. The positive effect of these interventions is likely to have modified the inflammatory, endocrine and metabolic response. The usual survivors will have had a less severe response but we will also see a cohort of non-survivors whose immune-endocrine response will be more severe. Our attention must now turn to therapeutic options in the days following injury.

To implement any treatments we need robust systems to characterise our patients both clinically and experimentally. Recording outcomes will be more difficult as mortality as an outcome becomes less valid as many more individuals who reach hospital survive. Ameliorating the immune-endocrine response, speeding up recovery and anabolic responses and improving the functional outcome for the injured will see more patients return to work and independent living.

The main aim of this thesis is the characterisation of the inflammatory response to severe injury. A cohort study is used identify targets that may ameliorate the immune-endocrine response to trauma.

The main hypothesis of the thesis is that the kinetics, characteristics and amplitude of the immune and endocrine response to trauma will relate to patient outcomes, including loss of muscle, sepsis and infection incidence. Moreover, that these will differ with the age of the patient.

The hypothesis will be tested by the following aims:

To characterise the immune-endocrine response following severe injury over time and with respect to age.

To interpret the data in order to identify possible treatment(s) to ameliorate the immune-endocrine response to injury.

To identify parameters to improve patient stratification and identify outcome measures for use in future trauma RCTs.

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Chapter 2: Materials and Methods

2.10 Clinical Environment

Military and Civilian trauma patients were and are still being admitted to the new QE Hospital in Birmingham (QEHB). Soldiers returning from Afghanistan arrived between 18 and 72 hours after injury; civilian patients were normally admitted directly from the scene of the injury, a practice re-enforced by the setting up of regional trauma centres. The patients were all resuscitated in a standardised manner, fluids and transfusion and an initial debridement within hours of their injury either in QEHB or in Camp Bastion before transfer to Birmingham. Once wounds are healed military patients are discharged to DMRC Headley Court where physiotherapy, prosthetic fitting and rehabilitation takes place. The civilian patients are generally admitted directly through the Emergency Department (ED), they are treated in a standardised way. When the civilian patients are discharged, they undertake their rehabilitation locally.

2.11 Collaboration with the Surgeon General's Casualty Nutrition Study

2.1.1 Background

When planning the Steroid and Immune Response to Injury (SIR) study, a military study was already running that was investigating nutrition in military personnel; The Surgeon General's Casualty Nutrition Study (SGCNS). Led by Dr Joanne Fallowfield the study had 3 phases. The first two phases were concerned with looking at soldiers before and during an operational deployment to Afghanistan in terms of nutrition, body habitus and strength. The final phase was looking at military personal that had had early measurements in phase 1 or 2 but following them to see what happened when they were injured. This yielded very few study participants, as casualty numbers were

thankfully low at this stage of the conflict in Afghanistan. The SGCNS changed their approach and began to recruit any military injured individual. Recruitment in to the SGCNS still remained low due to limited local resources. This presented an opportunity for collaboration.

It quickly became evident that the SGCNS had had aspirations to measure the inflammatory response and adrenal endocrine response to trauma but did not have the resources to do so. Another area that the CNS Team had had difficulty with was the timing of recruitment into their study. ICU staff had guided the SGCNS team to approach the relatives and patients at least 24 hours after arriving in the new QE, more than 48 hours after injury. ICU staff had reasoned that patient's relatives would be too distressed to be approached earlier. It was difficult for the SGCNS to reconcile this while relying on local staff to recruit and sample patients into their study. Two key events changed the timing of recruitment; the deployment of a research dietician into the UHB to support the SGCNS and the collaboration that formed with the current SIR study that forms the bulk of this thesis.

2.1.2 Recruitment into the combined study.

The SIR study was determined to collect samples as soon as possible to measure the initial inflammatory response to injury with an initial target of 100 patients. The Mental Capacity Act 2005 provides for non-Clinical Trial of an Investigational Medicinal Product (CTIMP) research using a 'Consultee Advice' process(396). Outlining this provision, ICU research and nursing staff reluctantly agreed when the combined study took the lead on identifying and recruiting patients. The SIR study would add an equal number of civilian patients to the combined study. At times clinical and nursing staff

still attempted to dissuade the research team from approaching relatives and patients immediately on arrival in to the hospital. They deemed it was inappropriate to approach relatives who appeared too 'distressed.' While this was handled sensitively, researchers explained to reluctant staff that this would be handled carefully and if the relatives did not want to speak to research staff they would quickly withdraw from any consultation. This did not happen and many saw the approach by research staff as a positive way relatives could participate in the on-going care of their loved-one. During the course of the recruited patients hospital stay, research staff would often be able to fill in gaps in knowledge that relatives had when they had had time to assimilate all the information they had been given by clinical staff.

All military and civilian trauma patients treated at QEHB were assessed for eligibility for entry in to the study. Those who met the following criteria were approached: adults (Age \geq 16 years old) with an ISS $>$ 15 or a thermal injury with \geq 20% TBSA. Traumatic Brain Injury (TBI) alone, or as the major component of an injury profile, was excluded. Patients with chronic inflammatory conditions such as Rheumatoid Arthritis, or life-limiting conditions such as Neoplasia were also excluded.

2.12 Ethical Considerations

Obtaining informed consent from patients was difficult. By the nature of their severe injury, patients often lacked capacity to consent under Mental Health Act (MCA) 2005. When an injured patient lacked capacity under section 3, the study team would gain advice from a 'consultee', personal or otherwise before entry into the study (under the terms of Section 32 and Section 33 of the Mental Capacity Act 2005). When an

individual regained capacity, full consent was obtained from the patient before they continued in the study.

The study was conducted according to Good Clinic Practice and complied with the Declaration of Helsinki, as adopted at the 52nd WMA General Assembly, Edinburgh, October 2000, and with the Draft Additional Protocol to the Council of Europe Convention on Human Rights and Biomedicine on Biomedical Research (CDBI/INF (2001) 5 dated 18 July 2001).

2.13 The Consent Process.

For military patients early notification was received from Camp Bastion to warn the clinicians at the QEHB of their potential arrival. Relatives were notified early by military liaison officers and were normally waiting for the patient to arrive in Birmingham. A rough assessment of eligibility was made and the study team were available on the arrival of the eligible patient from Afghanistan. Relatives were updated on the patient's condition by clinical staff and immediately afterwards the study team would approach the relatives for 'consultee' advice.

The civilian patients were generally recruited earlier into the study, 'relatives' at times could not be found or were unavailable and a professional 'consultee' was used to take initial samples until relatives arrived and were able to be consulted.

Briefing clinical staff to mention the study and introduce the team was a useful tactic. When this wasn't done it was noticeably more difficult to introduce the study to the relatives. A Patient Information Sheet for consultees was given and after they had had time to read and discuss the study if agreeable, a form to record the consultation was

completed. The consultee advice to enter the study was documented in the patient notes.

Only two sets of relatives described their injured relative as someone who would not want to be part of any research, one military and one civilian, and they were not recruited into the study.

2.14 Regaining Capacity to Consent

The introduction of the study to recruited patients that regained consciousness was handled carefully. Initially although patients were awake they still lacked capacity due to medications, head injury or other medical conditions. Verbal consent was sought to take samples or urine while patients had time to read the Patient Information Sheet (PIS) and ask more questions about the study. Only when the investigators were content that the patient had been correctly informed and had capacity did injured patients sign a consent form to formally continue in the study.

At times there was an opportunity to talk to patients immediately after injury before their condition worsened. A verbal consent was initially given and noted. Few were able to formally consent at this time. Even with verbal agreement from the patient, 'consultee' advice was sought before they were formally entered into the study. On one occasion a patient who had not been recruited into the study due to a relatives perception approached the team. The patient expressed disappointment that he had not been able to take part but at that time it was too late to recruit him.

2.15 Sampling Schedule

Blood, 24 hour urine collection and ultrasound thickness of muscle were asked of the volunteers the schedule for which is shown in Figure 2.1. Samples were taken as soon after injury as possible and at regular intervals to capture the inflammatory response to injury through to the rehabilitation phase of care. Due to the practicalities of neutrophil functional analysis, measures of phagocytosis were only performed as an inpatient.

Sampling Schedule for SIR Study

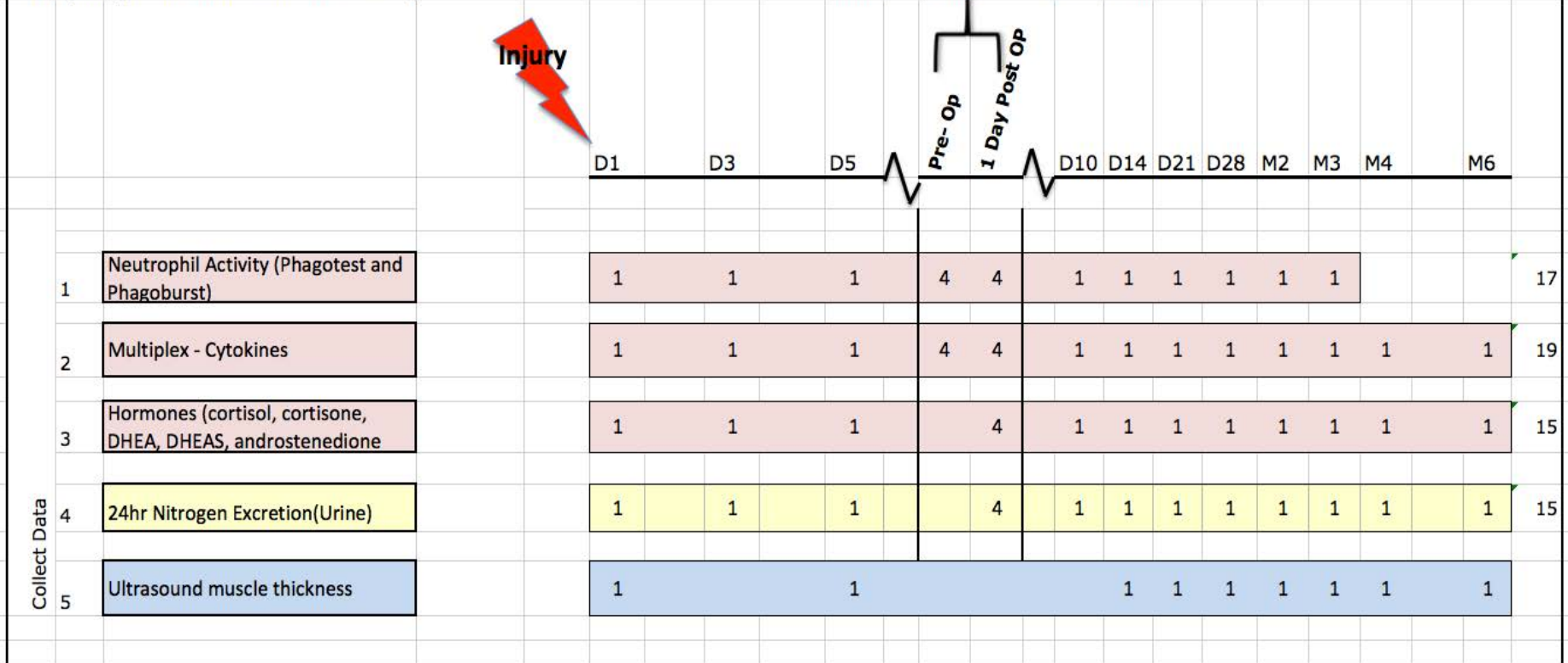


Figure 2.1. Blood and Urine collection and Measurement Schedule for the SIR Study. Day 1 is the first 24 hours following injury. The first sample was taken immediately after recruitment. Subsequent samples were taken between 0700-1000hrs on the day of sampling.

2.16 Patient characteristics.

Demographics of individual and injury were recorded at the time of injury. Initial debridement and later reconstruction were also recorded. Attempts to capture the clinical course were taken from ICU using physiological scoring systems; daily Sequential Organ Failure Assessment (SOFA), Acute physiology and chronic health evaluation II (APACHE2) and Simplified Acute Physiology Score II (SAPSII). Ventilatory days, length of ICU stay and hospital stay were also noted.

2.17 Routine Haematology, Biochemistry and Microbiology.

Normal laboratory results that were part of clinical treatment were extracted from the hospital informatics system. These included extra requests for CRP and Urinary Urea Excretion that was used to estimate Urinary Nitrogen Excretion.

2.18 Samples

Samples of whole blood were taken between 0700-0900hrs on the schedule in Figure 2.1. Serum and plasma were separated as soon as possible but always within one hour of being drawn. Neutrophil analysis and PBMC separation was done within 4 hours. Blood was collected as follows:

Whole Blood: 2 x 8ml Lithium heparinised whole blood tubes (Green Top Vacutainer™) were collected and used for: neutrophil function assays (phagocytosis, superoxide generation); isolation of Peripheral Blood Mononuclear Cells (PBMCs) and spun plasma to be stored at -80°C for further analysis outside the scope of the current work.

Serum: 8ml whole blood (Red Top) was centrifuged at 3000rpm (5804 Eppendorf centrifuge, Eppendorf UK Ltd., Stevenage, UK) for 15min and the serum separated, aliquotted and stored at -80°C:

450µl serum aliquotted in to wells of 3 microtitre plates for later estimation of mtDNA and cytokine analysis.

2 x 500µl serum for Steroid analysis by LC/MS.

500µl serum for NMR metabolomics.

2.19 Urine Samples.

24 hour urine sample collection was started on the day of blood sample collection at 0800hrs. If the first blood sample was taken after 1200hrs, the 24hour urine was started on the next day. The urine was emptied from a patient's catheter bag or patients would pass urine into a bottle that was then transferred to the 5 litre urine container. The containers were collected, volumes measured and then separated into smaller aliquots to be sent to the routine clinical laboratory. Urinary urea was measured in the routine laboratory and nitrogen excretion estimated from it.

2.20 Neutrophil Function

2.20.1 Phagocytosis - PhagoTest™

Using 100µl of heparinised whole blood phagocytosis was measured. Opsonised fluorescein isothiocyanate (FITC) labelled *Esch. coli* were used as the target in the commercial PhagoTest™ Kit (Orpegen Pharma GmbH, Heidelberg, Germany). The assay was performed in line with the manufacturers instructions (397). In this

quantitative test, heparinised whole blood is incubated with the FITC labelled *Esch. coli* at 0°C (control) or 37°C (test) for 10 min. After 10 minutes, 100µl of 'quenching solution' was added to stop the reaction. After washing the cells twice by addition of 3ml of wash buffer and centrifugation at 250g for 10 minutes at 4°C, the red blood cells (RBC) are lysed. A DNA solution is then added after another wash to allow exclusion of artefacts during flow cytometric analysis. During flow cytometry, 10,000 neutrophils were counted. The leukocytes were separated using the red fluorescence histogram and gating in FL2 (Figure 2.2). The forward scatter (FSC) and side scatter (SSC) plot was used to identify neutrophils based on their size and granularity (Figure 2.3), the doublets or triplets were excluded on width. Finally the percentage of neutrophils containing fluorescent bacteria and their mean fluorescence intensity was noted (Figure 2.4). Values for the control kept on ice were subtracted from the test sample.

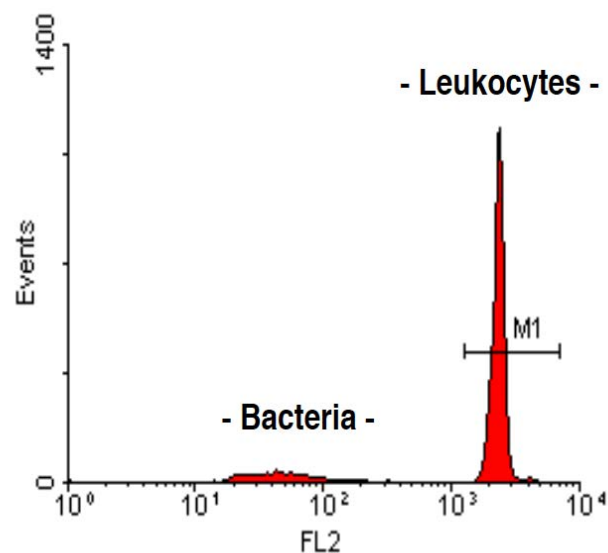


Figure 2.2: The Red Fluorescence Histogram for Phagotest. Gating on channel FL2 allows debris and bacteria to be excluded.

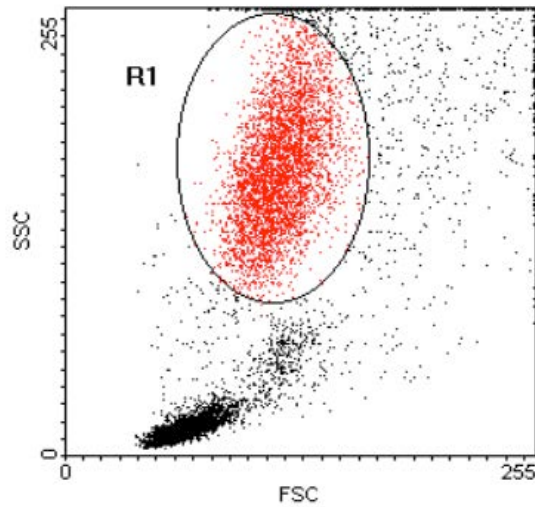


Figure 2.3: Granulocyte gating for Phagotest™. The forward scatter and side scatter histogram is used to isolate the neutrophils based on their size and granularity.

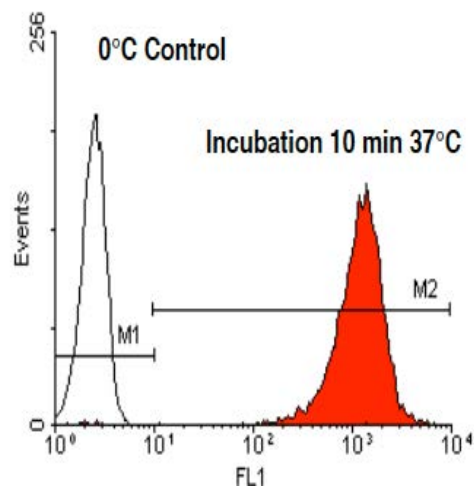


Figure 2.4. The green fluorescence histogram (FL1). F1 selects the neutrophils that have undergone phagocytosis and is able to calculate the percentage and the mean fluorescence.

To assess the phagocytic ability of neutrophils the number of cells that have ingested bacteria as a percentage were multiplied by the mean fluorescence intensity (MFI) divided by 100, known as the Phagocytic Index (PI).

$$\text{Phagocytic Index} = \frac{\% \text{ cells ingested bacteria} \times \text{MFI}}{100}$$

2.21 Superoxide Production - PhagoBURST™.

The PhagoBURST™ Kit (Orpegen Pharma GmbH,) was used to measure superoxide generation from neutrophil oxidative burst. The method relies on the conversion of dihydrorhodamine-123 (non-fluorescent) to its oxidized form, rhodamine-123 (fluorescent) by superoxide that can be quantified by flow cytometry.

The assay was performed as per the manufacturers instructions (398). 100 µL of heparinised whole blood was incubated for 10min at 37°C with 20 µL opsonized *E. coli*, phosphate buffered saline PBS (blank control) 20 µL N-formylmethionyl-leucyl-phenylalanine (fMLP), or 20 µL phorbol-12-myristate-13-acetate (PMA). The fMLP is the low, biological positive control and the PMA the high pharmacological positive control. 20 µL of substrate (dihydrorodamine-123 is converted to rodamine-123 by superoxide) was added and incubated for a further 10 mins at 37°C. The reaction was stopped after 10min by the addition of 2mls of a lysing and fixing solution of paraformaldehyde. The samples were centrifuged at 250g for 5 minutes at 4°C and washed with PBS twice. Finally 200 µL of a DNA staining solution was added and the samples were protected from the light and analysed using BD Accuri™ C6 flow cytometer (BD Biosciences). The gating of cells was the same as for PhagoTEST™, and the MFI in channel F1 was used to indicate the amount of superoxide production with opsonized bacteria after the blank MFI was subtracted as shown in Figure 2.5.

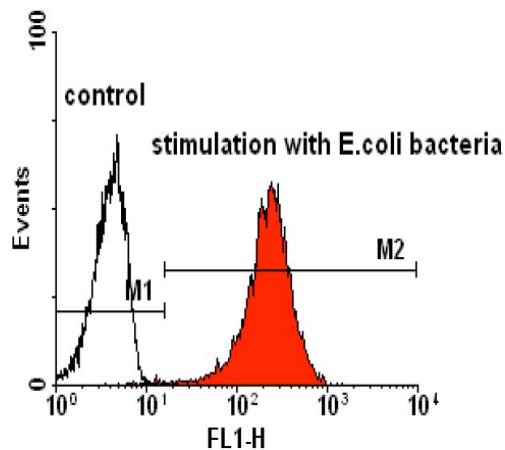


Figure 2.5. The production of Superoxide is captured by MFI on channel FL1. The conversion of dihydrorhodamine-123 (non-fluorescent) to its oxidized form, rhodamine-123 (fluorescent) is caused by the ROS generated by NADPH oxidase.

2.22 Determination of the phenotype of the neutrophil dual population

In order to identify the nature of a second population of neutrophils that appeared during Phagotest and Phagoburst in some injured patients the phenotype under light microscopy and presence of the following surface antigens were investigated: CD11b, CD11b(active), CD14, CD15, CD35, CD33, CD63 and CD66b.

2.23 Neutrophil differential count

A blood film was prepared by adding a 3-4mm drop of blood to a glass slide and backing a second slide held at 45° into the drop and smearing the blood to make a uniform cell layer. The smears were then air dried. The slide was then stained using the REASTAIN QUICK-DIFF Kit (Gentaur Molecular Products, Belgium). Briefly, the stock supplied solutions were poured into separated staining chambers. The slides were dipped into Quick-Diff FIX solution for 1 second 5 times. Then the slide was dipped in to Quick-Diff RED solution for 1 second 10 times. Then the slide was dipped into the Quick-Diff BLUE solution for 1 second 10 times. The slide was

then rinsed with the buffered water solution (Stock Phosphae Buffer diluted 1:20 with deionised water) and air-dried. The slide was then examined under the microscope and a modified differential count performed where 100 neutrophils were counted and the number of band cells and any early myeloid cells recorded.

2.24 Neutrophil cell surface antigen testing

These cell surface antibodies (Table 2.1), or their isotype-matched controls, were added to 100µl whole blood in polypropylene tubes at dilutions recommended by the supplier. Following incubation for 30mins on ice, BD FACS lysing solution (prepared from 10x stock from BD Biosciences) was added and samples incubated in the dark at room temperature(RT) for 20min. The lysate was then centrifuged at 250 x g at 4°C and then washed once in PBS (250 x g, 5 min at 4°C) and re-suspended in PBS.

The proportions of antigen surface expression was determined by flow cytometry using the BD Accuri™ C6 flow cytometer. Granulocytes were gated on FS/SS as described earlier. The isotype control was used to set the zero gate and then the subjects cells were gated on the whole neutrophil population and then the subpopulations separately so that the percentage of cells and MFIs could be compared for each population.

Antigen	Label	Source	Isotype Control	Concentration (µg/ml)
CD11b	PE	eBioscience	Mouse IgG1	1.25
CD11b (active)	FITC	eBioscience	Mouse IgG1	1
CD14	PB	Biolegend UK	Mouse IgG2a	4
CD15	FITC	Biolegend UK	Mouse IgM	0.31
CD33	PE	Dako	Mouse IgG1	0.3
CD35	PE	R&D Systems	Mouse IgG1	1.25
CD63	FITC	AbD serotec	Mouse IgG1	0.1
CD66b	FITC	Biolegend UK	Mouse IgM	2.5

Table 2.1: Details of human antibodies used in immunostaining.

2.25 Soluble CD16

Soluble CD16 was measured using a sandwich ELISA. 96-well microtitre plates were pre-coated with 100µl of 10µg/ml anti-CD16 (clone 3G8 Serotec) diluted in PBS and incubated overnight at 4°C. Plates were washed with PBS containing 0.05% Tween -20 and blocked with 300µl PBS containing 2% bovine serum albumin (BSA) at 37°C for 1 hour. After washing, 100µl of sample and recombinant human CD16 (R&D systems) as a standard were added and incubated at RT for 2 hours. Plates were then washed and 100µl of 0.5µg/ml biotinylated anti-CD16 (clone DJ130c;AbD Serotec) was added and incubated for 1 hour at RT. Plates were washed and 100µl of a 1:1500 dilution of streptavidin-horseadish peroxidase (HRP; Biolegend) was added and incubated at RT for 1 hour in the dark. Plates were washed and 100µl tetramethyl benzidine (TMB)(concentration defined by manufacturer) was added followed by 50ul H₂SO₄(2M) to stop the reaction. Absorbance was read using a spectrophotometer with a primary wavelength of 450 nm and a corrected reading using 570 nm (BioTek® Synergy HT, Northstar Scientific Limited, Leeds, UK). Soluble CD16 concentrations were extrapolated from the standard curve that was calculated from the known concentration standards using GraphPad Prism® software (GraphPad Software Limited, USA).

2.26 Serum DAMPS and Cytokines.

2.27 Mitochondrial DNA (mtDNA) – Polymerase Chain Reaction.

Mitochondrial DNA (mtDNA) was isolated from serum using commercial QIAamp blood DNA isolation mini kit (QIAGEN, Germany). 150µl of serum was removed from the storage plate after a slow thawing overnight at 4°C and placed in an eppendorf. The serum was then spun at 3000xg for 10 min at 4°C. 20µl of protease was aliquotted into a separate 1.5ml eppendorf tube. 50µl of serum was placed in to the eppendorf tube containing the protease and 150µl of Buffer AL was added and mixed by pulse-vortexing for 15s. The eppendorf was then incubated at 56°C for 10 min. 150µl of ethanol (100%) was added to the sample and mixed by pulse-vortexing for 15s. The mixture was then added to the QIAamp Mini spin column (in a 2ml collection tube). After centrifuging at 8000 rpm (5804 Eppendorf centrifuge) for 1 minute the QIAamp Mini spin column was placed in a clean 2ml collection tube and 500µl of Buffer AW1 was added. After centrifuging at 8000 rpm (5804 Eppendorf centrifuge) for 1 minute, the QIAamp spin column was placed into a clean 2ml collection tube. The QIAamp Mini spin column was carefully opened and after adding 500µl of Buffer AW2 was centrifuged at 13,000 rpm for 3 min. The QIAamp Mini spin column was then placed into a clean collection tube and centrifuged at 13,000 rpm for 1 minute to dry the column. Finally the QIAamp Mini spin column was placed into a 1.5ml eppendorf tube and 50µl of Buffer AE was added to the column. After incubating at room temperature for 5 min, the column was centrifuged at 8,000 rpm for 1 min (5804 Eppendorf centrifuge). The remaining liquid contained the DNA isolate.

The amount of mtDNA was measured by real-time quantitative polymerase chain reaction (PCR) using a LightCycler 480 (Roche). Primers for the Cytochrome B gene, which is present specifically in the mitochondrial genome, were used at a concentration of 0.5µM. The primers were supplied by Eurofin Genomics. The sequences of the primers targeting cytochrome B (CytB) were;

Forward: 5'-ATGACCCCAATACGCAAAT-3' and Reverse: 5'-CGAAGTTTCATCATGCGGAG-3'. 5µl of isolated DNA was amplified using the LightCycler 480 SYBR Green Master kit (Roche). The thermal profile was set up as follows: a first denaturation step at 95°C for 5 min followed by 45 cycles at 95°C for 10 seconds, 60°C for 20 seconds and 72°C for 20 seconds. A mtDNA standard curve was calculated using purified mtDNA, which was extracted from mitochondria isolated from peripheral blood mononuclear cells and quantified using a Nanodrop spectrophotometer. mtDNA concentrations in patient plasma were quantified in ng/ml.

2.28 High-Mobility Group Protein Box 1 (HMGB1).

HMGB1 was measured using a commercial sandwich ELISA according to manufacturers instructions (IBL-International, Hamburg). 100 µL of diluent buffer was added into the respective wells of the microtitre plate. 10 µL of diluent buffer for blank, standard provided in the kit, or each serum sample was then added into the respective wells and shaken for 350 rpm for 30s on plate shaker. After covering with an adhesive foil the plate was incubated for 24 hours at 37 °C. The adhesive foil was then removed and the incubation solution discarded. The plate was then washed with an automated plate washer 5 times with 400 µL of diluted Wash buffer. The excess solution was then removed by tapping the inverted plate on a paper

towel. 100 μ L enzyme conjugate was then added into each well and the plate covered with foil to protect from light and incubated for 2 hours at 25 °C. As before, the adhesive foil was removed and the incubation solution discarded. The plate was then washed 5 times with 400 μ L of diluted wash buffer and the excess solution was removed. 100 μ L colour solution(TMB) was add into each well and incubated for 30 min at RT (18-25°C). The colour reaction was stopped by adding 100 μ L of Stop solution into each well and the well contents gently mixed by shaking the plate. The optical density (OD) was measured with a spectrophotometer at 450 nm. A standard curved was constructed and the ODs of the samples used to calculate the concentration of HMGB1 in the sample using PRISM (GraphPad Software, Inc., California).

2.29 Cytokine assays – multiplex.

The 9-plex assay for IL-1 β , IL-4, IL-6, IL-8, IL-10, IL-17, TNF α and GM-CSF using luminex technology was used (Bio-Rad Laboratories, Germany). Calibration of the Bio-Plex® 200 reader was carried out using the Bio-Plex™ Calibration Kit (171-203060) prior to running the assay. Four-fold dilutions were used to generate a standard curve of the 'standards' supplied in the kit. Coupled beads were prepared by diluting stock beads 1:10 with assay buffer and placing in a falcon tube (Becton Dickinson, UK) and keeping in the dark. After 50 μ l of the prepared beads were added to the supplied 96 well black plate, two washes were performed with 100 μ l of wash solution using a magnetic plate washer (BioTek® Synergy HT, UK). 50 μ l of standards, blanks and serum was added to each well and the plate was covered with an adhesive strip and kept in the dark using foil. The plate was gently shaken at 1100 rpm for 30 seconds then left for 30 min on the shaker set at 300rpm. After incubation, the supernatant was removed and the plate was washed three times

with 100µl wash buffer. Detection antibody was prepared as per manufacturer's instructions and 25µl of diluted detection antibody was added to each well of the plate. The plate was again covered with an adhesive strip and foil and left on the plate shaker at 300 rpm for 30 minutes. The supernatant was again aspirated and plate was washed with 100µl wash buffer three times using a magnetic plate washer. 50µl of streptavidin-PE was added to each well and the plate was covered and shaken as previous described. In the last step, the plate was washed three times with 100µl of wash buffer and 125µl of assay buffer was added to each well and the plate was sealed and shaken at 850rpm for 30s on plate shaker before being read. Standard values were entered into the reader so that the Bio-Plex Manager software version 6.0. (Bio-Rad Laboratories, Germany) could plot the standard curves and calculate the concentration of cytokines.

2.30 Adrenal Steroids LC/MS

The Adrenal Steroids were measured using LC-MS by Dr Angela Taylor in the Centre for Endocrinology, Diabetes and Metabolism at the University of Birmingham. Cortisol, cortisone, testosterone, Androstenedione, DHEA and DHEAS were purchased from Sigma Aldrich, UK. Internal standards cortisol-d4, testosterone-d3, DHEA-d6 and DHEAS-d2 were used in the steroid measurements.

The steroids were extracted from 200µL of serum (after addition of an internal standard solution) via liquid/liquid extraction with 1mL of tert-butyl-methyl-ether (MTBE). The MTBE layer was evaporated to dryness, the samples were then reconstituted and analysed by LC-MS/MS initially for the quantification of cortisol and cortisone as previously described (399). Following this first assay the samples were again dried and derivatised to form oxime derivatives, which increases mass

spectrometry sensitivity to the delta-5 steroids such as DHEA. Serum androgens (testosterone, androstenedione and DHEA) were measured following oxime derivatisation as described previously(400,401). DHEAS was measured from 20µL of serum following protein precipitation as described previously (402,403).

For each of these methods a Waters Xevo mass spectrometer with Acquity uPLC system was used, fitted with a HSS T3, 1.8µm, 1.2x50mm column. Cortisol, cortisone and steroid-oxime analysis was carried out in positive mode and DHEAS analysis in negative mode. A gradient system of (A) water with 0.1% formic acid and (B) methanol with 0.1% formic acid was optimised for resolution of the steroids in each experiment.

All steroids were quantified with respect to a linear calibration series (calibrators were prepared in PBS with 0.1%BSA) with appropriate internal standards, ranging from 0.1 to 250ng/mL for steroid and steroid-oxime analysis and 250 to 10,000ng/mL for DHEAS analysis. Each steroid was identified by a matching retention time and 2 mass transitions in comparison to a reference compound. Each steroid was quantified relative to its deuterated analogue with the following exceptions androstenedione was quantified to testosterone-d3 and cortisone to cortisol-d4.

2.31 Lutenising Hormone (LH) and Sex Hormone Binding Globulin (SHBG)

As part of the SGCN Study, a serum sample was aliquoted from the military injured patients and sent to Newcastle University Hospital where the routine laboratory measured LH and SHBG. Dr David Woods made the data available to allow interpretation of measurements made by the SIRStudy.

2.32 Muscle Thickness (Ultrasound)

Ultrasound analysis of muscle thickness was used to estimate muscle loss over time. Measurements were taken from 4 different sites; Biceps Brachii, Radial Forearm Rectus Femoris and Rectus Abdominis using a portable ultrasound machine (SonoSite M Turbo Ultrasound machine with a HFL 38 High frequency linear transducer). The measurements were attempted weekly while in hospital and then at the same time as the blood sampling schedule as an outpatient.

Muscle thickness has been measured by portable ultrasound to assess lean body mass in a method previously described by Campbell *et al*(404,405). In the severely injured military patient, the degree of injury to limbs (including amputation), and/or bandaging of injuries results in difficulty taking of measures at the mid-upper arm, forearm and thigh and so Rectus Abdominis was added. The thickness of the muscle was measured on a frozen frame of the ultrasound image of the muscle group. This was repeated twice to give an average of 3 and a forth measurement was taken if any of the previous measures differed by more that 10%. Muscle thickness was taken weekly during admission and then at the blood measurement time intervals following discharge.

2.33 24-hour Nitrogen Excretion

Urinary Urea Excretion (UUE) was measured by the routine laboratory and is expressed in mmol/24hrs. Urinary Nitrogen excretion was chosen as faecal looses are unpredictable (226,406). The following calculation was used to estimate Urinary Nitrogen Excretion:

Urinary Urea Excretion (mmol/l) x 0.028¹ x.252² = Total Urinary Nitrogen excretion (g/l)

Urinary Nitrogen Excretion was used to estimate protein breakdown during catabolism. Other measures such as 3-methylhistidine have been used as measures of protein breakdown in sepsis and trauma(408). In this longitudinal study initial values would have been confounded by dietary meat intake which should be not be part of the diet 3 days prior to measurement (409).

2.34 Data management and Statistics.

The complexities of sample scheduling and the large amount of data recorded required a database to manage this. A database was designed in Filemaker, a cross-platform solution that can be used on Mac and Windows. This was double password protected and patient identifiers held in a separate link file. A sample schedule automatically reminded researchers when to take samples and complete measurements.

Analysis of variance (ANOVA), and Student t-tests were used for normal data, and Mann-Whitney tests were used for non-normal data. Categorical data is expressed in frequencies and proportions and continuous data in means and standard deviations. Statistical significance was accepted at the $p < 0.05$ level. Generalised linear mixed-effects models were used to examine the change in variables over time. Patients were included in the models as random effects to account for the possible correlation in serial measurements made on the same individuals. Time

¹ {Molecular Weight - N₂ = 28 (mol)}.

² Constant to account for urinary sources of nitrogen other than urea e.g. porphyrins, creatinine and other non-urea sources(407).

was modelled using restricted cubic splines to allow for flexible non-linear relationships. The distribution of each variable of interest was examined and log-transformed to normalise the distribution if necessary. SOFA and SAPSII scores were modelled as Poisson distributions due to their skewness and non-negative ranges. Plots of predicted average fixed effects with 95% confidence intervals were produced for the first 6 months and first 4 weeks after injury as required.

For the multivariable regression models that included Age, ICU Length of Stay, and NISS as explanatory variables, the strength of association with the outcome being modelled was assessed using F-statistics and associated p-values. The p-value cut-offs associated with the strength of evidence in the data are as follows: strong evidence ($p \leq 0.01$), moderate evidence ($0.01 < p \leq 0.2$), weak evidence ($0.2 < p \leq 0.5$) and no evidence ($p > 0.5$).

Predicted fixed effects were then treated as univariate time series' and pairs of these time series were then compared by evaluating their cross-correlation. This was examined to identify the lagged times at which maximum autocorrelation was obtained. Model selection was performed using AIC to determine the order of the spline used to model time. Analysis was conducted in R using libraries lme4, effects, rms, ggplot2, and SPSS. Median values are presented with interquartile ranges unless otherwise stated.

Chapter 3: The Clinical Course after Severe Injury

3.0 Introduction

Most of the trauma studies with significant patient numbers have focused on mortality and length of stay as a primary outcomes(410). Work has been focussed on the early measures of inflammation(411,412) and the endocrine response(413). Only studies in burn injury have characterised the longer term pathophysiological changes after severe injury(414,415). The progress in the management of severe trauma(416) highlighted the need for longitudinal studies with detailed sampling and measures to capture the clinical, immune and endocrine temporal changes that occur following injury. The inclusion of enough older injured patients would allow comparisons with younger severely injured patients to examine any significant differences in mortality and morbidity(362).

3.1 Patient population – Military and civilian severely injured patients

Military and civilian patients recruited into the SIR Study were followed from injury or as soon as possible on return from Afghanistan. This chapter summarises the demographics and the clinical journey of these patients. It will outline how the patients were selected, their recruitment and their progress within the study. Various clinical measurements, routine and study specific, were made to help the understanding of how the management of these severely injured patients goes far beyond the initial 24 hours of resuscitation that has had so much emphasis in the past.

3.2 Eligibility

Life threatening injury produces a systemic inflammatory response. The anatomically based Injury Severity Score (ISS) has been a long established method

for describing severe injury(417). An ISS >15 is considered a severe injury in polytrauma that evokes a SIRS response (418). It has been modified to take into account multiple injuries over one body site and called the New Injury Severity Score (NISS) (419).

While ISS and NISS can be calculated in thermal injury, they were not designed to compare burns and trauma and their use in this way is flawed (420,421). The size of the burn in terms of depth and %TBSA predicts outcome (422,423) and a thermal injury of over 20% produces a hypermetabolic and systemic inflammatory response (424). ISS>15 and/or burn injury with %TBSA>= 20 were therefore chosen as inclusion criteria in the SIR study. (425)

Traumatic brain injury (TBI) can leave patients in a permanent neurological deficit that hampers rehabilitation. The primary shearing effect on mid-line structures as well as the secondary local inflammatory reaction interferes with hypothalamic and pituitary function following traumatic brain injury (425). Including patients with severe TBI into the study would add many confounding factors with the potentially long sedation periods and the unique management protocols that can conflict with the resuscitation goals used for injuries in other anatomic regions. Patients with primary TBI or where TBI was a major contribution to their injury burden were excluded.

3.3 The SIR Study population

3.4 Recruitment

Recruitment for the study took place between May 2011 and July 2012 and is detailed in Figure 3.1. There were 995 patients screened and 106 patients were

eligible. Of the 106 patients approached, 4 patient family groups refused (2 military and 2 civilian). 102 severely injured patients estimated to have an ISS \geq 16 at the time of admission were entered into the study. Two patients were subsequently withdrawn from the study, one sustained an iatrogenic injury in Camp Bastion and the other patient had an undiagnosed carcinoma. Once a detailed examination of all the injuries was performed, many of the severely injured had extremity trauma that was not fully appreciated by ISS. NISS was therefore used to stratify the severely injured trauma patients. Five patients had a NISS <16 on later review and were excluded from any further analysis. All patients except the fatalities completed their in-hospital measurements, 35 patients failed to have their final 6 month measurements.

Three patients withdrew from the study but agreed to allow data already collected to be used; one found the measurements as an outpatient too cumbersome and the others were civilians who had large distances to travel for follow up. The cohort has been divided by age: young \leq 49 and old $>$ 49 years. A cut off of 49 years of age was used to separate young and old as age related differences in immune response are present by the 6th decade (426). The young patients were also separated into military and civilian to determine if the possible differences in health and fitness status of these groups may influence response to injury.

Data will be presented as medians with Interquartile Ranges (IQR) unless stated otherwise.

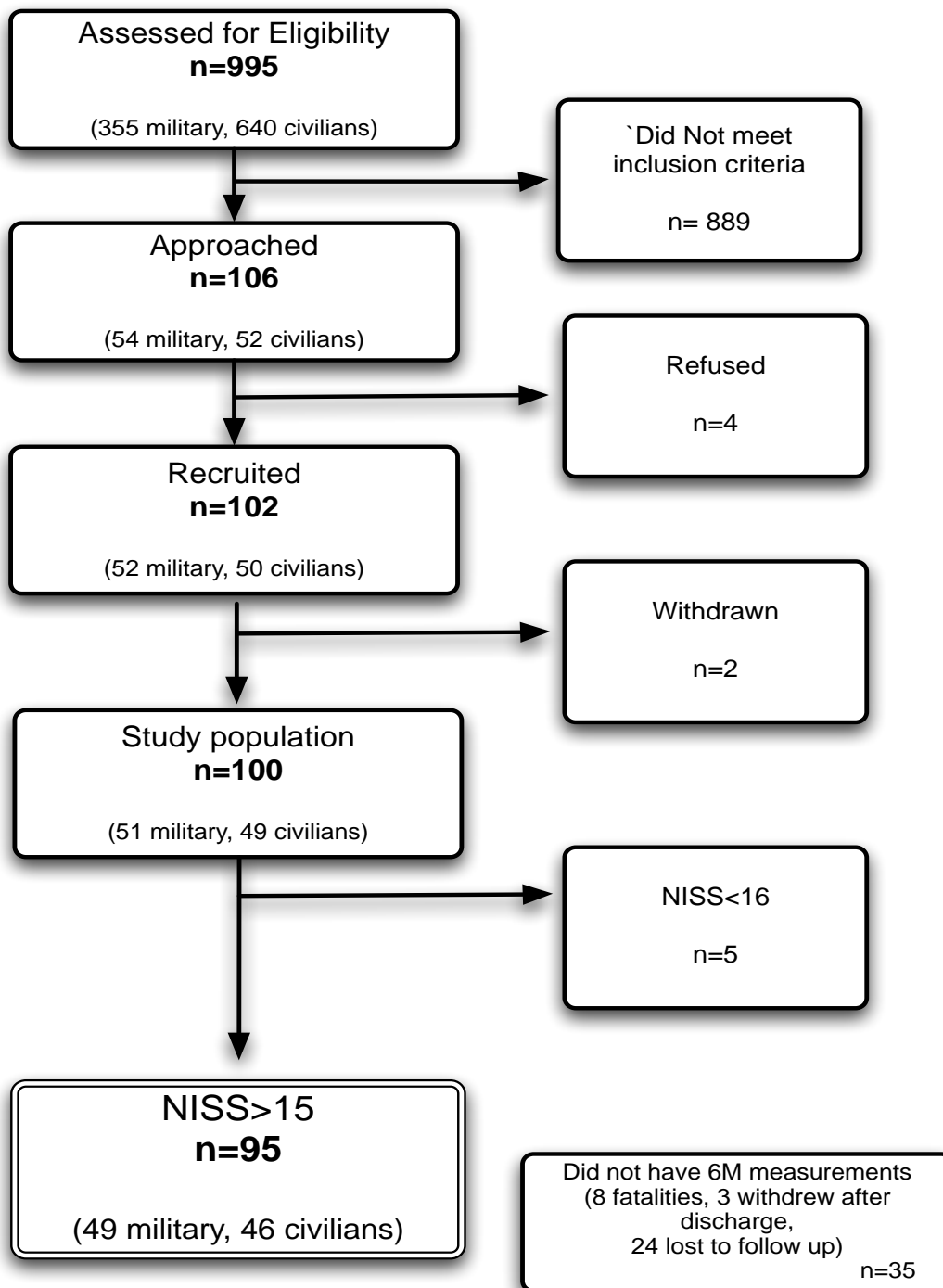


Figure 3.1. Consort Diagram of SIR Study recruitment.

3.5 Demographics

A summary of the cohort demographics and clinical course are shown in Table 3.1. Mortality was 8.4% (8 of 95) in those patients arriving at the Queen Elizabeth Hospital (QEH). The deaths all occurred later than 48 hours; median time 22 days (13-47) from injury. Total median ISS and NISS values were 26 (18-34) and 34 (29-44) respectively. The physiological and organ dysfunction scores of APACHEII, SAPS2 and SOFA after injury were 23 (16-28), 51 (36-57) and 9 (6-11) respectively.

The patients who survived remained ventilated for a median of 10 (6-17) days, remaining on ICU for a further day, (11, 5-18), and their median length of stay in hospital was 38 days (23-59). 23 patients had a septic episode in the first 48-hours following injury. 59 (62%) patients had at least one septic episode and most occurred during the second week following injury.

3.6 Young versus old severely injured patients.

Mortality rate was slightly lower in the young compared to the old patients, 5% vs. 19% (4 of 74 and 4 of 21; $p=0.047$). Differences in the young and old patients who survived were compared. The young patients had a higher NISS ($p=0.003$) and received more RBCs ($p=0.01$) and FFP ($p=0.001$) in the first 24hrs than the older cohort. The younger patient cohort also received less crystalloid ($p<0.001$). There were no significant differences in ISS, number of infections, number of operations, ICU scores (APACHEII, SOFA and SAPS) or ICU and total Length of Stay (LOS) between the young and old cohorts.

	Total	Young		Old	
		Survived	RIP	Survived	RIP
Patients	95	70 95%	4 5%	17 81%	4 19%
Male:Female	85:10	65:5	4:0	13:4	3:1
Age	28 (24 - 48)	27 (23 - 32)	23 (21 - 30)	60 (55 - 65)	73 (66 - 81)
GCS	14 (3 - 15)	14 (3 - 15)	3 (3 - 3)	14 (14 - 15)	15 (15 - 15)
ISS	26 (18 - 34)	26 (17 - 34)	49 (41 - 66)	24 (22 - 27)	27 (19 - 35)
NISS	34 (27 - 43)	36 (29 - 48)	49 (41 - 66)	27 (22 - 34)	31 (26 - 45)
Ventilator Days	10 (6 - 20)	10 ** (6 - 15)	36 (16 - 51)	16 ** (8 - 23)	13 (3 - 26)
Days on ICU	12 (6 - 19)	12 (6 - 18)	15 (5 - 44)	13 (2 - 20)	13 (4 - 26)
APACHE2 (Day1)	23 (16 - 28)	22 (17 - 26)	30 (28 - 32)	24 (11 - 31)	29 (26 - 34)
SAPS2 (Day1)	51 (36 - 57)	50 (39 - 55)	55 (51 - 59)	53 (28 - 67)	64 (55 - 76)
SOFA (Day1)	9 (6 - 11)	9 (6 - 11)	10 (7 - 12)	8 (5 - 11)	10 (9 - 11)
Infectious Episodes Per Patient	1 (0 - 4)	2 (0 - 4)	5 (1 - 16)	1 (0 - 4)	2 (0 - 6)
RBCs 1st24hr	8 (0 - 15)	10 (2 - 17)	22 (10 - 34)	2 (0 - 8)	0 (0 - 5)
FFP 1st24hr	6 (0 - 15)	10 ** (1 - 15)	22 (8 - 35)	1 ** (0 - 3)	0 (0 - 0)
Crystalloid/colloid (Litres) 1st24hr	1 (0 - 2)	0 ** (0 - 1)	1 (0 - 1)	2 ** (1 - 3)	2 (2 - 3)
Operative procedures/ patient, n	5 (3 - 8)	5 ** (3 - 8)	10 (5 - 18)	4 ** (1 - 5)	3 (0 - 7)
Length of hospital stay, days	37 (20 - 59)	41 (23 - 59)	44 (15 - 71)	37 (31 - 85)	20 (11 - 26)

Table 3.1: Summary of demographics and clinical course (median, IQR). Young and Old are ≤ 49 or > 49 respectively. (* $p < 0.05$, ** $p < 0.01$)

3.7 Mechanism of Injury

The mechanism of injury for all patients is shown in Figure 3.2. An explosion, primarily the Improvised Explosive Device (IED) (40, 42%) and Road Traffic Collision (RTC) (27, 28%) were the most common causes of injury. Patients injured by explosion or gunshot wound (GSW) were predominantly young, whereas patients injured by RTC were split between young (14, 15%) and old (13, 14%) as would be expected in civilian life. 8 (8%) of patients had a primary burn injury.

The age distribution of patients is shown in Figure 3.3. The military patients were younger (26 years, 22-28) and more commonly injured by IED (39, 41%). The civilian casualties were older (48 years, 33-60) and more commonly injured by Road Traffic Collision (27, 28%) ($p < 0.00001$).

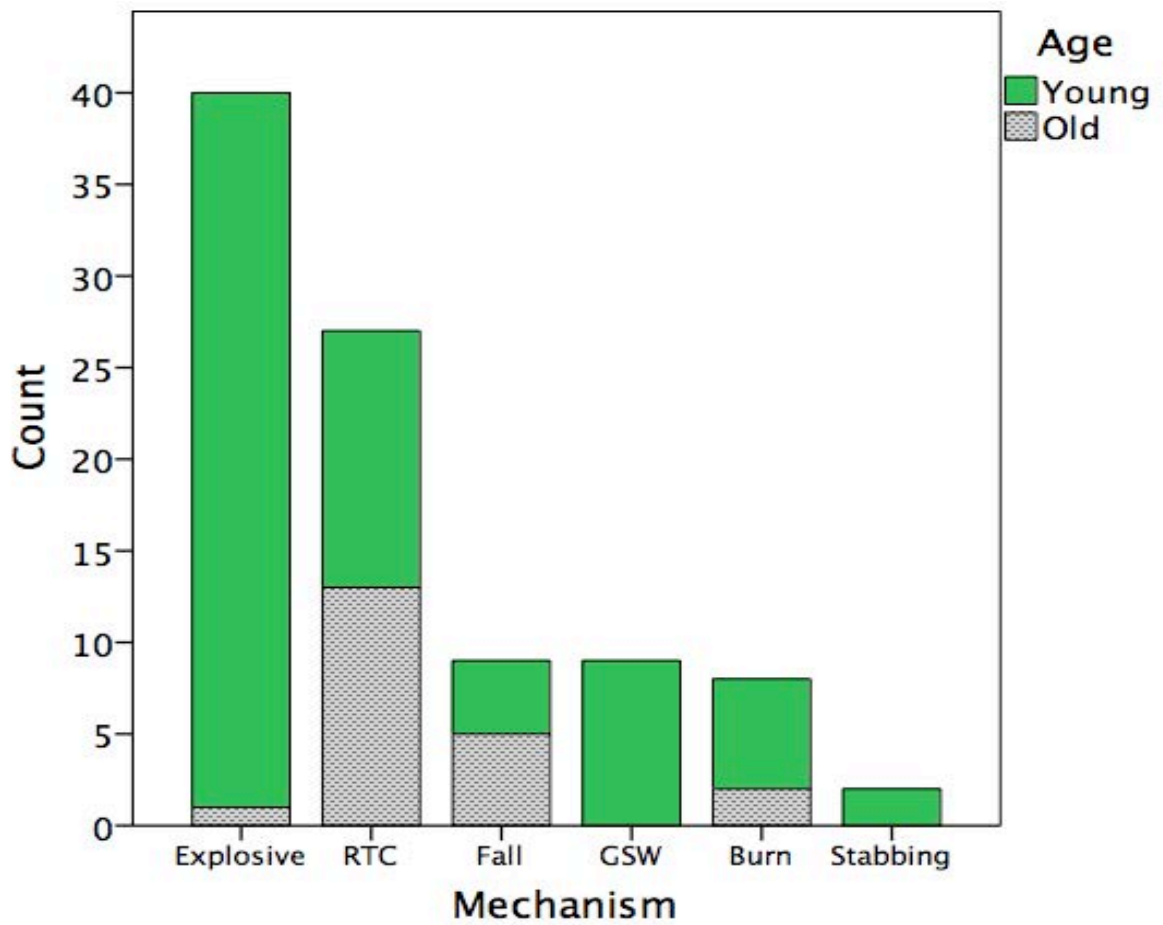


Figure 3.2. Mechanism of Injury for the severely injured patients (NISS>15) separated into young and old (n=95).

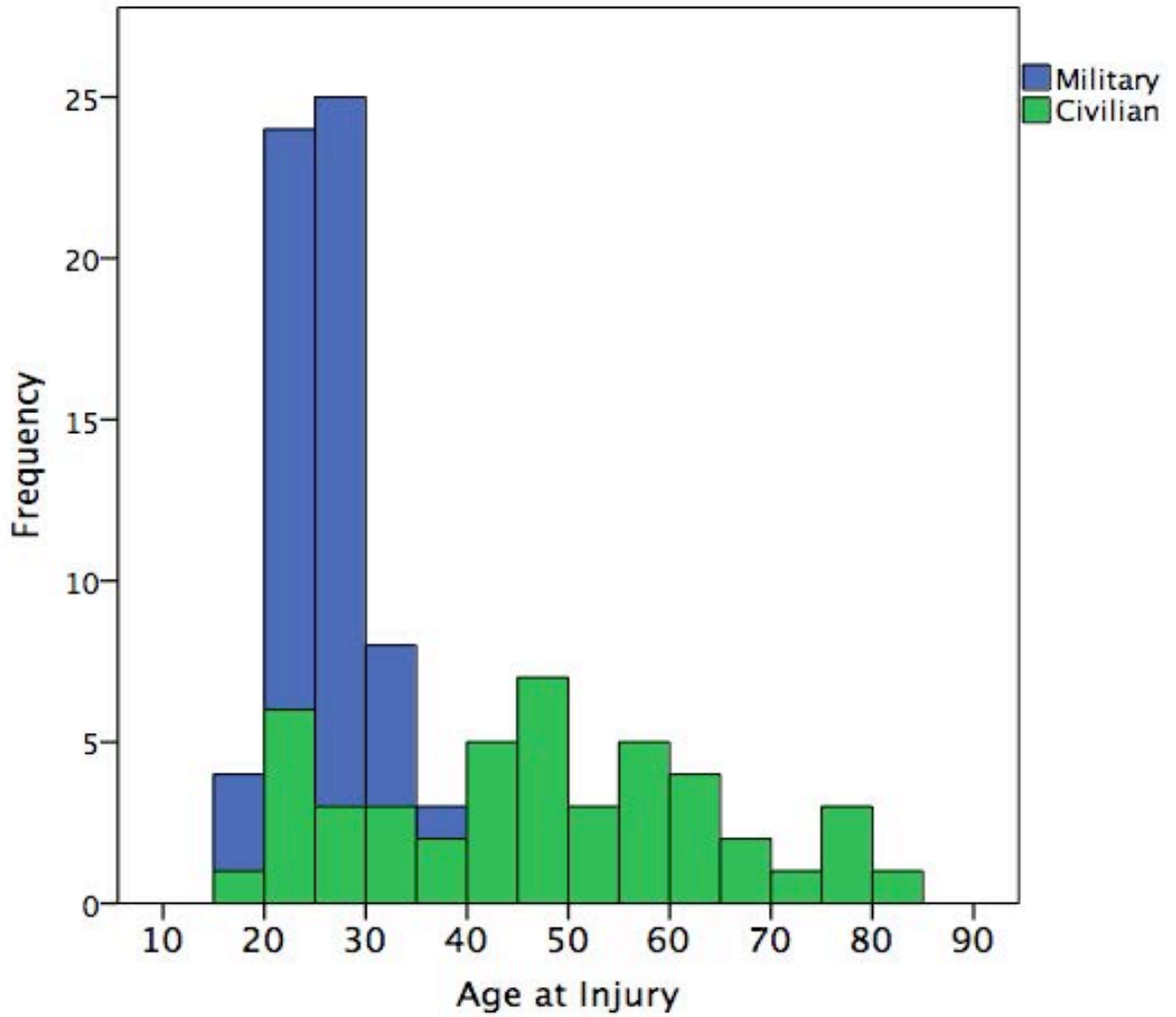


Figure 3.3. Age distribution at the time of injury for severely injured patients (NISS>15) separated into military and civilians (n=95).

3.8 Young military versus civilian casualties

The young patients were separated into military and civilian cohorts and the demographics are shown in Table 3.2. When comparing young military and civilians, there was again no difference in ISS scores but using NISS, military patients were more severely injured 41 vs. 34 ($p=0.007$). Military patients did receive more blood products during their resuscitation in the first 24 hours; RBCs 14 vs. 0 ($p<0.001$) and FFP 14 vs. 0 ($p<0.001$). Conversely, civilian patients received more crystalloid than military patients 0 vs. 3 litres ($p<0.001$). The military patients required more operative procedures than civilians, 7 vs. 3 operations ($p<0.001$) and stayed in hospital longer 50 vs. 24 days ($p=0.007$). Military patients demonstrated more organ dysfunction in the first 24 hours than civilian patients as described by SOFA score of 10 vs. 7 ($p=0.018$). No differences were seen between the young military and civilian patients with respect to the number infectious episodes, ventilatory days and ICU length of stay. There were also no differences in the physiological scores of APACHEII and SAPSII that were calculated in the first 24 hours.

	Military		Civilian	
Patients	49	RIP 2	25	RIP 2
Male:Female	49:0		20:5	
Age	26	(22 -28)	35	(24-42)
GCS	3	(3 -15)	15	(6 -15)
ISS	26	(18 -38)	27	(16-36)
NISS	41*	(30 -54)	34	(22-41)
Ventilator Days	9	(6 -12)	15	(8 -21)
Days on ICU	11	(6 -17)	12	(6 -21)
APACHE2 (Day1)	23	(19 -27)	20	(13-29)
SAPS2 (Day1)	50	(42 -56)	50	(24-55)
SOFA (Day1)	10*	(8 -12)	7	(5 -10)
Infectious Episodes Per Patient	2	(1 - 4)	1	(0 - 4)
RBCs 1st24hr	14**	(9 -26)	0	(0 - 5)
FFP 1st24hr	14**	(8 -23)	0	(0 - 2)
Crystalloid/colloid (Litres) 1st24hr	0**	(0 - 0)	2	(1 - 3)
Operative procedures/ patient, n	7**	(5 - 9)	3	(1 - 5)
Length of hospital stay, days	50*	(31 -59)	24	(16 -46)

Table 3.2. Demographics of young patients (<=49 years old) separated in to military and civilian casualties. Median values are shown with IQRs, significance levels * <0.05, ** <=0.001.

3.9 Intensive care scores

3.9.1 Sequential Organ Failure Assessment (SOFA)

SOFA score is used to assess the degree of organ failure/dysfunction on ICU(427). The score totals each organ system. The respiratory, cardiovascular, hepatic, coagulation, renal and neurological systems are scored from 0 - 4 based on physiological parameters and then totalled. Individual organ scores range from 0 for no dysfunction and 4 for severe organ dysfunction/failure.

SOFA scores are summarised for the severely injured patients (NISS>15) who survived. The daily SOFA scores are shown in Figure 3.4. Most injured patients had left ICU by 9 days but a small number had a prolonged ICU stay. SOFA score peaked at 48 hours after injury (10, 8-13).

3.1.1.1 Simplified Acute Physiology Score (SAPS) II

SAPSII is made up from 12 physiological measurements and 3 disease related scores(428). The physiological measures used are the worst in each 24hours period and started in the first 24 hours of admission to ICU. These can be followed daily and used to stratify the patients in terms of their clinical course later.

Modelled data (Figure 3.5) separated into young and old patients did not demonstrate a significant difference in scores. When the young patients were separated into military and civilian patients there was a significant difference: military patients had a higher SOFA on admission but also the rate of improving SOFA and SAPS was faster for the military patients as represented by the steeper slope.

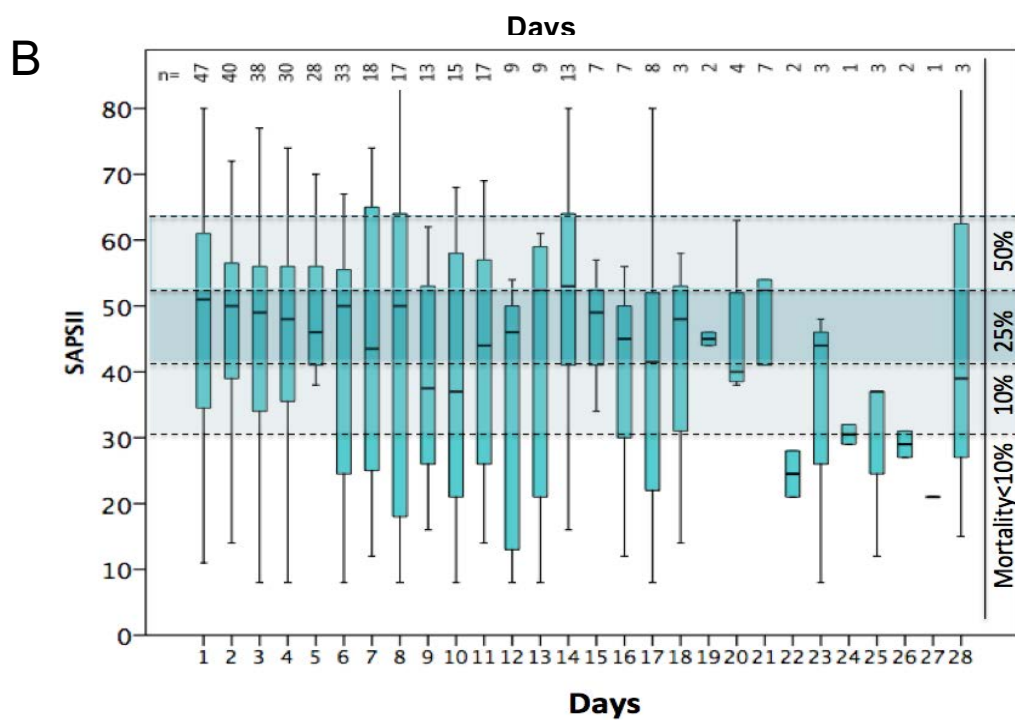
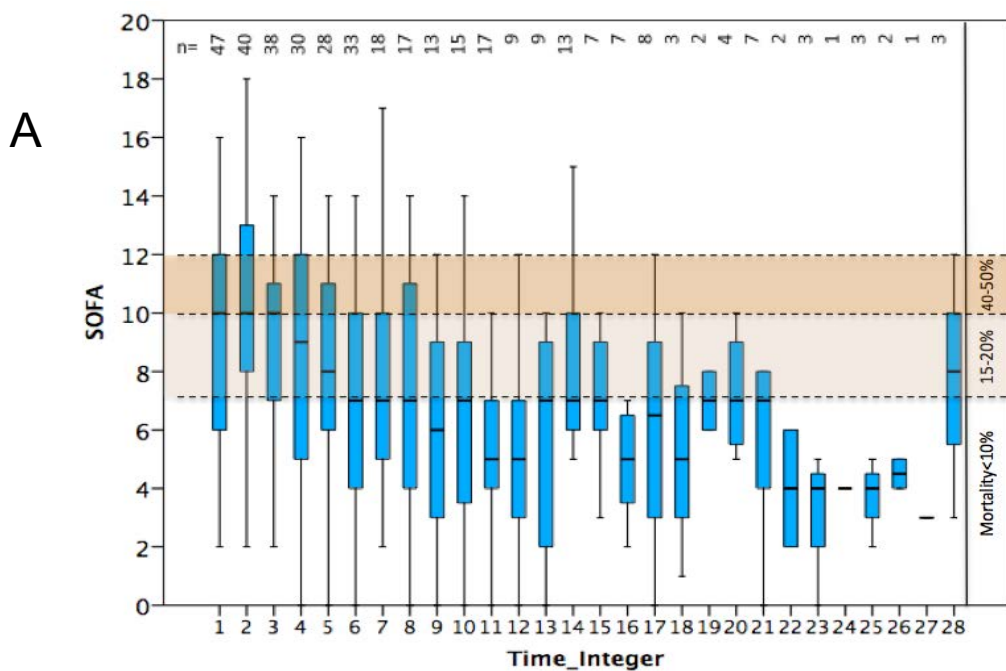


Figure 3.4. Daily (A) SOFA and (B) SAPSII values for injured trauma patients on ICU that survived. Median values with IQR are shown. % Mortality bands are shown as shaded bars.

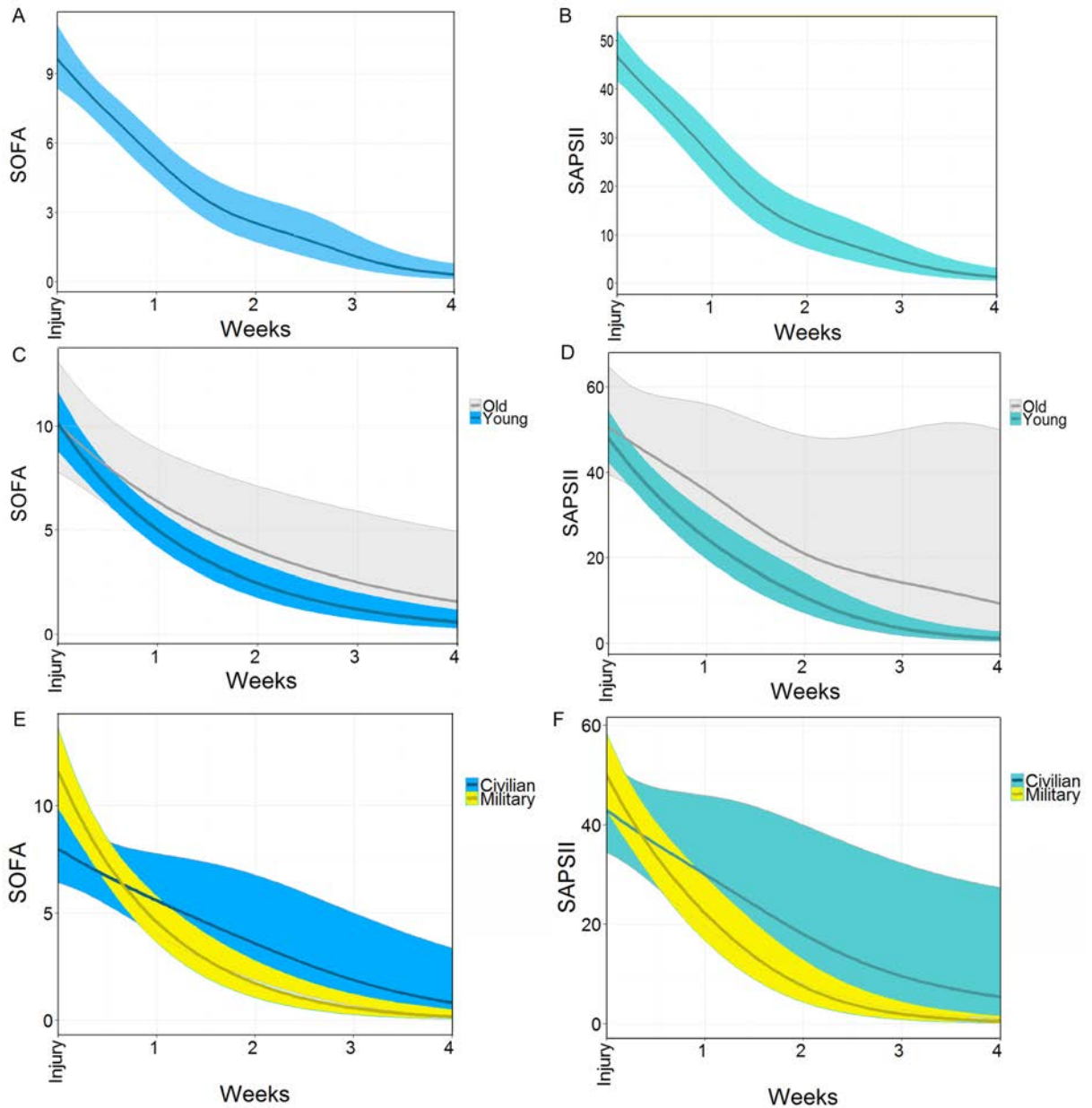


Figure 3.5. Modelled data of (A) SOFA and (B) SAPSII Scores in Severely injured patients on ICU with NISS>15 who survived. Young and old (C) SOFA and (D) SAPSII scores. Young patients were also divided into military and civilian (E) SOFA and (F) SAPS II. Data was modelled using a non-linearmixed effects technique; modelling time with a 4-knot cubic spline provided the best fit. Means and 95% confidence intervals of predicted fixed effects of time are shown.

3.10 Nitrogen Excretion

Catabolism is typified by muscle loss and a negative nitrogen balance. Nitrogen loss was estimated from total urea excretion as described earlier (Section 2.13). The changes in nitrogen excretion over time are summarised in Figure 3.6. Injured patients reached a median maximum excretion of 24.0 g/day (17.8 – 38.0) and this occurred in the second week following injury. The data was modelled using a mixed effects model including time as a 7-knot restricted cubic spline. The model confirms that the peak nitrogen excretion occurred in week 2 and the lowest nitrogen excretion occurred after 6 weeks. Past 6 weeks post injury nitrogen excretion increased again slowly before levelling off, as the number of observations drop the confidence intervals widen. Young patients had a higher median maximum nitrogen excretion than old patients, 33.8 (18.5-41.4) vs. 22.9 (14.4-23.8) $p=0.021$. The military patients did not differ significantly in their peak nitrogen excretion compared to the young civilians.

From the model (Figure 3.7) it can be inferred that there is strong evidence that nitrogen excretion values initially increase, peak in the second week before rapidly decreasing to a minimum between 6-8 weeks before rising and reaching a steady state by 3 months. After 3 months the confidence intervals widen and the strength of the relationship weakens significantly. There is moderate evidence that nitrogen excretion decreases with age (Figure 3.7 B) but increases with ICU length of stay (Figure 3.7 D). There is only weak evidence of a positive effect from injury severity (NISS) (Figure 3.7 C). The time taken to reach maximum nitrogen excretion was positively influenced by age (Figure 3.8) with older patients taking longer to reach maximum nitrogen excretion compared to younger patients.

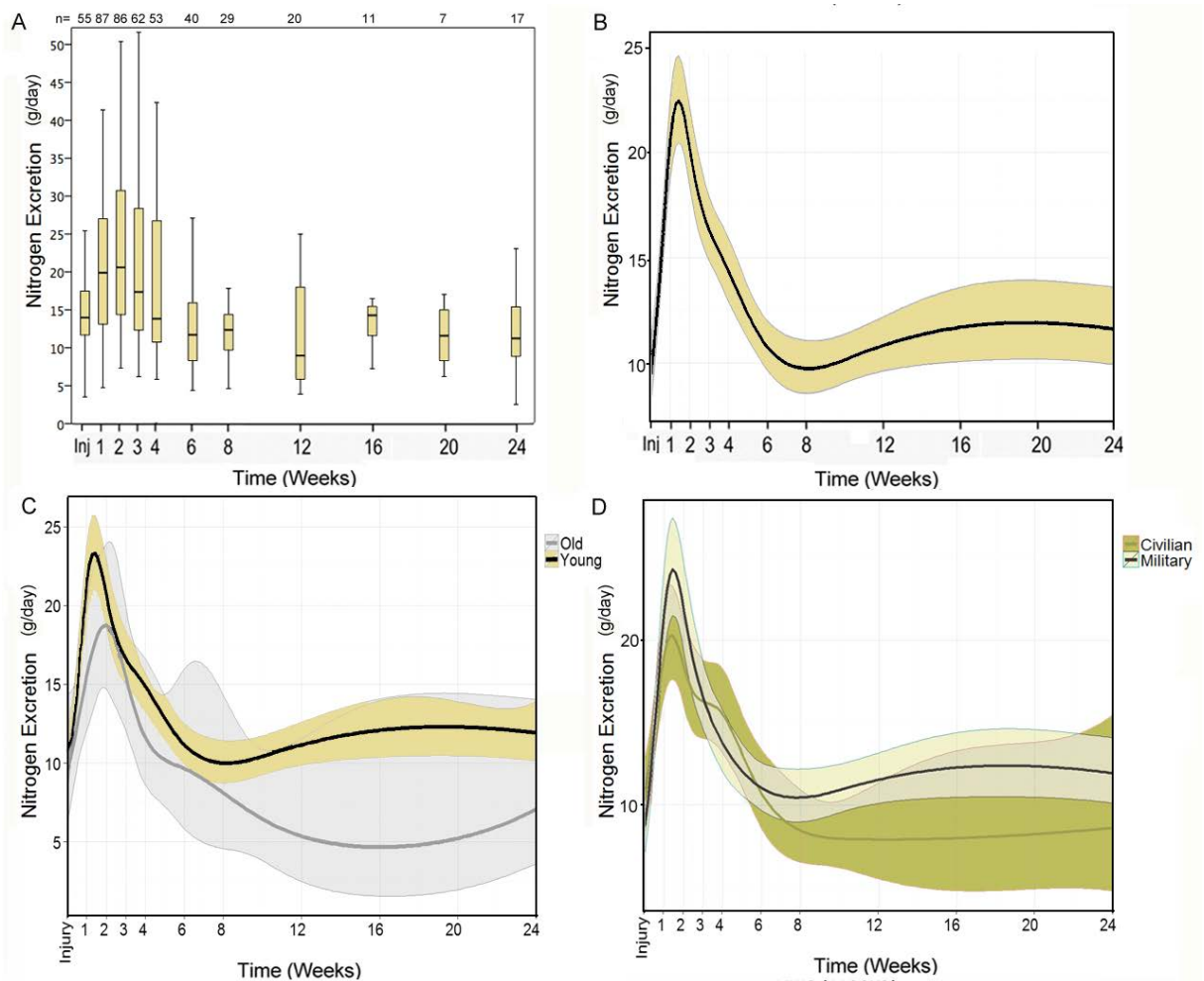


Figure 3.6. Nitrogen excretion of severely injured personal NISS>15 who survived. (A) Raw data displayed as median values with IQRs. (B) Modelled data displayed as means and 95% CI. Modelled data was separated into (C) young and old. Young patients were then separated into (D) military and civilian. Data was modelled using a mixed effects technique; modelling time as a 7-knot restricted cubic spline provided the best fit. Means and 95% confidence intervals for predicted fixed effects of time are represented.

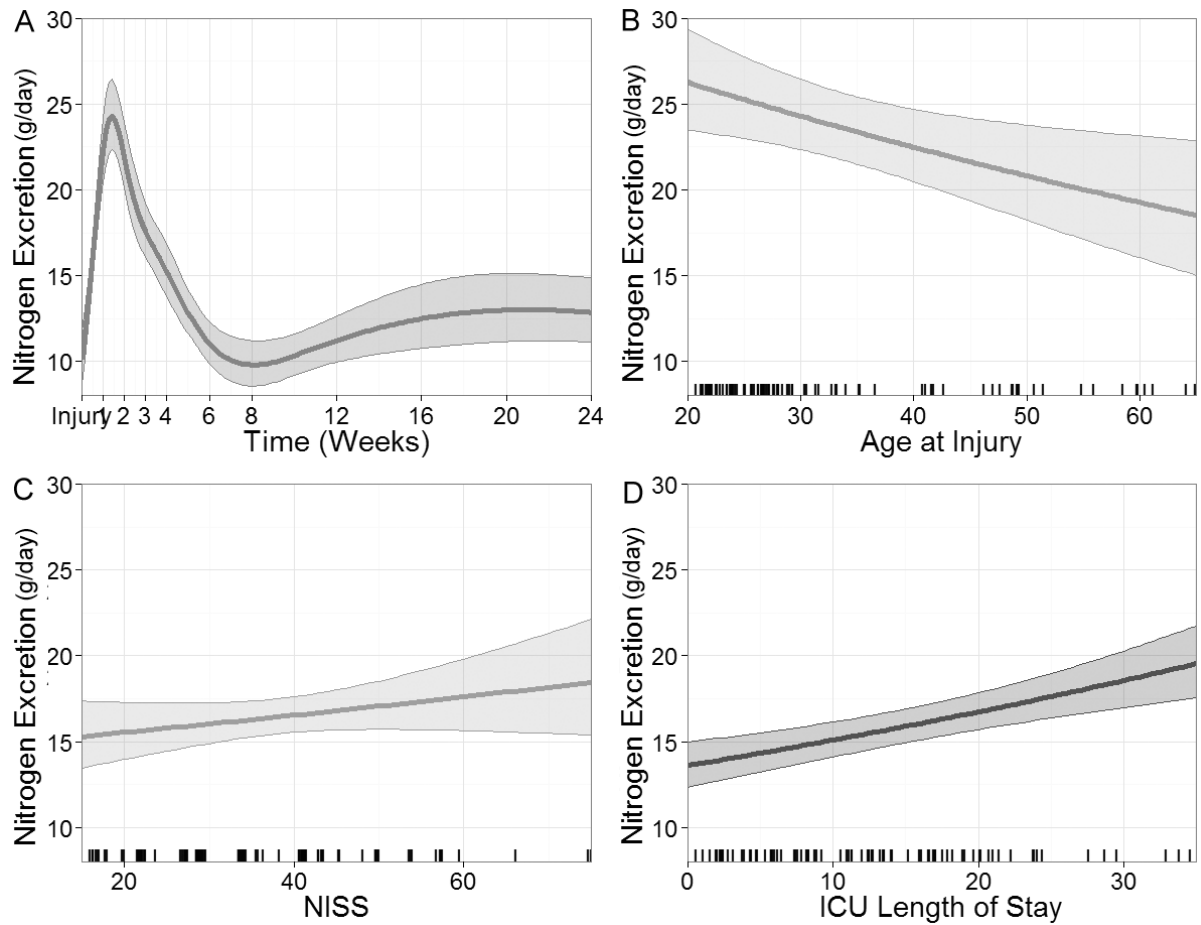


Figure 3.7. Nitrogen excretion levels after severe injury (NISS >15) were modeled against the covariates; Time, Age at Injury, NISS and ICU Length of Stay. Each have separate plots of their model predicted fixed effects; (A) Time, (B) Age at Injury, (C) NISS and (D) ICU Length of Stay. These predicted effects are calculated for each covariate assuming all other covariates remain fixed.

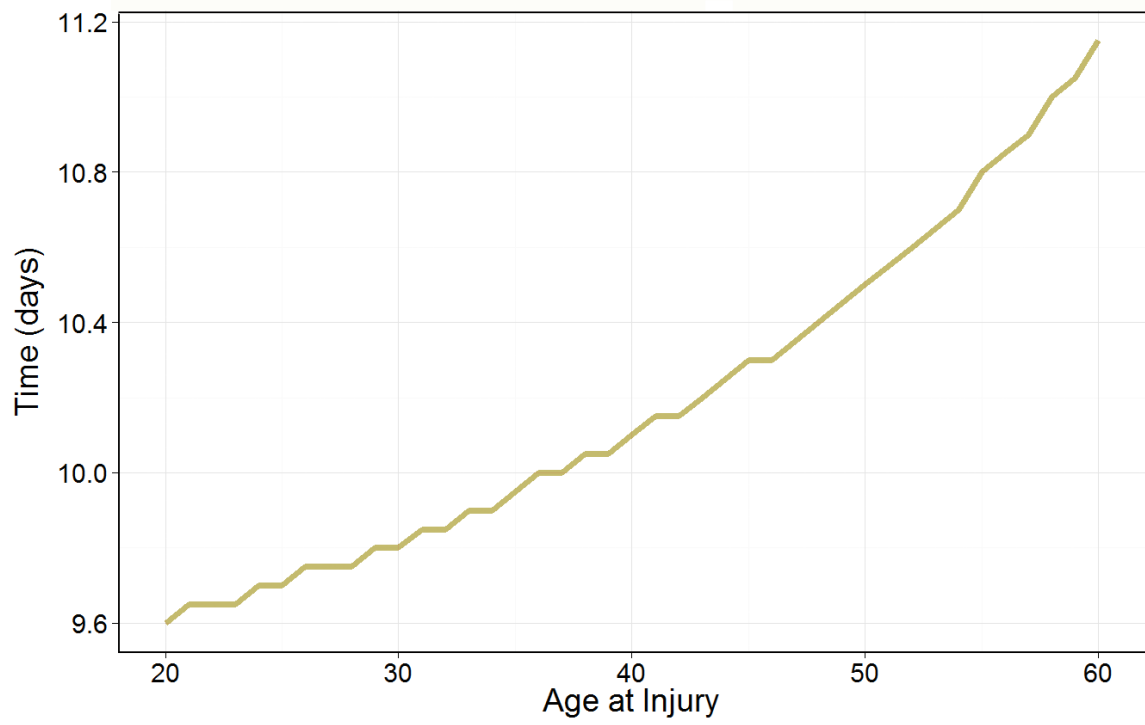


Figure 3.8. Time to reach maximum nitrogen excretion by age.

3.11 Muscle thickness

Muscle thickness was also used as a measure of catabolism and was estimated using a portable sonosite machine. Initially the measurements of 4 muscle areas/groups were taken; Biceps brachii, Rectus abdominis, forearm and quadriceps muscle groups. The results of the 4 areas assessed are shown in Figure 3.9. Different areas were chosen in an attempt to overcome the difficulties in using ultrasound in areas with extensive dressings and/or amputations. However the number of measurements achievable was found to be greatest for the Biceps brachii muscle site and the data followed a U shaped curve. This was consistent for the other sites but due to the lower measurement numbers the Biceps brachii muscle was used analysis in this study.

Biceps muscle thickness reduced after injury and reached a minimum thickness around 6 weeks. The median maximum loss was 26% (21-34%). 6 weeks after injury muscle thickness increased and returned to values measured at the time of injury by 6 months. The temporal changes are best seen in the modelled data in **Figure 3.10** where patients switch from muscle loss to muscle growth at an average of 47 days (95% CI, 40-54). Patients experienced the greatest % loss at day 20, at an average loss of 6.2%: 95% CI (3.8%, 8.5%).

Differences in muscle thickness were observed between the young and old and between the young military and young civilians (

Figure 3.11). The rate of decrease in muscle thickness was similar when comparing young and old and young military and young civilians. There was a slower muscle gain after the minimum was reached when comparing the young and the old modelled data. There was also a slightly quicker muscle gain in the young military compared to the young civilians.

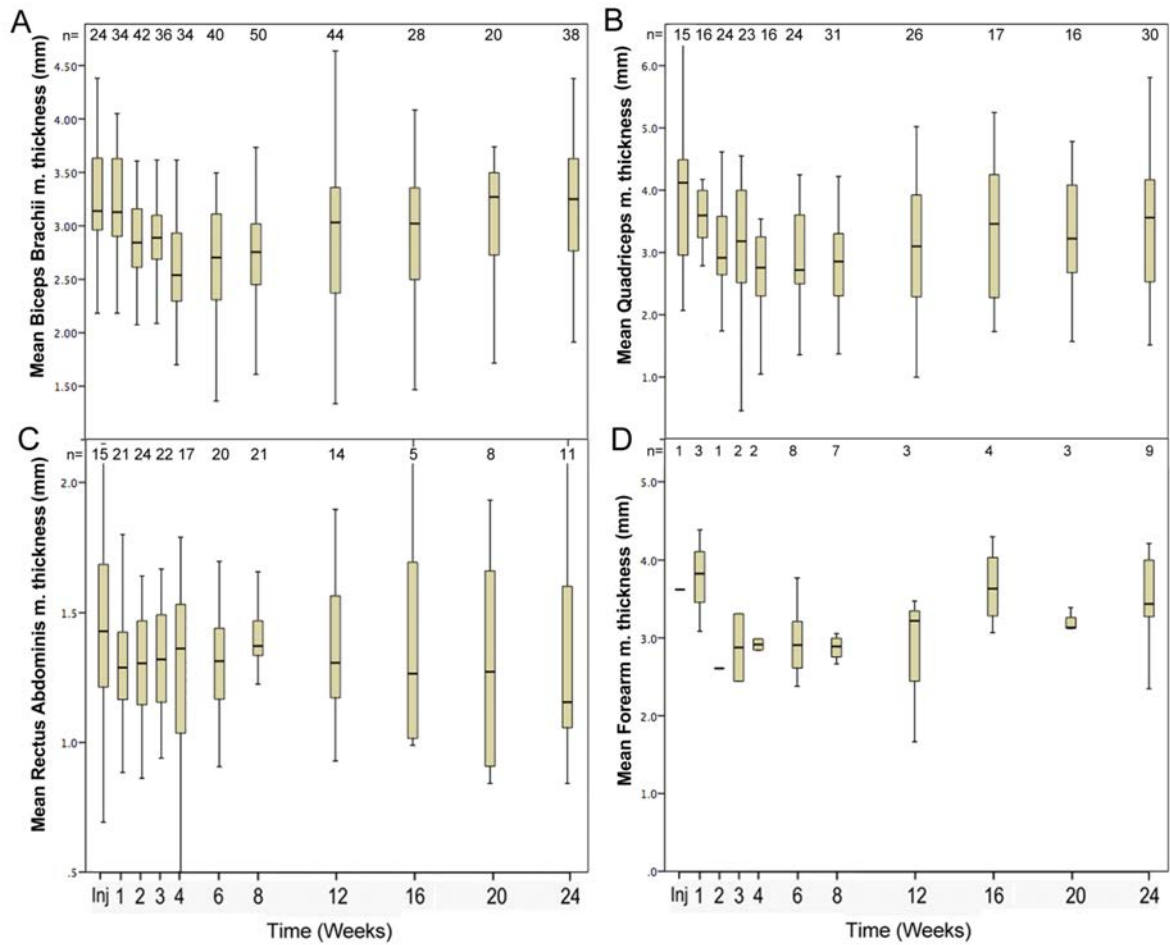


Figure 3.9. Muscle thickness as measured by portable Ultrasound machine in severely injured (NISS >15) patients who survived. Measurements were made of (A) Biceps brachii muscle, (B) Quadriceps muscle, (C) Rectus abdominis muscle and (D) Forearm muscle. Box and whisker plots describe median thickness (mm) with IQR.

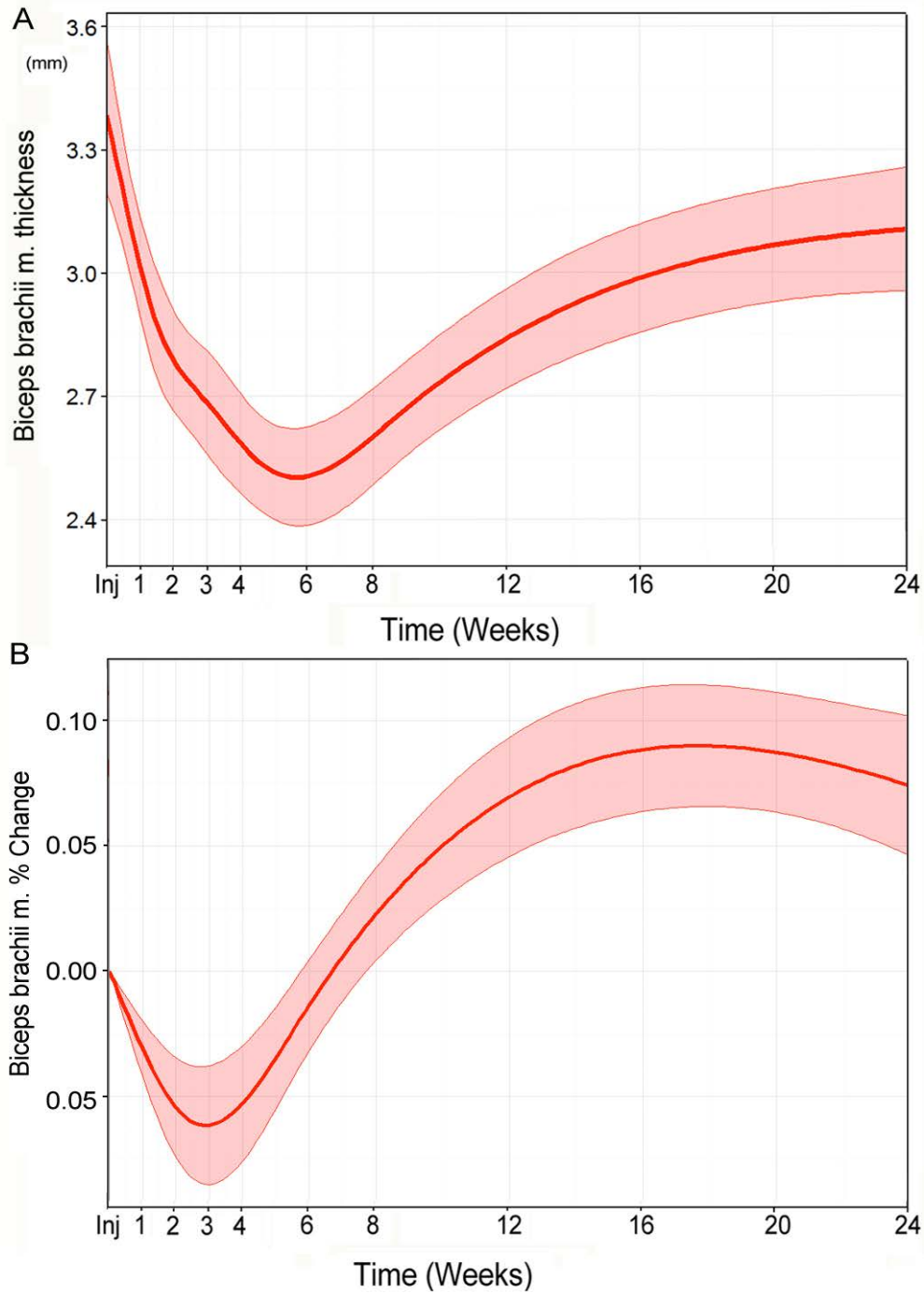


Figure 3.10. Modelling of biceps brachii muscle (A) thickness and (B) % change over time in patients with severe injury (NISS>16) that survived. Data was modelled with a mixed effects technique; time was modelled using 6-knot restricted cubic spline. Means and 95% confidence intervals for model-based predicted fixed effects of time are shown.

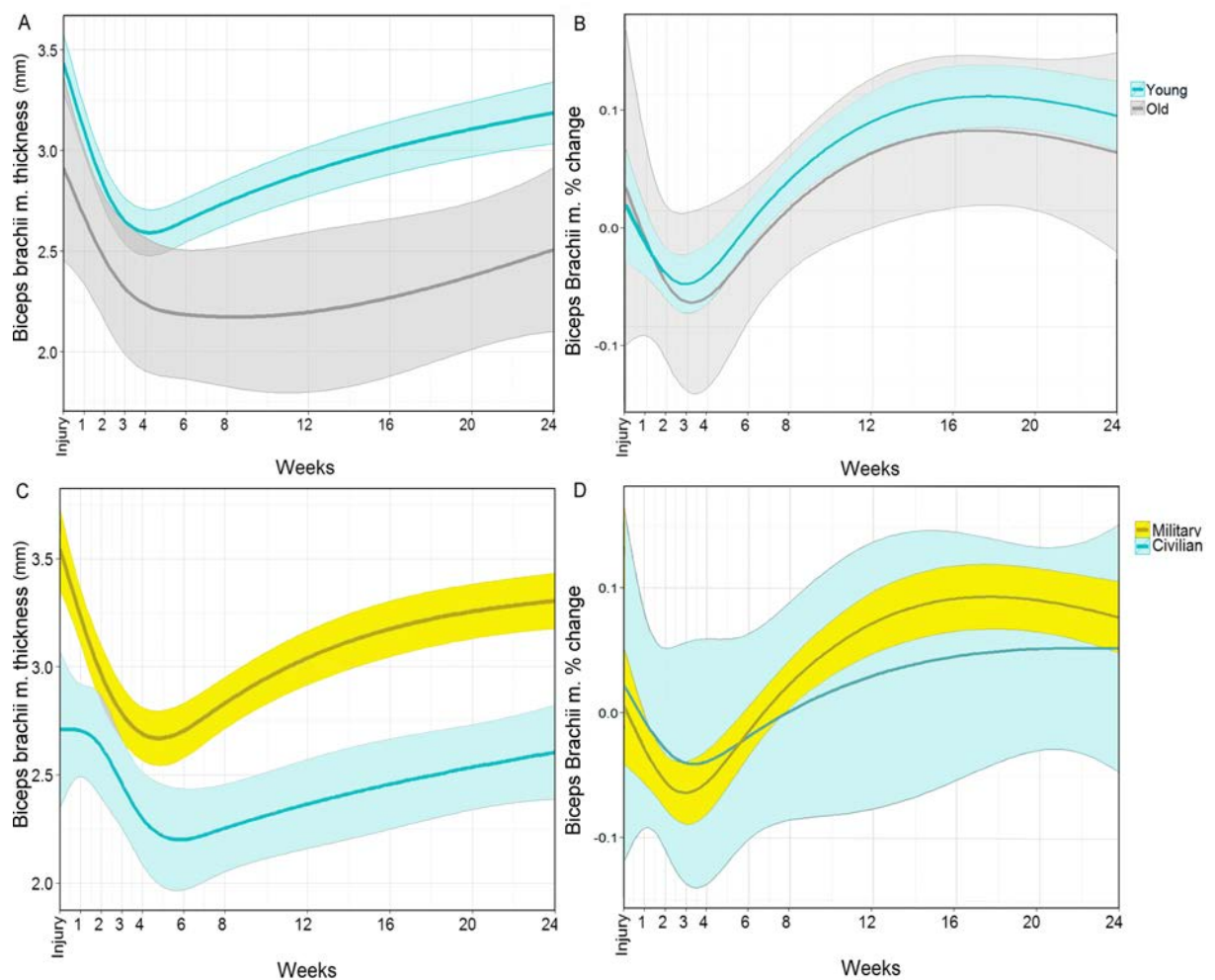


Figure 3.11. Modelled biceps brachii muscle thickness in patients with a NISS>15 that survived. Biceps brachii muscle thickness in mm was separated in to (A) young and old, and (C) young military and civilian severely injured patients. The percentage change over time was modelled for (B) young and old and (D) young military and civilian severely injured patients. Data was modelled with a mixed effects technique; time was modelled using a 6-knot restricted cubic spline. Means and 95% confidence intervals for predicted fixed effects of time are shown.

3.12 Injury Severity and Muscle Loss.

The relationship of muscle loss to injury severity is shown in Figure 3.12. ISS shows a linear positive relationship to loss of biceps thickness. A U shaped curve was observed for NISS and muscle loss, reaching the lowest levels at under 5 % at a NISS of 33. A similar picture was seen for daily %muscle loss and time to reach the minimum thickness of biceps brachii muscle.

When the modelled data of biceps thickness and nitrogen excretion were compared (Figure 3.13), during the steep rise and peak of nitrogen excretion in the first two weeks there was a rapid decline (50% of total loss) in muscle thickness. As nitrogen excretion starting decreasing after 2 weeks, the rate of muscle loss slowed, the remaining 50% of total muscle loss was lost over the next 4 weeks. Muscle thickness then stabilised before increasing again, muscle thickness was still increasing at 6 months.

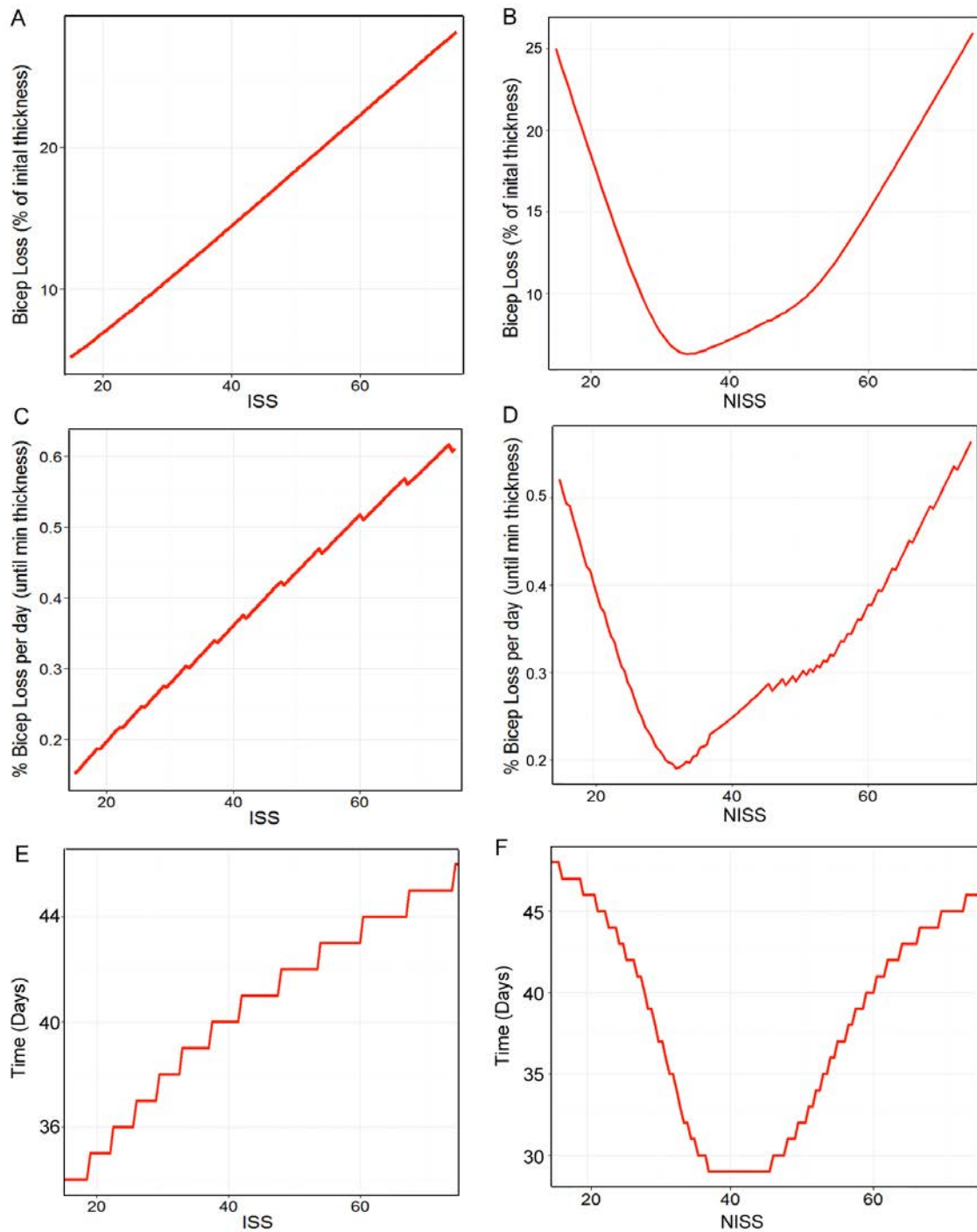


Figure 3.12. Relationship between Injury Severity and muscle thickness as measured by ultrasound of Biceps brachii muscle in patients with NISS>15 that survived. Total muscle loss as % of initial thickness against (A) ISS and (B) NISS. Daily % muscle loss against (C) ISS and (D) NISS. Time to reach minimum muscle thickness against (E) ISS and (F) NISS.

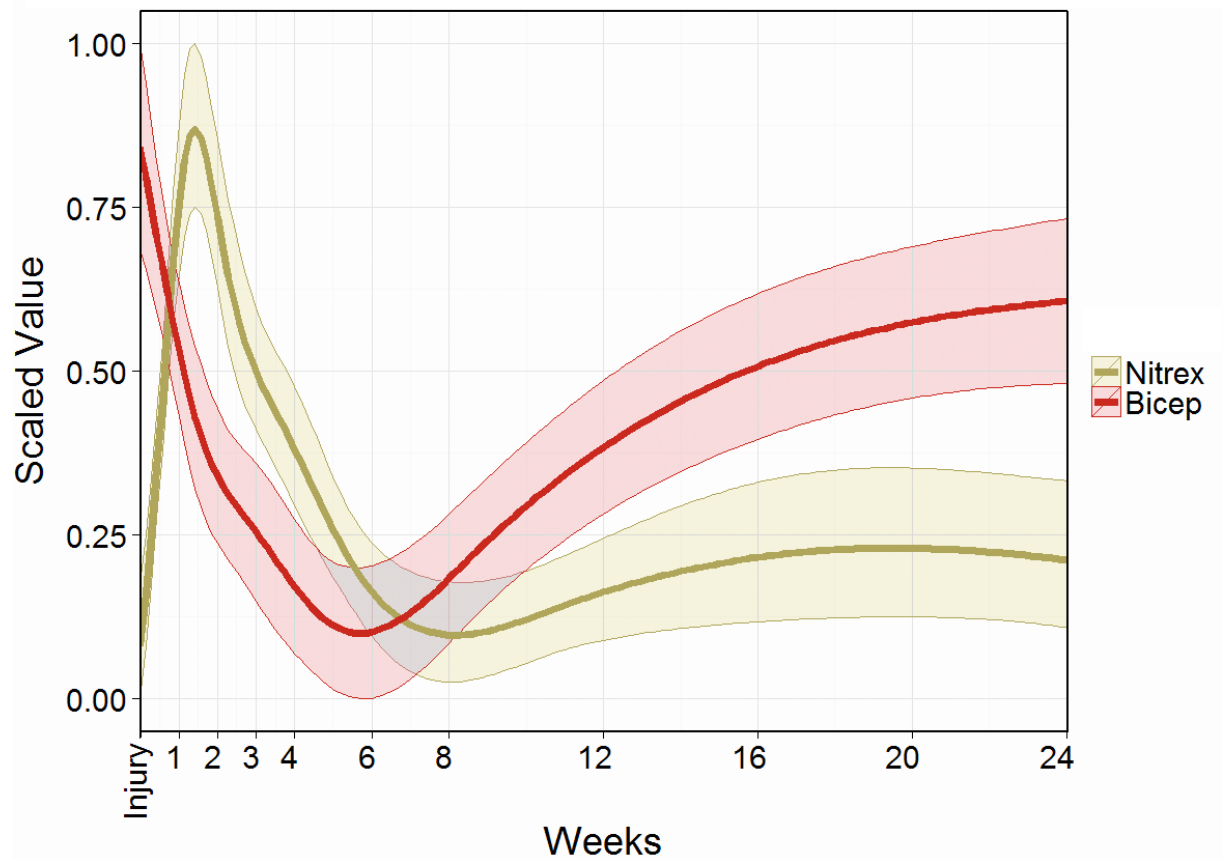


Figure 3.13. Comparison of nitrogen excretion and biceps muscle thickness in severely injured (NISS>15) patients who survived.

3.13 Inflammation and infection.

A more detailed examination of inflammatory response is reported in chapter 4 and only an overview of inflammation and septic episodes are described here.

The acute phase protein C-Reactive Protein (CRP) is a general non-specific measure in inflammation. The diagnosis of sepsis is challenging in the severe trauma patient with a background of on-going inflammation that may be sterile or non-sterile. A SIRS response with a positive culture is considered to be diagnostic (429) but this approach has been criticised (430).

CRP increased immediately after injury and on day 3 peaked at 240 mg/L (159-302mg/L), shown in Figure 3.14. CRP levels then slowly reduced but took over 2 months to fall below 50 mg/l and were still elevated at 6 months.

A SIRS response and a positive culture revealed that ten patients had an early septic episode in the first 48 hours following injury. Septic episodes peaked during the second week with 19 (22%) patients with severe injury suffering from sepsis (Figure 3.15). The timing of infection and the 'probability of sepsis model' against time showed that patients had a 6.2% chance of sepsis after injury and this decreased each day. The organisms that were cultured at the time of a septic episode are listed in Table 3.3.

While the septic episodes that occurred in the first 48 hours met the diagnostic criteria these results should be interpreted with caution. Many of the patients with blast injury are likely to have had positive tissue cultures as a result of contamination from the initial blast.

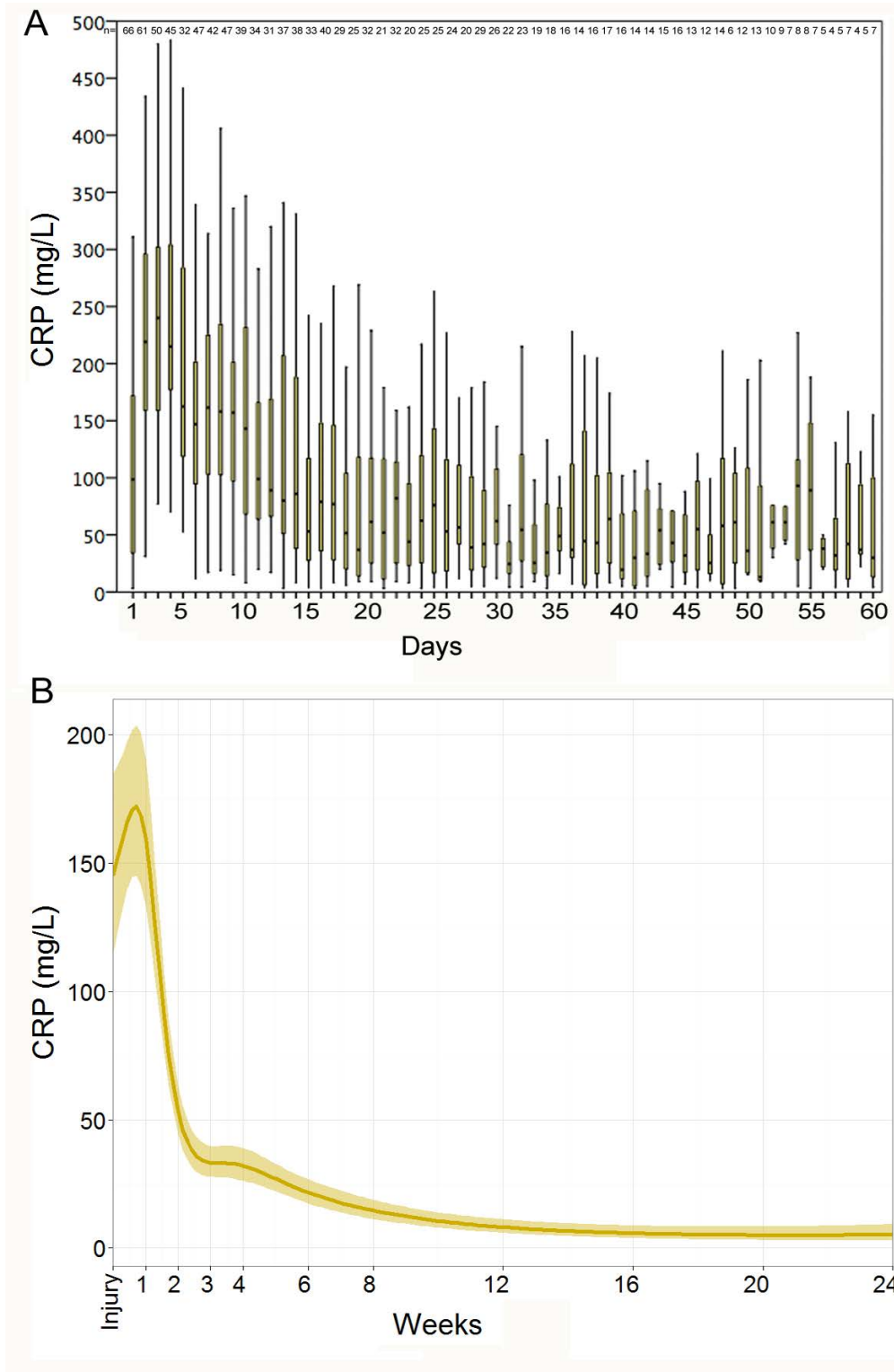


Figure 3.14. C-Reactive Protein of severely injured personal (NISS>15) who survived. (A) CRP levels daily over first 60 days displayed as median values with IQRs. (B) CRP modelled over time data displayed as means and 95% CI. Data was modelled with a mixed effects technique using a 6-knot restricted cubic spline to model time. Means and 95% confidence intervals for predicted fixed effects of time are shown.

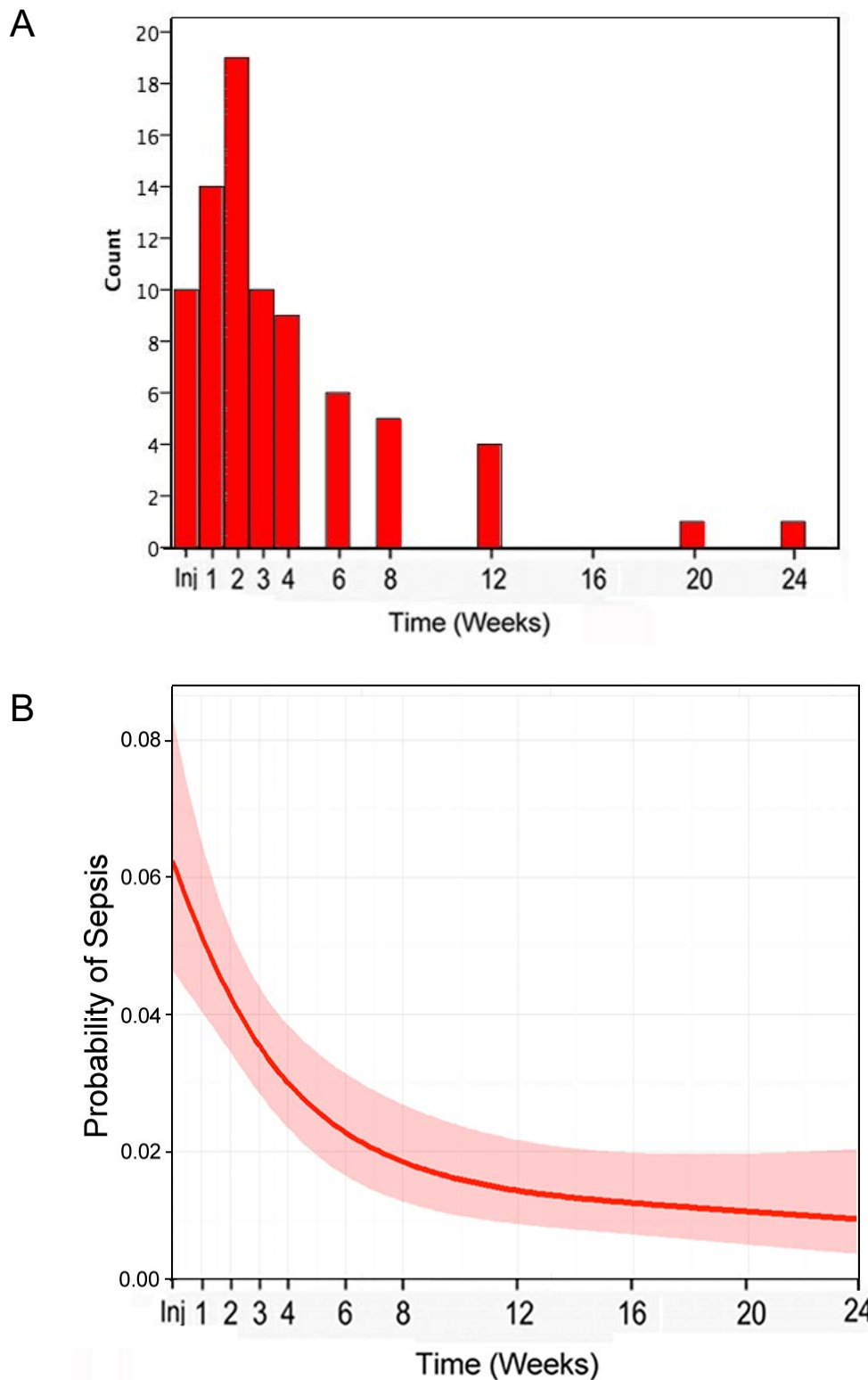


Figure 3.15. Septic Episodes following severe injury (NISS>15) in patients that survived over time. (A) Raw data of timing and number of episodes of sepsis. (B) Probability of sepsis modelled over time. Modelled data are displayed as means and 95% CI.

Organism	Gram Stain	Number of septic episodes	Number times organism cultured
Acinetobacter baumannii	Neg Bacilli	52	189
Pseudomonas species	Neg Bacilli	30	57
Mixed coliforms	Neg Bacilli	28	120
Candida species	Fungi	25	26
Staphylococcus aureus	Pos Cocci	20	135
Bacillus species	Pos Bacilli	15	72
Escherichia coli	Neg Bacilli	12	69
Pseudomonas aeruginosa	Neg Bacilli	10	221
Stenotrophomonas maltophilia	Neg Bacilli	10	116
Klebsiella pneumoniae	Neg Bacilli	9	49
Enterobacter cloacae	Neg Bacilli	8	78
Enterococcus species	Neg Bacilli	8	39
Coag negative Staphylococcus	Pos Cocci	6	14
Aspergillus terreus	Fungi	5	11
Aspergillus flavus	Fungi	4	8
Chry. indologenes	Neg Bacilli	4	10
Haemophilus influenzae	Neg Bacilli	4	8
Morganella morganii	Neg Bacilli	4	20
Pseudomonas putida	Neg Bacilli	4	9
Mixed anaerobic bacteria	Pos & Neg	5	6
Moraxella catarrhalis	Neg Bacilli	3	5
Proteus mirabilis	Neg Bacilli	3	8
Acinetobacter lwoffii	Neg Bacilli	2	4
Aeromonas sp.	Neg Bacilli	2	18
Aspergillus fumigatus	Fungi	2	5
Candida albicans	Fungi	2	50
Enterobacter aerogenes	Neg Bacilli	2	8
MRSA	Pos Cocci	2	2

Table 3.3. List of primary culture organisms, ranked according to the number of septic episodes the organism was cultured. The total number of times that organism was cultured from study patients irrespective of whether a diagnosis of infection or sepsis was made.

3.14 Discussion

A large cohort of severely injured patients young and old is described whose initial differences on appear to be injury severity as measure by NISS and the way they were resuscitated. The younger population were given more blood products and less crystalloid. Further examination revealed that it was young military patients who had a higher NISS and a more blood products. It has been difficult to make any significant conclusions as to whether this technique was beneficial to the patients in the longer term in this study but short term success could be extrapolated from Penn-Barwell JG *et al.* work who showed a year on year improvements with changing resuscitation techniques being cited as a possible factor(431). This technique was adopted well ahead of civilian practice and so is a significant factor in the military young patients.

The young military had higher early organ failure scores as measured by SOFA but interestingly the rate at which organ failure improved (i.e. the slope of change in SOFA) was significantly better in the military group compared to the young civilians. The reason organ failure scores improved quicker is multifactorial and may include pre-injury fitness, early use of tourniquet and consultant delivered timely care. Whether resuscitation with blood products had a direct impact of speed of recovery is difficult to say but although SOFA improved quicker, ICU length of stay was not different. The military also had a different rehabilitation regime with earlier resourced military physiotherapy in hospital and a discharge pathway directly to a rehabilitation centre specialising in muscular skeletal rehabilitation. The military injured had more procedures and a longer length of stay than the young civilians that may be explained by the fact that there were more military limb injuries from blast that required more reconstructive procedures.

Early enlightenment into the metabolic response to surgery and injury was made by Cuthbertson in 1932 and then built on by Moore who described classical 'ebb and flow' physiology relating muscle loss and altered nitrogen balance (198,199). The study demonstrates the high nitrogen excretion that correlates with muscle loss. Differences between young and old and military and civilian cohorts are difficult to make apart from the obviously fact that young and particularly young military have thicker biceps muscle than their older civilian or younger civilian counterparts.

Attempting to relate timing and amount of muscle loss to the severity of injury has met with mixed success as although ISS can correlate with time to maximum loss and amount of loss it doesn't take into account the extremity trauma. The relationship of NISS to muscle loss and timing is difficult to explain as the extremity trauma appears to have less of an effect of muscle loss. This maybe due to early use of tourniquets and the military effect in this cohort, where if blood loss is kept to a minimum those with a relatively major injury are less catabolic and loss less muscle.

The well-documented timing of sepsis following injury is again described in this study as the majority of infections happen in the second week providing early contamination of tissues is excluded. Rising CRP and the growth of pathogenic bacteria support this.

The short-term and long-term stress response that has evolved to protect an individual from life threatening injury has consequences to the recovery individual who survived their injury. Catabolism and infection along with the recovering anabolism appear the natural clinical course of patients. What is about the immune response affecting this cohort that leads to more infections and does the underlying endocrine profile contribute to this?

Chapter 4: The Inflammatory Response to Severe Trauma

4.0 Introduction

The inflammatory response to injury is initiated through the release of nuclear and mitochondrial-derived DAMPs from damaged or necrotic tissue. Of these, HMGB1 and mtDNA have been heavily implicated in initiating this sterile inflammatory response (31). Measuring cytokines and acute phase proteins allows for the assessment of the local and systemic response to DAMPs. To date, the temporal changes in these molecules have been studied in the immediate post injury period but not during the later catabolic and anabolic phases of recovery (432) and age differences in this response have not been reported.

The neutrophil, once considered a simple phagocyte of homogenous phenotype, now boasts an array of sophisticated mechanisms that allows this innate immune cell to not only deal directly with foreign material but communicate with other immune cells including those of the adaptive immune system (111). In addition it is now clear that these cells display more than one phenotype in the circulation (433). These include immature granulocytes with reduced bactericidal function(434) and a newly emerging neutrophil phenotype is one of suppression; a granulocytic MDSC(146). The exact function of MDSC or immature granulocytes have not been fully characterised in the setting of trauma thus consequences of their increased frequency for the clinical course of the trauma patient is not fully understood.

This chapter studied the inflammatory response to severe traumatic injury across a six-month time period, in the SIR cohort, by measuring the circulating concentrations of the DAMPS, mtDNA and HMBG1, pro and anti-inflammatory cytokines and via the assessment of neutrophil phenotype and function.

The small number of deaths (8 of 95) in the study prevented the use of mortality as an outcome measure. Thus, the results presented in this chapter are based on the responses of surviving patients with a NISS>15 and attempts to differentiate inflammation with respect to age (Figure 4.1). A cut off of 49 years was used to separate young and old patients as age related differences in immune response are present by the 6th decade (426). The main differences between the young and old population were injury severity as described by NISS (p=0.003) and the amount of fluids received in the first 24 hours; RBC (p=0.012), FFP (p=0.001) and crystalloid (p<0.001). These results are summarised in Table 3.1.

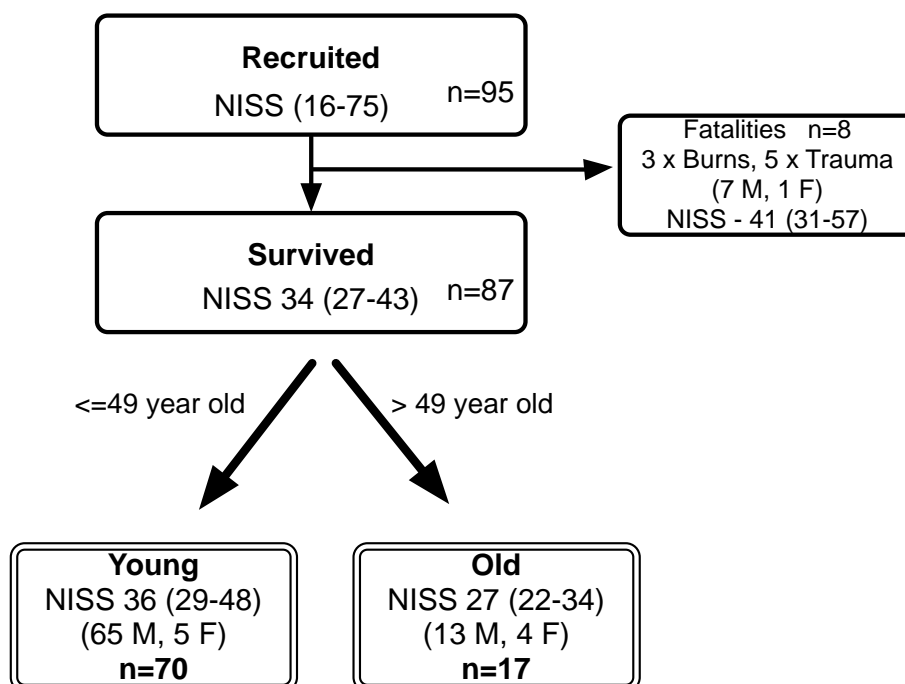


Figure 4.1. Cohort selection to examine the inflammatory response after severe injury. 70 young and 17 old injured adults were used for the subsequent analysis in this chapter unless otherwise stated.

4.1 Results

4.2 Mitochondrial DNA (mtDNA)

Mitochondrial DNA released from damaged cells is thought to be an early initiator of the inflammatory response. MtDNA levels are also thought to be related to clinical outcome(64,435). In the SIR cohort immediately after injury, mtDNA rose and peaked in the second week with a median concentration of 31 ng/ml (IQR, 8-109; **Figure 4.2**). Levels approached control values after two months. When comparing young and old patients, mtDNA levels were lower in young patients immediately after injury ($p < 0.001$). In older patients, mtDNA levels peaked at a higher level in week 2 ($p = 0.001$) after injury compared to the young.

From the model of co-variants (Figure 4.3), it can be inferred that following severe traumatic injury, mtDNA values initially increase and peak in the second week prior to returning to baseline levels over time (Figure 4.3A). There is moderate evidence that mtDNA increases with age (Figure 4.3B), but mtDNA does not appear to influence ICU length of stay (Figure 4.3D) and was not significantly affected by injury severity assessed by NISS (Figure 4.3C).

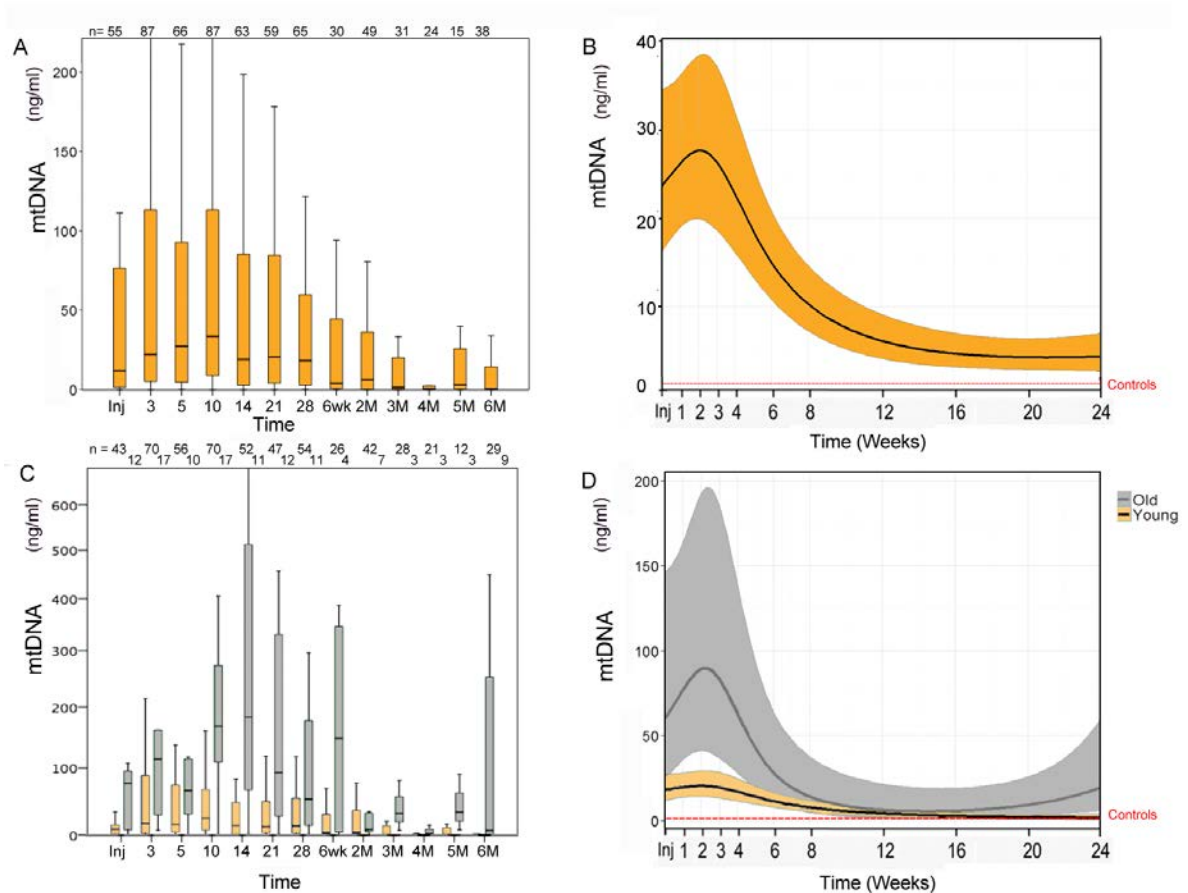


Figure 4.2. Mitochondrial DNA concentration over time in severely injured patients with a NISS>15. (A) MtDNA concentration (ng/ml) over time in days, weeks (Wk) and months (M). (B) Log mtDNA concentration (ng/ml) modelled over time in days to help normalise the extremely skewed data. (C) Raw mtDNA data differentiated by age. (D) Modelled Log mtDNA differentiated by age. Raw data box and whisker plots show median and interquartile ranges (A and C). Data was modelled using a non-linear mixed effects model that accounts for unbalanced repeated measures using a 4-knot cubic spine (B and D). Modelled data is represented by means and 95% confidence intervals.

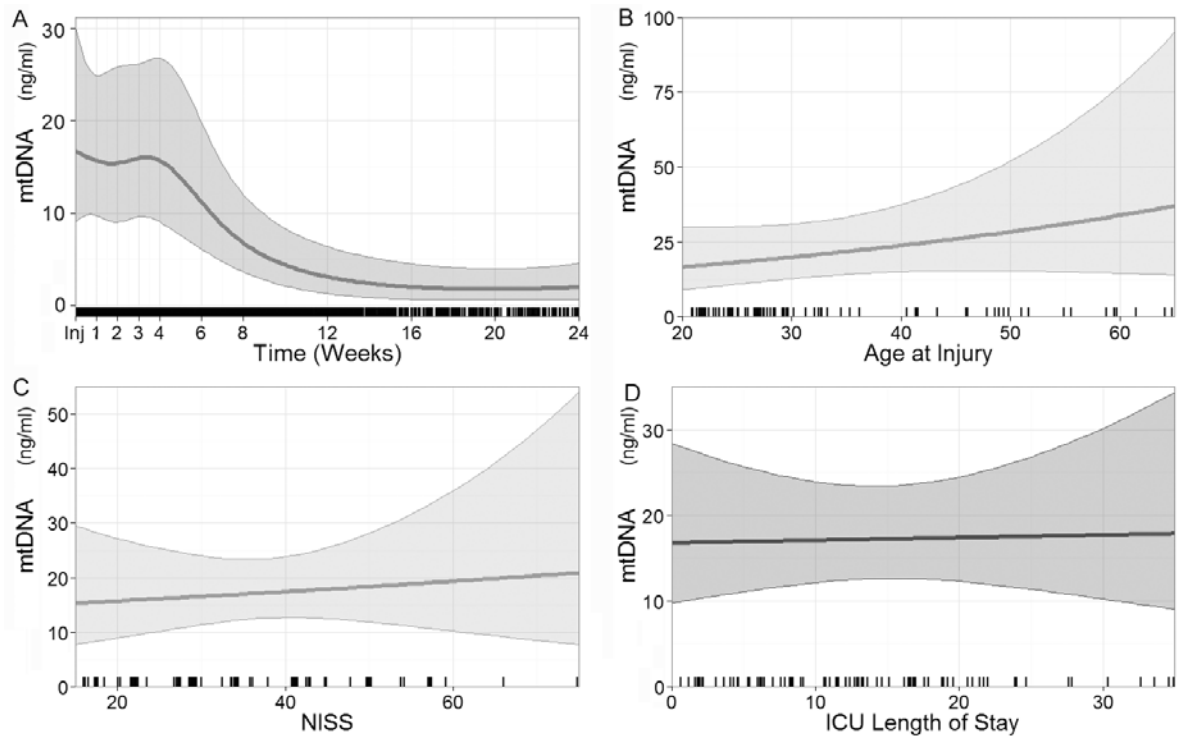


Figure 4.3. Plasma MtDNA concentration (ng/ml) following severe traumatic injury (NISS >15) were modeled against the co-variants; Time, Age at Injury, ICU Length of Stay and NISS. Each have separate plots of their model predicted fixed effects; (A) Time, (B) Age at Injury, (C) NISS and (D) ICU Length of Stay. These predicted effects are calculated for each covariate assuming all other covariates remain fixed. Short vertical lines on the x-axis represent distribution of occurrences to make up the model.

4.3 HMGB1

HMGB1 is also released from damaged cells in response to trauma and is thought to have a pivotal role in the initiation and propagation of the inflammatory response to injury (436). HMGB1 results are displayed in Figure 4.4; values are given as medians with IQR unless otherwise stated. HMGB1 was elevated at the time of injury and peaked in the second week at 6.7 ng/ml (range 3.9-10.1 ng/ml). Levels decreased over the next 6 months but did not return to the normal range (<1.4ng/ml). HMGB1 concentrations peaked later in older injured patients (day 14) compared to younger subjects (day 10). Like mtDNA, the median peak levels of HMGB1 were higher in the older patients at 9.7 ng/ml (5.1-12.4 ng/ml) compared to the young at 6.7 ng/ml (3.9-9.9 ng/ml) but this did not reach significance (p=0.13).

From the model output (Figure 4.5 A), we can infer that there is strong evidence that HMGB1 values initially increase over the first 7 days post injury before decreasing over the next 50 days and then remain stable. There is strong evidence that log HMGB1 concentrations influence ICU length of stay (Figure 4.5 D), moderate evidence that HMGB1 increases with age (Figure 4.5 B), but only weak evidence that HMGB1 decreases with injury severity NISS (Figure 4.5 C).

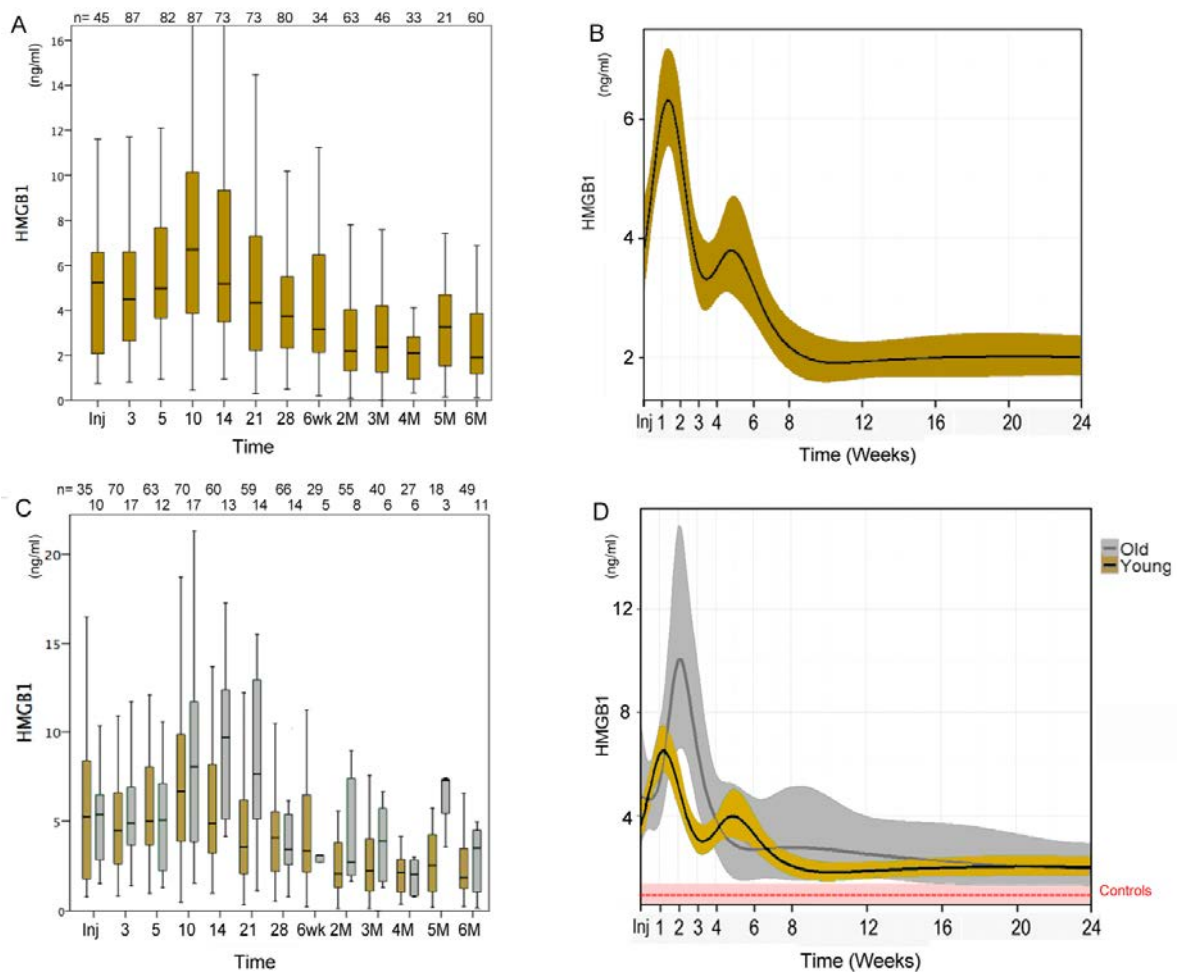


Figure 4.4. HMBG1 concentrations (ng/ml) in severely injured patients (NISS>15) over time. (A) HMBG1 concentrations (ng/ml) over time in days, weeks (Wk) and months (M). (B) HMBG1 concentrations (ng/ml) modelled over time in days to help normalise the extremely skewed data. (C) HMBG1 concentrations (ng/ml) differentiated by age. (D) Modelled HMBG1 concentrations (ng/ml) differentiated by age. Raw data box and whisker plots show median and interquartile ranges (A and C). Data were modelled using a mixed effects model that accounted for unbalanced repeated measures. Time was modelled using a 8-knot restricted cubic spline (B and D). Modelled data are represented by means and 95% confidence intervals for predicted fixed effects of time.

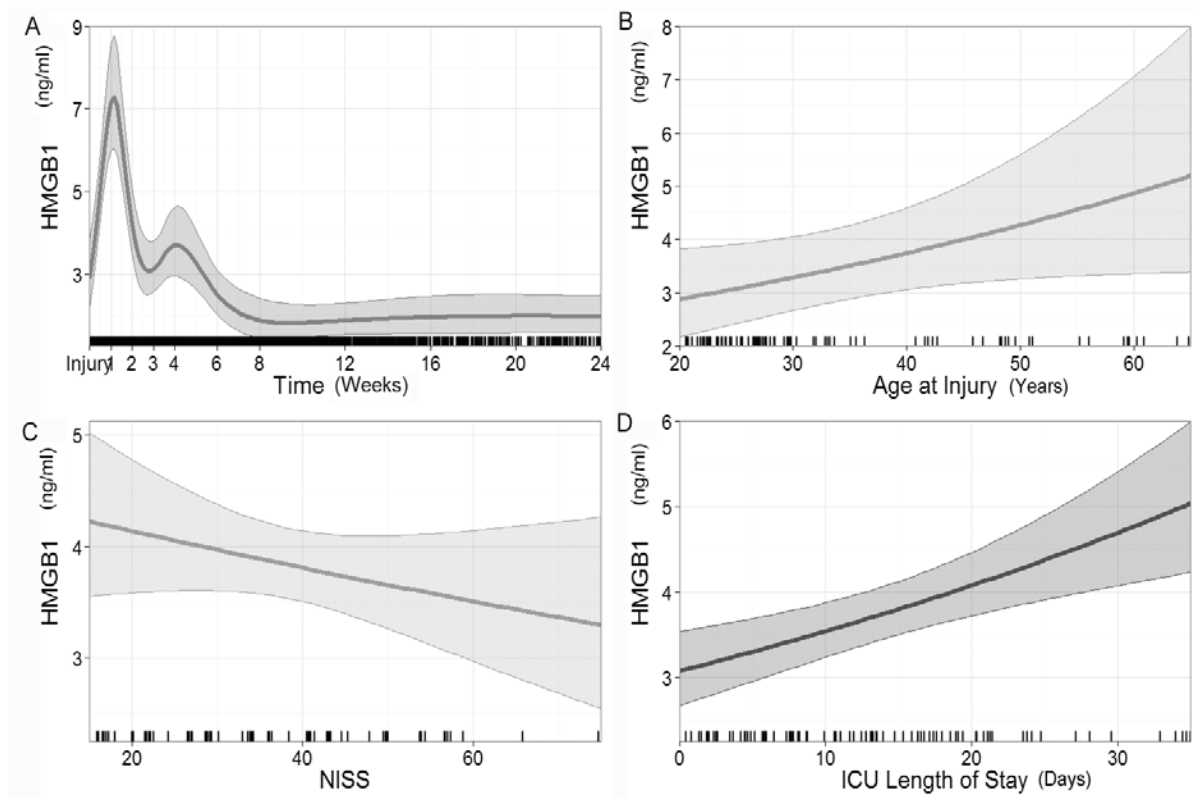


Figure 4.5. HMBG1 concentration (ng/ml) in injured adults over time was modelled against the covariates; Time, Age at Injury, ICU Length of Stay and NISS. Each covariant has separate plots of their model against predicted fixed effects; (A) Time, (B) Age at Injury, (C) ICU Length of Stay and (D) NISS. These predicted effects were calculated for each covariate assuming all other covariates remain fixed. Short vertical lines on the x-axis represent distribution of occurrences to make up the model.

4.4 Cytokines

The 9-plex luminex assay gave results for IL1- β , IL-4, IL-6, IL-8, IL-10, IL-13, IL-17, TNF- α and GM-CSF. The data are presented in Figure 4.6. Only IL-6, IL-8 and IL-10 showed uniformly significant results, these were modelled as described earlier and are shown in Figure 4.7. IL-6, IL-8 and IL-10 all show an immediately raised level that rapidly falls after injury. The young patients showed higher cytokine levels compared to the older patients but due to wide confidence intervals failed to reach statistical significance. Older patients also exhibited a slightly increased level of IL-6, IL-8 and IL-10 later in their recovery, with IL-6 increasing slightly after 4 weeks, IL-8 from 6 weeks until after 6 months and IL-10 between 12-16 weeks.

The data for IL1 β , IL4, IL17, TNF α and GM-CSF could not be interpreted further due to a large number of undetectable values for cytokine levels.

The modeled data allowed the examination of IL-6, IL-8 and IL-10 with respect to the co-variants of Time, Age at Injury, ICU LOS and NISS (Figure 4.8, Figure 4.9 and Figure 4.10). There is strong evidence that IL-6, IL-8 and IL-10 values initially decrease over time before leveling off (Figure 4.8) and that IL-6, IL-8 and IL-10 levels decrease with age (Figure 4.9). Log IL-6, log IL-8 and IL-10 had a positive effect on ICU length of stay (Figure 4.10). There was only weak evidence that NISS has a positive influence IL-6 and IL-8. There was no evidence to support the effect of NISS on IL-10 production. NISS correlated slightly better than ISS with early (<48hours) IL-6, $R=0.351$ ($p=0.001$) and $R=0.339$ ($p=0.002$) respectively. With respect to IL-8, ISS was slightly better at predicting than NISS in early levels of IL-8, $R=0.330$, ($p=0.003$) and $R=0.304$ ($p=0.006$). NISS and ISS failed to significantly correlate with early IL-10 levels.

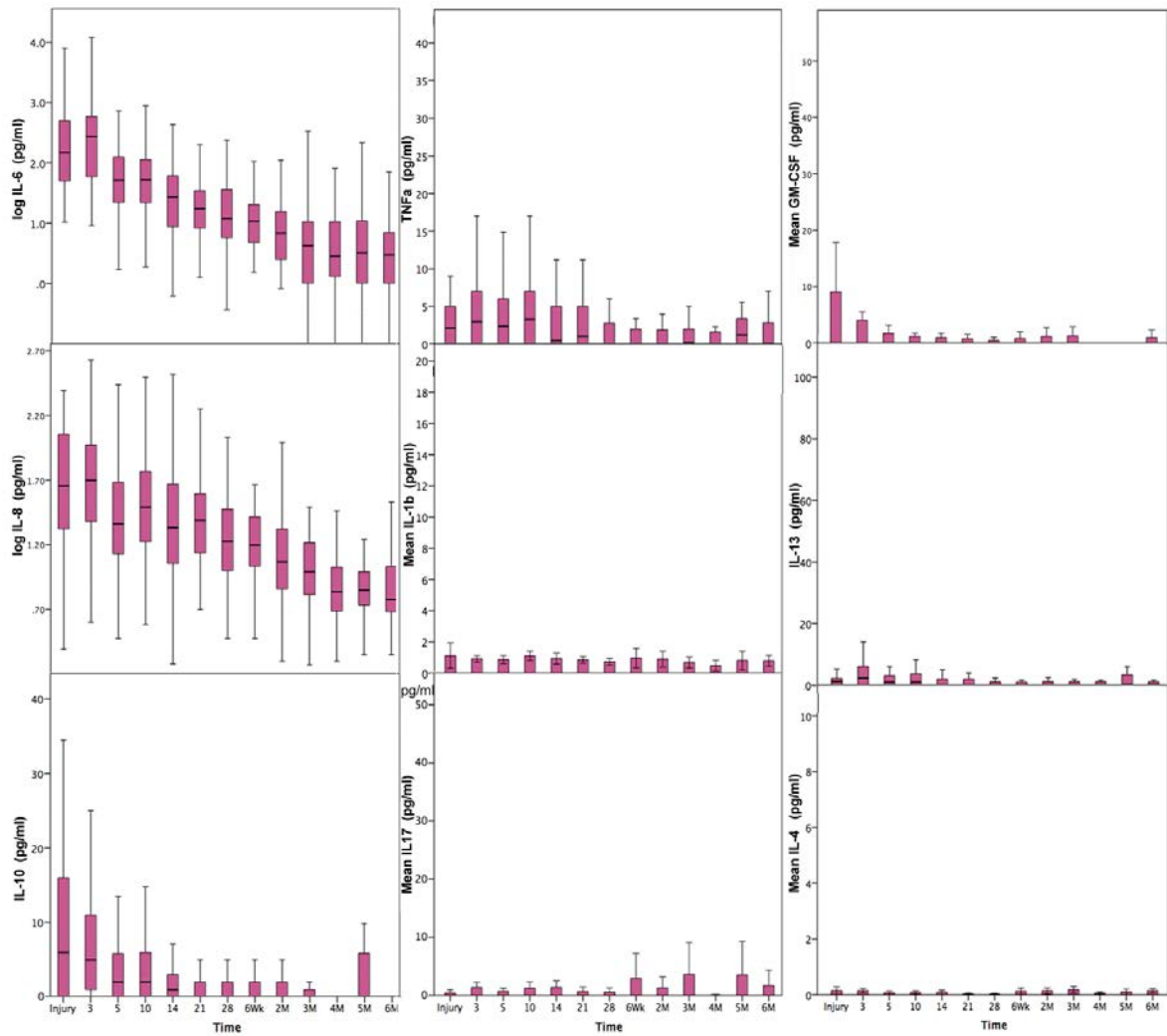


Figure 4.6: Temporal changes in serum cytokine concentrations in severely injured trauma patients (NISS>15). Log values are displayed for IL-6 and IL-8. Where box and whisker plots with median values and IQR were not possible mean values with 95% confidence intervals are shown as labelled.

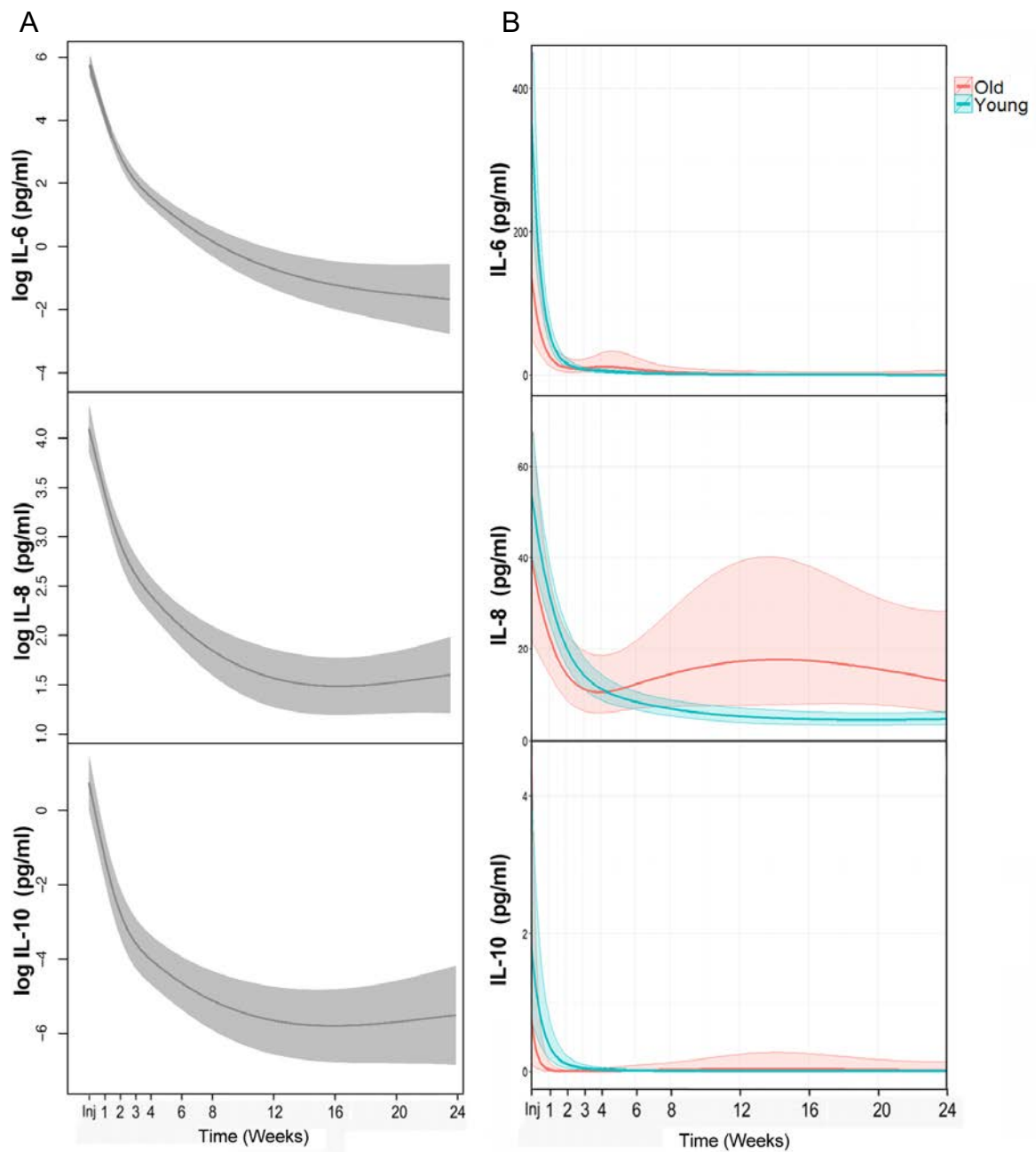


Figure 4.7. Temporal changes in serum IL-6, IL-8 and IL-10 concentrations.

(A) Modelled Log values for IL-6 IL-8 and IL-10. (B) Modelled IL-6, IL-8 and IL-10 comparing young and old patients. Data was modelled using a mixed effects model that accounts for unbalanced repeated measures. Time was modelled using a 4-knot cubic spline. The predicted fixed effects of time are represented by means and 95% confidence intervals.

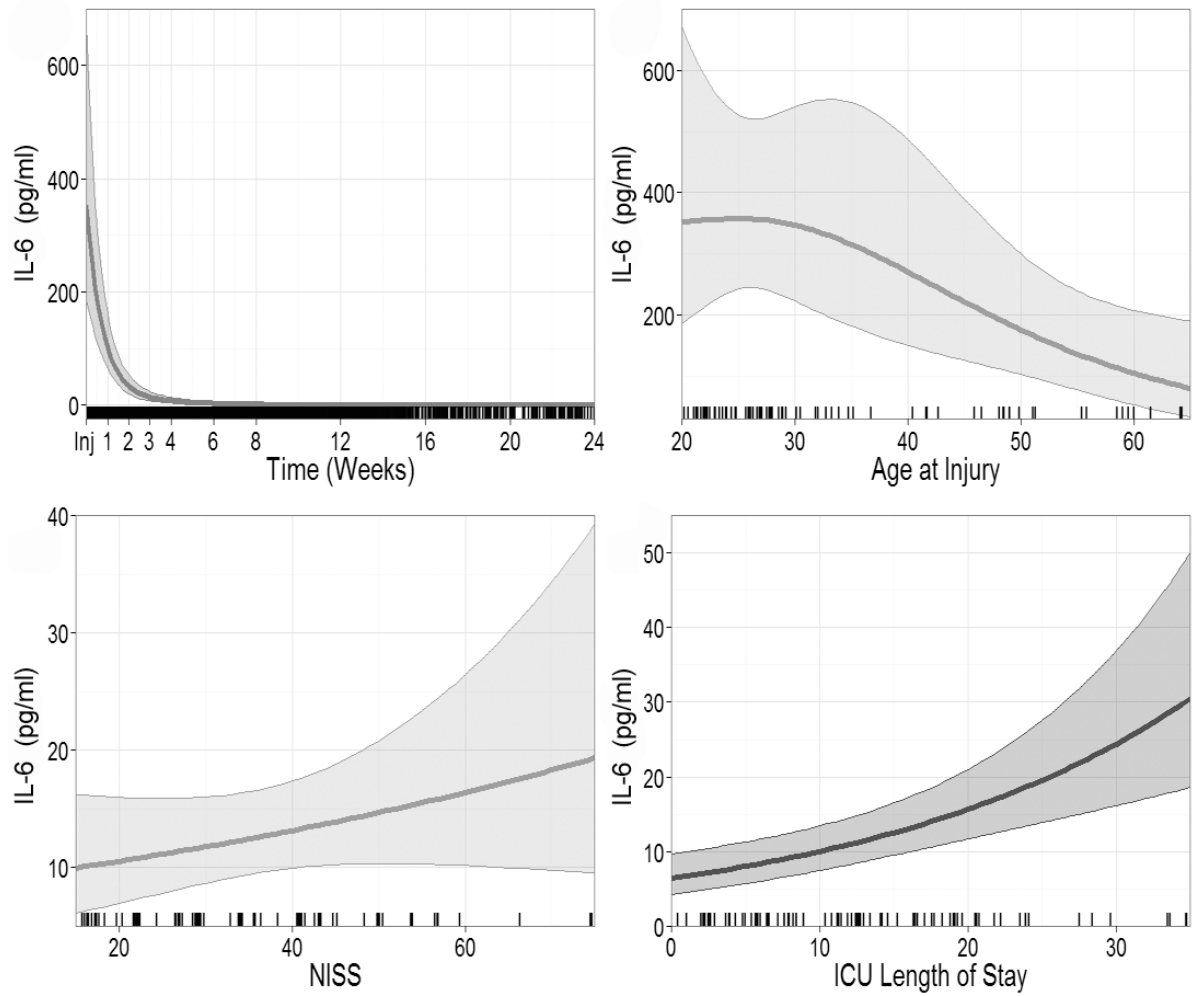


Figure 4.8. Serum IL-6 concentrations modeled against the co-variants: Time, Age at Injury, ICU Length of Stay and NISS. Each co-variant has separate plots of their model predicted fixed effects. These predicted effects are calculated for each covariate assuming all other covariates remain fixed. Short vertical lines on the x-axis represent occurrences to make up the model.

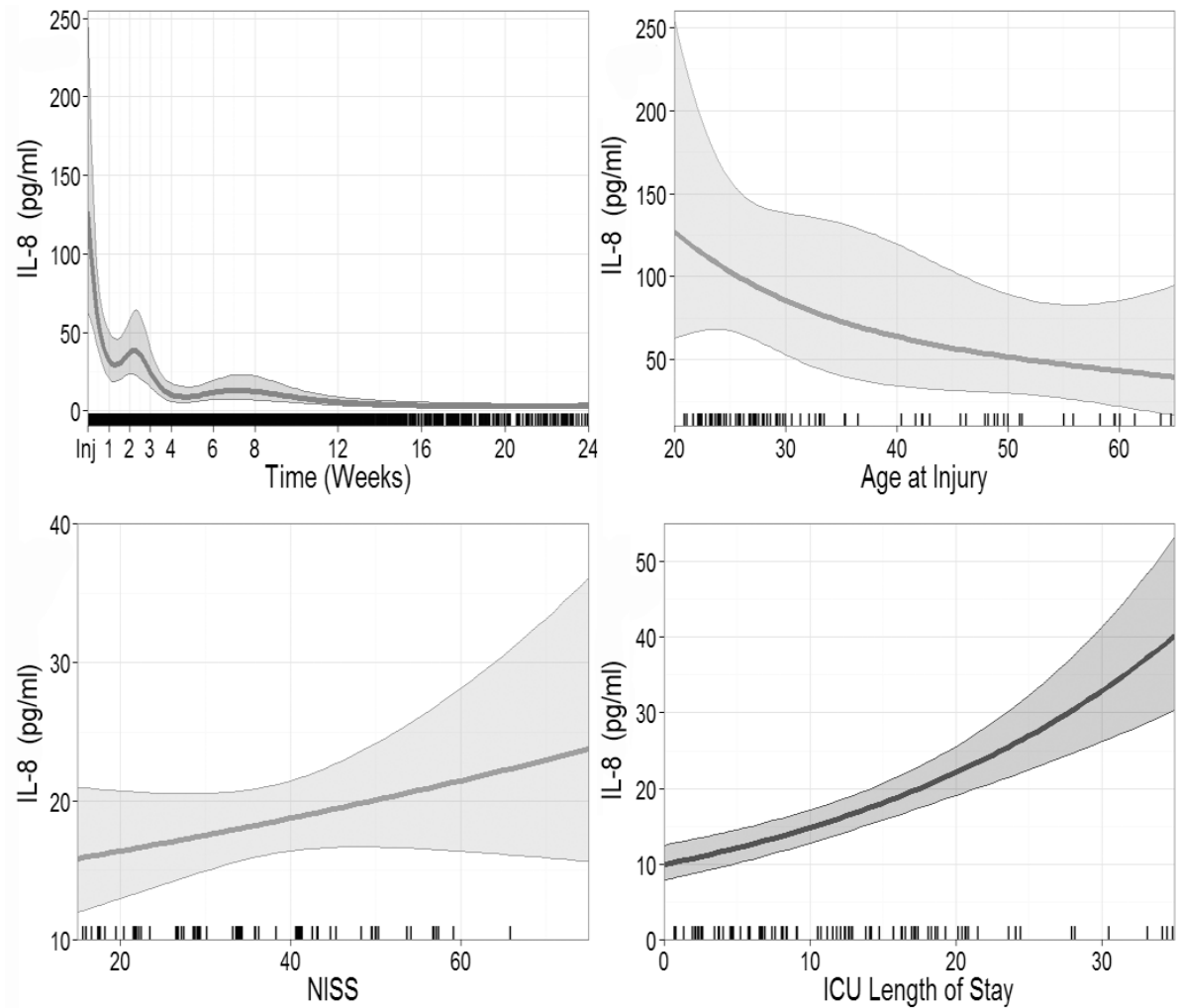


Figure 4.9. Serum IL-8 modeled against the covariates: Time, Age at Injury, ICU Length of Stay and NISS. Each co-variant has separate plots of their model predicted fixed effects. These predicted effects were calculated for each covariate assuming all other covariates remain fixed. Short vertical lines on the x-axis represent occurrences to make up the model.

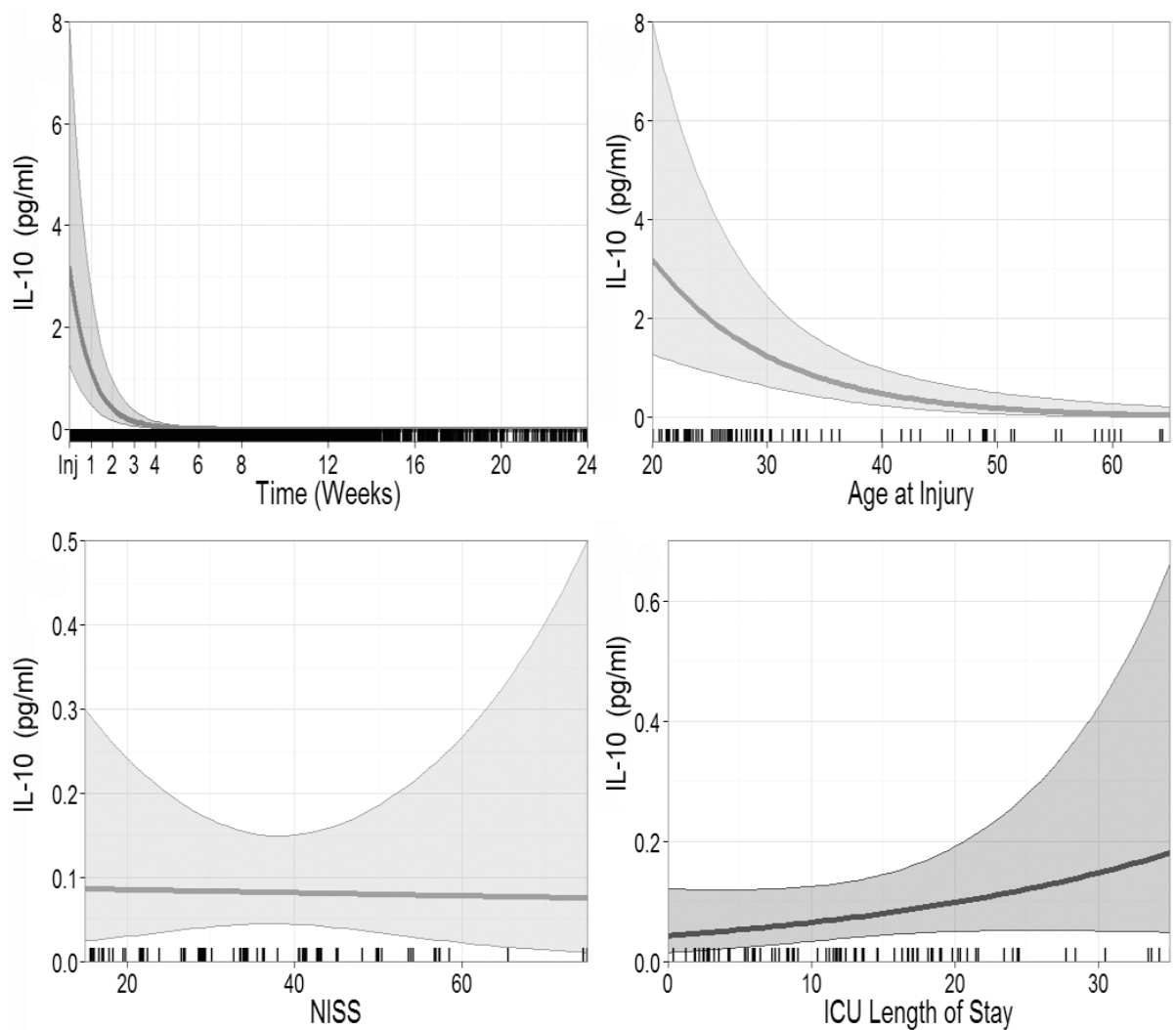


Figure 4.10. Serum IL-10 modeled against the covariates: Time, Age at Injury, ICU Length of Stay and NISS. Each co-variant has separate plots of their model predicted fixed effects. These predicted effects were calculated for each covariate assuming all other covariates remain fixed. Short vertical lines on the x-axis represent occurrences to make up the model.

4.5 Neutrophils

As the most abundant leukocyte in blood and the first immune cell to arrive at a site of infection or injury, neutrophils are a key initial defence against invading organisms. The ability of the injured patient to fight infection can be represented in part by the ability of their neutrophils to phagocytose bacteria and produce ROS.

4.5.1 Neutrophil Count

The mean neutrophil count on Day1 was $10.3 \times 10^9/l$ (± 5.7) and was significantly higher than normal values ($5.0 \times 10^9/l$, ± 2.5), ($p < 0.000001$). The initial increased counts were followed by return to normal counts for the rest of the first week before increasing again during the second week. Neutrophil counts returned to normal over the next 6 weeks (Figure 4.11). Younger patients had significantly lower neutrophil counts on Day 3 ($5.4 \times 10^9/l$, ± 2.9), 2 Months ($5.4 \times 10^9/l$, ± 2.9) and 3 Months ($4.3 \times 10^9/l$, ± 1.5) following injury compared to older patients ($p < 0.000001$). Normal values are taken from UHB laboratory reference ranges.

Neutrophil Superoxide Production Superoxide production was measured in neutrophils while in hospital. The production of superoxide by the neutrophils of injured patients is described by a U-shaped curve (Figure 4.12). Initial values were reduced, reached a minimum during the second week after injury and returned to control values after 2 months. There were no differences in the amount of superoxide produced (MFI) between younger and older patients despite there being a significant difference between the young and controls ($p < 0.0001$). 19 uninjured adults (13 young and 6 old) were sampled and analysed to act as controls.

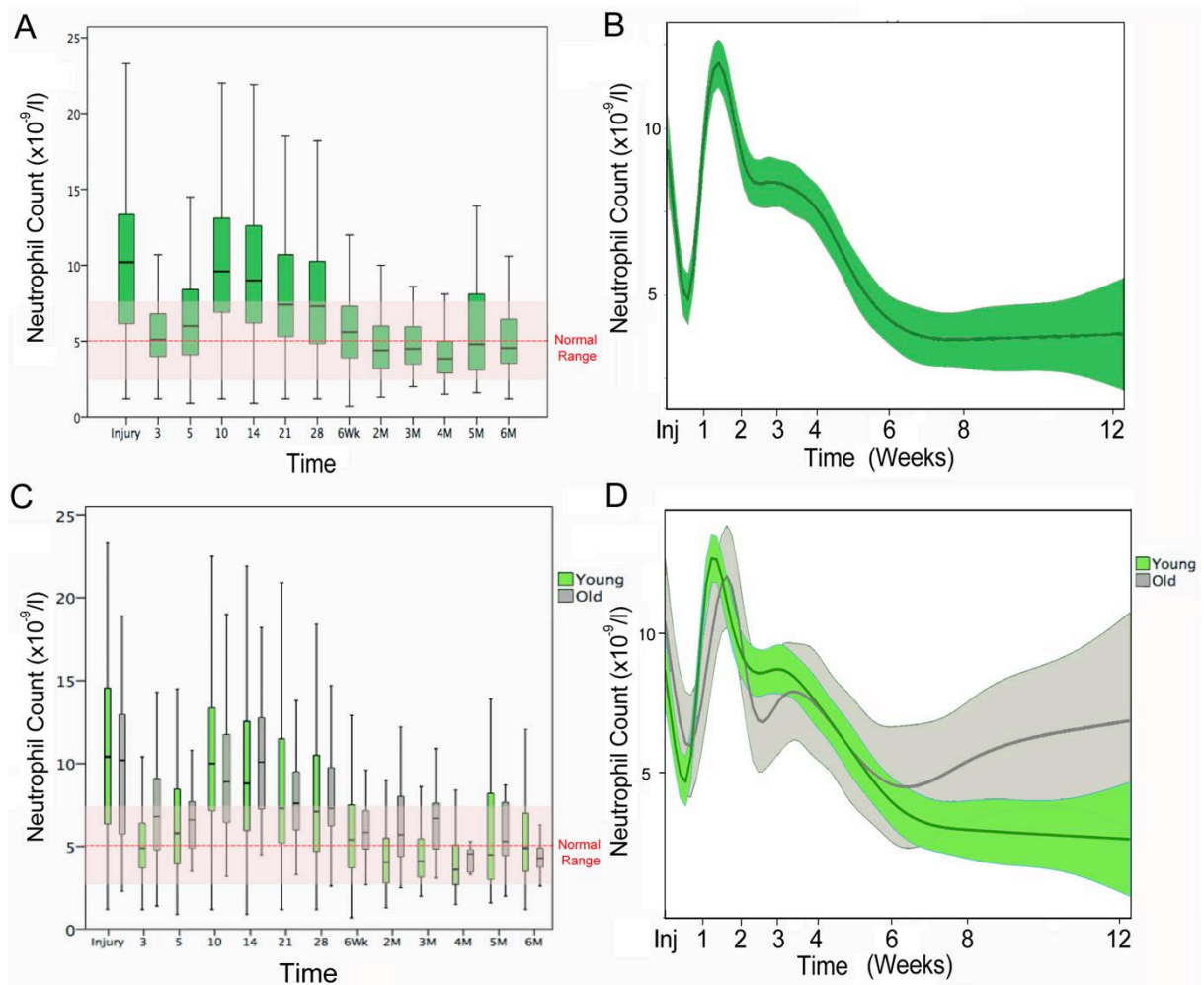


Figure 4.11. Neutrophil count from injury through to 6 months following severe injury (NISS>15). (A) Raw data of neutrophil counts. (B) Modelled data of neutrophil counts. (C) Raw data of neutrophil counts separated into young and old patients. (D) Modelled data separated into young and old injured patients. UHB laboratory normal ranges are shown. Median values with IQR are represented by box and whisker plots. Modelled data are represented by means and 95% confidence intervals. Data was modelled using a non-linear mixed effects model that accounts for unbalanced repeated measures using a 5-knot cubic spine.

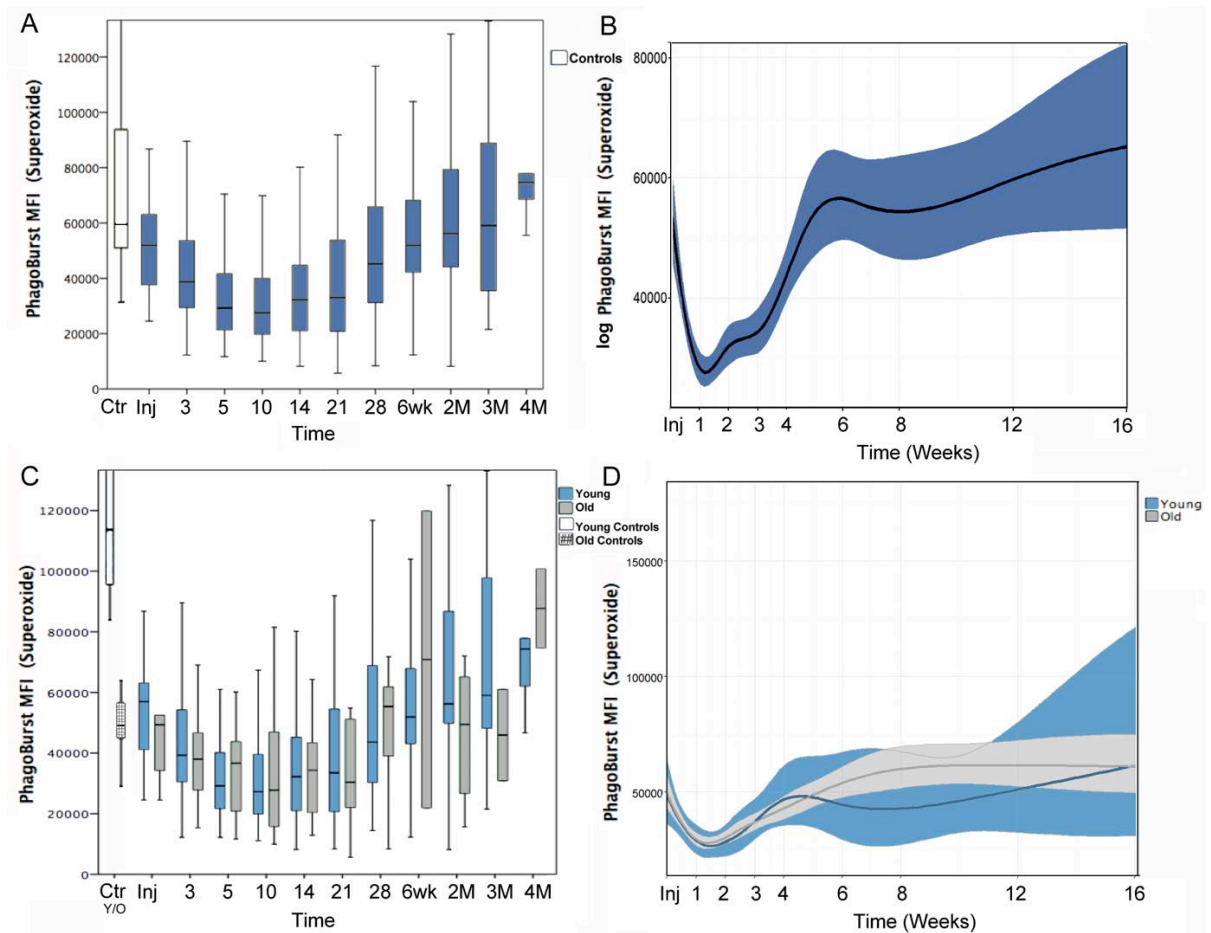


Figure 4.12. Neutrophil production of superoxide following severe injury as measured by PHAGOBURST™. (A) Neutrophil superoxide production in patients that survived with NISS >15. (B) Neutrophil superoxide modelled using a non-linear mixed effects model. (C) Neutrophil superoxide production (raw data) separated into young and old. (D) Neutrophil superoxide production modelled for young and old. 19 adults controls were measured, 13 young and 6 old. Median values with IQRs are represented by box and whisker plots. Model-based predicted fixed effects of time are represented by means and 95% confidence intervals. Data was modelled using a mixed effects model that accounts for unbalanced repeated measures. Time was modelled using a 4-knot restricted cubic spline.

4.5.2 Neutrophil Phagocytosis

The ability of neutrophils to phagocytose is represented by the phagocytic index, a calculation of the percentage of cells involved in phagocytosis and the proportion of bacteria (*Esch. coli*) that have been engulfed (Figure 4.13). The temporal profile of neutrophil phagocytosis for injured patients followed a fluctuating course. Initially the levels were low compared to data for healthy controls, improved over the first week, and decreased to reach the lowest level in the second week before increasing to normal levels by 4 months. The increase in phagocytic index from the lowest point slowed between 3-4 weeks before increasing again.

When phagocytic index data was separated in to young and old patients, phagocytic index at 2 weeks post injury was significantly lower in the younger cohort ($p < 0.001$). The older patients results were much more variable, with wider confidence intervals and they did not follow a consistent pattern (Figure 4.13).

To examine which parts of the phagocytic index were responsible for the variability in the older injured patients, the percentage of cells that underwent phagocytosis and the number of bacteria ingested per cell are shown in Figure 4.14. The data suggest that the older injured person has more variability in the number of bacteria that their neutrophils can ingest but that the pattern of the percentage of cells involved in phagocytosis is similar to younger injured patients. At two weeks, the younger cohort had significantly lower % of cells involved in phagocytosis ($p = 0.006$) and lower number of bacteria ingested per cells (MFI; $p < 0.001$).

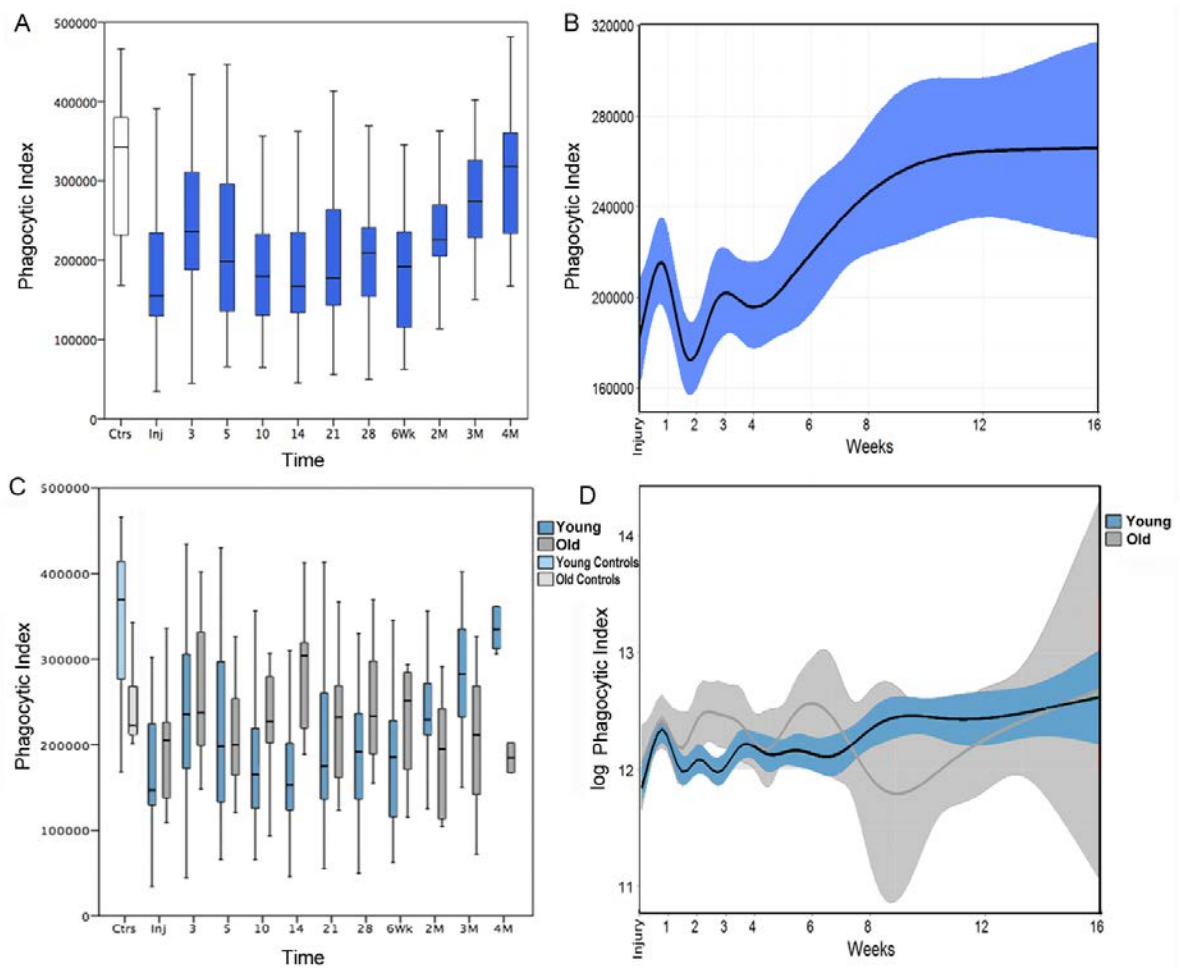


Figure 4.13. Phagocytic Index in severely injured patients (NISS>15) that survived as measured by PHAGOTEST™. Phagocytic index in trauma patients that survived with NISS >15, (A) raw data and (B) modelled data. Phagocytic Index separated into young and old, (C) raw data and (D) modelled data. Median values with IQRs are represented by box and whisker plots. Model-based predicted fixed effects of time are represented by means and 95% confidence intervals. Data was modelled using a mixed effects model that accounts for unbalanced repeated measures. Time was modelled using a 9-knot restricted cubic spline.

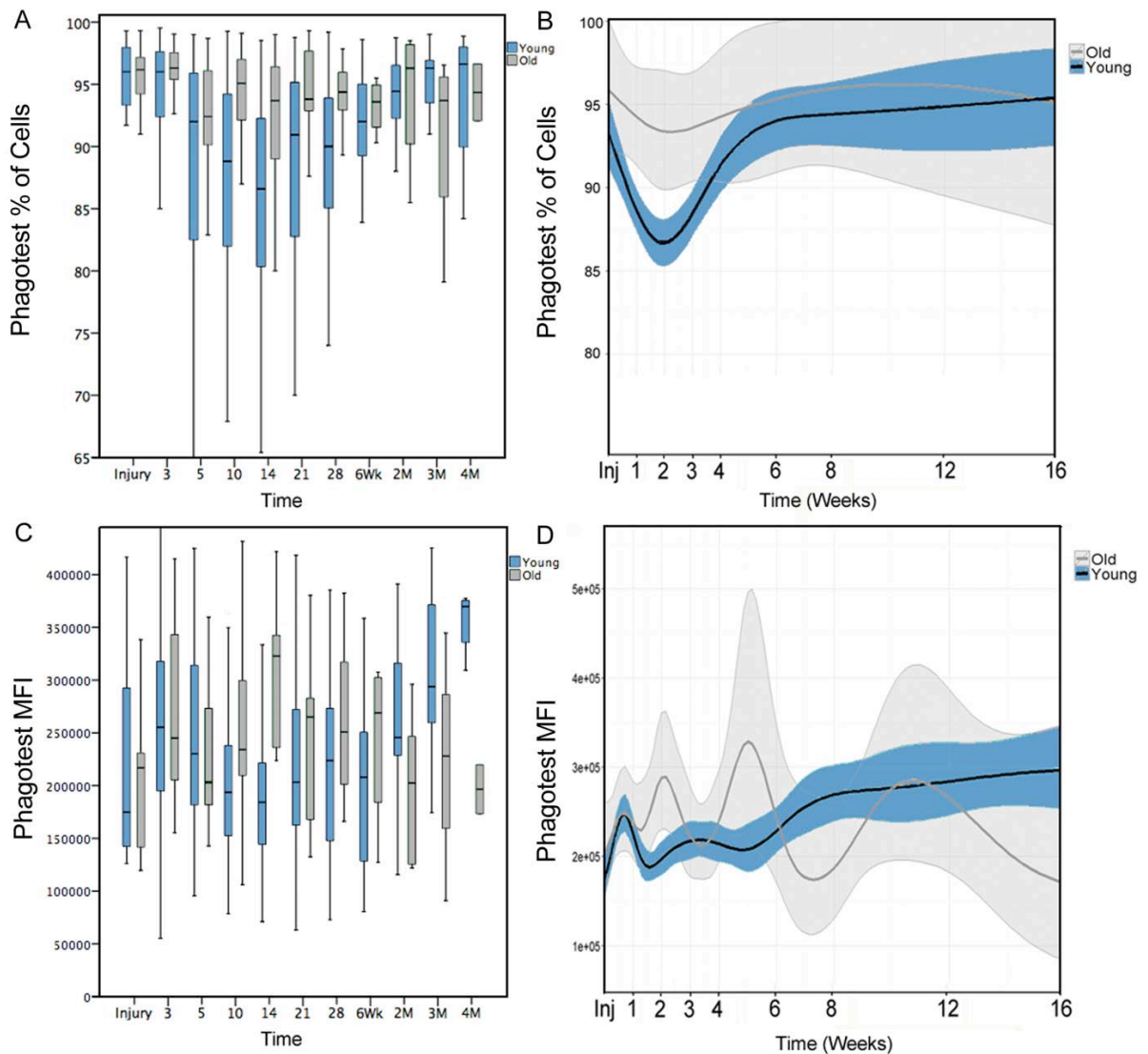


Figure 4.14. Components of phagocytic index separated in to young and old cohorts. PHAGOTEST™ % of cells ingesting bacteria shown as (A) raw data and (B) modelled data. PHAGOTEST™ MFI for amount of bacteria ingested shown as (C) raw data and (D) modelled data. Median values with IQRs are represented by box and whisker plots. Model-based predicted fixed effects of time are represented by means and 95% confidence intervals. Data was modelled using a mixed effects model that accounts for unbalanced repeated measures. Time was modelled using an 11-knot cubic spline.

4.5.3 Dual population of Neutrophils

During flow cytometry analysis of PHAGOBURST™ and PHAGOTEST™ data, two distinct populations of neutrophils were noted in some patients at some time points. Flow cytometry revealed a slightly larger and less granular population of cells on the forward scatter and side scatter plots (Figure 4.15). There was a reduced ability for these neutrophils to produce superoxide as detected by PHAGOBURST™ (Figure 4.16). This second neutrophil population also demonstrated a reduced phagocytic ability. In one example only 29% of neutrophils in the second population were able to phagocytose (reduced PHAGOTEST™ % of cells) and those that did engulfed a lower number of bacteria (reduced MFI) shown in Figure 4.17.

The timing of the appearance of the second population is shown in Figure 4.18. Most commonly a second population was seen in the second week after injury. The presence of a second population of neutrophils was seen in forty-six (48%) of patients. The demographics of patients who had a second population are collated in Table 4.1. A predictive model was created using logistic regression in SPSS using SOFA (Day1), NISS, ICU LOS, infectious episodes and number of operations and the strength of prediction ranked. In order of significance, the number of operative procedures, SOFA score (Day1) and ICU LOS were the largest independent predictors that an injured patient would demonstrate a second population of neutrophils. Other significant differences were that those with a second population were younger, more severely injured, had more blood products (RBCs and FFP) in the first 24 hours after injury, had more infections and longer LOS.

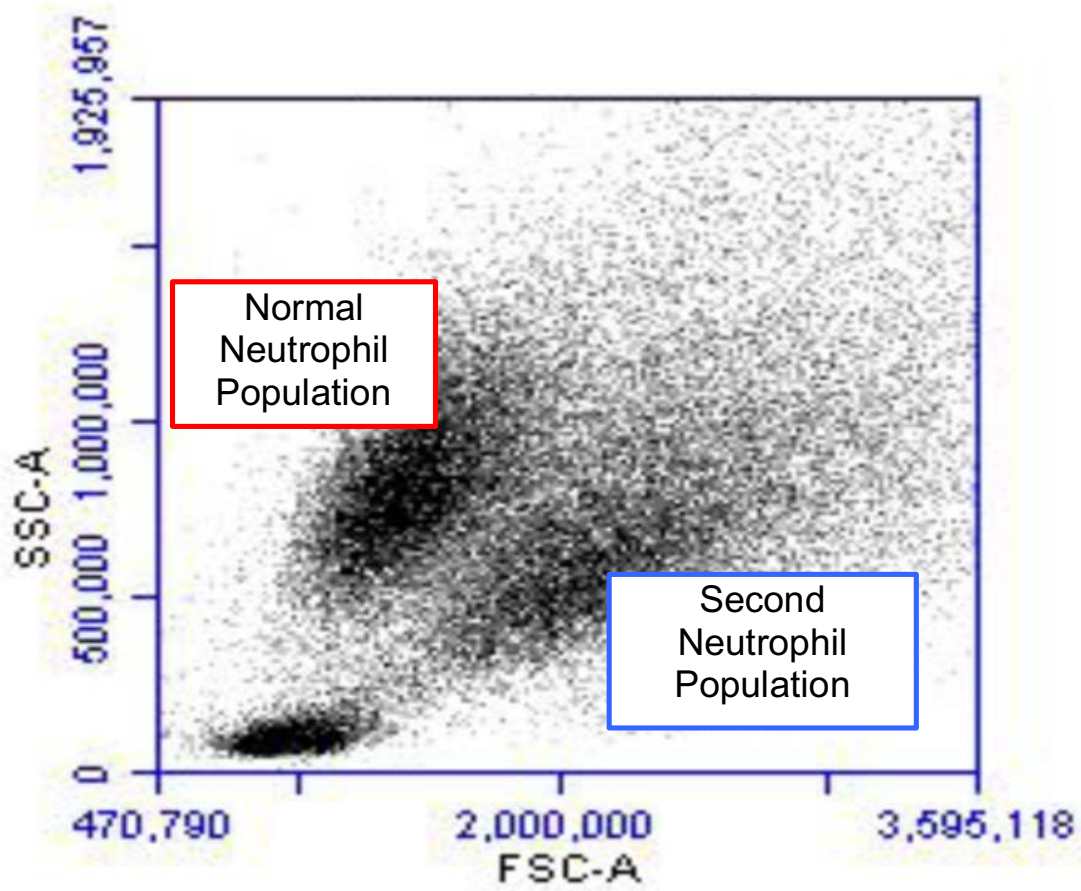


Figure 4.15. A second population of neutrophils was observed in some patients during the neutrophil analysis. Forward Scatter (FSC) and Side Scatter (SSC) reveal a slightly larger and less granular population of cells.

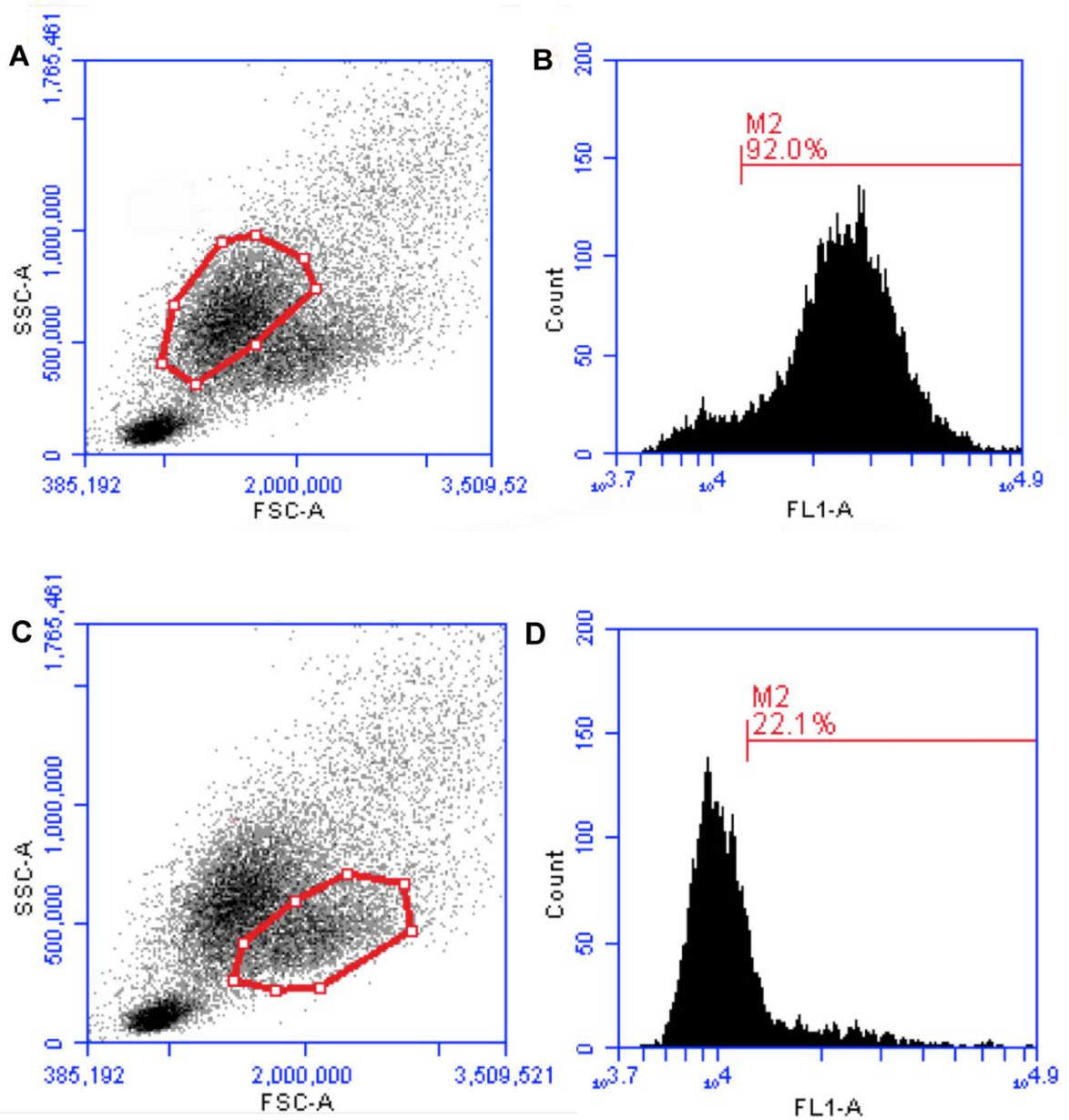


Figure 4.16. PHAGOBURST™ analysis of neutrophil populations: FSC and SSC demonstrated a second population of cells that have reduced ability to produce of superoxide.

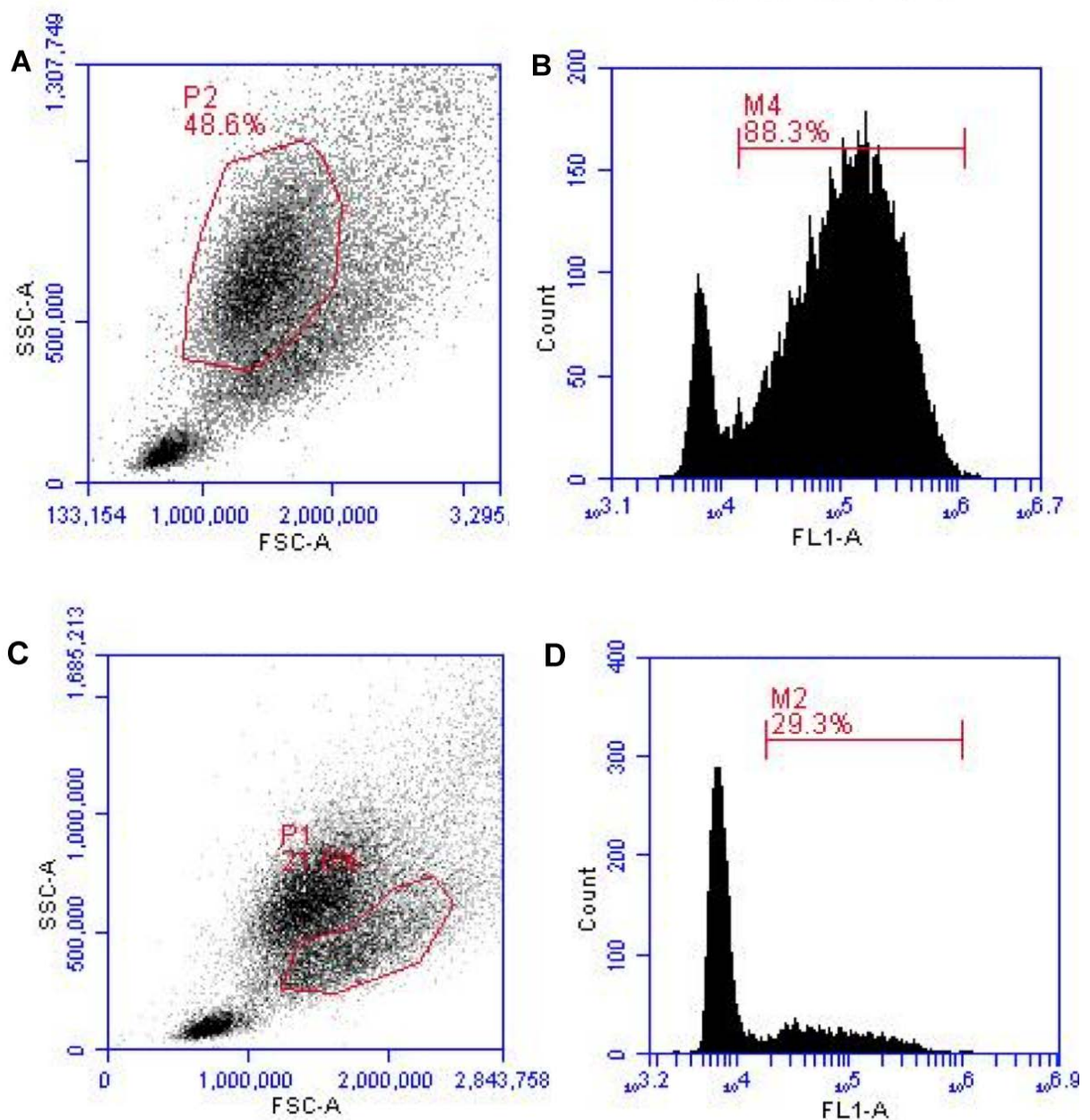


Figure 4.17. PHAGOTEST™ analysis demonstrating a second population of neutrophils with a reduced ability to phagocytose. (A) Gating on the usual population of neutrophils reveals the vast majority of cells were able to phagocytose (B). (C) When gating on the second population, the FS and SS plot reveal a slightly larger and slightly less granular population of neutrophils where most of the cells (71%) do not phagocytose bacteria and those that do, engulf less bacteria (D).

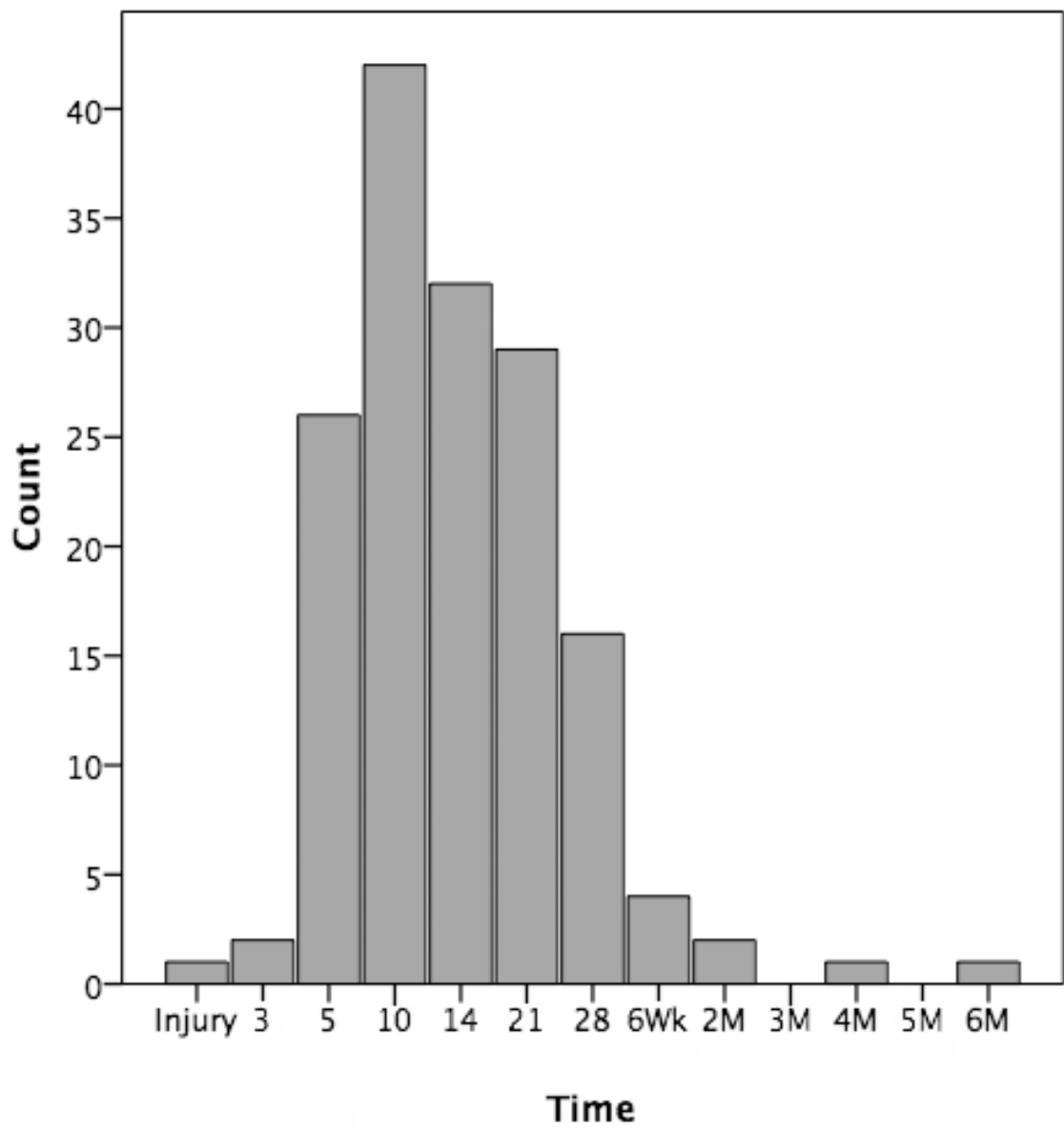


Figure 4.18. Timing of the appearance of a second population of neutrophils in severely injured patients (NISS>15).

Neutrophil Population	Dual	Normal	p=
n=	46	49	
Survived/RIP	41/5	46/3	0.405
Gender M/F	45/1	40/9	0.01 *
Age ^a	27 (23-32)	33 (26-49)	0.03 *
GCS	13 (3-15)	14 (6-15)	0.064
ISS	27 (20-38)	24 (17-34)	0.319
NISS ^a	41 (29-50)	29 (22-41)	0.033*
TRISS	85 (31-96)	91 (77-98)	0.013*
Ventilator Days	11 (8-21)	8 (4-19)	0.033*
Days on ICU ^a	17 (10-22)	8.6 (4-15)	0.000**
APACHE2 (Day1)	25 (19-29)	21 (13-25)	0.008*
SAPS2 (Day1)	54 (46-59)	51 (24-55)	0.046*
SOFA (Day1) ^a	10 (8-12)	9 (5-10)	0.001**
Infectious Episodes Per Patient ^a	2 (1-5)	1 (0-3)	0.005*
RBCs 1st24hr ^a	10 (5-16)	6 (0-13)	0.006*
FFP 1st24hr	8.5 (4-15)	3 (0-14)	0.003*
Crystalloid/colloid (Litres) 1st24hr	0 (0-1)	0.8 (0-2)	0.008*
Operative procedures/ patient, n ^a	6.5 (4-9)	4 (1-6)	0.000**
Length of hospital stay, days	51 (32-65)	33 (16-49)	0.005*

Table 4.1. Demographics of severely injured patients in whom a second population was observed.

^a Predictors used in Logistical Regression analysis to rank the independent strength of association.

To examine the neutrophil phenotype by differential staining and light microscopy, a whole blood smear was prepared and Giemsa stained. The white cell differential revealed a slightly increased number of band cells (10-20%) and metamyelocytes (1-2%) within the neutrophil population. Examples of blood films are shown in Figure 4.19. These proportions did not explain the large number of cells within the second population. Further characterisation of the second population of neutrophils was performed by staining for cell surface antigens.

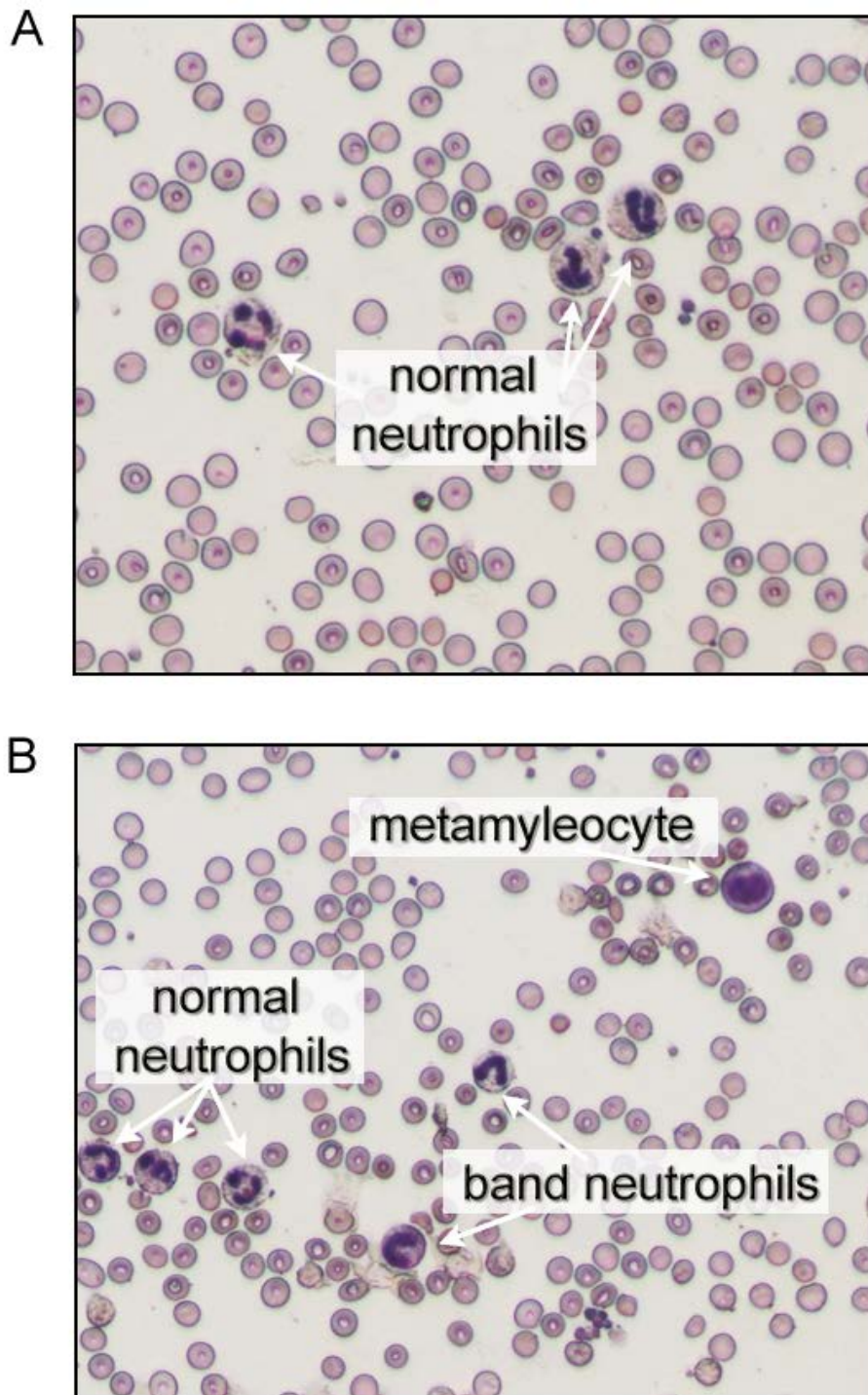


Figure 4.19. Examples of stained blood smears from severely injured patients showing (A) normal neutrophils and (B) band neutrophils (left shifted) and a precursor granulocyte (metamyelocyte).

4.5.4 Neutrophil Surface Antigens

CD11b is involved in numerous neutrophil processes including adhesion, migration and phagocytosis (437) as well as neutrophil recognition of opsonised pathogens and complement activation (438). CD14 is a co-receptor with TLR-4 that is strongly expressed on monocytes and much more weakly on neutrophils, a characteristic used to differentiate between these cell types (331). CD15 is involved in phagocytosis and chemotaxis and is used to identify neutrophils (439). CD33 is a myeloid marker that is slowly shed as cells mature. CD35 is a complement receptor that initiates degranulation. CD63 and CD66b are vesicle membrane receptors of primary and secondary granules respectively that are increased on the cell surface as neutrophils degranulate. These markers can also be used to help identify granulocytic MDSCs that are CD11b⁺/CD33⁺/CD14⁻/HLADR⁻/CD15⁺. MDSC, as mentioned earlier, are variable in their antigen expression and the only strong identifying factor is their ability to cause immune suppression in other cells such as T cells (147).

A summary of the antigen profile used to identify the two neutrophil populations seen in trauma patients is shown in Table 4.2. Examples of staining for each of the antibodies for CD11b, CD11b (active), CD15, CD33, CD63, CD66b and CD35 are shown in Figures 4.19-4.22. Although the staining for MDSC was positive in both populations, further work in our laboratory found that an increase in MDSC was not seen in the neutrophil populations but was seen in the monocyte area on FSC and SSC (J Hazeldine, personal communication). The significant finding from the surface antigen staining was that the second population of neutrophils demonstrated a reduced expression of CD11b and CD35.

Antigen	Population	MFI	% of Stained Cells	Phenotype
CD11b	Normal	3722.68	68.4	CD11b++
	<i>Second</i>	<i>1727.53</i>	<i>23.9</i>	<i>CD11b+</i>
CD11b (active)	Normal	156852.89	97.0	CD11b (active)++
	Second	59250.22	89.0	CD11b (active)++
CD15	Normal	12033.69	95.8	CD15++
	Second	7774.86	69.5	CD15++
CD33	Normal	1407.19	79.0	CD33+
	Second	2009.54	89.2	CD33+
CD63	Normal	3693.16	49.4	CD63+
	Second	3406.79	62.1	CD63+
CD66b	Normal	124184.19	99.5	CD66b++
	Second	102478.92	98.3	CD66b++
CD35	Normal	59213.26	99.5	CD35++
	<i>Second</i>	<i>17035.7</i>	<i>38.3</i>	<i>CD35+</i>

Table 4.2. Summary of antibody staining of severely injured patients neutrophil population. Incubation of patient neutrophils with antibodies and an isotype-matched control to surface antigens was performed on neutrophils in an attempt to characterise them. ‘Normal’ represents the usual population of neutrophils and ‘Second’ represents the additional population of neutrophils seen on forward scatter and side scatter flow cytometry. The results in red show where there are large differences in the normal and second population of neutrophils seen in the surface staining.

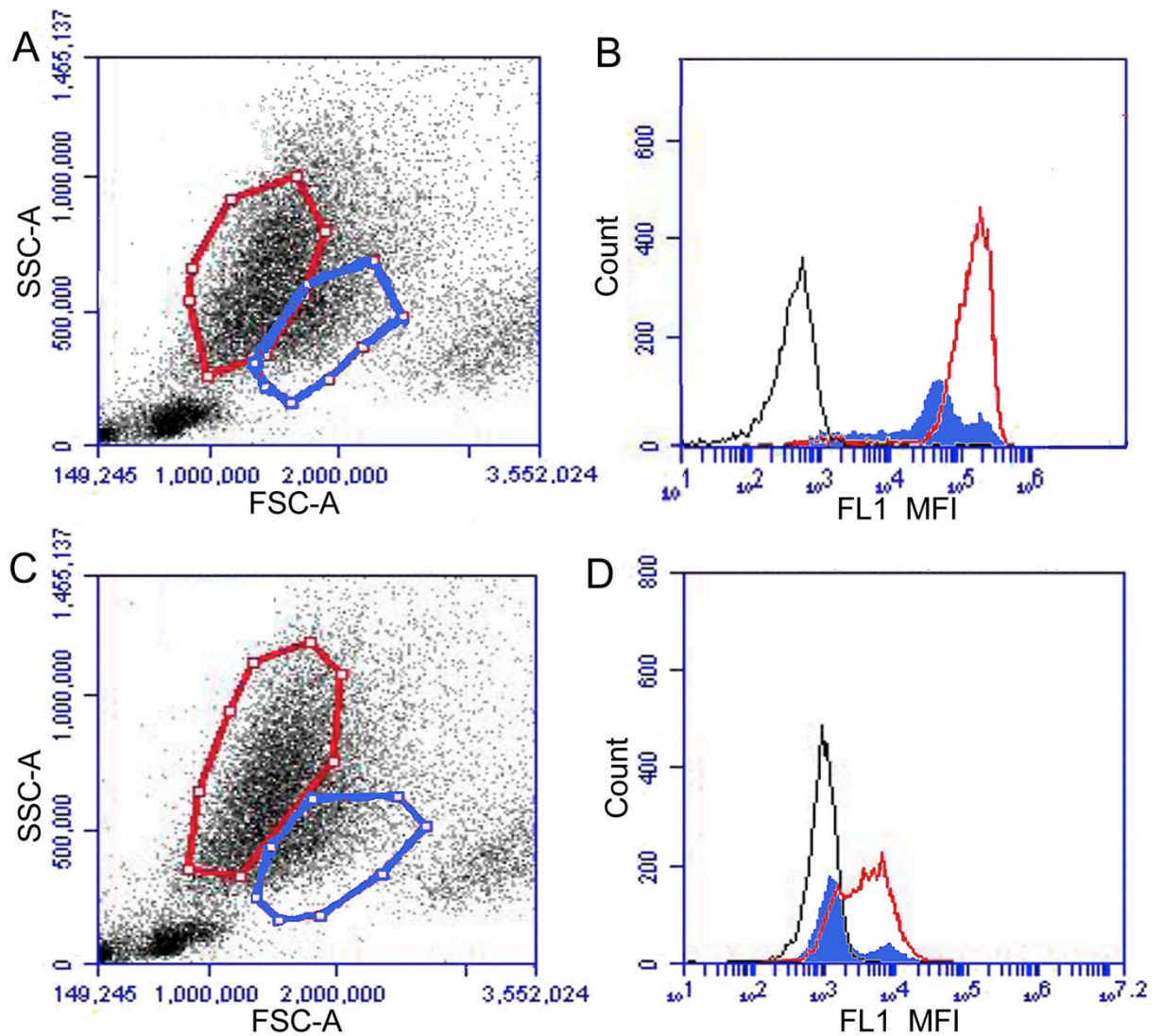


Figure 4.20. Surface antigen staining for CD11b gated on the normal and second neutrophil populations. (A) Neutrophil gating on the normal (red) and second population (blue) for CD11b (non-active). (B) CD11b expression (MFI) for the normal and second population of the non-active form. (C) Neutrophil gating on the normal (red) and second (blue) population for staining with CD11b (active). (D) CD11b (active) expression (MFI) for the normal and second population of the active form.

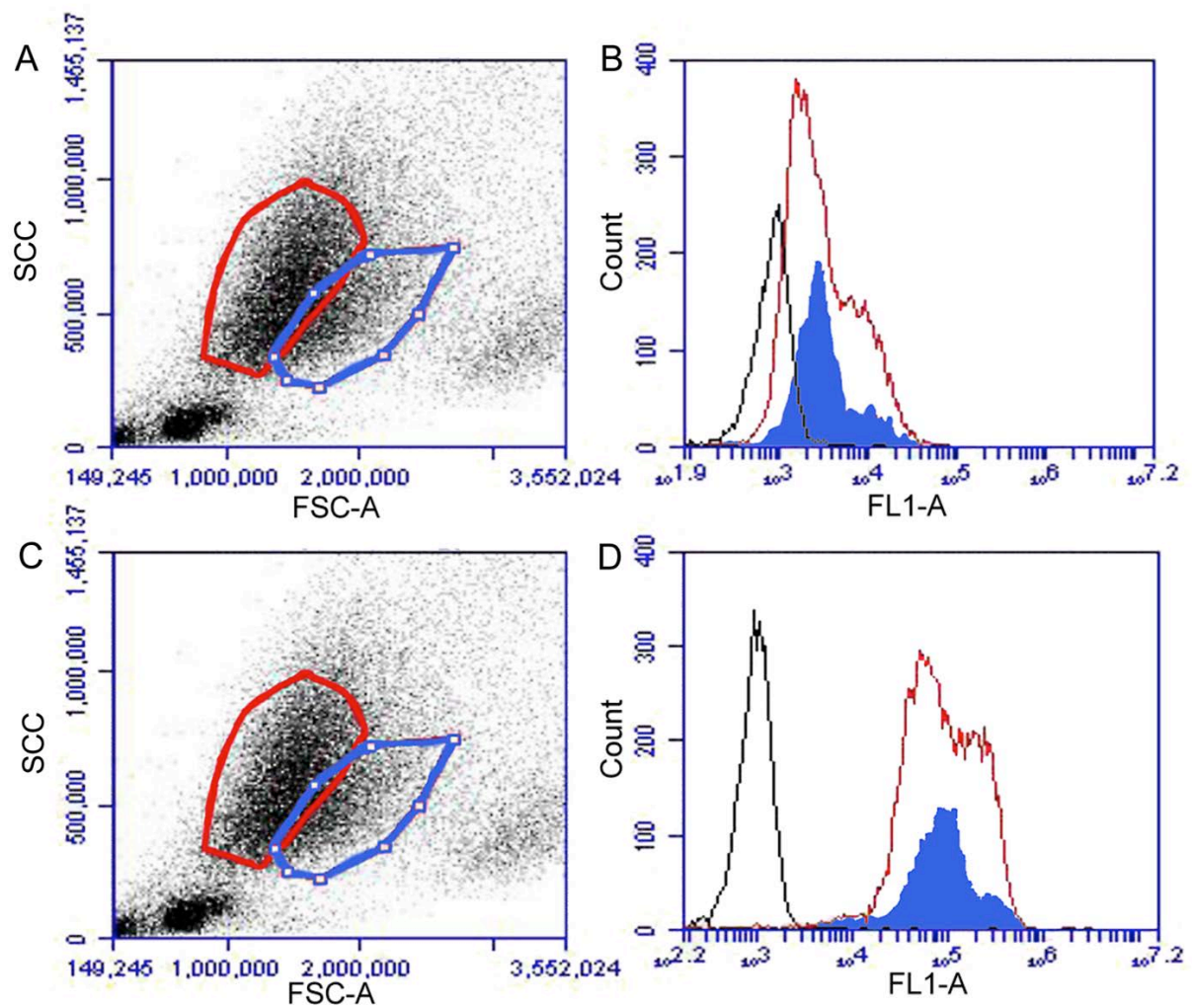


Figure 4.21. Surface antigen staining for CD63 (azurophilic granules) CD66b (specific secondary granules) gated on the normal and second neutrophil population. (A) Neutrophil gating on the normal (red) and second population (blue) for CD63. (B) CD63 expression (MFI) for the normal and second population of neutrophils. (C) Neutrophil gating on the normal (red) and second (blue) population for staining with CD66b. (D) CD66b expression (MFI) for the normal and second population of neutrophils.

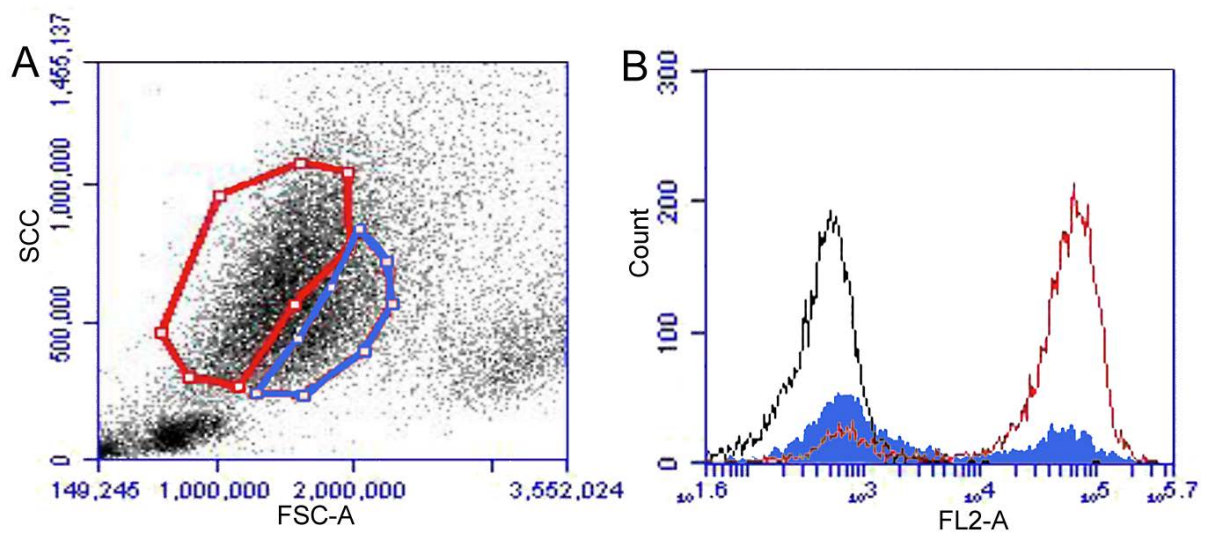


Figure 4.22. Surface antigen staining for CD35 gated on the normal and second neutrophil population. (A) Neutrophil gating on the normal (red) and second population (blue) for CD35. (B) CD35 expression (MFI) for the normal and second population of neutrophils.

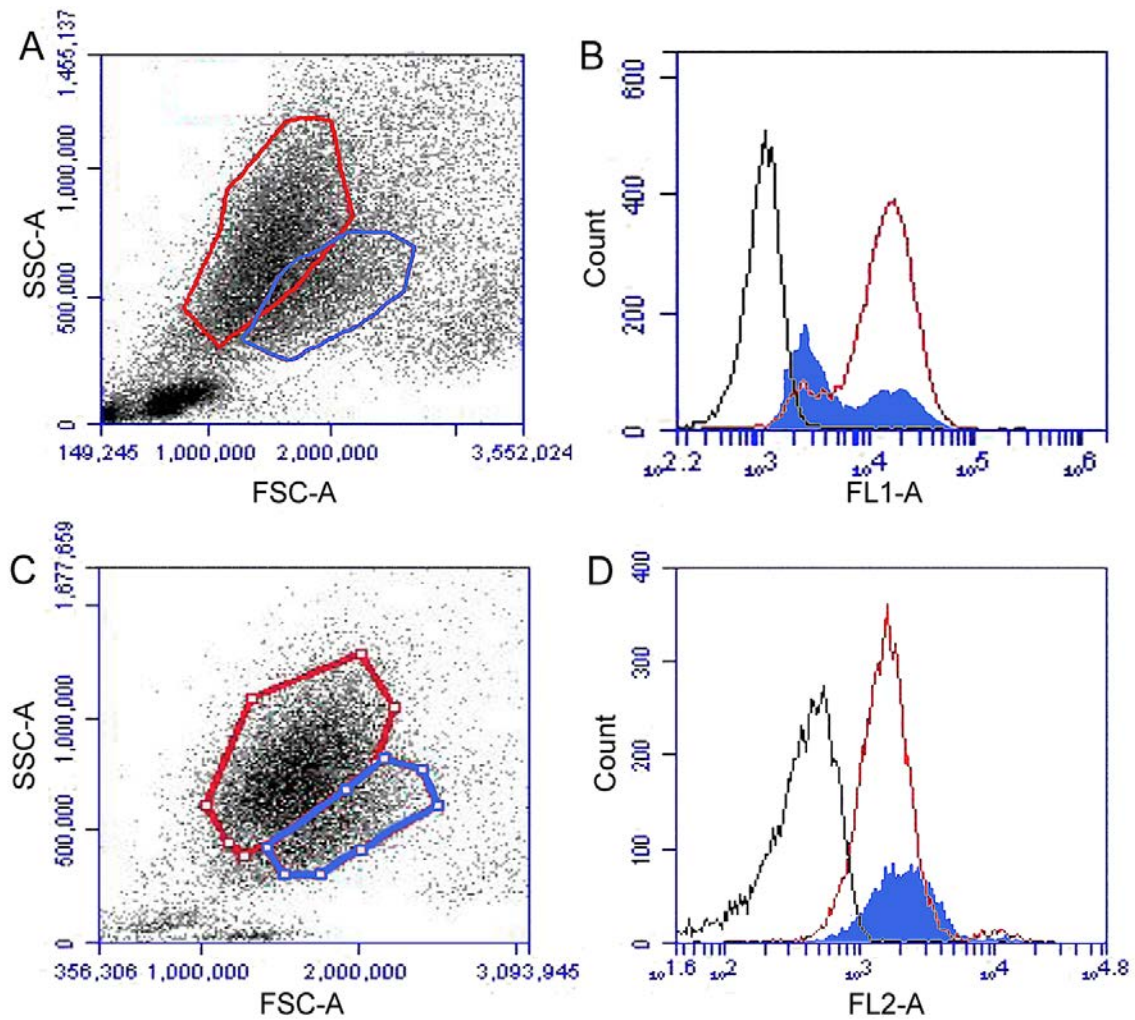


Figure 4.23 Surface antigen staining for CD15 and CD33 gated on the normal and second neutrophil population. (A) Neutrophil gating on the normal (red) and second population (blue) for CD15. (B) CD15 expression (MFI) for the normal and second population of neutrophils. (C) Neutrophil gating on the normal (red) and second (blue) population for staining with CD33. (D) CD33 expression (MFI) for the normal and second population of neutrophils.

4.6 Sepsis and the second neutrophil population

One explanation for the presence of a different phenotype of neutrophils could be exposure to complement and bacterial infection. Support for this could be made by the timing of the appearance of this phenotype and detection of infection in the patients.

The probability of developing a second population of neutrophils peaked in the second week following injury in a similar way to the number of septic episodes. When the cohort was separated into those who had a septic episode and those who did not during their admission, there was an increased chance of them having a second population of neutrophils (Figure 4.24).

To investigate whether there was a relationship between the diagnosis of sepsis and the appearance of a second population of neutrophils an autocorrelation technique was used. Time was used as a factor to see whether any significant correlation was present. An auto-correlation factor (ACF) of 0.76 was found at day 8. In other words, 8 days after the diagnosis of sepsis a second population of neutrophils appeared in the blood of severely injured patients (NISS>15) that had survived. This was performed for sepsis and the appearance of a second population of neutrophils and is shown in Figure 4.25.

The diagnosis of true sepsis appearing earlier than 48 hours after injury in the study could be doubted despite meeting diagnosis. To examine this, the autocorrelation was repeated after removing the septic episodes that occurred in the first 48 hours following injury. An ACR of 0.56 was found at day 4, that is when a septic episode occurred a second population of neutrophils was found 4 days later.

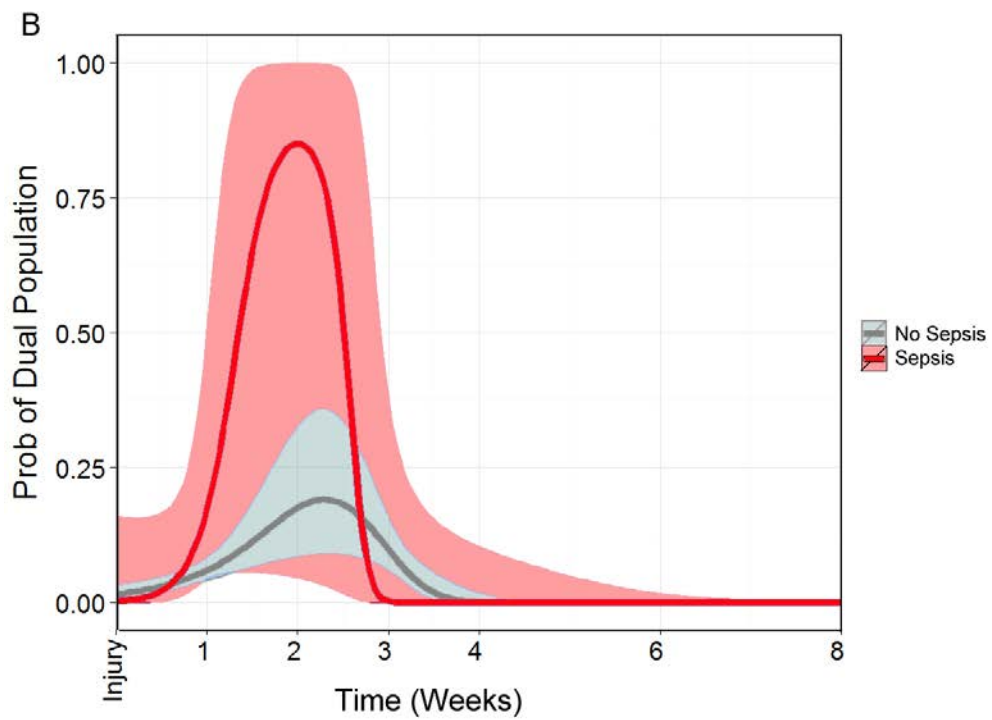
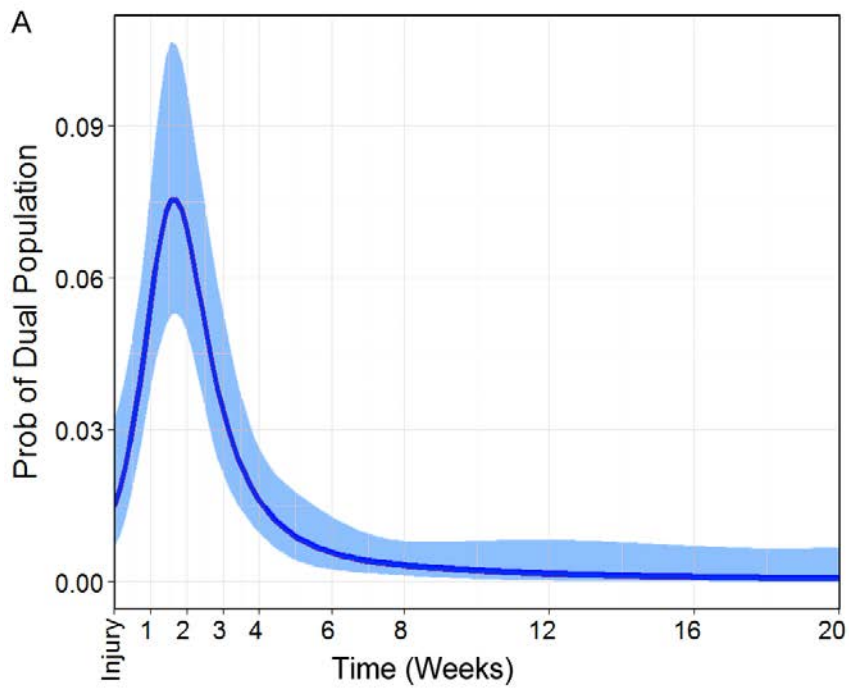


Figure 4.24. (A) The probability of a severely injury patient (NISS>15) that survived demonstrating a second population of neutrophils over time. (B) The probability of a second population of neutrophils separated into whether the patient had an episode of sepsis or not.

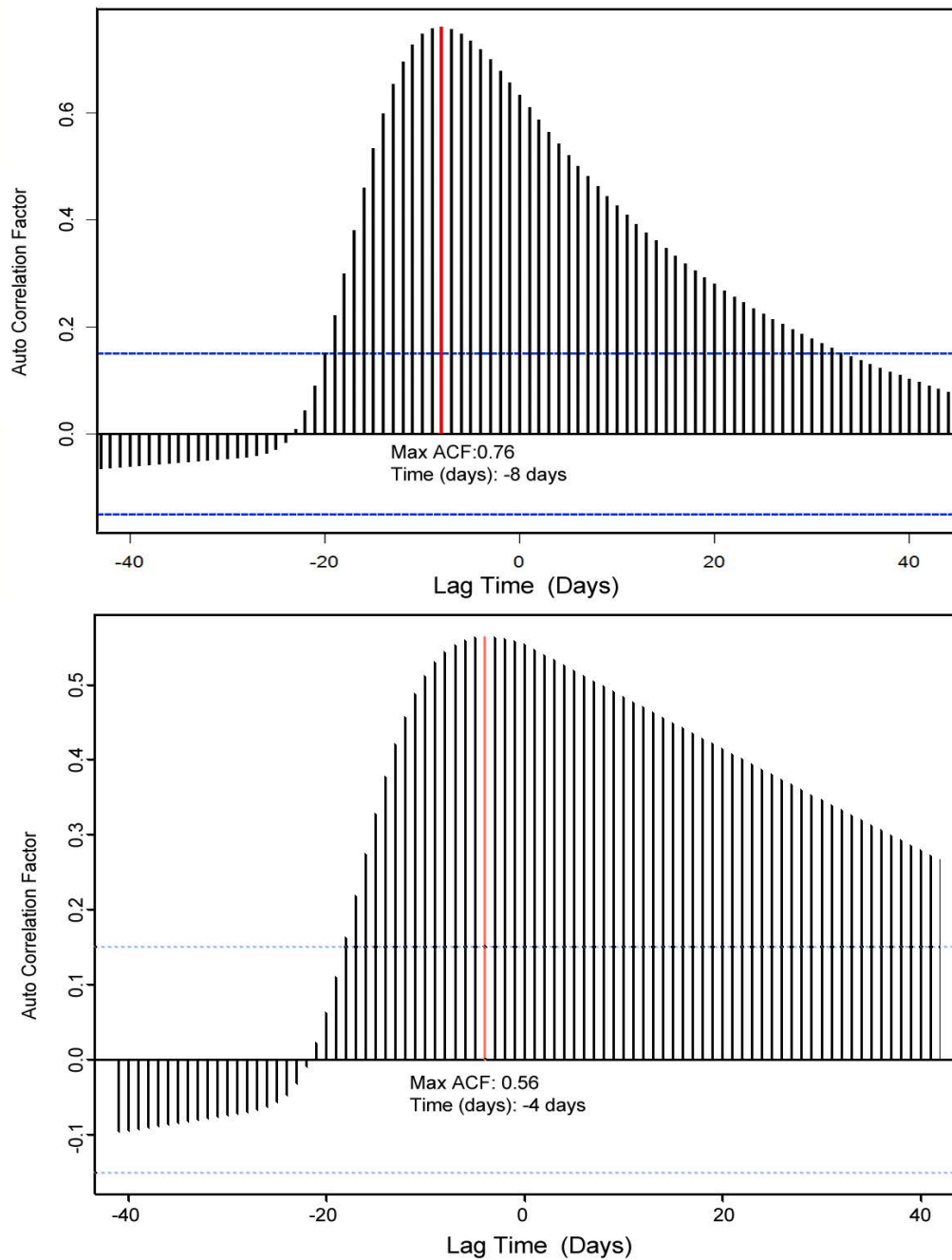


Figure 4.25. Autocorrelation using Time with Septic episode and the timing of the appearance of a second population of neutrophils. (A) For all data the maximum correlation occurred 8 days following sepsis. **(B)** When septic episodes from the first 48hours were removed the maximum correlation occurred at 4 days following the septic episode.

4.7 Discussion.

The ability to quantify DAMPS and relate absolute levels to the amount of cells damage is an attractive concept. Xiaoling Gu *et al.* was able to show how mtDNA was related to an increase in post injury SIRS but could only crudely demonstrate the differences in mtDNA levels between the severely injured (ISS \geq 16) and not severely injured (ISS $<$ 16)(440). Others have also positively correlated mtDNA levels with injury severity but only by separating patients into mild, moderate and severe injury cohorts(441). Yamanouchi and colleagues on the other hand did correlated mtDNA levels with ISS ($R^2=0.362$) in a study comparing infections in trauma and non-trauma with 37 trauma patients (64). Our study has allowed levels to be followed over time but has only shown a weak association with injury severity. The lack of strong correlation with injury severity could be attributed to a weakness in the way injury severity scores characterise patients or a failure in the detection of early DAMPS to give the full picture of tissue damage. Other factors that should be considered are that blood measurements after trauma can be confounded by the half-life of DAMPS, blood loss, dilution and the timing of samples not capturing the temporal changes in analytes.

It is interesting that the levels of DAMPS after trauma differ with age. Itagaki *et al.* found similar significant differences in mtDNA levels between young and old patients that were severely injured; mtDNA levels were higher in the elderly but in their study their ability to produce NETs when stimulated was reduced(442). It is generally accepted that the mitochondrial DAMPS initiate the inflammatory response locally(31) and that it is probably formyl peptides associated with the break up of the mitochondrion that are the initiator and that detection of mtDNA itself is a surrogate marker of the quantity of mitochondrial derived DAMPS present(432).

The half-life of the various DAMPS differs but is generally longer than the short acting cytokines(443). HMGB1 half-life can range from the 17 minutes to 30 hours in serum but the literature is at odds as HMGB1 can bind to other proinflammatory cytokines and increase its duration of action, as well as the fact that there are various isoforms that may also interfere with detection (444). To complicate matters further, while still present in the serum HMGB1 action can be degraded by thrombomodulin more evidence of the link between immunity and coagulation (445). Circulating DNA in young healthy volunteers has a half-life of 10-15 minutes in the circulation it is unlikely that mtDNA would be handled any differently in the elderly when it is cleared by the liver(446,447)

The increased release of DAMPS in contrast to the immunesenscence(190) that occurs with increasing age is difficult to explain. A review of the role of senescent cells in ageing by van Deursen highlights the presence of 'aberrant tissue architecture', impaired tissue regeneration as well as the reduced numbers of stem and other mitotically active cells that contribute to older diseases (448). The role of cellular senescence can be advantageous as they help to limit excessive scarring during wound repair(449). There may be a difference in clearance of DAMPs in the elderly but there is no evidence to suggest this. Another possibility is that older injured cells do not recover from injury when cells are ischaemic or mechanically injured as well as younger ones. The mechanisms are not clear but the role of Nitric Oxide (NO) may contribute as there is a reduced bioavailability and a build up of Reactive Oxygen Species(ROS) during hypoxia that is increased in the elderly(450,451).

The response to injury can be monitored through cytokine production. The classical production of the proinflammatory cytokines IL6 and IL8 seen in this study are

consistent with the literature (452-454). Contrast then the increase release in DAMPs (mtDNA and HMGB1) with the reduced cytokine production seen in the elderly compared to the young following injury. One might expect a more exaggerated cytokine response with the significantly increased presences of DAMPS observed. It is likely is that the immune-senescence seen in other diseases affecting old age are also seen here in major trauma(455).

The neutrophil numbers and their response decrease with age after injury. In the young, neutrophil function recovered to normal levels after 4 weeks during the study but the older patients took much longer; supporting the opinion that there is a decreased ability for older adults to fight infection(456,457). The reduced function of neutrophils from older adults has been also shown by Vanzant and colleagues (458) who describe the immune-senescence contributing to a diminished response and possibly worse outcomes. In our own laboratory, older neutrophils and macrophages have been shown to produce less superoxide and have reduced phagocytic ability in an elderly uninjured population(181) as well as in a elderly hip-fracture study (412,459,460) The immunesenescence described is in contrast to the chronic pro-inflammatory state that is seen in the elderly and contributes to many diseases such and diabetes and cardiovascular disease seen in old age(461).

The curious appearance of a second population of neutrophils with a different phenotype during the first 6 weeks following severe injury lead to investigations to ascertain the role of these cells. In response to infection or trauma a neutrophillia is often seen as the marrow responds to the on-going threat(462). Indeed when observed macroscopically an increase in banded (left-shifted) neutrophils was seen but this was not to the proportions seen in the second population of neutrophils and did not fit with a population of cells that failed to undertake any phagocytosis. Another

idea was that these neutrophils were MDSCs. The MDSC role was first investigated in its role in tumour growth but a different phenotype was being observed in trauma and sepsis(463). This concept was initially encouraging, the role of MDSCs, adapted granulocytes and monocytes was being highlighted in the literature. Koendermans *et al*'s work in demonstrating a population of neutrophils which actively suppress the immune system would fit with the clinical picture of an exaggerated inflammatory response seen in our patient group (464). Attempting to identify these cells still is debated, the only sure way is to test their suppressive nature *in vitro*(147). The surface staining (CD11b⁺/CD33⁺/CD14⁻/CD15⁺) was consistent but their proportion to standard neutrophils did not fit with our findings(146). The location on the forward scatter and side scatter plot of the second population of neutrophils also did not fit this hypothesis as MDSC appear within the monocyte area(130).

Further surface antigen testing of the neutrophils using labelled antibodies and flow cytometry was used to try and identify their phenotype. CD63 and CD66b are useful in identifying the loss of granules for neutrophils as the antigen becomes incorporated into the surface cell membrane(465). The second population of neutrophils did reveal a strong expression of CD63 and CD66b with the surface membrane but no difference was seen between the usual and the second population of neutrophils. Similarly the cells in the second population of neutrophils had normal CXCR1 levels so were not reverse migrated from tissues(466) or activated neutrophils (CD11b active) distinct from the normal neutrophil population(467). Interestingly though, surface staining did reveal two markers, CD11b and CD35 that are thought to be involved in activation and bacterial opsonisation, were down-regulated. Studies have shown up regulation of CD35 and CD11b in response to bacterial infection. In a study of 135 patients with either viral or bacterial infection by Lilius *et al*. used CD64, CD35 and CD11b to

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differential bacterial from viral infections where a CRP/CD11b ratio was able 76% sensitivity and 80% specificity for the diagnosis of bacterial over viral infection. Conversely other groups have found lower levels of neutrophil surface markers. Babcock *et al.* demonstrated lower CD16 and CD11b in 34 severely burnt patients related to infection and lower CD35 expression but not related to infection(468). In a study from Finland, Nuutila *et al.* used CRP and CD11b ratio as a predictor of sepsis and in particular gram positive bacterial infection(469). Nuutila *et al.* go on to suggest mechanisms behind the reduced detection of CD11b is due to binding of fibrinogen, intercellular adhesion molecule 1, complement component C3bi, *Candida albicans*, and neutrophil inhibitory factor. Other suggestions by Nuutila *et al.*'s group of why CD11b was low are due to a failure of degranulation that would also be consistent with the lower CD35 expression. Against this is that other markers of degranulation such as CD66 should also be affected. Finally the idea that the complement receptors (CD11b and CD35) are shed from the neutrophil cell surface sometime after they are incorporated in to the cell surface membrane post degranulation or after activation would explain the loss of phagocytic activity but loss of CD11b and CD35 has not been proved in sepsis before. Other complement receptors such as C5a have been shown by Xu *et al.* in a study of 34 patients with sepsis via rapid internalisation of the receptor following exposure to C5a in patients with infection. The presence of different neutrophil receptors has been explored in an attempt to differentiate gram positive and negative infections from fungi and viruses to some extent but this has not reached the clinical environment(136).

There is some support for the idea that the second population of neutrophils seen during neutrophil function testing (PhagoTEST and PhagoBURST) may be a surrogate marker of neutrophil contact with bacteria or indeed exposure to sepsis. The patients

who demonstrated a second population of neutrophils were sicker with higher SOFA scores and spent more time of ICU and more operative procedures. Significant differences are also seen in the severity of injury (NISS), infection, blood transfusion rates and age that will all have some relationship with how severe the initial injury was, where it was and how the patient responded. Previous exposure to infection would explain why the neutrophils poorly phagocytose when they were tested in vivo. The appearance of the second population of neutrophils in the sicker patients and commonly during the second week following injury also supports this hypothesis. When modelling the probability of the appearance of a dual population and sepsis and strong relationship was seen. By using an autocorrelation technique an attempt was made to look at the diagnostic value of this second population, suggesting there may be a delay between the start of any infection and the first appearance of the second population of neutrophils. Exposure to complement particularly C3b may represent previous exposure of opsonised bacteria and this second population of neutrophils represent an activated or primed neutrophil that has already functioned.

The diagnosis of infection in the presence of an on-going SIRS response remains a challenge. In our population, diagnosing infection was confounded further because many of the injuries were accompanied by severe contamination or impregnation of material in to the tissues from blast. Animal and human excreta are used as fertiliser in areas of Afghanistan where IEDs were placed that produced severe blast injuries in the military cohort. The timing and presence of a reduced expression of complement receptors that are involved in phagocytosis does hint at a link with infection but this is unproven by this work.

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Chapter 5: The Endocrine Response to Severe Trauma

5.10 Introduction

Afferent somatic and autonomic stimulation from the area of injury has an immediate affect on the sympathetic nervous system that in turn will influence the immune and endocrine systems (223). The immediate stimulation of 'flight or fight' is followed by a longer co-ordinated response that not only deals with immediate threats such as blood loss but also prepares the injured individual to repel foreign material and begins the repair process (17). The role of the endocrine system specifically during this time can have profound effects for the severely injured patient, with hypermetabolism, catabolism, immune dysfunction and organ dysfunction leading to critical illness and even death (229,470).

Adrenaline and nor-adrenaline are released in to the circulation but as the stress response continues cortisol become more important (471). The dominant catabolic drive allows substrates to be mobilised for repair. After some time catabolism subsides and anabolic hormones dominate. The repair of injured tissues moves into stages of remodelling and then maturation. The lost muscle and tissue is slowly replaced (472).

The immediate response to injury has been studied in detail but less is known about the response in the days and weeks that follow. Much of the evidence comes from elective surgical studies and also from burn injuries (473-475). This chapter focuses on the adrenal cortex-derived hormones and their temporal changes that occur from injury to 6 months following injury. The relationship of these adrenal hormones to the immune system and other outcome measures will then be explored.

5.1 Results

A cohort of young (age <50 years old), severely injured (NISS>15) males who survived but were not given steroids were selected in an attempt to remove the bias from females and older adults with co-morbidities (Figure 5.1.). Sixty patients, a mix of young military and civilian patients, with no co-morbidities that were treated at the new QE hospital from 2011-2012 were analysed.

A summary of the cohort demographics, the mechanism of injury and the distribution of septic episodes are shown in (Figure 5.2.) Median age was 27 (24-31), median NISS was 34 (29-44) and their Day 1 APACHE was 21 (14-25). Patients remained ventilated and stayed on ICU for a median of 9 days. Median length of stay was 36 days (19-56). Improvised Explosive Device (IED) and Road Traffic Collision (RTC) were the most common causes of injury. Ten patients (17%) had an apparent septic episode in the first 48-hours following injury. Twenty-five (42%) of patients had at least one septic episode and most occurred in the 2nd week following injury.

Data are displayed as medians with IQR unless stated otherwise. Control data were generated from normal age match volunteers; 16 were recruited for the current study and the data from another 21 was included from a previous study that was analysed by Angela Taylor.

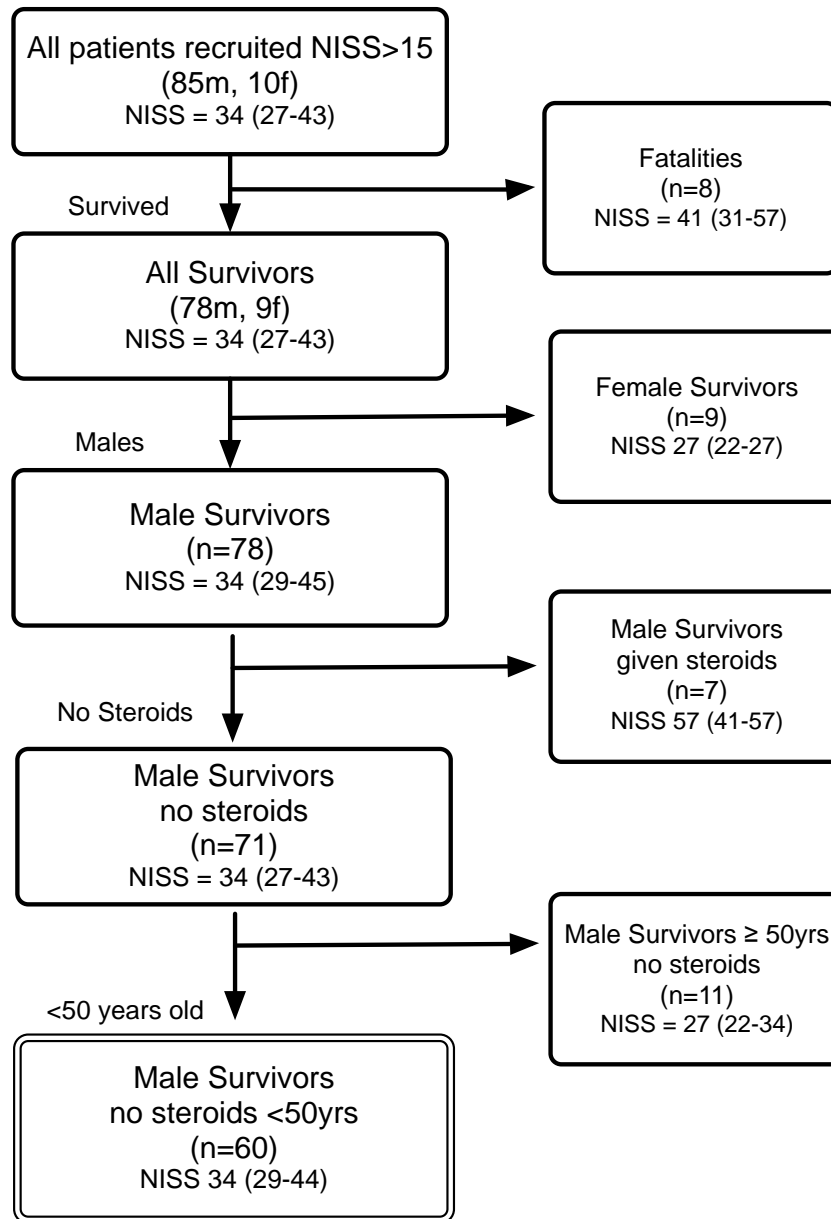


Figure 5.1. Subgroup Selection for Analysis. Sixty male survivors of severe injury (NISS>15) under 50 years of age, who had not been given exogenous steroids.

A

Demographics	
Patients n=60	Median (IQR)
Age	27 (24-31)
GCS	14 (3-15)
ISS	25 (17-32)
NISS	34 (29-44)
TRISS	94 (56-98)
APACHE 2 (Day1)	21 (14-25)
SAPS 2 (Day1)	48 (26-54)
SOFA (Day1)	9 (6-10)
Septic Episodes	1 (0-2)
RBCs (Day1), Units	10 (2-17)
FFP (Day1), Units	9 (1-15)
Crystalloid/colloid (Day1), L	0 (0-1)
Ventilator Days	9 (5-12)
ICU Length of Stay, Days	9 (5-16)
Operative procedures	5 (3-7.5)
Hospital Length of Stay, Days	36 (19-56)

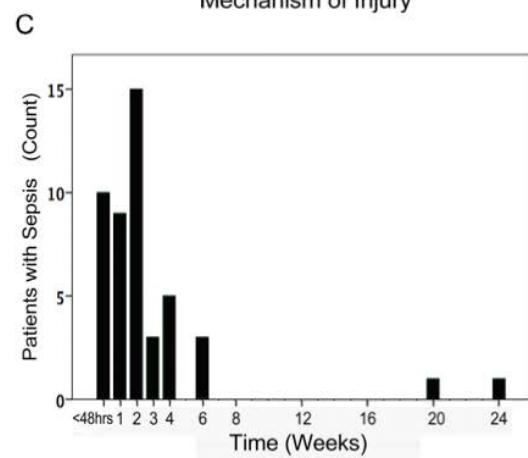
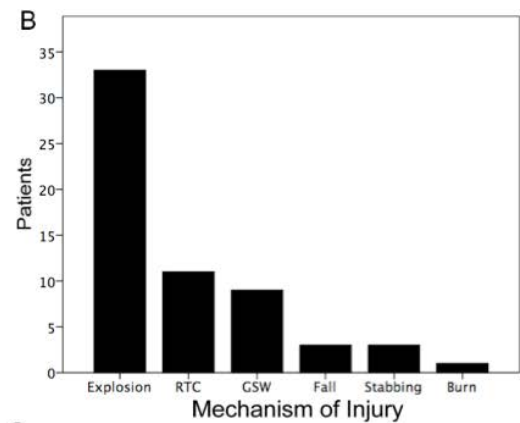


Figure 5.2. Patient Characteristics: Demographics (A), Mechanism of Injury (B) and the distribution of Septic episodes (C) for 60 male survivors from severe injury (NISS>15) under 50 years of age, who had not been given exogenous steroids.

5.2 Glucocorticoids

The glucocorticoid data along with the respective modeling is shown in Figure 5.3. Serum concentrations of the major active glucocorticoid cortisol (F) increased after injury peaking at 369 (261.7-521.1) nmol/l by week 4. There was a difference between the cortisol levels immediately after injury (208, 132.1-331.8 nmol/l) and week 4 ($p=0.001$), but no significant differences between age matched controls and changes in cortisol level 1-4 weeks after injury. Serum concentrations of the inactive metabolite cortisone (E) levels were lower after injury and increased slowly over time but this difference was not significant ($p=0.08$). The Cortisol/Cortisone ratio (F/E) peaked after 2 weeks (6.8, 5.5 - 8.2) and was significantly raised compared to controls ($p<0.0001$). The F/E ratio returned to normal at around 6 weeks.

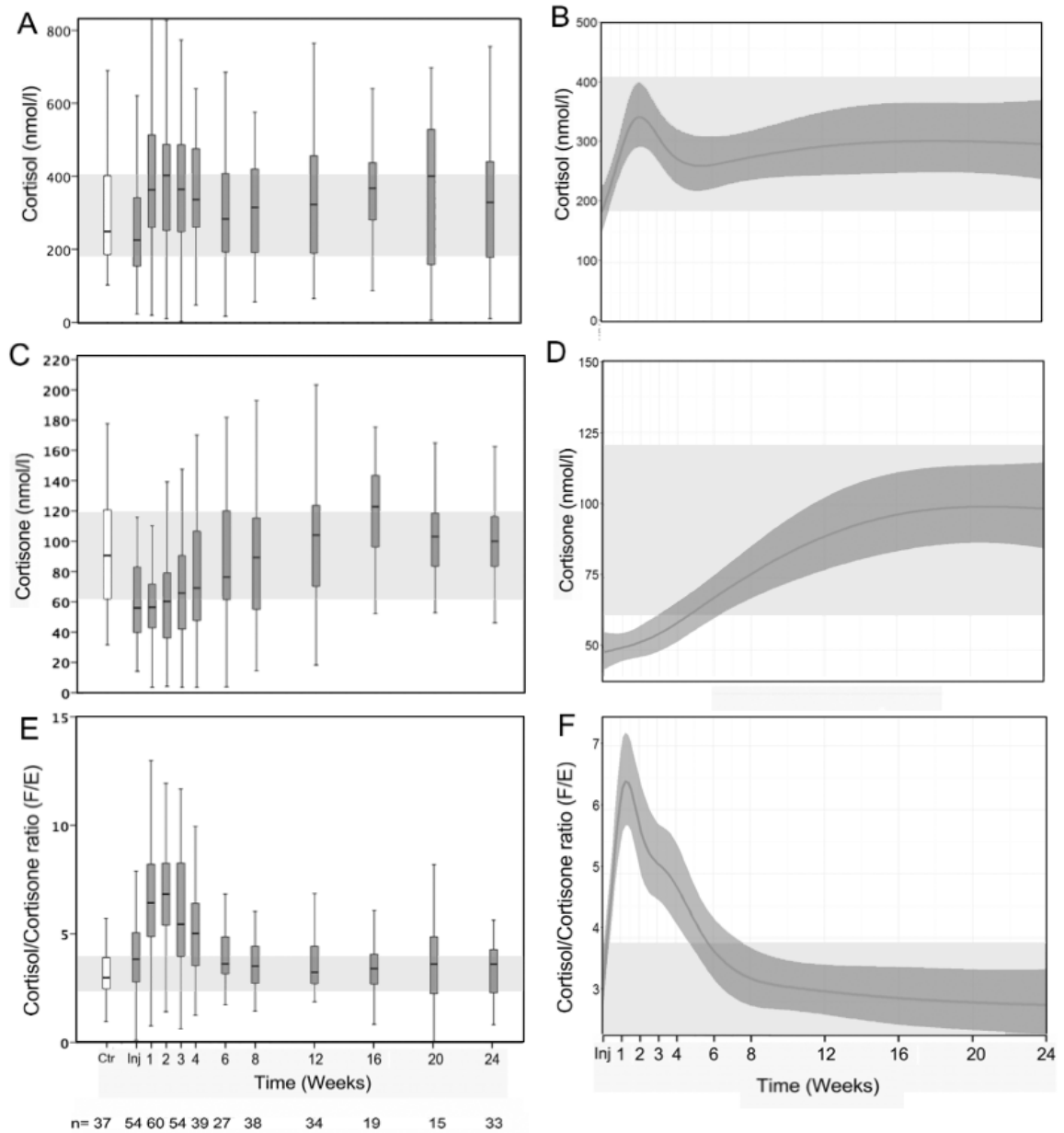


Figure 5.3. Serum glucocorticoids in 60 male survivors from severe injury (NISS>15) under 50 years of age, who had not been given exogenous steroids. Serum cortisol levels over time – (A) raw data and (B) modelled data. Serum cortisone over time – (C) raw data and (D) modelled data. Cortisol/Cortisone ratio (F/E) over time – (E) raw data and (F) modelled data. Raw data are represented by box and whisker plots showing median and interquartile ranges. Data were modelled using a non-linear mixed effects model that accounts for unbalanced repeated measures using a 4-knot cubic spine. Modelled data are reported as means and 95% confidence intervals.

5.3 Adrenal Androgen Precursors

The adrenal androgen precursor results along with the respective modeling are shown in Figure 5.4. Dehydroepiandrosterone (DHEA) was very low after injury (4.9, 2.1-6.6 nmol/l; $p < 0.0001$) compared to controls but recovered 3 months after injury. Serum dehydroepiandrosterone sulphate (DHEAS) concentrations were significantly lower compared to controls at the time of injury and continued to decrease during the following 2 weeks. DHEAS levels were at their lowest at week 2 (2.6, 1.5-3.9 $\mu\text{mol/l}$) and had not returned to control values by 6 months ($p < 0.00001$). Serum androstenedione was below the reference range at the time of injury (2.8, 1.8-6.0 nmol/l) and significantly increased back to the mid reference range at 2 weeks after injury (4.2, 2.8-7.7 nmol/l; $p < 0.05$ compared to baseline after injury), exhibiting a much faster recovery than serum DHEA and DHEAS.

The catabolic/anabolic ratio can be represented by the proportion of adrenal catabolic hormones to the adrenal anabolic hormones as exemplified by the cortisol to DHEA or DHEAS ratio or also the ratio of active androgenic DHEA over inactive DHEA sulphate (DHEA/DHEAS) that is shown in Figure 5.5. The Cortisol/DHEA ratio peaked in week 1 (113, 41.3-184.3; $p < 0.000001$), plateaued and then decreased after week 4. No difference was seen in Cortisol/DHEA ratio between the severely injured and controls by 4 months. Cortisol/DHEAS ratio increased after injury and peaked at 2 weeks compared to controls (0.13, 0.09-0.22; $p < 0.01$). The Cortisol/DHEAS ratio then declined slowly over the study period but did not return to the controls values by 6 months (0.05, 0.03-0.08; $p = 0.003$). The DHEA/DHEAS ratio increased to 0.0025 (0.0015-0.0037; $p = 0.001$) by week 2 compared to controls but failed to return to normal during the study period.

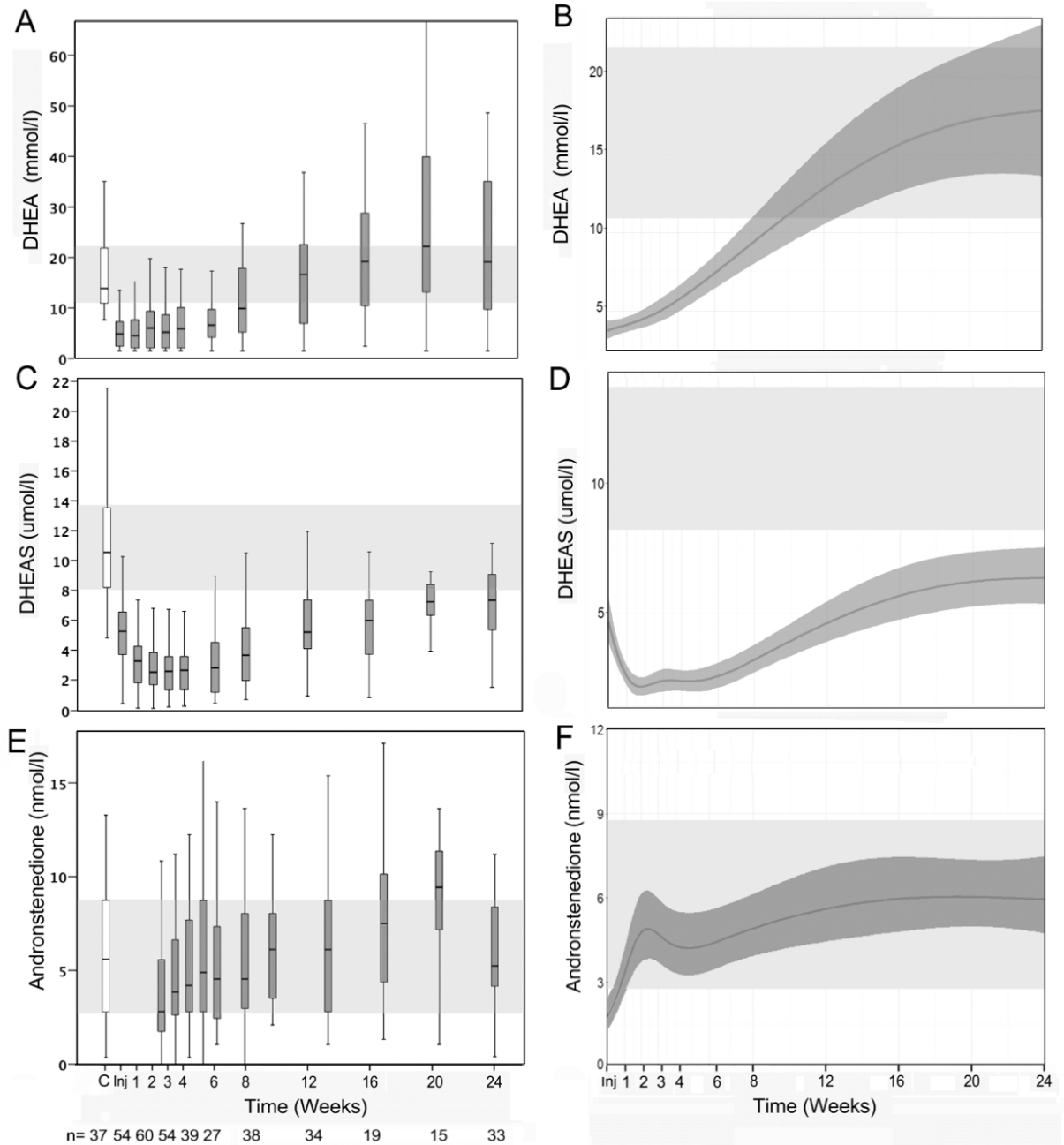


Figure 5.4. Serum DHEA, DHEAS and Androstenedione in 60 male survivors from severe injury (NISS>15) under 50 years of age, who had not been given exogenous steroids. DHEA against time raw data (A) modelled data (B), DHEAS against time raw data (C) and modelled data (D), Androstenedione raw data (E) and modelled data (F). Data were modelled using a non-linear mixed effects model that accounts for unbalanced repeated measures using a 4-knot cubic spline. Modelled data is reported as means and 95% confidence intervals. The grey shaded areas represent the sex-specific reference ranges.

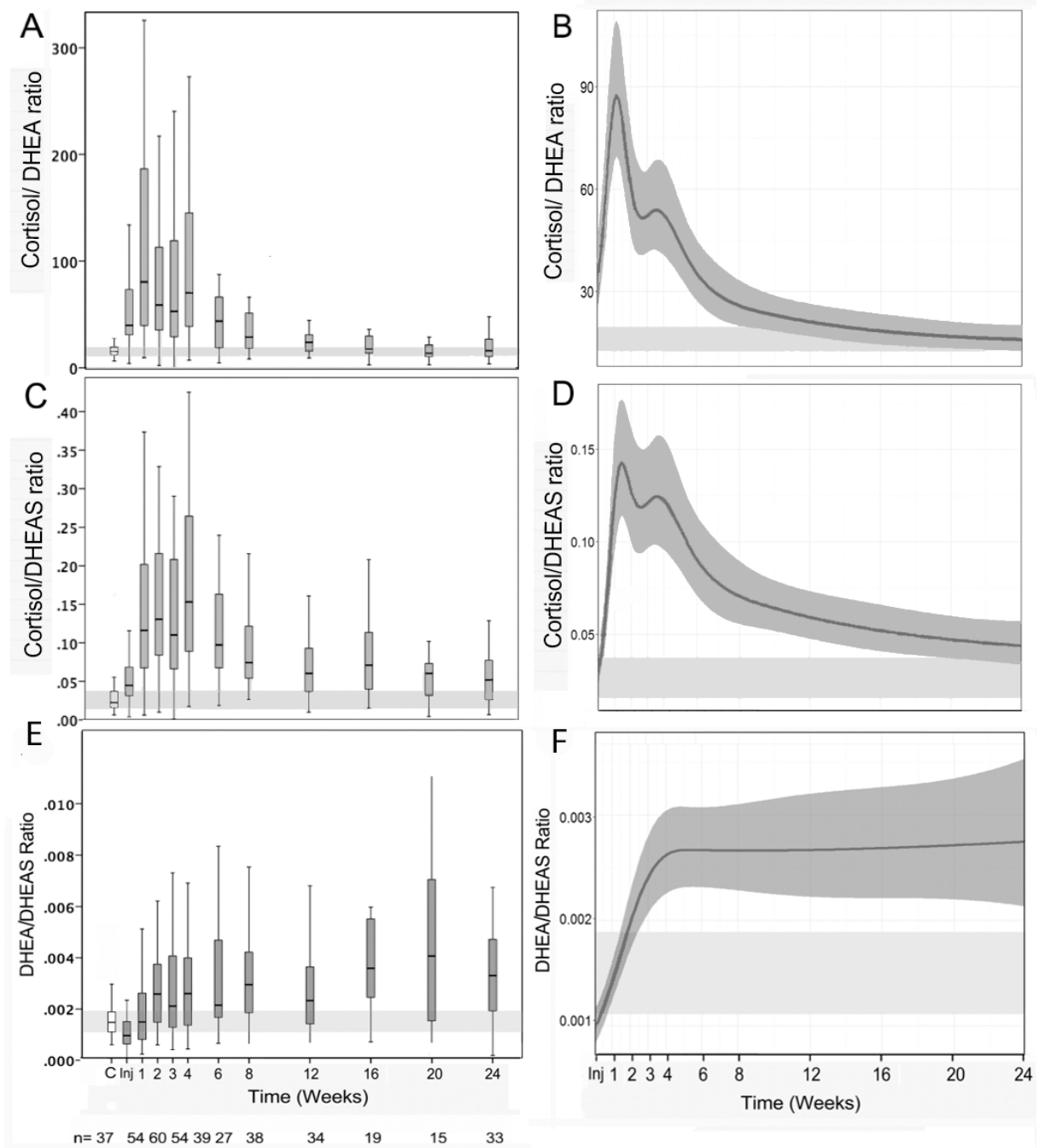


Figure 5.5. Serum Cortisol: DHEA ratio, Cortisol DHEAS ratio and DHEA/DHEAS ratio in 60 male survivors from severe injury (NISS>15) under 50 years of age, who had not been given exogenous steroids. Cortisol: DHEA ratio against Time raw data (A) modelled data (B), Cortisol DHEAS Ratio against Time raw data (C) and modelled data (D). DHEA/DHEAS ratio raw data (E) and modelled data (F). Data were modelled using a non-linear mixed effects model that accounts for unbalanced repeated measures using a 4-knot cubic spine. Modelled data is reported as means and 95% confidence intervals.

5.4 Active Androgens

The serum androgen testosterone from injury through to 6 months is shown in Figure 5.6. Testosterone was low, undetectable in some cases, following injury (0.7, 0.2-1.4 nmol/l; $p=0.0001$). Testosterone quickly increased after the first week following injury to reach the aged matched reference range by 4 weeks. Testosterone remained within aged matched reference range from 4 weeks to 6 months following injury.

The gonadotrophic response to traumatic injury was assessed through measuring the luteinising hormone (LH) and sex hormone-binding globulin (SHBG) is shown in Figure 5.7 with serum testosterone shown again for comparison.

Serum LH was acutely suppressed immediately after injury and increased back to the normal reference range between injury (1.2, 0.5-2.2 U/l) and Day 7 (2.8, 1.3-5.9, $p=0.046$), and between Day 7 and Day 14 (7.9, 4.6-12.7, $p=0.002$). Concentrations continued to increase by Day 28 relative to Day 7 (10.3, 6.6-14.1, $p=0.001$). Normal range = 1.8-8.2.

Serum SHBG concentrations were subnormal immediately after injury (thereby increasing the fraction free testosterone) and increased back to the normal reference range between Injury and Day 7 (20.0, 17.0-24.0, $p=0.001$), and then attained a plateau between Day 7 and Day 28. Normal Range = 15 – 48 nmol/L⁻¹

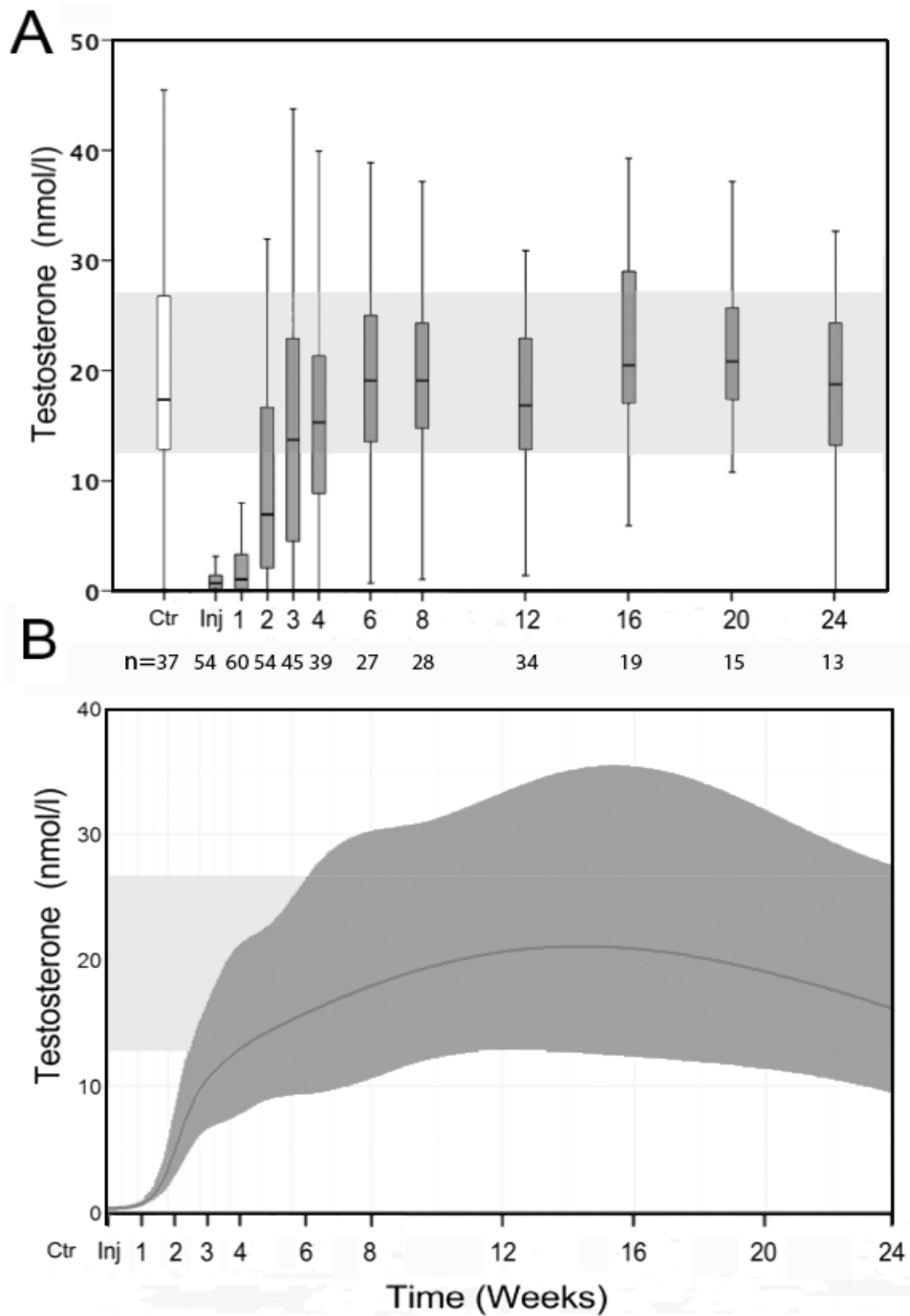


Figure 5.6. Serum testosterone concentrations in 60 male survivors after severe injury (NISS>15) who were not given exogenous steroids. Testosterone levels over time - (A) raw data and (B) modelled data. Data were modelled using a non-linear mixed effects model that accounts for unbalanced repeated measures using a 4-knot cubic spine. Modelled data is reported as means and 95% confidence intervals.

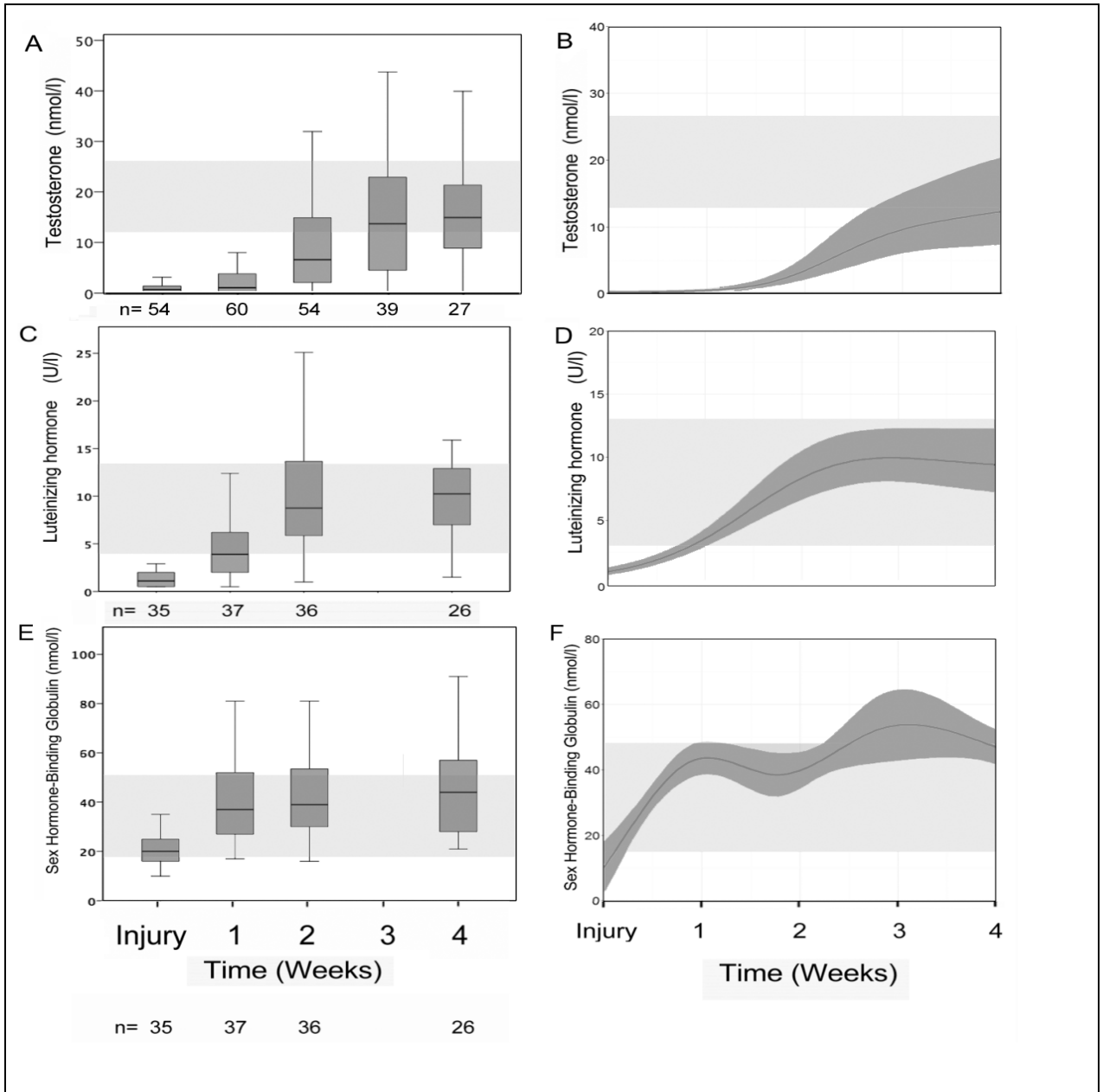


Figure 5.7. Serum testosterone, luteinising hormone (LH) and Sex hormone binding globulin (SHBG) levels in male, survivors after severe injury (NISS>15) who were not given steroids. Testosterone concentration over time – (A) raw data and (B) modelled data. LH – (C) raw data and (D) modelled data. SHBG over time – (E) raw data and (F) modelled data. LH and SHBG were only measured in the military cohort. Raw data is represented by box and whisker plots shows median and interquartile ranges. Data were modelled using a non-linear mixed effects model that accounts for unbalanced repeated measures using a 4-knot cubic spline.

5.5 *In vivo* catabolic response - Nitrogen excretion and muscle thickness

The effect of injury on protein metabolism was captured using 24-hours urinary urea excretion and ultrasound assessment of muscle thickness and is shown in Figure 5.8. Mean urinary nitrogen excretion increased during the first week to peak at 25.0 (+/- 16.1) g/day after the first week and returned to below 15 g/day by week 4. The mean maximum nitrogen excretion measured was 33.0(+/-21.3) g/day.

The thickness of biceps brachii muscle was found to be the most reliable measure; dressings, amputations and other wounds hampered the measurements in other areas. Changes in biceps brachii muscle thickness followed a U-shaped curve from injury that flattened after 4 weeks following injury ($p=0.001$). The mean maximum loss was 22.7% (+/-12.5). During recovery from injury biceps brachii muscle thickness slowly returned to their first recorded values, no significant differences could be detected any more after 3 months.

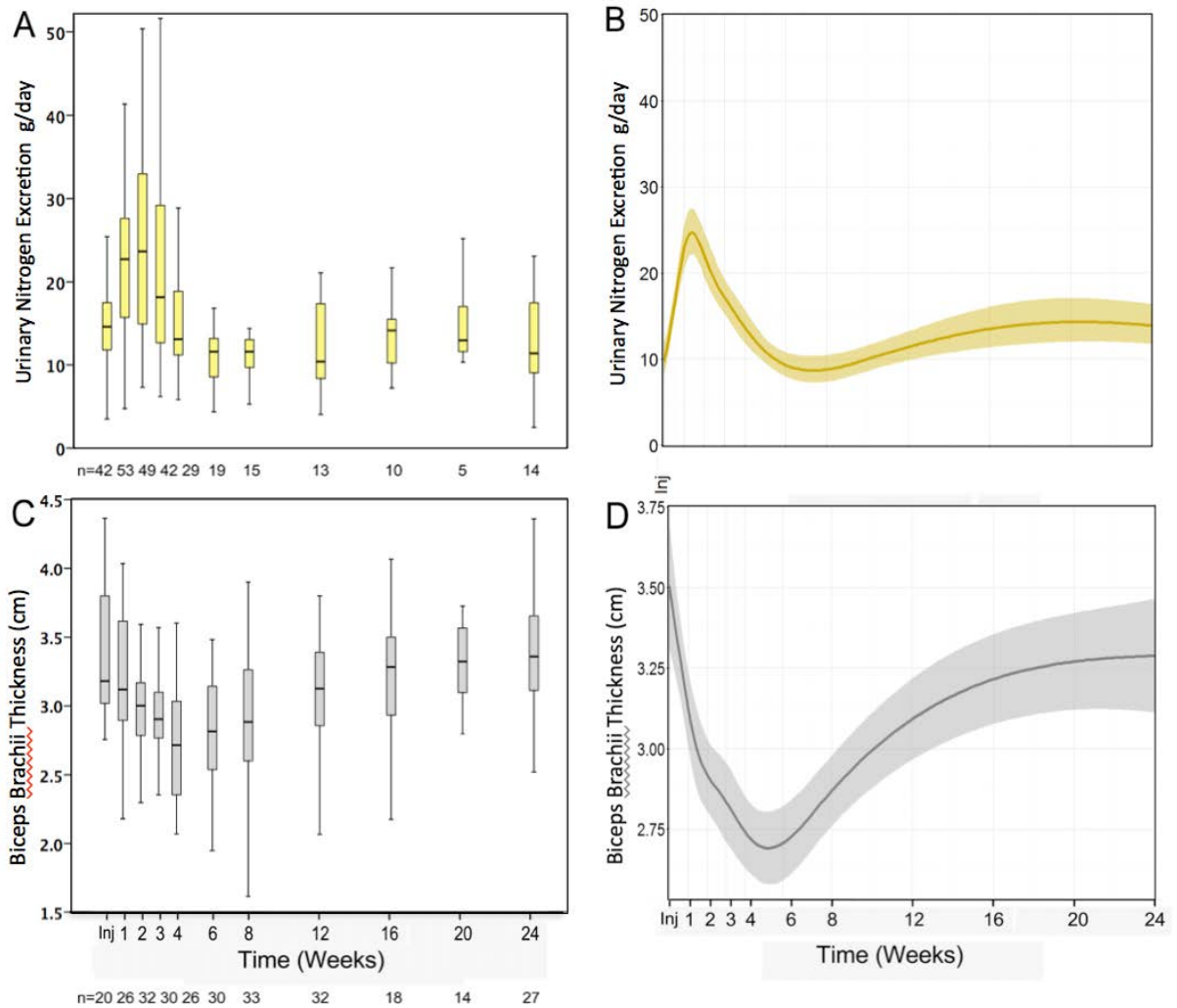


Figure 5.8. Urinary nitrogen excretion and biceps brachii muscle thickness in 60 male survivors from severe injury (NISS>15), under 50 years old who had not been given exogenous steroids. Urinary Nitrogen Excretion (A) raw data and (B) modelled data. Ultrasound thickness of biceps brachii muscle thickness (C) raw data and (D) modelled data. Box and whisker plots are displayed as median values with IQR. Data were modelled using a non-linear mixed effects model that accounts for unbalanced repeated measures using a 4-knot cubic spine. Modelled data is reported as means and 95% confidence intervals.

5.6 Adrenal steroid in relation to sepsis and organ failure

The relationship between sepsis and changes in the endocrine system are summarised in Figure 5.9. An autocorrelation technique was used to test whether time influenced the correlations between variables. The cortisol/cortisone ratio (F/E) showed a correlation with the probability of sepsis that was the strongest at a lag time of 6 days (ACF= -0.929). DHEA and DHEAS, and testosterone levels all showed strong auto-correlations with the probability of sepsis at lag time 0; ACF=-0.852, 0.852 and 0.969 respectively.

This approach was also used to interpret the SOFA and SAPSII scores over 4 weeks for patients admitted to ICU and they showed similar correlations. The probability of SOFA scaled data with adrenal steroids response are shown in Figure 5.10. Moderate correlation is seen with SOFA score and F/E ratio (ACF=0.566) at a lag time of 7 days. SOFA score strongly negatively correlated with DHEA (ACF= -0.848) and testosterone (ACF= -0.786). Positive correlations were seen with SOFA score and DHEAS (0.901). The SAPSII data is not shown.

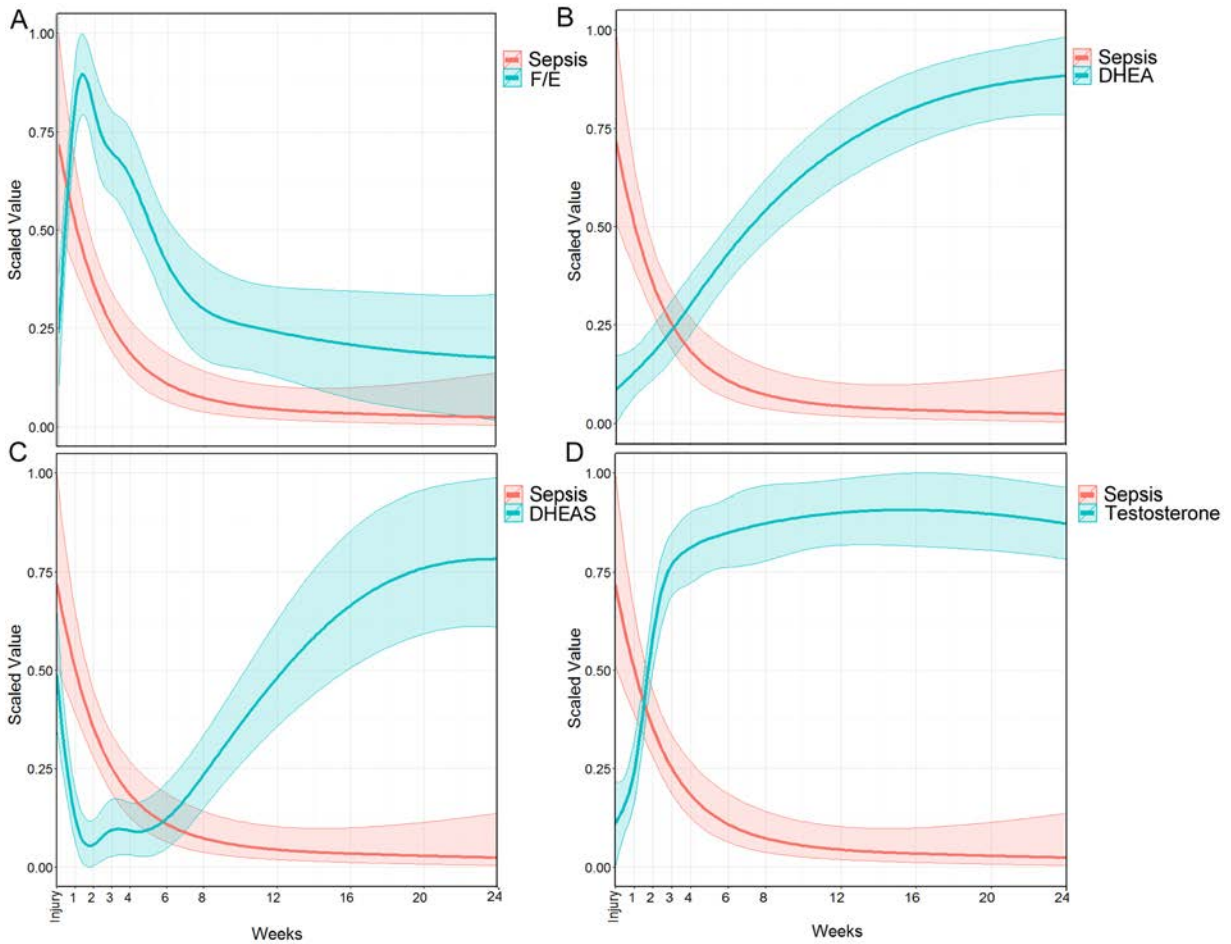


Figure 5.9: The probability of sepsis in relation to endocrine response. The probability of sepsis compared to serum (A) cortisol/cortisone ratio (F/E), (B) DHEA, (C) DHEAS and (D) testosterone. Data were modelled using a non-linear mixed effects model that accounts for unbalanced repeated measures using a 4-knot cubic spine. Modelled data are reported as means and 95% confidence intervals.

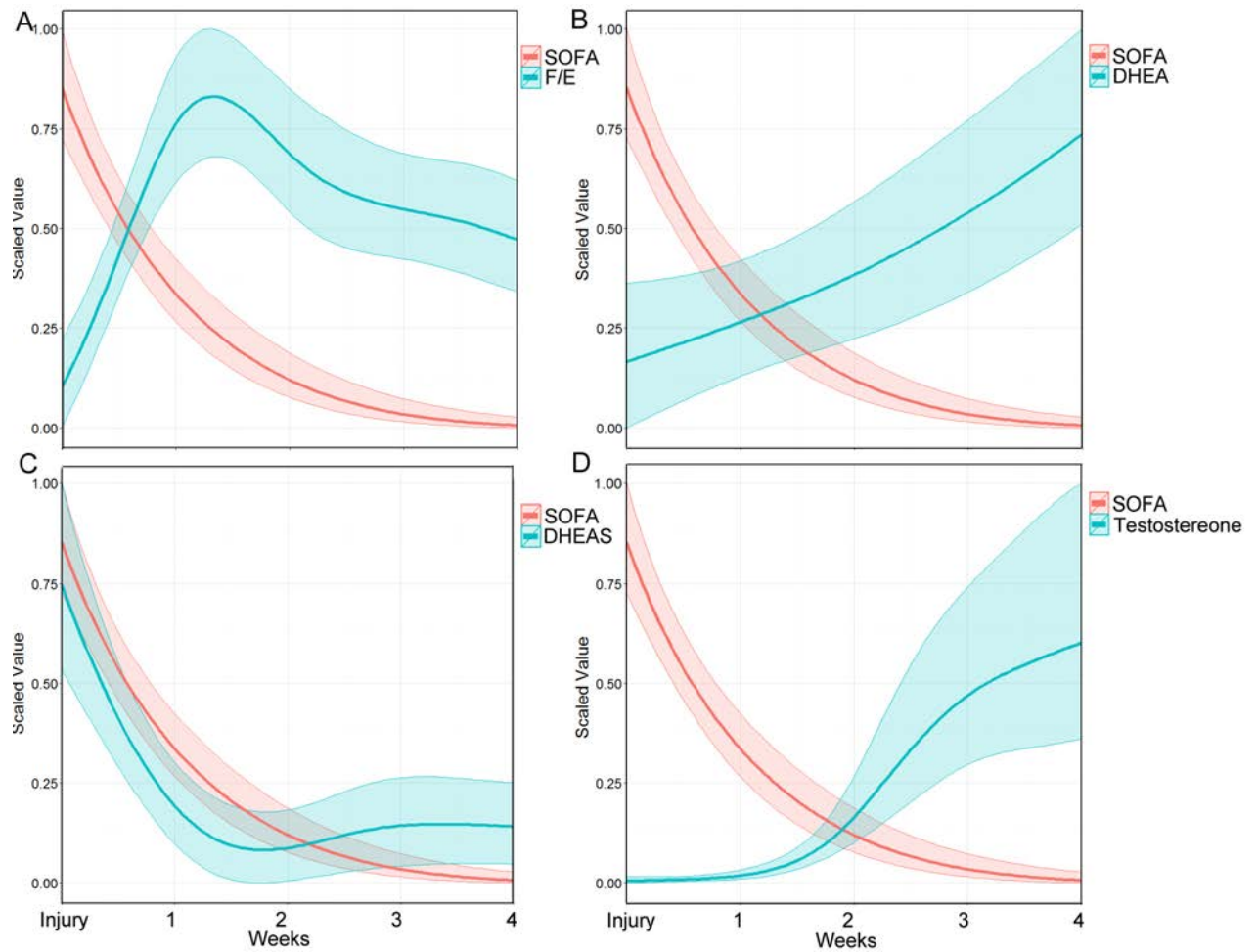


Figure 5.10. The relationship between probability of organ failure (SOFA) and endocrine response. The probability of SOFA compared to serum (A) cortisol/cortisone ratio (F/E), (B) DHEA, (C) DHEAS and (D) testosterone. Data were modelled using a non-linear mixed effects model that accounts for unbalanced repeated measures using a 4-knot cubic spline. Modelled data is reported as means and 95% confidence intervals.

5.7 Correlation of *in vivo* catabolic response and serum androgens

Serum DHEA levels, urinary nitrogen excretion and biceps brachii muscle thickness were compared and the results plotted in Figure 5.11. An autocorrelation technique revealed a maximum correlation at -2 days although similar values were observed to +20 days.

As DHEA levels start to increase nitrogen excretion peaks and then rapidly decreases. Biceps muscle thickness decreases to reach its lowest level at 6 weeks after which DHEA increases further and muscle thickness increases again. DHEA continues to rise throughout the study period in line with muscle gain. Maximum correlation of Biceps thickness to DHEA level was seen at -14 days with an ACF = 0.79.

Serum testosterone levels, urinary nitrogen excretion and biceps brachii muscle thickness were compared and the results plotted in Figure 5.12. An autocorrelation technique revealed a maximum correlation at -3 days.

As testosterone levels start to increase nitrogen excretion peaks and then rapidly decreases. Biceps muscle thickness decreases to reach its lowest level at 6 weeks. Testosterone starts to increase faster than DHEA, a head of increases biceps muscle thickness. Testosterone continues to rise throughout the study period in line with muscle gain. Maximum correlation of biceps thickness to testosterone level was seen at -20 days with an ACF = 0.84.

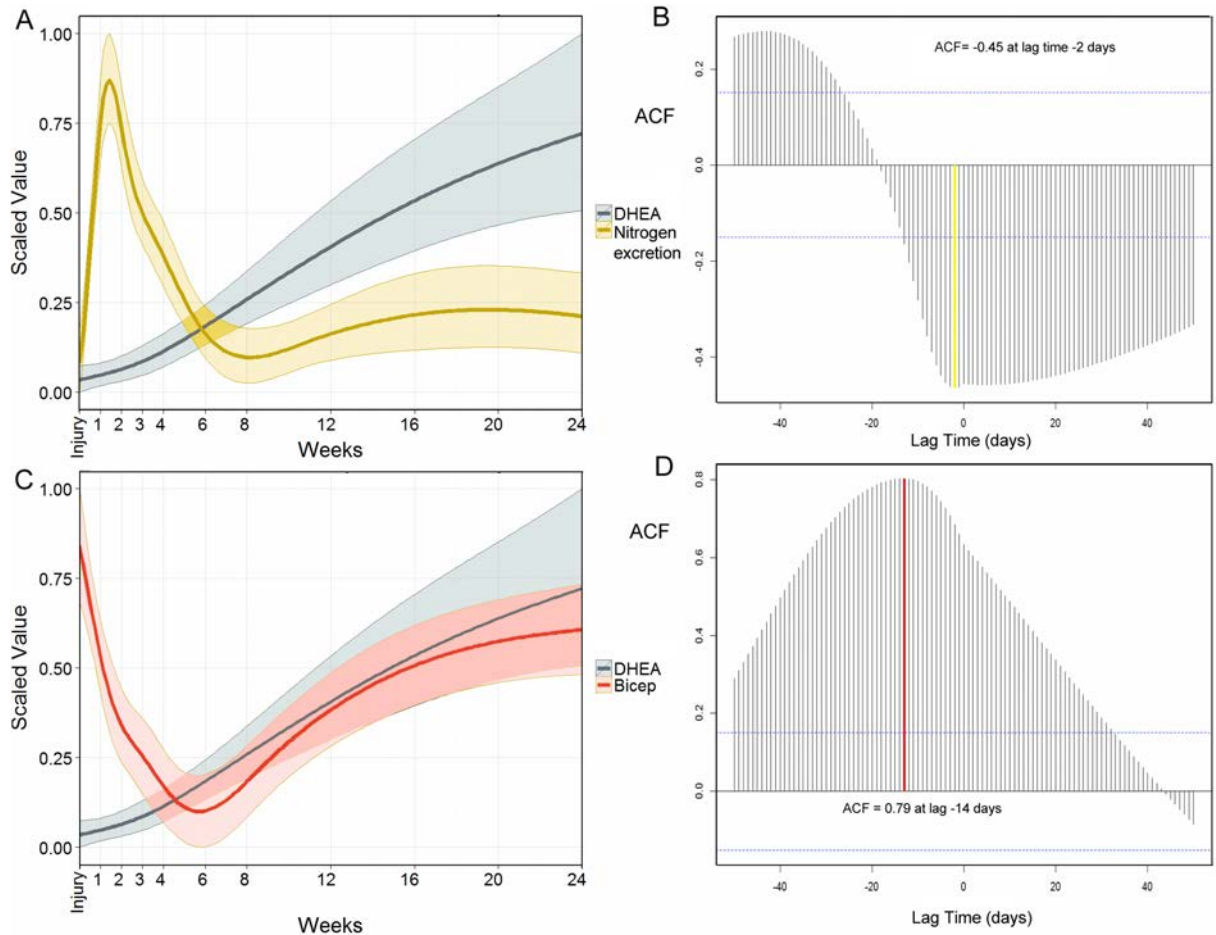


Figure 5.11. The relationship between DHEA, urinary nitrogen excretion and biceps muscle thickness over time for young (<50), severely injured (NISS>15) males who had survived and not been given anabolic steroids. Modelled data is displayed on a scaled x-axis for (A) urinary nitrogen excretion and (C) biceps brachii muscle thickness. Autocorrelation date is shown highlighting the autocorrelation factor (B) urinary nitrogen excretion and (D) biceps brachii thickness. The data were modelled using a non-linear mixed effects model that accounts for unbalanced repeated measures using a 4-knot cubic spine. Modelled data is reported as means and 95% confidence intervals.

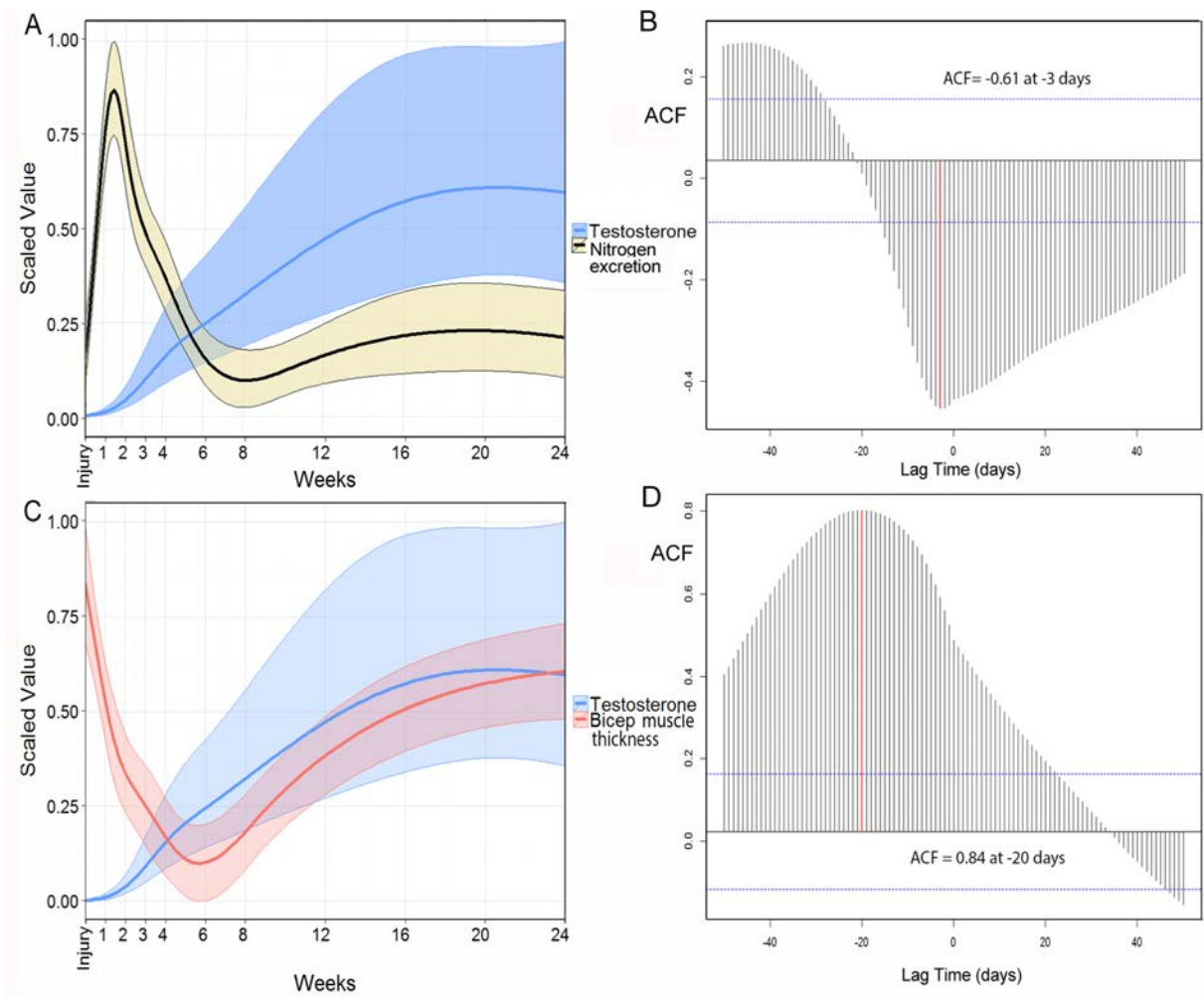


Figure 5.12. The relationship between testosterone, urinary nitrogen excretion and biceps muscle thickness over time for young (<50), severely injured (NISS>15) males who had survived and not been given anabolic steroids. Modelled data is displayed on a scaled x-axis for (A) urinary nitrogen excretion and (C) biceps brachii muscle thickness. Autocorrelation date is shown highlighting the autocorrelation factor (B) urinary nitrogen excretion and (D) biceps brachii thickness. The data were modelled using a non-linear mixed effects model that accounts for unbalanced repeated measures using a 4-knot cubic spine. Modelled data is reported as means and 95% confidence intervals.

5.8 Discussion

Early enlightenment into the metabolic response to surgery and injury was made by Cuthbertson in 1932 and then built on by Moore who described classical 'ebb and flow' physiology relating muscle loss and altered nitrogen balance (198,199). The major endocrine response to injury is to produce catecholamines and corticosteroids. These groups of molecules produce a short-term and long-term stress response that has evolved to protect an individual from life threatening injury. As glycogenolysis, gluconeogenesis, proteolysis and lipolysis dominate and protein anabolism is inhibited, a profound muscle loss occurs.

Cortisol is released during the stress response and can be associated with the degree of surgical insult(229). Cortisol levels can vary indicated by the wide normal range, diurnal fluctuations, protein binding and the difficulties in diagnosing adrenal insufficiency(476). A more consistent measure of stress response is reportedly the Cortisol/Cortisone ratio (F/E) (477) that represents the activity of 11 β -hydroxysteroid dehydrogenase (11 β -HSD) (218). 11 β -HSD type 1, the major enzyme activating cortisone to cortisol, has been shown to be upregulated systemically and locally in response to inflammation in sepsis and trauma to dampen the inflammatory response and regulate excessive glucocorticoids (478,479). 11 β -HSD type 1 activity as well as its expression has been shown to act to increase intracellular cortisol in immune cells such as monocytes and macrophages to dampen inflammation(480). Pro-inflammatory cytokines can also up regulate 11 β -HSD type 1 activity in human epithelial cells(481). There is not much in literature with relation to 11 β -HSD activity and severe injury. There some evidence in its role in regulating immune function in burns (482). The widespread use of corticosteroids to dampen excessive inflammation in sepsis and

critical illness has fallen out of favour. Two meta-analyses were only able to confirm reversal of shock but not outcomes(300,483). The surviving sepsis campaign looked at over 17K patients in an observational study and showed that shock could be reversed with corticosteroids but that this did not improve mortality(302).

The glucocorticoids are produced at the expense of the sex steroid precursor DHEA that is low after injury. The sulphated form of DHEA, DHEAS is initially maintained and attempts to recover DHEA levels via hydroxysteroid sulfotransferase (SULT2A1). This idea that there is up regulation of SULT2A1 is supported by the increased DHEA/DHEAS ratio that rises in the first 2 weeks following injury and remains above controls for beyond the duration of the study. Cortisol/DHEAS is increased in septic shock and trauma. Arlt *et al.* found DHEA to be increased in septic shock, in a study of 181 patients with septic shock and 31 trauma patients, while DHEAS concurrently was low, resulting in an increased serum DHEA/DHEAS ratio. DHEA and DHEAS levels were found to be low in the trauma cohort (484).

As the F/E ratio recovers to control values, DHEA levels increase and the clinical effect is a reduction in nitrogen excretion and an increase in biceps brachii thickness. This reversal of catabolism/anabolism is represented by the Cortisol/DHEA ratio and mirrors the time course. This change to anabolism is an important outcome measure as it then determines time to recovery. In this study the severity of injury, as presented by ISS and NISS did not significantly change the duration of catabolism and hence the timing of the switch from catabolism to anabolism.

A correlation of DHEA and F/E ratio to infection rate has been shown in this study but the data is unable to definitively prove a cause and effect relationship. Increased infection rate and mortality has been previously associated with high cortisol and low

DHEA levels in trauma studies (459,485-487). Could DHEA be beneficial as an anti-glucocorticoid during the inflammation seen following severe injury? As well as having anabolic properties, DHEA and DHEAS also have been shown to be immunogenic, probably through direct and indirect action on oestrogen receptors(241,488). Hazeldine *et al.* reviewed the role of DHEA and highlighted the mechanism through a NF- κ B pathway in some studies(488). Interestingly, Radford *et al.* described how DHEAS can enhance the activity of neutrophils to increase superoxide production and so enhance immunity by via a protein Kinase C- β (489). This ability for DHEA to overcome the suppressive nature of cortisol, that may be detrimental in prolonged inflammation, was shown in an elderly hip-fracture study that highlights its possible uses in all traumatic injuries(459). DHEA administration in animal models has already been shown to be beneficial in trauma and/or sepsis. As a prophylactic agent, or during treatment, DHEA has been shown to enhance recovery and improve survival in animal studies (490,491).

The effect of severe injury on the gonadotrophic axis has been studied in the literature. In traumatic brain injury (TBI), a significant proportion of patients go on to develop anterior pituitary dysfunction(492). Another study reported testosterone suppression in all of the males patients within 10 days of head injury(493). In patients admitted to critical care with acute illness graded according to APACHE II score mean testosterone concentrations were lower on admission than in controls, with testosterone concentrations being lower in those patients with more severe illness(235). More recently Gang *et al.* reported low testosterone immediately after injury in a study with 95 patients who had suffered severe injury(494). While burns and critical care studies have shown a central pattern hypogonadism(235,495) there is not a great deal of evidence. The current study clear shows a central cause of suppression to the

gonadotrophic axis. The most likely cause would be a reduction in luteinizing-hormone releasing hormone (LHRH). This is supported from head injury data(425). Other cause could be opiates used on ICU which are known to induce a degree of central hypogonadism(496).

Testosterone may be a biomarker of severity of injury(221) although this was not supported by the current study. Androgens have important roles in healing, erythrocytosis, maintenance of bone density and muscle mass(346). The catabolic state that occurs following trauma presents a significant challenge, and the uses of the synthetic testosterone analogue, *oxandrolone*, has already been discussed.

The chance to reduce the catabolic effects of the glucocorticoids and enhance the immune responses particularly to sepsis using DHEA is attractive. Analysing a young severely injured population has reduced the confounding effects that occur with reducing levels of adrenal steroids in older age. Being able to measure the catabolic effects of severe injury and to a lesser extent the incidence of infection could shape the design of a study that would ameliorate the inflammatory response, reduce muscle loss and enhance the ability to fight infection for severely injured patients in the future.

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Chapter 6: Discussion - The Response to Severe Injury

6.0 Overview of the SIR Study

The Steroids and Immunity from Injury through to Rehabilitation (SIR) study examined the immunoendocrine response to severe injury. Young military and young and old civilian patients were followed from the initial release of DAMPS following injury, through the subsequent cytokine storm and changes in immune function and endocrine parameters. The temporal changes in immune function were interpreted against a background of on-going inflammation and a profound stress response and further related to the physical consequences for the patient. More specifically, by prospectively following patients, the SIR Study was able to monitor the development of critical illness during the catabolic and immunocompromised phases of recovery and identify the endocrine changes that occurred during the transition from catabolism to anabolism.

6.1 Stratification of patients and utility of the injury severity score (ISS)

Stratifying the SIR study patients was difficult using anatomical based scoring systems such as ISS, NISS and even TRISS, which does include physiological parameters (497). Despite the mechanism of injury in the study patients ranging from a fall from height to the explosive power of an IED, their ISS was similar. It was the NISS that detected a difference between injuries in young and old patients and thus gave a more accurate stratification of patients related to injury severity and ultimate outcome. The military also had a higher NISS than their civilian age-matched counterparts, due to the military sustaining a higher proportion of extremity trauma.

The time injured patients spent on ICU was similar in all groups highlighting that the older, less severely injured patients required more time on ICU relative to their injury severity and that post ICU, the younger patients remained in hospital longer when more

reconstruction and in-hospital rehabilitation was needed. These variations are confounded by the geographical location of the patient's home and the availability of rehabilitation services. The difficulties in patient stratification using the existing scoring systems may also explain why the correlation of injury severity with duration and magnitude of catabolism is weak. The study did show that on average a severely injured patient is catabolic, and lost muscle for 6 weeks. That said, injury severity did affect daily muscle loss. Thus, across the range of ISS (16-75) muscle was lost at a rate varying from 0.2 to 0.8% muscle lost per day, however importantly the maximal total loss was achieved between 4 and 6 weeks irrespective of injury severity (ISS 16-75). Therefore, using catabolism as an outcome measure may be more useful in the development of future scoring systems. The traditional outcome measures of using MOD/F and particularly mortality, that was low in this study (8 patients), are less useful.

Other studies have been able to separate patients when broadly characterised under mild, moderate and severely injured but direct correlations with outcome are not strong(170,498). New possibilities to predict mortality and MODS with combinations of integrins have recently been investigated by Furmaga *et al.* in a trauma study of 17 patients with hemorrhagic shock (499), but these stratifiers need broader testing in large patient cohorts to establish validity. The analysis of genomics, via high-throughput microarray systems, is the latest stratification tool. mRNA analysis can be used to determine patterns of change in gene expression following severe injury, termed the "genomic storm" (500). Interestingly, the problems being encountered with genomics are similar to those encountered in the current study, in that using injury severity scores to stratify patients is inadequate. That said, this approach can use the genomic score to predict worse outcomes (501).

6.2 Immunoendocrine response to severe injury

The initial release of DAMPS as measured by mtDNA and HMGB1 appeared to occur at the same time as the release of early cytokines, potentially confirming current thinking that they initiate the sterile inflammatory response. However, this study was unable to capture the pattern of DAMP and cytokine release in the minutes and hours immediately following injury. By the time the first samples of the study were taken cytokine levels and DAMPs were already increased. Interestingly the anti-inflammatory cytokine IL-10 was also raised in the first samples after injury, which supports the changing literature that describes the release of pro and anti-inflammatory mediators occurring simultaneously after injury (170,502). A model encapsulating the kinetics of DAMPS and cytokine release, developed from the SIR study data, is shown in Figure 6.1. By 24 hours following injury, DAMPS and cytokines follow a similar kinetic pattern. What is surprising is the continued rise of mtDNA and HMGB1 levels in the days following injury. This may represent delayed clearance of DAMPs or continued cell necrosis in the injured tissues, possibly due to poor tissue perfusion and hypoxia, which continues for days and weeks following injury.

The supposition that a SIRS response is followed directly by a CARS response originates from studies that have followed the clinical course of sepsis(503), where a later immune suppression can hamper recovery. In trauma uncomplicated by sepsis, inflammation is not usually prolonged and recovery is shorter. A concept proposed by Gentile *et al*, characterised a 'Persistent inflammation, immunosuppression and catabolism syndrome (PICS)' that complicates some severely injured patients (153). Their PICS criteria cites ICU>10 days, high CRP, low lymphocyte count and excessive signs of catabolism. The conundrum is how to predict which patients will go on to develop sepsis and MOD/S. The severity of injury was used in the past but these

anatomical methods are now poor predictors of survival (431). Another factor to consider, as suggested by the SIR data, is the magnitude of DAMPS and/or cytokines as another potential predictor of outcome(25,504,505), together with degree of catabolic response as discussed above. One very recent paper suggests that the while there is an association between outcome and mtDNA level in critically ill patients this strengthened by an increased expression of TLR-9(506).

One of the major interests of the thesis was to try and understand the poor outcomes of the older trauma patient. The age-related differences in DAMPs and cytokine release seen in the study, namely lower cytokine levels and higher DAMPs compared with young patients, are modelled in Figure 6.2. The increased DAMPS from the older cohort may represent a delayed clearance of dead and damaged cells, it is well established that macrophages from older subjects do not phagocytose dead cells and debris as efficiently as those from young adults(507). Another explanation is that cells and tissue in the older adults are less stress resistant and more fragile and there is certainly support for this notion in the biogerontology literature (508-510). In addition, the normal response to DAMPs is the release of cytokines from macrophages, dendritic and other immune cells as well as the endothelial cells when alerted to on-going necrosis. The reduced cytokine release seen in the older patients in response to increased DAMPS may be another consequence of the age-related decline in immune responses, immunosenescence, also discussed earlier. Certainly the ability of macrophages and DCs to respond to TLR ligands, many of which also bind DAMPs, is reduced with age(457). A recent study from the Glue Grant project team also found lower cytokine levels in the elderly. Their large cohort study young (age <55, n = 1395) and old (age ≥55, n = 533) revealed a unique 'genomic storm' that was associated with worse outcomes in the elderly(458). Importantly, differences in release of DAMPS and

cytokines observed with age highlights that any model using biomarkers should include age within the algorithm (442).

Looking forward then, attempts to improve outcomes for the older trauma patient will need to encompass approaches to enhance immune responses. This is now a definite possibility as the immunosenescence field is now reporting pharmacological agents able to combat age-related immune decline, including inhibitors of the p38 MAP Kinase and PI3 kinase pathways (426,511).

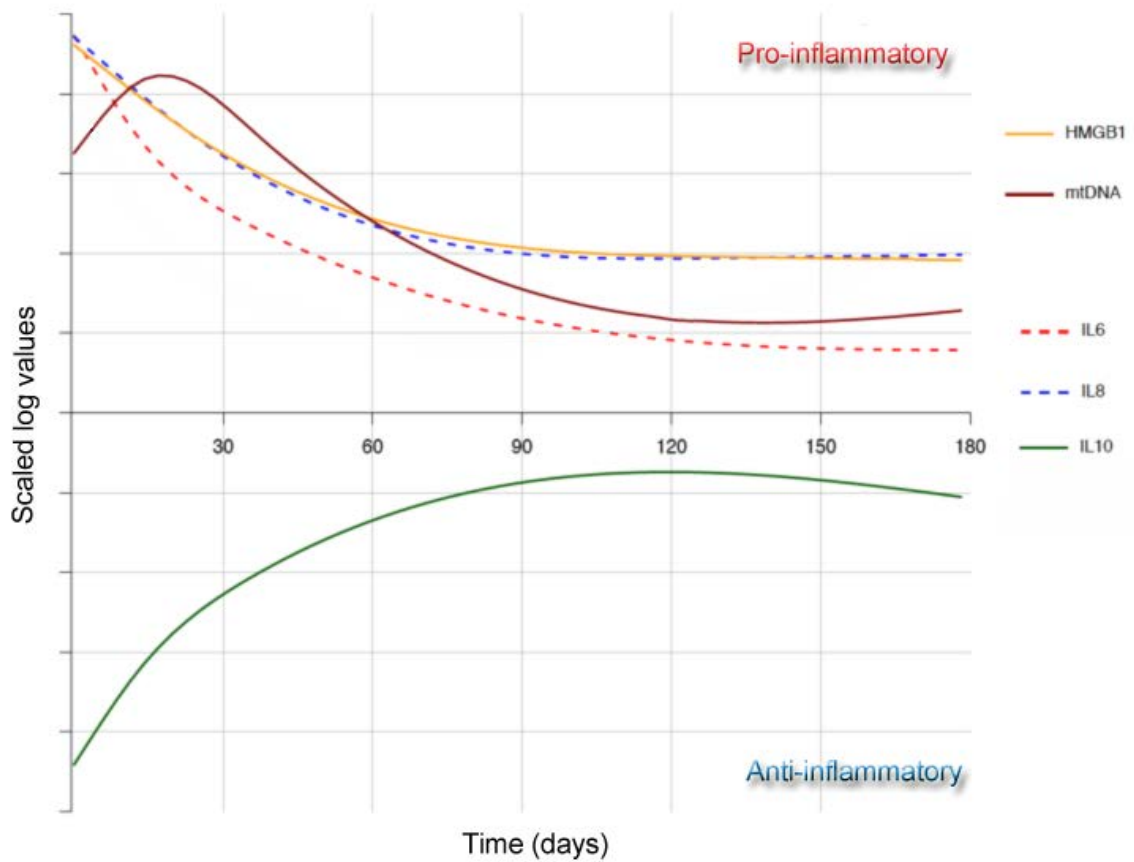


Figure 6.1. Pattern of DAMPs and cytokine generation following injury. The model shows that pro and anti-inflammatory cytokines are released at the same time and are concomitant with the release of the DAMPs HMGB1 and mtDNA. This model is based on the totality of the SIR data for young and older patients.

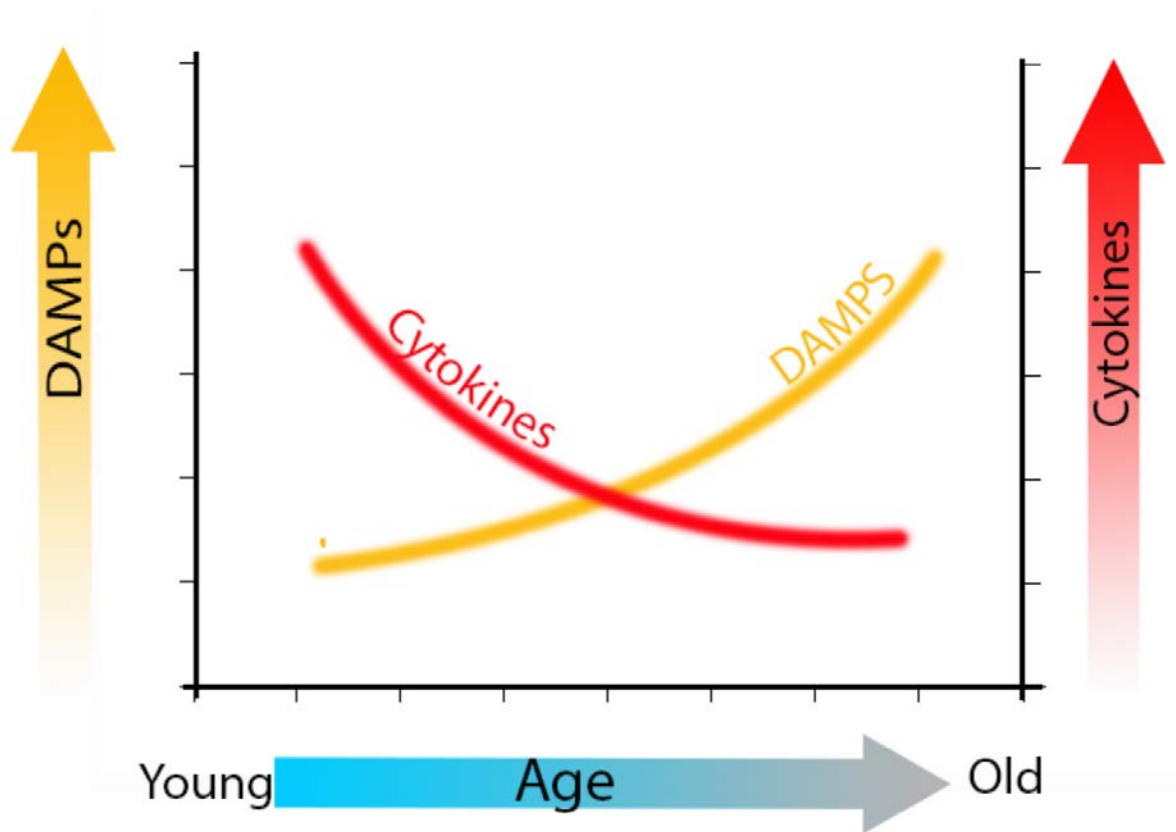


Figure 6.2. DAMPs and Cytokine release with age. The model depicts age-related differences in the response to injury, showing reduced cytokine generation per unit DAMP production with age.

Alongside the initial immune response, neuronal and endocrine signalling produces catecholamines and corticosteroids in response to tissue damage through direct activation of efferent pathways, as well as early central detection of inflammatory cytokines. These groups of molecules produce a short-term and long-term stress response that has evolved to protect the individual from life threatening injury. As glycogenolysis, gluconeogenesis, proteolysis and lipolysis dominate and protein anabolism is inhibited, a profound muscle loss occurs. The increased F/E ratio continues for several weeks until anabolic hormones begin to dominate and the stress response subsides. Later, adrenal based sex hormones and other anabolic hormones increase as catabolism is reversed.

In the SIR study, it was shown that injury severity has a relationship with the magnitude and duration of catabolism (as measured by muscle and nitrogen losses) as well as other factors such as age. The contribution of other co-variants such as genetics and pre-morbid condition were not addressed in this study. As mentioned above, rather surprisingly the switch from catabolism to anabolism seemed to occur after 4-6 weeks, irrespective of the degree of muscle loss. Interventional paradigms to reduce muscle loss after injury may thus need to aim to address the rate of muscle loss rather than the timing of the return to anabolism as that latter may turn out to be harder to influence.

A model to summarise the complex relationship that the immune and endocrine response has on the injured host is shown in Figure 6.3. In the background of inflammation, a reduction in immunity renders the host more susceptible to sepsis and the consequences MOD/F. The SIR Study has captured the high proportion of injured patients that go on to develop a septic episode. The reduction in immune function does appear to correspond with the appearance of sepsis. It is still to be proved that

the different phenotype of neutrophils, observed in this study, which also peaked around two weeks following injury is related to the onset of MOD/F and/or sepsis, or is a consequence of these outcomes. An excess of poorly functioning or pro-actively immune suppressive neutrophils would certainly predispose to infection. Alternatively, onset of sepsis could put increased pressure on the haemopoietic system leading to the release of immature neutrophils from the bone marrow with compromised function.

Also relevant is the question of whether sepsis is a consequence of MOD/F or could it be that MOD/F is caused by the inflammation generated by the septic patient and/or the activity of the pathogen? Hampering the investigation in to this is the diagnosis of sepsis that still relies too much on clinical suspicion or rather non-specific parameters. Improved biomarkers for sepsis would make diagnosis more objective. In this respect, if the frequency of immature neutrophils is shown to be associated with sepsis this would provide a readily measured method of diagnosis(512). This was suggested in a recent paper by Mare *et al*(512,513). There are also molecular methods emerging that have promising predictive power, in this realm I would specifically list metabolomics. Several studies have analysed the metabolic response to injury and metabolomic analysis has been shown to be able to differentiate between sterile and non-sterile inflammation(233,514). Although not currently available for routine diagnostics, the method is being adapted for bedside use.

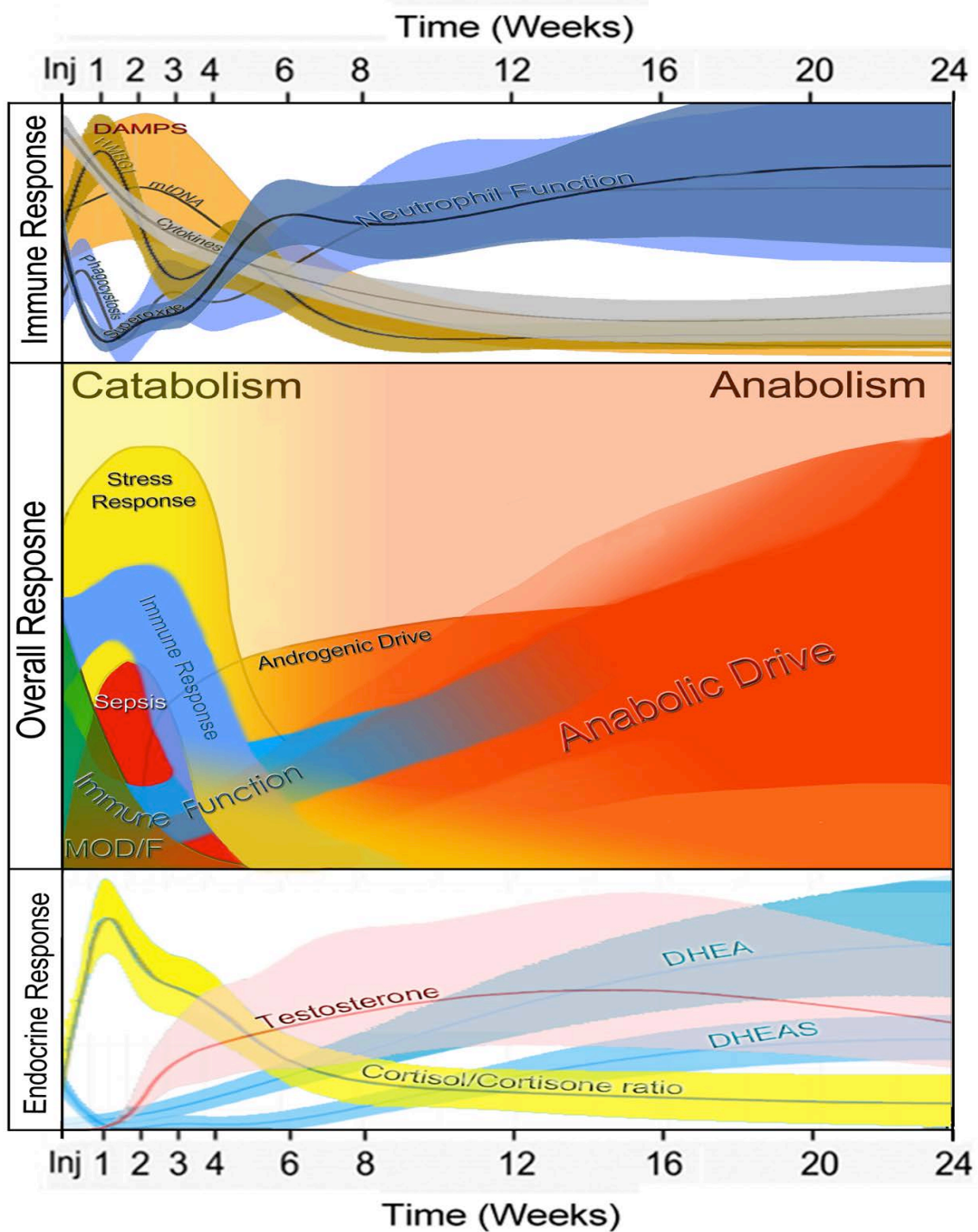


Figure 6.3. Immunoendocrine response following severe injury. In combining the immune and endocrine response the overall response is shown; as the immune function improves, sepsis and MOD/F resolve and the androgenic drive dominates with improve immune function.

6.3 Therapeutic options.

6.3.1 DAMPS and cytokines.

There have been many attempts to create an anti-inflammatory effect by blocking early cytokine responses since the discovery of anti-inflammatory cytokines. Many years of sepsis trials have failed to appreciate the fine balance between a pro and anti-inflammatory responses and it is thus not surprising that suppression of the inflammatory response has not yet been successful in turning promising animal work into successful clinical treatments(515). DAMPS operate through TLRs and so logically blocking the relevant TLRs and/or preventing accumulation of the DAMP would be efficacious(516). Mitochondrial derived DAMPS such as mtDNA but more importantly other associated molecules may also be important in initiating inflammation(517). Piccinini *et al.* reviewed this approach and found a contrasting ability of mono-clonal antibodies to HMGB1 to reduce inflammation *in vivo*(518,519). Another study by Runkuan Yang *et al.*, demonstrates the value of blocking antiHMGB1 in an animal model following haemorrhage(520)(Runkuan Yang).The use of blocking histones another DAMP has been successful in the animal model but yet to reach clinical trials (521). Lord *et al.* reviewed some of the suggested strategies for ameliorating the inflammatory response(48) and highlights experimental treatments such as using IFN- γ or immunoglobulin has not shown any benefit when evaluating infection rate or pneumonia. Lulong Bo and colleagues in a meta-analysis reviewed G-CSF and GM-CSF for the treatment of sepsis and couldn't recommend its use(522). In a review of the pro- and anti-inflammatory properties of IL-6, care was suggested before attempting to block IL-6 due to complex modes of action. With all this in mind if one considers that these processes may be beneficial to the host, blocking a pathway in 'danger signalling' may have non-immune consequences. This is exemplified by an

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interesting paper by Yasunori Shintani *et al*, who discovered that activation of TLR-9 was beneficial to neurons and cardiac myocytes as protective pathways allowed the cells to operate in more hypoxic challenging environments (523) .

That said, strategies that are likely to be more successful are using these treatments in a more stratified way in selected patients who have been predicted to go on to sepsis and MOD/F. This may for example take age in to account and would in this case rather try to promote a more robust inflammatory response rather than reducing it. Appreciating the whole system and the vital role of the neuro-endocrine system in the generation and maintenance of the inflammatory response will hopefully anticipate any deleterious downstream consequences of therapeutic strategies.

6.3.2 Endocrine balance

Reducing the physiological effects of inflammation particularly the adrenergic drive has yielded success in burns particularly with the use of beta-blockade in children and adults (524).With the failure of trials like BALTI-2 (326) to show improved outcomes using beta agonist in sepsis, the focus has now turned to see if beta-blockade (525) can ameliorate the response following sepsis and eventually trauma.

There are limited data describing the effect of major trauma on the gonadotrophic axis. Studies from traumatic brain injury (TBI) are variable reporting 9-100% of central depression(493). Testosterone levels, be it after surgery (486), burns (236) or acute severe illness fall within 24 hours. The recovery of testosterone appears to relate to the severity of illness or injury (221,494). Although the current study does show a dramatic reduction in testosterone, there was not a strong relationship with severity of injury.

The underlying mechanism for the pattern of central hypogonadism observed in the present study is not completely understood. In keeping with the evidence from head injury, it would seem possible that a reduction in hypothalamic gonadotropin-releasing hormone (GnRH), is the most likely explanation (526). However attempts to prove this in patients with prolonged critical illness have been unconvincing (527). The use of opiate analgesia has also been shown to induce a degree of central hypogonadism (496,528) although this is unlikely in the current study with opiate analgesia requirements remaining high and testosterone returning to normal.

6.3.3 Androgens.

6.3.3.1 Testosterone.

The implications of the reduction in testosterone concentrations reported in the present study are potentially far-reaching. Testosterone may have potential as a biological marker of trauma severity and the response to treatment. Low testosterone concentrations may also offer a therapeutic target. Not only does testosterone have important roles in erythrocytosis, conservation of bone density and lean muscle mass (with a direct anabolic effect on skeletal muscle), there is some evidence that androgens mediate immune suppression following major trauma (529). Trials using testosterone antagonists have shown some benefit (530,531). The advantages are attributed to the blocking of androgen receptors but an indirect action of these agents may be to increase up-stream steroids and oestradiol that in the tissues may enhance immune response and improve outcomes. The synthetic testosterone analogue – *oxandrolone* – has already been used with a degree of success in combating the reduction in lean body mass that occurs in severe burn injuries (532). Supporting the anabolic drive has yielded success in burns which has now resulted in Oxandrolone,

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the testosterone analogue, being considered 'Standard of Care' in severe burns (>30%TBSA)(533). Oxandrolone has also been used in two trauma studies whose methodology and conclusions were not helpful and have already been discussed (356,357,534).

6.3.3.2 DHEA.

DHEA is the most abundant circulating steroid hormone, a natural hormone of downstream metabolism and the major secretory product of the adrenal gland (535). As a sex hormone precursor, DHEA modulates many physiologic processes including metabolism and cardiovascular function.

To date there are no clinical trials for the use of DHEA in humans trauma or sepsis, despite very encouraging results in rodent models of sepsis and traumatic injury. For example, in mice after haemorrhage, DHEA supplementation enhances immune responses and reduces mortality and sepsis (536,537), reduces hepatic damage (538), restores cardiac dysfunction (539) and improves splenocyte function via direct T cell stimulation (540). In the presence of bilateral femoral fractures in mice, DHEA administration was associated with a reduced systemic inflammatory response (541), and reduced mortality (542). There is a suggestion that DHEA is organ specific in some of its actions that was suggested by Litche *et al.* (541) and supported in trauma sepsis models (542). DHEA administration has been found to accelerate wound healing in hypogonadal mice (543). DHEA can also be protective in soft tissue ischaemia re-perfusion injury, reducing leucocyte activation and improving capillary perfusion (544). When administered in thermal injury models DHEA has been shown to exhibit similar properties to that of the haemorrhage/ischaemia; normalising hepatocellular

metabolism (545), preserving immune function, resisting infective episodes and preventing tissue destruction (546-548).

There have been a small number of studies that examined the use of DHEA for immune function in humans. In vaccination, DHEA has been used to augment seroconversion. Studies are conflicting with Danenberg *et al.* and others were unable to demonstrate any improvement in influenza vaccine antibody titre when DHEA was given to the elderly (549,550). In contrast DHEA supplementation in the elderly did increase influenza vaccine titres(551). A finding also supported by Degelau *et al.*, they attributed higher influenza titres to lower circulating DHEAS (552). Human studies in both sexes during middle age have shown enhanced NK cell function, increased numbers of monocytes and stimulate response enhanced response in T and B cells(553,554). That said, a study of middle age men failed to show any improvements in immune function after 4 weeks(555).

The potential utility of DHEA to enhance immune function has been suggested by work in our group. For example, we have highlighted that the adrenal response to stress is linked to reduced neutrophil bactericidal function, specifically superoxide production with the cortisol:DHEAS ratio higher in hip fracture patients who went on to develop serious infections (459). Moreover, treatment of neutrophils with DHEAS at physiological levels was able to enhance neutrophil superoxide generation, suggesting that it could improve immunity in these patients (489). That DHEA and DHEAS levels decline with age, adrenopause, may well contribute to poor outcomes in older patients after injury.

The protective effects of oestrogens on immune cells and organ function also highlights the potential role of sex hormones in modulating trauma related immune

dysfunction(393-395,556-559). The relationship between low DHEA levels and probability of sepsis supports the proposition that DHEA supplementation might reduce the incidence of infection. As an intervention DHEA supplementation would support early anabolic drive as well as help the immune system at a time when it is suppressed. The DHEA side-effect profile is low and it is well tolerated, but its efficacy is much debated(560,561).

The early treatment with an early sex steroid such as DHEA will have immunogenic properties as well as supporting anabolism with fewer side-effects than pure anabolic agents such as oxandrolone (378). The ratio of catabolic and anabolic hormones appear to contribute to muscle loss and increased risks of infection. Whether the direct anabolic effects of DHEA act to reduce muscle loss or the resultant reduction in the inflammation results in less sepsis/MOD and that that itself causes the trauma patient to lose less lean muscle mass is not known. However, it is hard to avoid the possible benefits that DHEA supplementation may offer the severely injured; reducing catabolism, enhanced immune function, improved anabolism that would result in shorter hospital stays, quicker recovery and potentially a higher proportion of return to independent living and work. The population that are likely to yield the greatest benefit are the older injured; even moderate injuries are known to have poorer outcomes(368). Normal DHEA levels are in decline with older adults and supplementation with DHEA in a trauma context may prove extremely important.

6.4 Strengths and Limitations

The strengths of the study are the good number of young male patients with no-comorbidities. The inclusion of civilian trauma patients allowed some translation across the patient groups. The 6 month follow up with a reasonable number completing the

study has allowed the temporal changes to be recorded with confidence. The additional sampling to that for clinical need has allowed the study of the endocrine profile over six months.

The limitations of this observational study are not unique, particularly the missing values of potentially important covariates and other unmeasured but potentially important confounders and effect modifiers. The population studied is diverse; with a wide variation in the mechanism of injury, age, occupation and the injured patient's potential function reserve. It is particularly important to draw attention to the wide distribution of age in the civilian cohort compared to the young military cohort. The military patients also had more rehabilitation than their civilian counterparts.

The collection of 24-hours urines relies heavily on the clinical staff and patients accurately recording and including all samples in the collection particularly later in the study. The measurement of muscle thickness is difficult in the clinical environment with wounds and dressings hampering measurements. The frequency of measuring muscle thickness may not have been sensitive enough to capture the timing of protein loss accurately.

The study has demonstrated the current limitations in scoring methods used to stratify the severely injured that are largely designed to predict mortality. The mechanism, age and co-morbidities, all have the potential to change the response to trauma that is also complicated by a genetic response that is partly hard-wire and partly adaptive. Where possible sub-group analysis was undertaken to remove some of the variability to study the ongoing changes in response over time. The study was limited by the inability to diagnose accurately infection in patients with ongoing SIRS, but current clinical criteria were used.

There were significant funding and resource limitations. Measures of genomic patterns of change in trauma would have added to the scientific value but were not possible due to sample and resource constraints. The metabolomic pattern of change not presented in the thesis is still to be analysed.

6.5 Future work.

6.5.1 DHEA Supplementation.

The use of a sex steroid to correct the balance of catabolic/anabolic drive and support the immune system is attractive. DHEA has been explored in other patients groups such as rheumatoid arthritis (562), sarcopenia (563) and others(564) but not in a clinical trauma studies. Much of the recent studies involving the administration of DHEA have been in the setting of adrenal insufficiency that has either been associated with non-trauma disease states or advancing age (565-568). The next phase of work would be a pharmokinetic study in a trauma cohort to establish the dosage of DHEA required to restore serum levels of DHEA, DHEAS and downstream androgens in severely injured patients back to those observed in healthy age-matched population. We envisage a cross-sectional, open, single dose pharmacokinetic approach and are currently planning such a study.

6.5.2 Diagnosis of infection

The diagnosis of infection is still hampering researchers and clinicians alike. We have chosen to apply Bone's criteria of a SIRS response with positive culture. In our cohort we have a number of blast injuries from Afghanistan. Here, contaminated debris is driven deep into tissue and body cavities. It is difficult to decide whether the positive

cultures from deep tissues and blood are contributing to the SIRS response. We found a strong correlation between the adrenal hormone temporal changes and the probability of sepsis. This does not imply causality but represents an endocrine environment that is present when there is an increased probability of infection.

Metabolomics, discussed earlier, is the study of the pattern of change of metabolic products that has been successful as a prognostic tool in identifying patients who respond well to treatments in inflammatory conditions such as Rheumatoid arthritis(569). One area of future interest is using metabolomics to identify products of metabolism from bacteria and the host response to infection that may help distinguish between sterile and non-sterile inflammation, namely SIRS and infection. This will aid the rational provision of antibiotics and aid in selection of patients for intervention trials. It will also complement any mechanistic work that will be needed in demonstrating the immune enhancing effects of DHEA supplementation in severely injured patients.

6.5.3 Reversal of Catabolism

As stated above, DHEA supplementation will be an early interventional follow up to this study, with the aim of not only reducing the nadir of muscle breakdown but perhaps also reducing the time taken to return to anabolism. Again thinking of the older patient, this approach may be especially beneficial but we need to be prepared for the need to take additional measures. In particular research in the metabolic physiology field has shown that older adults are more resistant to anabolic stimuli from protein and exercise, termed anabolic resistance (570). Thus, we may need to combine DHEA supplementation with amino acids such as leucine, perhaps via nutritional supplements already in use in ICU, or exercise. The latter may need to be through techniques such as Electrical Muscle Stimulation as exercise is not practical for many injured

patients(571). Metabolomics may also be useful in stratifying trauma patients through the use of early identification of metabolic products that can predict the clinical course, for example catabolism vs. anabolism. Understanding the point where anabolism dominates will be useful as an outcome measure in RCTs and as a target of intervention.

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