UNIVERSITY OF SALFORD

Interleukin-6 and Interleukin-10 concentrations as predictors of patient outcome following major traumatic injury.

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MSc by Research

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Abbreviations

AECC - American-European consensus conference

AIS - Abbreviated Injury Scale

APACHE - Acute Physiology and Chronic Health Evaluation

ARDS - Acute respiratory distress syndrome

BP - Blood pressure

CARS - Compensatory anti-inflammatory response syndrome

CPAP - Continuous positive airway pressure

CRP - C reactive protein

CVVH - Continuous Veno-Venous Heamofiltration

DAMPS - Damage associated molecular patterns

DIC - Disseminated intravascular coagulopathy

DNA - Deoxyribonucleic acid

eGFR - Estimated Glomerular Filtration Rate

EI - Electron ionisation

ELISA - Enzyme linked immunosorbent assay

ESI - Electro spray ionisation

FBS - Foetal Bovine serum

FiO^2 - Fraction of Inspired Oxygen

Gp130 - Glycoprotein 130

HMDB - Human Metabolome Database

HMGB1 - High mobility group box 1

HNP - Human neutrophil peptide

HR - Heart rate

HSP - Heat shock protein

ICAM - Intercellular adhesion molecule

ICU - Intensive care unit

IL-10 - Interleukin-10

IL-10R1 - Interleukin-10 receptor 1

IL-1α - Interleukin-1 alpha

IL-1β - Interleukin-1 beta

IL-4 - Interleukin-4

IL-6 - Interleukin-6

IL-6R - Interleukin-6 receptor

IL-8 - Interleukin-8

IRAK - Interleukin-1 receptor-associated kinase

ISS - Injury Severity Score

JAK - Janus associated kinase

JNK - Jun N-terminal kinase

kDa - Kilodalton

LC - Liquid chromatography

LFA-1 - Lymphocyte function-associated antigen 1

LL-37 - Cathelicidin antimicrobial peptide

LRR - Leucine rich repeats

MAC-1 - Macrophage-1 antigen

MALDI - Matrix-assisted Laser desorption/ionisation

MAP - Mean arterial pressure

MAPK - Mitogen-activated protein kinase

MCP-1 - Monocyte chemotactic protein 1

mM - Millimolar

mmHg - Millimetres of mercury

MODS - Multi-organ dysfunction syndrome

MOF - Multiple organ failure

MRI - Manchester Royal Infirmary

MS - Mass spectrometry

NF-κB - Nuclear factor kappa-light-chain-enhancer of activated B cells

NHS - National Health Service

NISS - New injury severity score

NIV - Noninvasive ventilation

NMR - Nuclear magnetic resonance

PAMPs - Pathogen associated molecular patterns

PaO^2 - Partial pressure of oxygen

PBMC's - Peripheral blood mononuclear cells

PBS - Phosphate buffered saline

PCWP - pulmonary capillary wedge pressure.

PE - Phycoerythrin

PECAM - Platelet/endothelial cell adhesion molecule 1

PLT - Platelet count

PRR - Pathogen recognition receptor

PT - Prothrombin time

qSOFA - Quick Sequential Organ Failure Assessment

RAGE - Receptor for advanced glycation end products

ROS - Reactive oxygen species

SAA - Serum Amyloid A

sIL-6R - Soluble Interleukin-6 receptor

SIRS - Systemic inflammatory response syndrome

SOCS 3 - Suppressor of cytokine signalling 3

SOFA - Sequential Organ Failure Assessment

SR - Salford Royal Hospital

STAT - Signal transducer and activator of transcription

TF - Tissue factor

TGF - Transforming growth factor

Th-1 - Class 1 T helper cells

Th-2 - Class 2 T helper cells

TIC - Trauma induced coagulopathy

TIR - Toll/Interleukin-1 receptor

TIRAP - Toll-interleukin 1 receptor (TIR) domain containing adaptor protein

TLR - Toll like receptor

TNFα - Tumour necrosis factor alpha

TRAF - TNF receptor-associated factor

TRAM - TRIF-related adaptor molecule

Treg - Regulatory T cells

TRIF - TIR-domain-containing adapter-inducing interferon-β

VCAM - Vascular cell adhesion protein

vWF - von Willebrand Factor

WCC - White cell count

Abstract

Trauma is one of the main causes of death worldwide, accounting for 4.8 million deaths per year. This death rate has led to trauma being classed as the top cause of death for males, aged between fifteen and twenty-nine. More recently, however, the pattern of major trauma is reported to be changing, with elderly cohorts and falls from less than 2 meters emerging as the dominant presenting complaint. In addition to these deaths, directly caused in the early phase following major trauma, a second peak of deaths resulting from the complications including sepsis and multiple organ failure, occurs in the days and weeks following the initial traumatic insult.

These complications develop due to an imbalance between the pro-inflammatory and anti-inflammatory response to traumatic injury. This imbalance results in the counter inflammatory response becoming dominant. This results in complications including sepsis and multi-organ failure occurring due to the resultant immunosuppression. Thus, the ability to monitor the pro- and anti-inflammatory responses through the measurement of interleukin-6 and interleukin-10, may allow an early prediction of patient outcome and the likelihood of developing complications.

Blood samples and clinical data were taken on days 1, 3 and 5, with additional clinical data taken on day 8, following admission. Patient blood serum was analysed for their interleukin-6 and interleukin-10 concentrations, using cytometric bead arrays, in sequential samples, over a five-day period following traumatic injury.

The concentrations of interleukin-6 and interleukin-10 for these patients were then compared to their clinical data and scoring systems. This evaluated the use of interleukin-6 or interleukin-10 as potential biomarkers for the early detection of complications and poor clinical outcome following trauma. Metabolomic analysis was also conducted in parallel to validate methodology and identify new molecules and patways involved in the response to trauma.

Interelukin-10 concentration was further utilised to cluster liquid chromatography/mass spectrometry metabolomic analysis to identify significant metabolites that are higher in patients with elevated interleukin-10.

Preliminary results show that both interleukin-6 and interleukin-10 differentiate between good and poor outcome. Median interleukin-6 concentrations were found to be at their peak in day 1 (54.28 pg/ml ± 214.48), decreasing in day 3 (29.43 pg/ml ± 300.19) and further decreasing in day 5 (10.90 pg/ml ± 673.74). A similar pattern was observed following analysis for interleukin-10 with the peak on day 1 (5.87 pg/ml ± 20.21), decreasing in day 3 (2.59 pg/ml ± 4.96) and decreasing further in day 5 (1.99 pg/ml ± 8.82). Furthermore, Day 1 interleukin-10 concentrations were used to cluster the metabolomic analysis. With this grouping, a significant change in penicillin based antibiotic metabolites was observed in day 5 metabolomic analysis of trauma patient's serum samples, identifying day 1 interleukin-10 a predictive marker for the need for long term antibiotic usage.

This study indicates that the balance between interleukin-6 and interleukin-10 has potential predictive value for the early detection of complications following trauma and provide early guidance towards optimal therapeutic intervention.

Chapter 1 - Introduction

1.1 - An introduction to trauma

Major trauma is classed as any injury that has the potential to cause prolonged disability or death. It refers to physical injuries of sudden onset and severity that require immediate medical interventions, such as surgery, resuscitation therapy and therapeutic options to return bodily functions back to homeostatic levels. Major trauma results in life changing injury that can affect either a single organ system, such as during severe brain injury or involve multiple body systems in a process called polytrauma (McCullough *et al*., 2014).

Due to the complex nature of trauma injuries, patients are required to stay in the hospital for extended periods of time and may require specialist care in an intensive care unit (ICU). These necessities for patient care have a major financial impact on health care services throughout the world. Each trauma patient admitted to hospital in the UK costs on average £50,000, with trauma injuries resulting in death costing approximately £750,000 (National Institute for Health and Care Excellence, 2015). This cost of treating traumatic injury, results in an annual estimated cost of £1.6 billion, accounting for 7% of the total National Health Service (NHS) budget in 2008 (Christensen *et al*., 2008).

Continuing advancements in medical technology, treatment, trauma care and the implementation of trauma audits, mean that patients can now survive for extended periods following traumatic injury (Lecky, 2015). However, these patients still live with high levels of disability following the initial traumatic injury, along with the risk of developing complications such as sepsis and multi-organ failure (Lord *et al*., 2014). This has been shown in a recent World Health Organisation study that trauma accounts for between 10 and 11% of all disability worldwide (World Health Organisation, 2014). Traumatic injury fulfils the disease classification for a global pandemic, due to it being a recurrent and significant cause of morbidity and mortality over time and occurring across numerous continents, despite best efforts to prevent and control its impact (Lecky *et al*., 2010 in Pope *et al*., 2010).

1.1.1 - Incidence and epidemiology of trauma

Trauma is one of the main causes of death worldwide and is the leading cause of death for those under the age of 40, accounting for approximately 10% of all deaths in this age bracket. Of the 55 million people that died worldwide during 2013, 4.8 million (8.7%) deaths were attributed to trauma (both unintentional and intentional) (Parker & Magnusson, 2016).

Based on a recent World Health Organisation report, injury/trauma is amongst the main cause of deaths, causing 9% of total mortality worldwide and became number one cause of death in 15-29 years old group (World Health Organisation, 2012). Within England, there are at least 20,000 cases of major trauma each year, resulting in 5,400 deaths and significant numbers resulting in permanent disability requiring long-term care (England National Audit Office). Figure 1 shows the change in trauma incidence in the UK over the past 25 years. Despite being the number one cause of death in the population under the age of 40 worldwide, a change in age distribution has been observed recently in developed countries such as the UK. This shift has caused trauma to be a major cause of death in people over 60 years of age (Figure 2) (Kehoe *et al*., 2015).

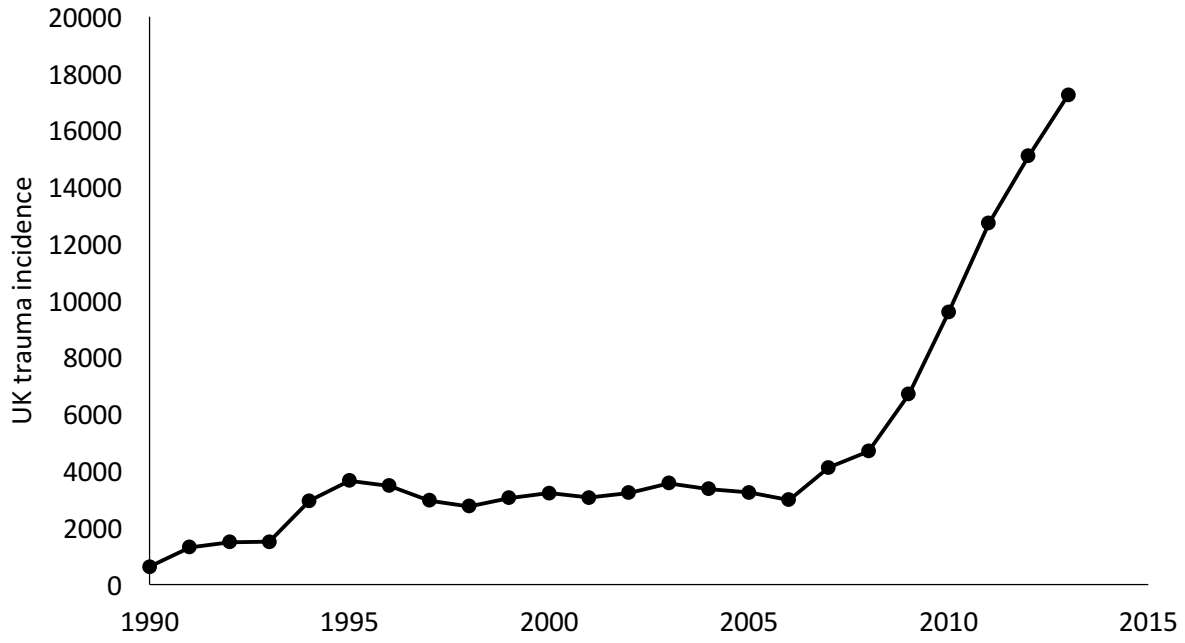

Figure 1) The change in UK trauma incidence between 1990 and 2013. Adapted using data from Kehoe *et al*., 2015.

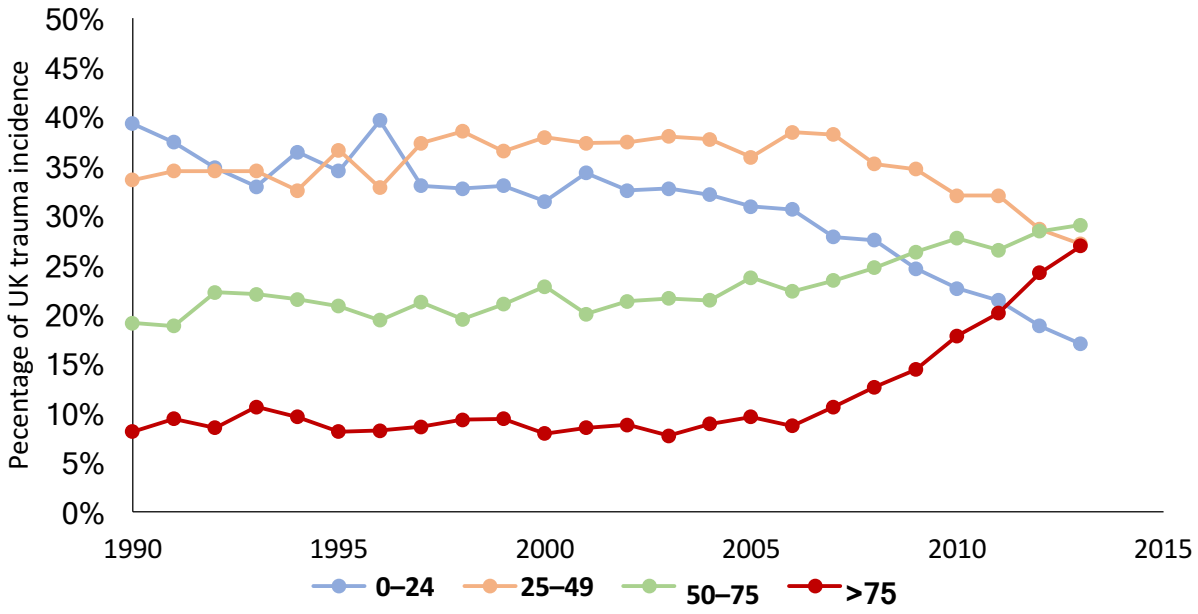

Figure 2) The change in trauma incidence amongst age groups, using data from between 1990 and 2013. Adapted using data from Kehoe *et al.*, 2015.

This is a problem in the developed world due to the expansion in numbers in the ageing population attributed to a higher life expectancy because of advancements in medical care. This has also resulted in a change in the gender distribution with the incidence of trauma shifting from a predominantly male population to a more mixed gender population. This change (Figure 3) mainly occurs in developed, higher economic countries, such as the UK.

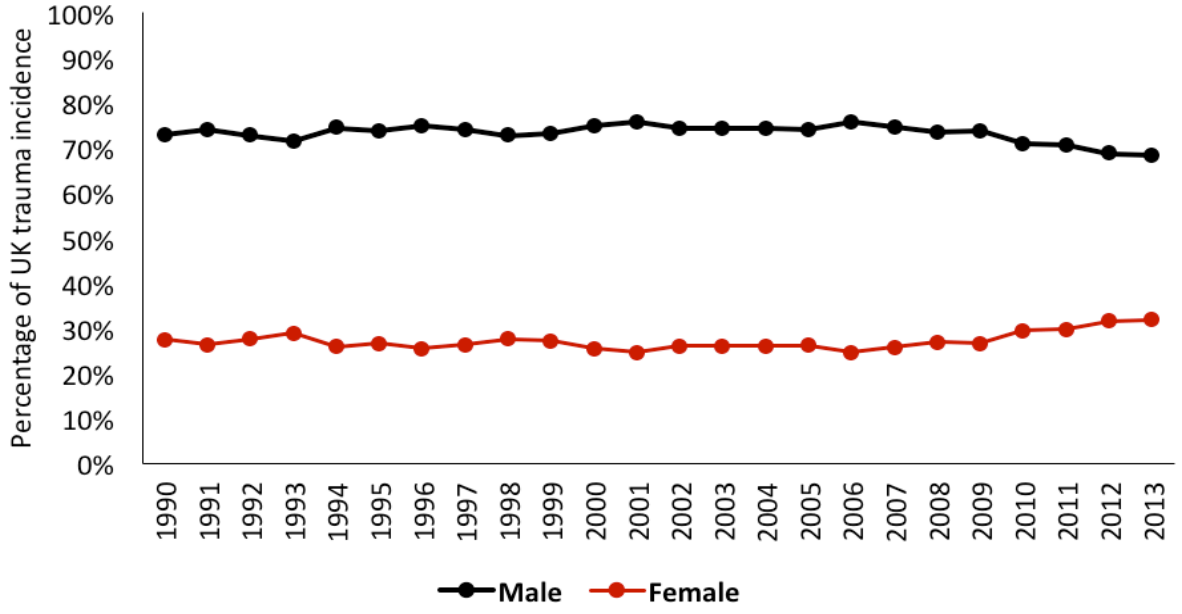

Figure 3) The change in trauma incidence between men and women, between 1990 and 2013. Adapted using data from Kehoe *et al.*, 2015.

This age-related change in trauma incidence has not occurred in middle to low-income countries, with trauma predominantly occurring and increasing in the classical, under 40 population. This increase is due to these countries undergoing rapid industrialisation, a dramatic rise in the number of motor vehicles, and recurrent armed conflicts (Alberdi *et al.*, 2014).

Lower income countries that are currently undergoing industrialisation have a major source of occupation in the farming and manufacturing sectors of industry. These groups of industries are high risk for the incidence of traumatic due to the use of large machinery and the reliance of motor vehicles on underdeveloped road infrastructure (Alberdi *et al*., 2014). The incidence of traumatic injury differs significantly among lower income countries and higher income countries. This was presented in a World Health Organisation study discussing the impact of injury and violence worldwide (World Health Organisation, 2014). This study identified that the mortality rate per 100,000 of the population, is higher in lower income countries, compared to high-income countries (Figure 4).

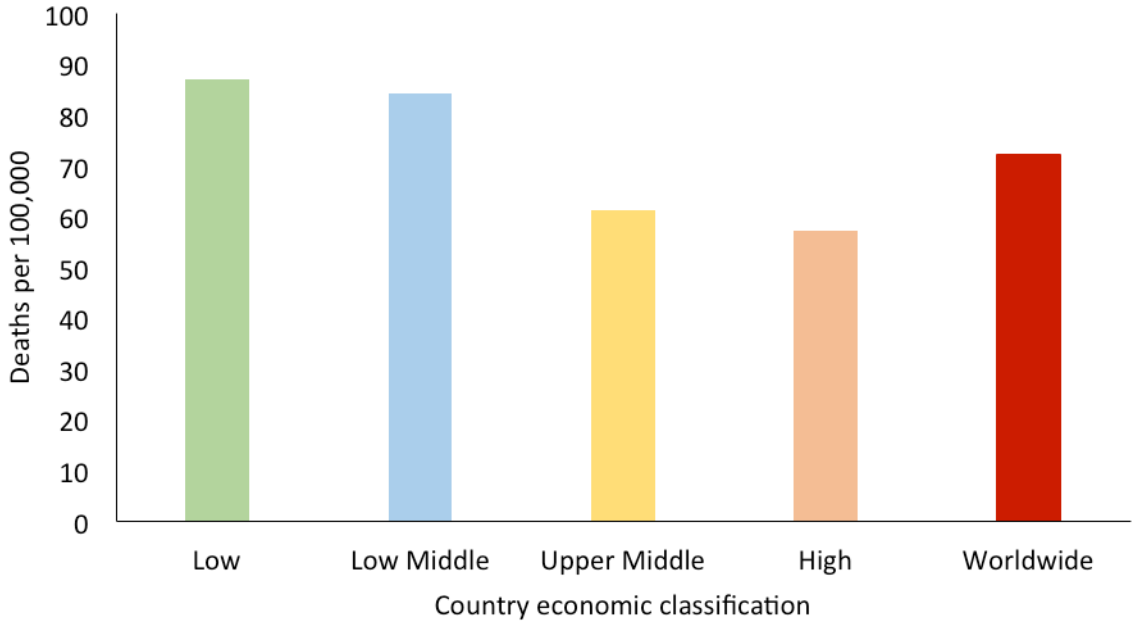

Figure 4) The mortality rate of traumatic injury in countries grouped on their economic classification. Adapted from World Health Organisation, 2014.

This difference in mortality rate is primarily due to the availability of rapid response medical treatment and improved road safety measures in higher income, more developed countries, allowing the victims of traumatic injury to receive medical intervention significantly quicker, thus decreasing their risk of death due to traumatic injury (Vos *et al*., 2015).

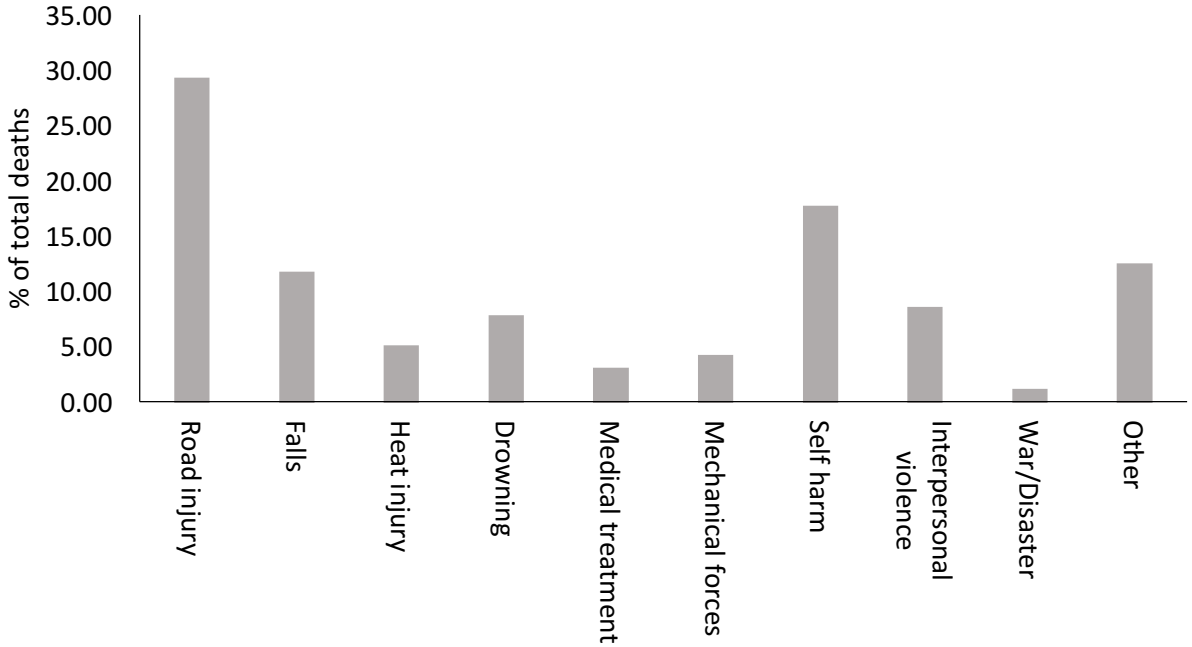

Figure 5) The main causative mechanisms for trauma worldwide and the percentage of total trauma deaths associated with each main causative mechanism. Adapted using data from (Haagsma *et al.*, 2016).

Globally, major trauma is most commonly caused by motor vehicle accidents (Figure 5). This is due to poor road safety in low-income countries, differences in driving regulations in comparison to developed countries, along with poor road infrastructure, despite the increasing number of vehicles (Søreide, 2009; Nantulya & Reich, 2002). However, in higher economic countries, a new main cause of trauma incidence is developing, in the form of falls from a height of lower than two meters. This is due to the ageing population associated with higher developed countries. These groups of individuals are at a greater risk of having poor clinical outcomes because of the trauma despite the relatively short distance of the fall. This is due to this cohort having significant levels of comorbidities and increased susceptibility to nosocomial infections in addition to poor healing.

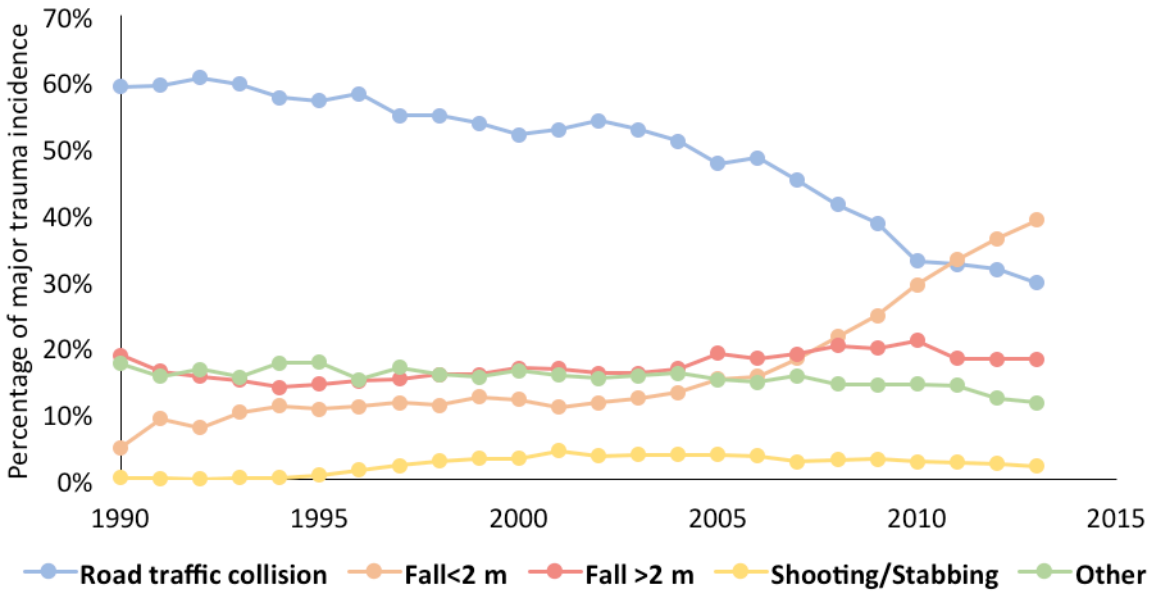

Figure 6) The main causative mechanisms of major trauma in the UK and the change in incidence between 1990 and 2013. Adapted using data from Kehoe *et al.*, 2015.

Over the previous 25 years, the cause of trauma in the UK has dramatically changed (Figure 6). In this 25-year period, there has been a dramatic decrease (30%) in road traffic related trauma, with falls from a height of less than two meters becoming the most common cause of trauma in the UK (Kehoe *et al.*, 2015). Overall, the incidence of trauma is increasing year on year, in both the developed and developing regions of the world. This identifies the need to conduct research into preventative measures, diagnostics and therapeutic options, to decrease the incidence of trauma and lower the mortality rate in both developed and developing countries.

1.2 - The pathophysiology of trauma

Traumatic injury causes damage to tissues and organs. This damage induces several responses by the body, both local and systemic to maintain immune integrity and stimulate repair mechanisms (Keel & Trentz, 2004). The response to injury involves the activation of both the innate and adaptive immune system to initiate recovery mechanisms. The innate immune system acts as the first line of defence for the body against potential pathogenic agents. It does this by detecting a potentially pathogenic substance and triggering responses. The innate immune response activates the adaptive immune response allowing the recruitment of B and T cells to the site of injury and the production of antibodies and cytokines. These responses are characterised by the local and systemic production and release of different mediators, including cytokines, complement factors, coagulation proteins, acute phase proteins, neuro-endocrine mediators and the initiation of immunocompetent cells at the site of tissue damage (Keel & Trentz, 2004).

1.2.1 - Pathogen and damage associated molecular patterns

An immune response following trauma is induced by molecules released from damaged cells (DAMP's) or pathogen cell surface structures (PAMP's). These molecules interact with receptors on the surface of immune cells, inducing the immune responses associated with traumatic injury.Pathogen associated molecular patterns are structures on the surface of bacteria that are recognised by innate immune cell receptors (Mogensen, 2009). These structures play a key role in the survival of bacteria or the pathogenicity of the bacteria (Kumar, Kawai & Akira, 2011). They bind to pathogen recognition receptors on the surface of innate immune cells and trigger an inflammatory response, shown in Figure 8) (Kumar, Kawai & Akira, 2011).

Damage-associated molecular patterns are molecules found within cells that avoid recognition by the immune system under normal physiological conditions (Land, 2015). However, when cells become stressed or following tissue damage, these molecules are then released or exposed on the surface of cells (Figure 7) (Land, 2015).

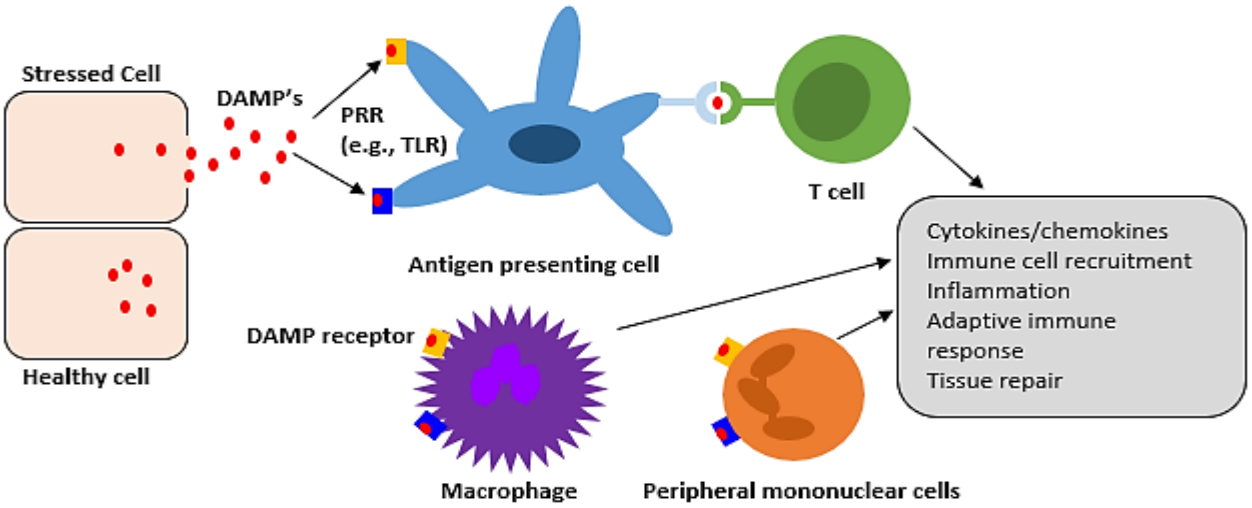

Figure 7) The interactions that DAMP's can undergo, following their release from stressed cells and the potential responses that can occur following their binding to receptors on

DAMP's include intracellular proteins, numerous nuclear, cytosolic, mitochondrial molecules along with components from the extracellular matrix have been identified as DAMPs. The first molecule to be identified as a DAMP was the High mobility group box 1 (HMGB1) and since, numerous different molecules have been identified including Mitochondrial DNA, Histones, Heat shock proteins, Genomic DNA and S100 proteins (Sharma & Naidu, 2016). These molecules can also be passively released into the extracellular environment from the extracellular matrix because of undergoing necrotic cell death or extracellular damage. These molecules trigger an inflammatory response in a similar fashion as PAMP's, by binding to PRR's on the surface of innate immune cells (Figure 7) (Krysko *et al.*, 2011).

1.2.1.1 - High mobility group box 1

High mobility group box 1 is a nuclear protein, which is described as a DNA binding protein and has a function in the transcription process (Park *et al.*, 2006). Within the nucleus, HMGB1 binds non-specifically to the minor groove of the DNA helix, causing the structure to bend (Bianchi & Agresti, 2005). When a cell undergoes necrosis, following damage, HMGB1 is released into the environment, allowing it to interact with molecules and receptors it is not usually in contact with due to its location within the nucleus (Bianchi & Manfredi, 2007).

High mobility group box 1 has been shown to interact with Toll-like receptors 2 and 4 resulting in a cell signalling cascade that results in the production of pro-inflammatory cytokines. However, other studies have shown HMGB1 to act as a chemoattractant for proinflammatory cytokine secreting cells, rather than directly causing elevations in cytokine secretion (Rouhiainen *et al.*, 2007). This ability to act as a chemoattractant is due to the binding to the receptor for advanced glycation end products (RAGE) (Hori *et al.*, 1995). The binding of HMGB1 to RAGE results in the activation of Mitogen-activated protein (MAP) kinases and NF-κβ signalling pathways that are known to be involved in the production of pro-inflammatory cytokines (Bianchi & Manfredi, 2007).

1.2.1.2 - S100 Proteins

S100 proteins are a collection of intracellular proteins categorised by their calcium-binding motifs (Donato *et al.*, 2013). Their normal functions include calcium homoeostasis, cell cycle regulation, cytoskeletal interactions, protein phosphorylation, cell growth and migration, and the regulation of transcription factors (Srikrishna & Freeze, 2009). The S100 proteins are passively released from cells following cellular damage allowing them to undertake extracellular activities (Donato *et al.*, 2013). When released into the extracellular environment S100 proteins act as DAMP's, sounding the alarm to the immune system that something is abnormal in this region of the body.

Specific S100 proteins have been shown to be involved in processes that cause a pro-inflammatory response, these S100 proteins are known as S100A8, S100A9 and S100A12 (Srikrishna & Freeze, 2009). They can induce both pro-thrombotic and pro-inflammatory responses such as chemokine stimulation, production of adhesion molecules and pro-inflammatory cytokine stimulation (Donato *et al.*, 2013). S100A8 and S100A9 can form a complex known as Calprotectin (S100A8/A9), which can activate pro-inflammatory cytokine production by monocytes and macrophages via the NF-κβ and p38 MAP kinase pathways (Sunahori *et al.*, 2006). This complex can also activate RAGE, mediating the production of inflammatory mediators (Gebhardt *et al.*, 2008). S100A9 has also been reported to independently induce the production of TNF α, IL-1β, IL-6 and IL-8; via the NF-κβ signalling pathway (Donato *et al.*, 2013).

S100A12 induces the production of pro-inflammatory cytokines from mast cells, particularly IL-6 and IL-8 (Yang *et al.*, 2001). S100A12 can also prompt the manufacture of chemokines important for neutrophil, lymphocyte and monocyte recruitment to the site of inflammation, along with the release of TNF α from preformed stores (Yang *et al.*, 2007).

1.2.1.3 - Heat shock proteins

Heat shock proteins (HSP) were originally discovered in 1962 by Ferruccio Ritossa during an experiment on *Drosophila* in which they were stressed with heat to study gene expression (Ritossa, 1962). These proteins under normal physiological conditions fulfil an important intracellular role relating to protein folding and transport (Grundtman *et al.*, 2011). These proteins are expressed as cognate proteins and inducible forms released under stressful conditions (Tsan & Gao, 2004). Heat shock proteins can be released following several different stimuli other than heat, including environmental, pathological and physiological stimuli (Tsan & Gao, 2004).

Heat shock proteins are evolutionarily conserved and are abundantly expressed in both prokaryotic and eukaryotic organisms (Hauet-Broere *et al.*, 2006). There are six main families of heat shock proteins each distinguished by their molecular weight. These include: HSP100, HSP90, HSP80, HSP60, HSP40 and small heat shock proteins (sHSP's) (Hauet-Broere *et al.*, 2006).

Heat shock proteins are highly immunogenic and have been shown to play a role in the regulatory function of T-cells and the induction of anti-inflammatory cytokines during chronic inflammation (Hauet-Broere *et al.*, 2006). In addition to T-cells, heat shock proteins have also been shown to act on macrophages, dendritic cells, B-cells and natural killer cells (Quintana & Cohen, 2005). The regulation of autoimmunity can be indirectly by activating regulatory T-cells that control pathogenic T-cells, specifically for self-antigens other than heat shock proteins. Heat shock proteins have interactions with Toll-like receptors inducing an immune response (Quintana & Cohen, 2005). Specifically, human HSP60 can directly inhibit chemotaxis and anti-inflammatory responses via TLR-2 and suppress cytokine signalling (Quintana & Cohen, 2005).

Damage-associated molecular patterns, such as High mobility group box 1, S100 proteins and heat shock proteins, share structural and functional similarities with the exogenous conserved microbial surface structures, which are released from invading pathogens termed PAMP's. These molecules are recognised by a group of receptors called pathogen recognition receptors (PRR's) including Toll-like receptors (Hirsinger *et al.*, 2012; Sharma & Naidu, 2016).

1.2.2 - Toll-like receptors

The release of DAMP's activates Toll-like receptors (TLR's) triggering the activation of T cells and causing an immune response. Toll-like receptors are a conserved family of receptors that trigger a pro-inflammatory signalling cascade in response to either PAMP's or the DAMP's released during tissue damage.

Toll-like receptors are type 1 transmembrane protein characterised by an extracellular domain containing leucine-rich repeats (LRR's) and a cytoplasmic tail that contains a conserved region called Toll/IL-1 receptor domain (Nishiya & DeFranco, 2004). These receptors are predominantly expressed in tissues involved in the immune response, such as the spleen and peripheral blood leukocytes, along with those exposed to the external environment including the lungs and gastrointestinal tract (Nishiya & DeFranco, 2004).
To date, ten human variants of toll-like receptors have been identified, called TLR-1 to TLR-10. Most of these receptors are expressed on the plasma membrane of cells, apart from TLR's -3, -7, -8 and -9, which are located within an endosomal compartment (Nishiya & DeFranco, 2004). These endosomal TLR's scan for nucleic acids of bacteria, viruses or parasites, which have been phagocytosed/endocytosed into the cell. The detection of nucleic acids triggers signalling pathways that result in the production of inflammatory mediators (Gibbard, Morley & Gay, 2006).
Toll-like receptors act as receptors for intracellular signalling and the expression of genes. Initially, a ligand such as a PAMP or DAMP binds to a Toll-like receptor, either extracellularly or to internal receptors (Figure 8). Once bound, the ligand allows the recruitment of signalling adaptor molecules, in the form of MyD88, TIRAP, TRAM and/or TRIFF, following the activation of Toll/Interleukin-1 receptor(TIR) domains on the TLR (Kawai & Akira, 2010; Lim & Staudt, 2013).

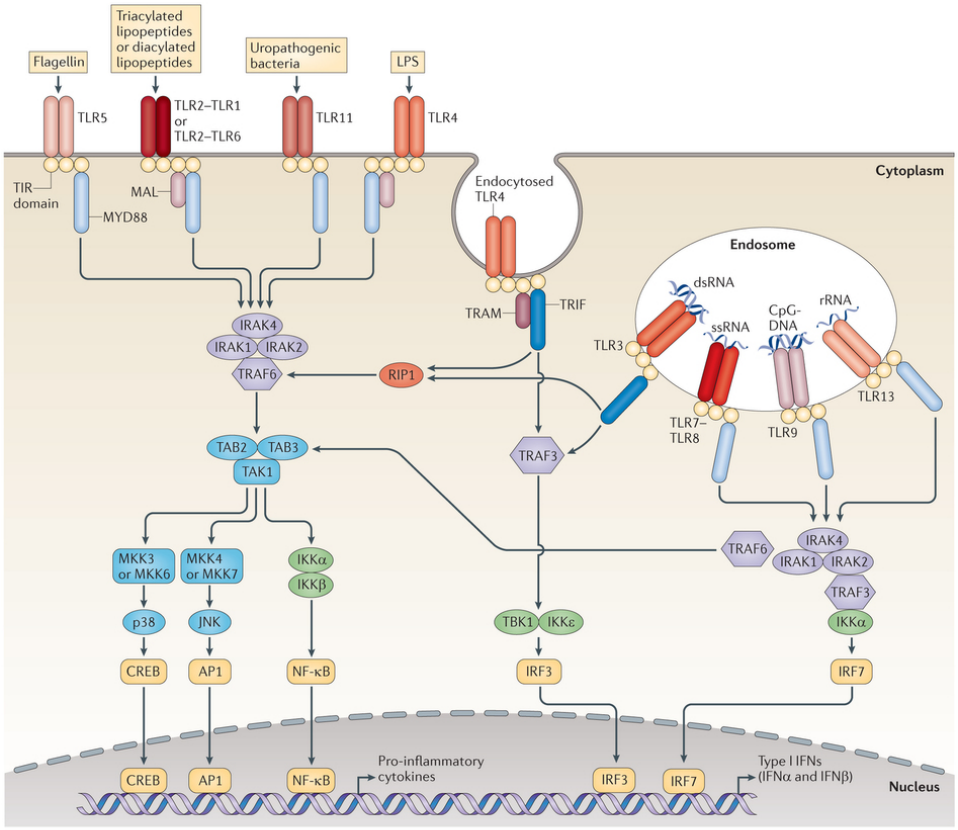

Figure 8) The variety of Toll-like receptors and the vast array of signalling pathways involved in initiating the inflammatory response following the detection of DAMP's and PAMP's. (O'Neill, Golenbock & Bowie, 2013)

Depending upon the signalling adaptor molecule used in the signalling pathway, various kinases and ubiquitin ligases are recruited and activated, including IRAK4, IRAK1, IRAK2, TBK1, IKKe, TRAF6 and pellino 1 (Lim & Staudt, 2013).

This signalling pathway culminates in the engagement of NF-kb, Type 1 interferon, P53 MAP kinase and JNK/MAPK pathways (Figure 8) (Kawai & Akira, 2010; Lim & Staudt, 2013; Morrison, 2012). These pathways cause the expression of genes relating to pro-inflammatory modulators, cell survival, immune cell proliferation and immune regulation. These changes in gene expression allow the inflammatory process to begin and induce the changes associated with an inflammatory response.

1.2.3 - The coagulation response during major traumatic injury

Coagulation is the process used to prevent the loss of blood from cuts and damage, to maintain haemostasis and limit blood loss following major injury (Thornton & Douglas, 2010). This involves blocking the ruptured blood vessel using a combination of activated platelets and fibrin (Lasne, Jude & Susen, 2006).

Platelets become activated by binding to collagen, which is exposed to the blood circulation following damage to the endothelium of the blood vessel (Andrews & Berndt, 2004). This binding of platelets to the exposed collagen, results in the activation of integrins on platelets, resulting in a strong adhesion of platelets to the site of damage or rupture (Heemskerk, Bevers & Lindhout, 2002).

The process to activate fibrin involved numerous clotting factors, which activate one another until it ultimately results in the activation of fibrin (Palta, Saroa & Palta, 2014). The activation of fibrin is a result of the coagulation cascade, this can occur through two pathways, the contact activation (intrinsic) and the tissue factor pathway (extrinsic). The tissue factor pathway is commonly activated following tissue damage, due to its ability to rapidly generate activated fibrin (Figure 11).

The extrinsic pathway is initiated by stable factor (factor VII) leaving circulation and interacting with tissue factor (TF), generating an activated complex (TF:VIIa) (Riewald & Ruf, 2002). This complex activates Christmas factor (factor IX) and Stuwart-Prowler factor (factor X) to form IXa and Xa. Factor Xa activates prothrombin, converting it to thrombin (Owens & Mackman, 2010). Thrombin can then act on fibrinogen converting it to fibrin and activating fibrin stabilising factor (factor XIIIa). This allows the formation of a stable clot through the binding of strands to the site of vascular injury, strengthened via the support of factor XIIIa (Schenone, Furie & Furie, 2004).

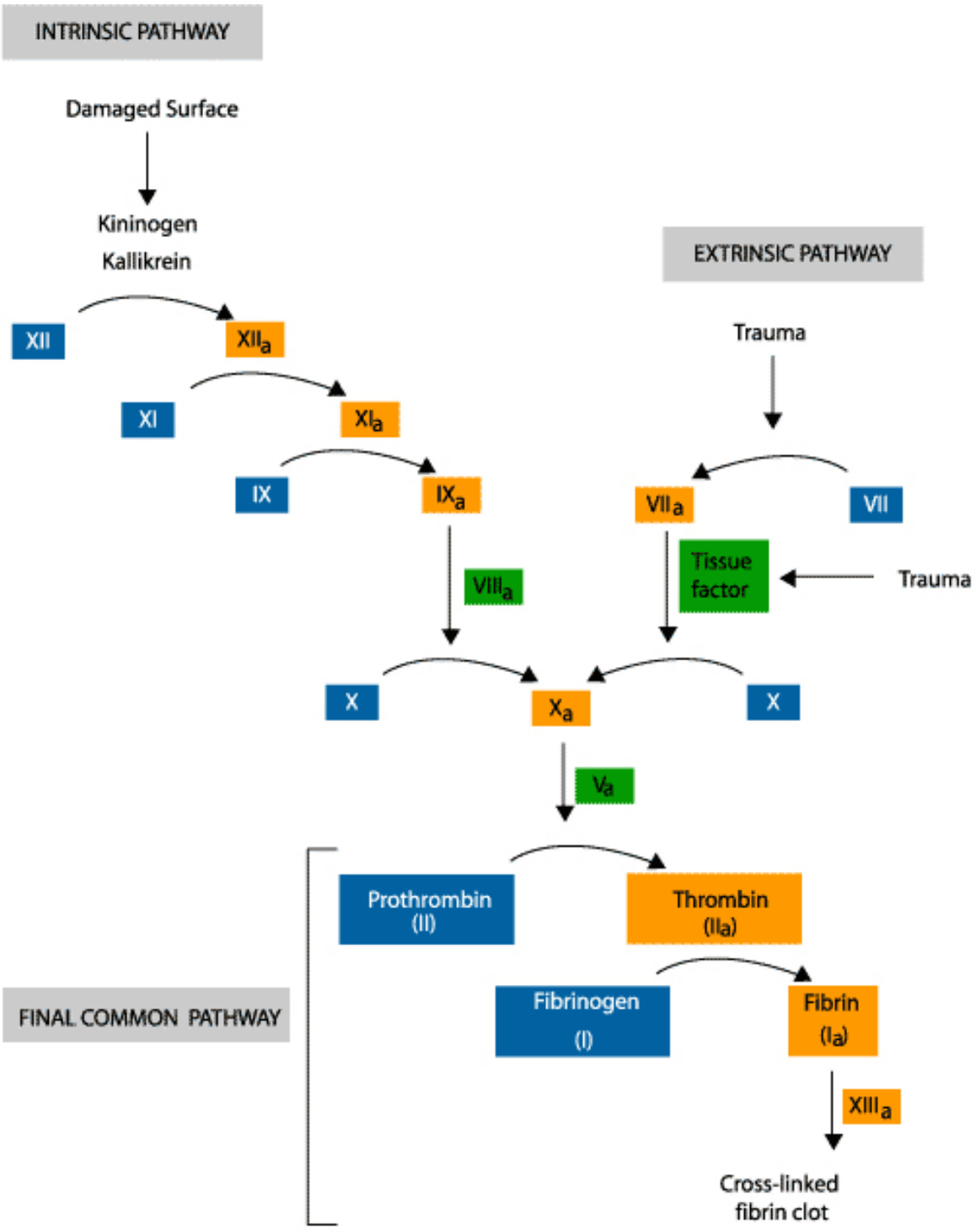

Figure 11) The clotting cascade involved in in the generation of fibrin. This either involves the intrinsic pathway or the extrinsic pathway to form activated factor X.

An alternative pathway of activating the coagulation cascade is through the contact activation pathway. This is initiated by the binding of plasma proteins Hageman factor (factor XII) and prekallikrein on collagen, mutually converting each other to XIIa and kallikrein (Green, 2006). Factor XIIa converts plasma thromboplastin (factor XI) to XIa. Factor XIa activates factor IX, which interacts with a co-factor VIIIa to form the tenase complex (Gailani & Renne, 2007). The tenase complex then activates factor X. The pathway then merges into the common pathway, resulting in the formation of fibrin (Adams & Bird, 2009; Owens & Mackman, 2010).

Kallikrein has several functions including the ability to convert plasminogen to plasmin and stimulate the formation of kinins from kininogen (Wu, 2015). Plasmin is an enzyme that degrades blood plasma products, including fibrin clots. This allows blood clots to be degraded once they are no longer required to prevent bleeding (Bryant & Shariat-Madar, 2009). Kinin molecules such as bradykinin and kallidin are peptides responsible for the regulation of blood pressure and activation of inflammation (Bryant & Shariat-Madar, 2009).

In the period following trauma, clotting factors can become depleted if there is a large volume of blood loss because of the patient's injuries (Brummel-Ziedins, 2013; Callum & Rizoli, 2012). This over utilisation can result in the depletion of clotting factors, causing an inability of blood to clot properly, causing further blood loss and potentially fatal consequences if external clinical intervention is not given (Shaz *et al*., 2011). However, if the clotting response becomes overactive, commonly observed following traumatic injury, intravascular fibrin clots can form (Lippi & Cervellin, 2010). These clots can lead to microcirculatory disturbances, blocking blood flow and resulting in hypoxia induced cellular damage (Levi, 2007; Kumar & Gupta, 2008).

1.2.4 - The inflammatory response to trauma

Inflammation is the reaction the body invokes when it detects potentially pathological material. This reaction is done, to prevent the causative agent from spreading, to aid in the elimination of the pathological material, and trigger healing responses, to repair damage caused by the pathological agent or the inflammatory response itself.

This inflammatory response causes a variety of symptoms including, redness and warmth at the site of inflammation, swelling, and the presence of oedema. These external symptoms are side effects of the inflammatory response attempting to clear the pathological material. The inflammatory response involves a variety of different immune cells, signalling pathways, cytokines, chemokines and receptors. These all work together to elicit a successful immune response and clear the causative material.

1.2.4.1 - Cellular and vascular changes in inflammation

Once a potentially pathogenic molecule, in the form of PAMPS or DAMPS, has been detected by sentinel tissue cells, such as dendritic cells, macrophages and mast cells, it triggers the release of inflammatory mediators, causing a local inflammatory response (Nourshargh & Alon, 2014). This inflammatory response causes changes to the local cellular environment and blood vessels. The purpose of these changes is to allow effector immune cells to migrate from the blood system into tissues, to eliminate the pathogenic material.

The key events that occur to allow the transfer of effector immune cells from the blood into tissues are vasodilation, increased vascular permeability and elevated expression of vascular adhesion molecules (Nourshargh & Alon, 2014). These processes allow the rapid delivery of blood-borne defences to the site of the inflammatory response, the leakage of soluble proteins into tissue from the blood, and the attraction and migration of leukocytes to the inflamed tissue (Kolaczkowska & Kubes, 2013). Migration of leukocytes into inflamed tissue is a multi-step process involving several molecular and vascular changes, allowing leukocytes to pass from the blood into tissue and invoke their effector functions. These changes are split into four main steps, rolling, crawling, adhesion, and transmigration (Figure 9).

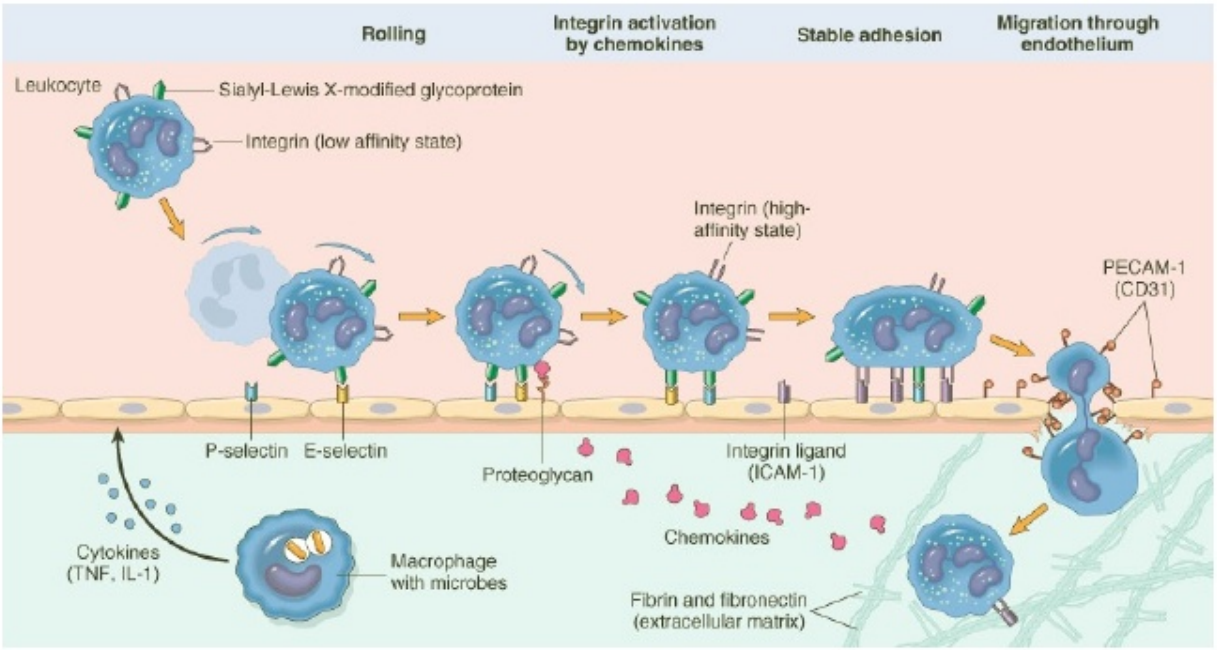

Figure 9) The processes involved in the transmigration of leukocytes from the blood, through the vascular endothelium, into the tissue containing the causative agent of the inflammatory response.

The process of rolling begins following the detection of a potentially pathogenic molecule by tissue based dendritic cells and macrophages. Once a molecule is detected, dendritic cells and macrophages release mediators (TNFα and IL-1) to activate local endothelial cells within blood vessels (Phillipson & Kubes, 2011). The activation of endothelial cells causes an expression of adhesion molecules, namely P-selectin and E-Selectin, on their surface (Kolaczkowska & Kubes, 2013). These selectins can then bind to glycosylated structures on leukocytes, causing them to interact with the endothelial surface, so they can interact with further receptors on endothelial cells (Ley, 2003). These interactions are rapidly broken and reformed, as the leukocyte travels along the endothelial wall, causing it to slow down on the endothelial surface (Rossiter, Alon & Kupper, 1997).

Once slowed, stronger binding of leukocytes to the endothelial surface can occur. This is triggered by the binding of positively charged chemokines to G-coupled chemokine receptors on the leukocyte surface (Sun & Richard, 2012). These chemokines are immobilised on the surface of the endothelium, by interactions with negatively charged heparin sulphates.

These interactions serve as anchors to prevent the sheer force of blood flow, from washing leukocytes away from the endothelial surface (Kolaczkowska & Kubes, 2013). The binding of chemokines to chemokine receptors, causes a conformational change on the leukocyte surface integrin's, allowing them to firmly attach to endothelial cells (Herter & Zarbock, 2013). The integrin's on the surface of leukocytes (LFA-1 and MAC-1), following the conformational change, can bind to ICAM-1, ICAM-2 and VCAM-1 cell adhesion molecules on the surface of endothelial cells (Kolaczkowska & Kubes, 2013). The binding of LFA-1 to ICAM-1 is a key component of triggering the firm adhesion of leukocytes to endothelial cells, allowing the leukocytes to begin the process of migrating through the endothelial border and into the inflamed tissue (Harris *et al*., 2000).

Transmigration of leukocytes through the endothelium is a complex process, involving the interaction of leukocytes in three dimensions with endothelial cells, the subendothelial basement membrane, pericytes and interstitial tissue (Muller, 2013). The transmigration process involves key interactions of platelet/endothelial cell adhesion molecule 1 (PECAM). These interactions are expressed diffusely on the surfaces of most leukocytes and are concentrated to the borders of endothelial cells, the main site of transmigration through the endothelial membrane (Muller, 2013).

Studies have shown that leukocytes migrate through the subendothelial basement membrane at regions where the expression of collagen IV and laminin 10 is at a low density. This allows the least amount of proteolysis to be conducted, to allow the cell to pass through the basement membrane (Wang *et al*., 2006). To conduct this process, CD11a on leukocytes interacts with ICAM-1 on pericytes, to guide them towards the regions basement membrane with a low density (Proebstl *et al*., 2012). In an ideal situation, leukocytes pass through the endothelial membrane at a site where there are gaps between endothelial cells, a low density of basement membrane and a gap between pericytes, to allow the leukocytes to efficiently pass through the endothelial membrane (Kolaczkowska & Kubes, 2013).

Once through the endothelial membrane, leukocytes need to migrate through the tissue to the site that induced inflammation. This is done using a chemotactic gradient from the site of leukocyte entry into the tissue increasing towards the focus of inflammation. Potential chemotactic factors include bacterial peptides, complement proteins (C5a), extracellular matrix degradation products, arachidonic acid metabolites (Leukotriene B4), other lipid mediators (Platelet-activating factor) and several small molecular weight (<10KDa) cytokines (CXC chemokines) (Moreland, 2004).

When the leukocyte reaches the focus of the inflammation, it acts to clear the causative agent. This is done through phagocytosis of the causative agent, the release of antimicrobial molecules (neutrophil peptides LL-37, HNP-1, HNP-3 and Nitric oxide) and the production and release of extracellular traps in the form of Neutrophil nets (Kaplan & Radic, 2012; Dale, Boxer & Conrad Liles, 2008; Aarbiou *et al*., 2006; Mariano *et al*., 2012).

The transmigration of leukocytes into inflamed tissue is a key component in the elimination of pathogenic sources and the repair of damage following injury. Once in the tissues, they have other key roles including the removal of causative agents, the recruitment of further immune cells, the secretion of cytokines and chemokines, activation of complement pathways and the acute phase response.

1.2.4.2 - The acute phase response to major trauma

The acute phase response is a physiological process that occurs during inflammation, triggered by infection, trauma and malignancy (Gruys *et al*., 2005). The response is caused by the release of pro-inflammatory cytokines, secreted during an inflammatory response (Gruys *et al*., 2005). Pro-inflammatory cytokines (TNFα, IL-1 and IL-6) act on hepatocytes to induce the production of acute phase proteins (Figure 10) (Moshage, 1997; Cray, Zaias & Altman, 2009). These proteins act as inflammatory mediators, scavenger molecules and tissue repair molecules, to allow enhancement of tissue protection and antimicrobial mechanisms (Sander *et al*., 2010).

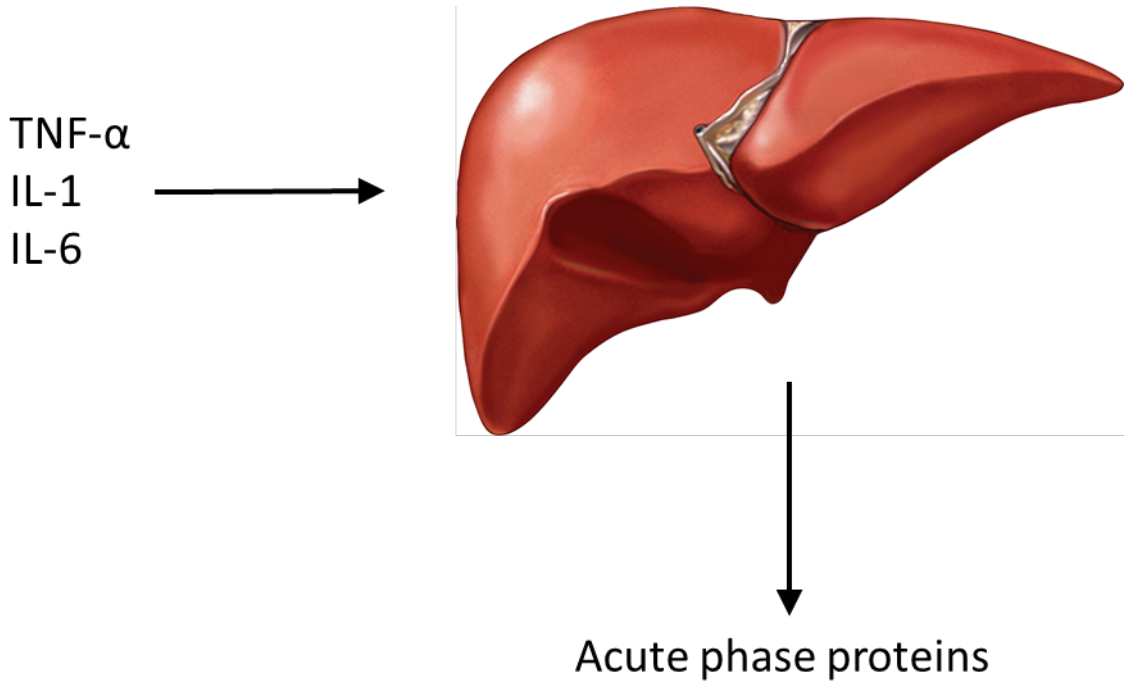

Figure 10) The interaction between pro-inflammatory cytokines and liver hepatocytes, induces the acute phase response and the production of acute phase proteins.

There are several acute phase proteins that play a role in the immune response following traumatic injury including C reactive protein, fibrinogen, α2 macroglobulin, serum amyloid A and complement factors (Ceciliani, Giordano & Spagnolo, 2002; Cray, Zaias & Altman, 2009).

C reactive protein is an opsonin, which binds to damaged and dying cells (Pepys & Hirschfield, 2003; Black, Kushner & Samols, 2004). Once bound, these molecules activate the complement system, resulting in the removal of the damaged cell by phagocytosis (Du Clos, 2000; Volanakis, 2001).

Fibrinogen is a glycoprotein produced by the liver. It plays a key role in the coagulation cascade (Lowe, Rumley & Mackie, 2004). Fibrinogen acts as a precursor molecule to fibrin, which is produced following the conversion of fibrinogen using thrombin (Kamath & Lip, 2003). Both fibrinogen and fibrin can form bridges between platelets, strengthening blood clots. However, fibrinogen is predominantly utilised as a precursor molecule for fibrin (Weisel, 2005).

α2 macroglobulin is a plasma protein. It acts as an anti-protease, which inactivates a vast array of proteinases (Ebersole & Cappelli, 2000; Werner *et al*., 2003). Its major function is the inhibition of fibrinolysis, by inhibiting plasmin and thrombin (Gouin-Charnet *et al*., 2000). This inhibition of thrombin has a major role in the coagulation cascade, as it results in limited fibrin production and weaker clot formation. α2 macroglobulin can also act as a transporter molecule, with it being shown to transport numerous growth factors and cytokines, such as platelet-derived growth factor, basic fibroblast growth factor, IL-1β, TGF-β, and insulin (Rehman, Ahsan & Khan, 2013).

Serum amyloid A (SAA) is secreted during the acute phase of inflammation as a part of the acute phase response in the liver (Richard & Sun, 2015; Uhlar & Whitehead, 1999). It has several different functions including the transport of cholesterol and the induction of enzymes that degrade extracellular matrix. Its main role in the inflammatory response is to recruit immune cells to the site of inflammation, through chemotactic action (Eklund, Niemi & Kovanen, 2012). SAA also has the capability to induce cytokine production, further enhancing the inflammatory response (Eklund, Niemi & Kovanen, 2012).

The acute phase response also produces proteins of the complement system. The release of DAMPS and PAMPs induce the activation of the complement system (Huber-Lang, Kovtun & Ignatius, 2013). The complement cascade involves a series of cleavages, via the classical and alternative pathways, leading to the formation of C3 and C5, which are further cleaved to form the functional components of the complement system (Amara *et al*., 2008; Murphy & Weaver, 2016). The main functions of these components are opsonisation, inflammation and direct destruction of cells (Sinno & Prakash, 2013).

Opsonisation of molecules targeted for phagocytosis involves the binding of complement proteins to targeted cells (Tosi, 2005). Complement proteins C3b and C4b covalently bind to pathogens, apoptotic cells and antibody-antigen complexes, allowing phagocytic cells to recognise cells and phagocytose them (Sharma & Ward, 2011).

Anaphylatoxins are also produced by the complement cascade (Peng *et al*., 2009). Complement proteins C3a and C5a support different inflammatory mechanisms including the enhancement of the acute phase response (Markiewski & Lambris, 2007), chemotaxis, the recruitment and activation of phagocytic cells (DiScorpio & Schraufstatter, 2007), and the degranulation of mast cells and basophils (Ali, 2010), resulting in increased vascular permeability and oedema (DiScorpio & Schraufstatter, 2007). C5a also plays a role in the coagulation cascade, by increasing the expression of tissue factor, activating the tissue factor pathway of the coagulation cascade (Rittirsch, Flierl & Ward, 2008).

1.3 - The role of cytokines in inflammation

Cytokines are small, secreted molecules released by cells, which have a specific effect on the interactions and communications between cells (Zhang & An, 2007). Cytokines can be termed as lymphokines (made by lymphocytes), monokines (made by monocytes), interleukins (made by leukocytes that act on other leukocytes) and chemokines (molecules that possess chemotactic activity). These groups of cytokines vary, not only in their function but also their molecular weight, which can range from 6-70kDa (Stanken & Poschenrieder, 2015). The word cytokine is a general term used to describe a secreted molecule. This can be narrowed down based on the type of cell which secretes the molecule.

Cytokines have multiple modes of action on how they reach their target cell. They can work in an autocrine fashion, where the cytokine released by a cell also bind to a receptor on the secretory cell. Cytokines can also act on cells in the area surrounding the secretory cell, termed paracrine action. Or cytokines can enter the bloodstream and act on cells at a great distance from the cell that secreted the cytokine, this is termed endocrine action (Libby *et al.*, 1995; Dembic, 2015).

The dysregulation of cytokine secretion causes the pathogenesis of many inflammatory conditions (Elshaer & Begun, 2016), and has been shown to play a key role in the pathogenesis of trauma complications. Therefore, the balance of pro and anti- inflammatory cytokines is an important part of homoeostasis, when it becomes abnormal, can lead to dire consequences for the patients.

1.3.1 - Interleukin-1

Interleukin-1 (IL-1) is a central mediator of innate immunity and inflammation (Garlanda, Dinarello & Mantovani, 2013). The interleukin-1 family of cytokines contains eleven different molecules, with the key members being Interleukin-1 alpha (IL-1α) and Interleukin-1 beta (IL-1β). IL-1α and IL-1β are potent inflammatory cytokines that activate the inflammatory process (Di Paolo & Shayakhmetov, 2016).

IL-1α and IL-1β, are encoded by separate genes, bind to the same receptor (IL-1R1) and have similar biological properties. IL-1α is found as a precursor in the epithelial cell lining of the gastrointestinal tract, lung, liver, kidney, endothelial cells and astrocytes (Chen *et al*., 2007). Following cellular damage, such as after traumatic injury, the precursor is released, acting as an alarmin for the immune system and inducing an inflammatory cascade (Rider *et al*., 2011). IL-1α localises to the nucleus and functions as a component of transcription. The activation of transcription stimulates the release of TNFα from endothelial cells (Saperstein *et al*., 2009), induces the proliferation of CD4+ T cells (Ben-Sasson *et al*., 2013) and increases the abundance of blood neutrophils (Rider *et al*., 2011).

IL-1β is produced by hematopoietic cells such as blood monocytes, tissue macrophages, skin dendritic cells and brain microglia in response TLR, activated complement components, other cytokines (such as TNF-α) and IL-1 itself (Dinarello, 2011). *In vivo*, IL-1β can evoke fever, hypotension, release of adrenocorticotrophic hormone and production of cytokines which in turn induce various inflammatory and immune responses (Li *et al*., 2008). IL-1β has been shown to be elevated in the period following traumatic injury (Li *et al*., 2008). It has also been shown to be a major cytokine in the development of auto inflammatory conditions, such as rheumatoid arthritis and Type 2 diabetes (Dinarello, 2009; Lopalco *et al*., 2015). Correlations have also been observed, that link the serum concentration of IL-1β to patient survival following the development of septic shock (Casey, Balk & Bone, 1993).

1.3.2 - Interleukin-4

IL-4 is produced mainly by activated T cells but also by mast cells, basophils, and eosinophils (Nelms *et al*., 1999). A typical cytokine structurally, with molecular weight varying between 12 and 20 kDa because of variable natural glycosylation. (Luzina *et al*, 2012). Functionally, IL-4 regulates cell proliferation, apoptosis, and expression of numerous genes in various cell types, including lymphocytes, macrophages, and fibroblasts, as well as epithelial and endothelial cells (Luzina *et al*, 2012).

IL-4 is a key cytokine in the development of allergic inflammation. It is associated with induction of the epsilon isotype switch and secretion of IgE by B lymphocytes (Steinke & Borish, 2001). It is also involved in the differentiation of Th-2 lymphocytes leading to Th-2 cytokine release (Vella *et al*., 1997) and the induction of VCAM-1 expression on the vascular epithelium (Moser, Fehr & Bruijnzeel, 1992). Interleukin-4 can subsequently produce further IL-4 through a positive feedback loop on Th2 cells.

1.3.3 - Interleukin-6

Interleukin-6 (IL-6) is a polyfunctional cytokine that plays a key role in host defence. IL-6 was first identified in the mid-1980's as a small glycoprotein that is relatively unique compared to other cytokines, due to it being produced by a broad spectrum of cells in response to a variety of different stimuli (Rincon, 2012).

Human Interleukin-6 is encoded for by a gene, located on chromosome 7p21, spanning approximately 5 kilobases (Kb) in length and is composed of four introns and five exons (Keller *et al.*, 1996). These exons encode for an 1197 base pair mRNA transcript, which is then translated into the Inteleukin-6 protein (NCBI GenBank, 2017). This protein is made up of 212 amino acids including a 28-amino acid signalling peptide (Tanaka *et al.*, 2014). The core protein is 20kDa in mass, however, it has varying degrees of glycosylation leading to a natural product with a mass of between 21-26kDa (Tanaka *et al.*, 2014).

Interleukin-6 was first identified in 1986 and was initially called B-cell stimulatory factor-2 before further analysis was conducted and identified that Interleukin-6 had the same protein structure as several other molecules, allowing it to be known as a pleiotropic cytokine with a wide range of functions (Kishimoto, 2010). Interleukin-6 has been described as relatively unique compared to other cytokines, in that it is produced by a broad spectrum of cells in response to a variety of different stimuli (Rincon, 2012).

Under normal physiological conditions, the concentration of interleukin-6 is relatively low at a concentration of between 0-5pg/ml in human serum (Hunter & Jones, 2015). However, under disease conditions, such as post trauma, the concentration can rapidly rise to significantly higher levels than normal, allowing a disease condition to be determined compared to homeostatic conditions (Hunter & Jones, 2015).

Interleukin-6 is mainly secreted by T cells and macrophages, to stimulate an immune response following events such as: during infection and after traumatic events, namely burns and tissue damage leading to inflammation. However, it is also secreted from other lymphoid and non-lymphoid cells including B cells, fibroblasts, keratinocytes, endothelial cells, astrocytes and mesangial cells (Figure 12) (Kishimoto, 1989). It has several functions within the body that allow IL-6 to control systemic responses such as the acute phase response, the regulation of leukocyte recruitment, numerous effector functions and increasing the retention of inflammatory cells within inflamed tissue (Liu *et al.*, 2016).

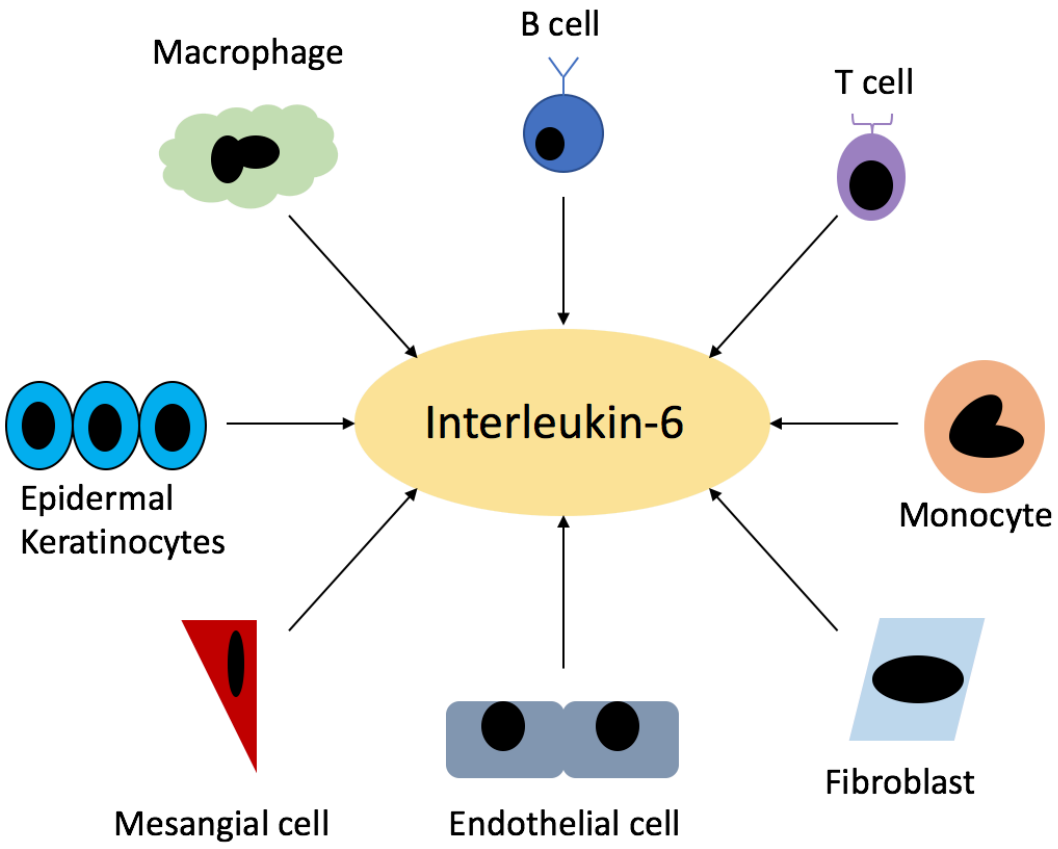

Figure 12) The range of cells, which produce interleukin-6.

Interleukin-6 has also been shown to act as a warning signal to the immune system, by indirectly acting as a DAMP due to its rapid synthesis following the damage to cells or the presence of a pathogenic organism (Tanaka *et al.*, 2014). The presence of Interleukin-6 tells the body that there has been damage and triggering an immune response at the location at which the causative factor has bound to the PRR (Tanaka *et al.*, 2014).

Interleukin-6 yields its biological activity on cells through interaction with two receptors: IL-6R and Glycoprotein 130 (Gp130) (Figure 13). Initial binding of interleukin-6 to IL-6R cannot itself initiate a signalling cascade due to its short cytoplasmic domain (Hassan *et al.*, 2014). Thus, the IL-6/IL-6R complex needs to bind to another cell membrane receptor known as Gp130. The dimerization of Gp130, following the binding of the IL-6/IL-6R complex, allowing the activation of protein kinases promoting the phosphorylation of downstream signalling molecules (Hunter & Jones, 2015).

The cytoplasmic domain of Gp130 is significantly larger than IL-6R, therefore it can contain many potential motifs that induce signal transduction such as the Signal transducer and activator of transcription (STAT) and Mitogen-activated protein kinase (MAPK) pathways (Kishimoto, 2010). However, Gp130 requires the further addition of a protein kinase such as Janus kinase (JAK) to deliver downstream signalling through these two pathways (Hassan *et al.*, 2014).

Once phosphorylated by the JAK, STAT can dimerise and pass into the nucleus, leading to the expression of genes by binding to promoter regions of target genes (Horn *et al.*, 2000). Gp130 is expressed on all cell types, while IL-6R is limited to a group of lymphoid cells including monocytes, macrophages, neutrophils, B-cells and some populations of T-cells, along with non-lymphoid cells such as hepatocytes (Chalaris *et al.*, 2011).

However, IL-6 signalling can take place on cells that do not possess IL-6R due to a process called trans-signalling through a soluble version of IL-6R (sIL-6R), produced following proteolytic cleavage or alternative splicing of the IL-6R molecule (Drutskaya *et al.*, 2015). This process of trans-signalling allows significant expansion of the types of cells that IL-6 can interact with, preventing it from exclusively acting on cells of the immune system (Drutskaya *et al.*, 2015).

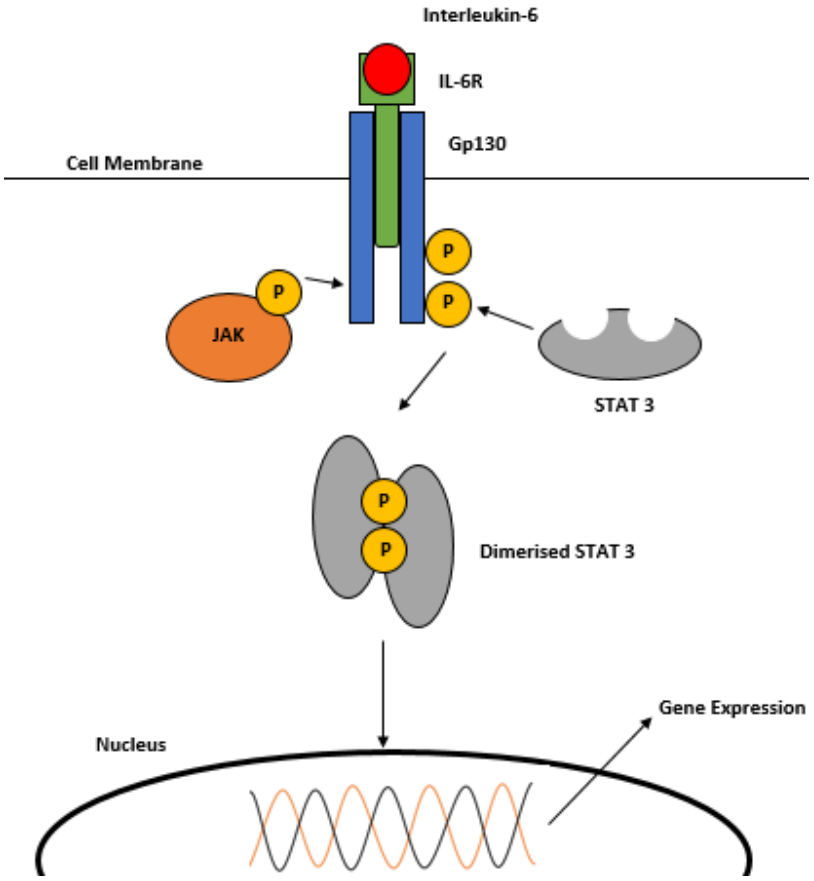

Figure 13) The process by which Interleukin-6 induces gene expression within cells possessing Gp130 on their cell membranes.

The binding of interleukin-6 to cells can induce several functional effects mainly to control the systemic response during inflammation, namely: the recruitment of leukocytes, effector functions and the retention of inflammatory cells within inflamed tissues (Liu *et al.*, 2016). Interleukin-6 causes the recruitment of leukocytes due to the binding of the IL-6/IL-6R complex to endothelial cells, activating them, resulting in the secretion of Interleukin-8 and monocyte chemoattractant protein 1 (MCP-1), and leading to the expression of adhesion molecules on the endothelial cells (Gabay, 2006).

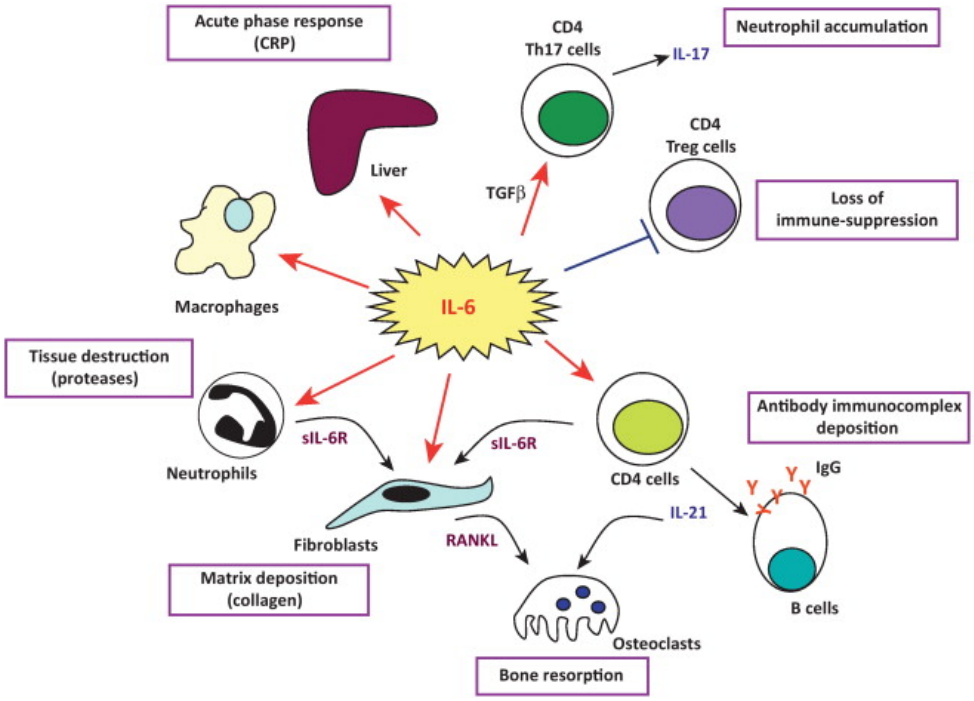

Figure 14) The key biological functions of Interleukin-6 and its effects on cell functions throughout the body. Image from Rincon, 2012.

Another key function of Interleukin-6 is to act as a regulator of the acute phase response (Figure 14). It does this by inducing the expression of acute phase response proteins such as C-Reactive Protein (CRP), Fibrinogen and Serum Amyloid A (Chalaris *et al.*, 2011). This process aids in the recruitment of immune cells to the site of inflammation and the clearance of any causative agent, which may have triggered the inflammatory response.

1.3.5 - Interleukin-10

Interleukin-10 (IL-10) is a major anti-inflammatory cytokine. It is classed as a type II cytokine (Th-2) and is a founding member of a family of cytokines including, interleukins-19, 20, 22, 24, 26, 28 and 29 (Mosser & Zhang, 2008). Interleukin-10 was first described as a product of T-helper class II cells, to inhibit cytokine synthesis in class I T-helper cells (Th-1) (Couper, Blount & Riley, 2008).

IL-10 is known to be produced by a variety of cells both immune and non-immune cells. It is produced by macrophages, dendritic cells, B cells and various subsets of CD4+ and CD8+ cells (Figure 15) (Couper, Blount & Riley, 2008). Additionally, non-immune effector cells, such as epithelial cells and keratinocytes are also capable of producing IL-10 in a response to infection or tissue damage (Iyer & Cheng, 2012).

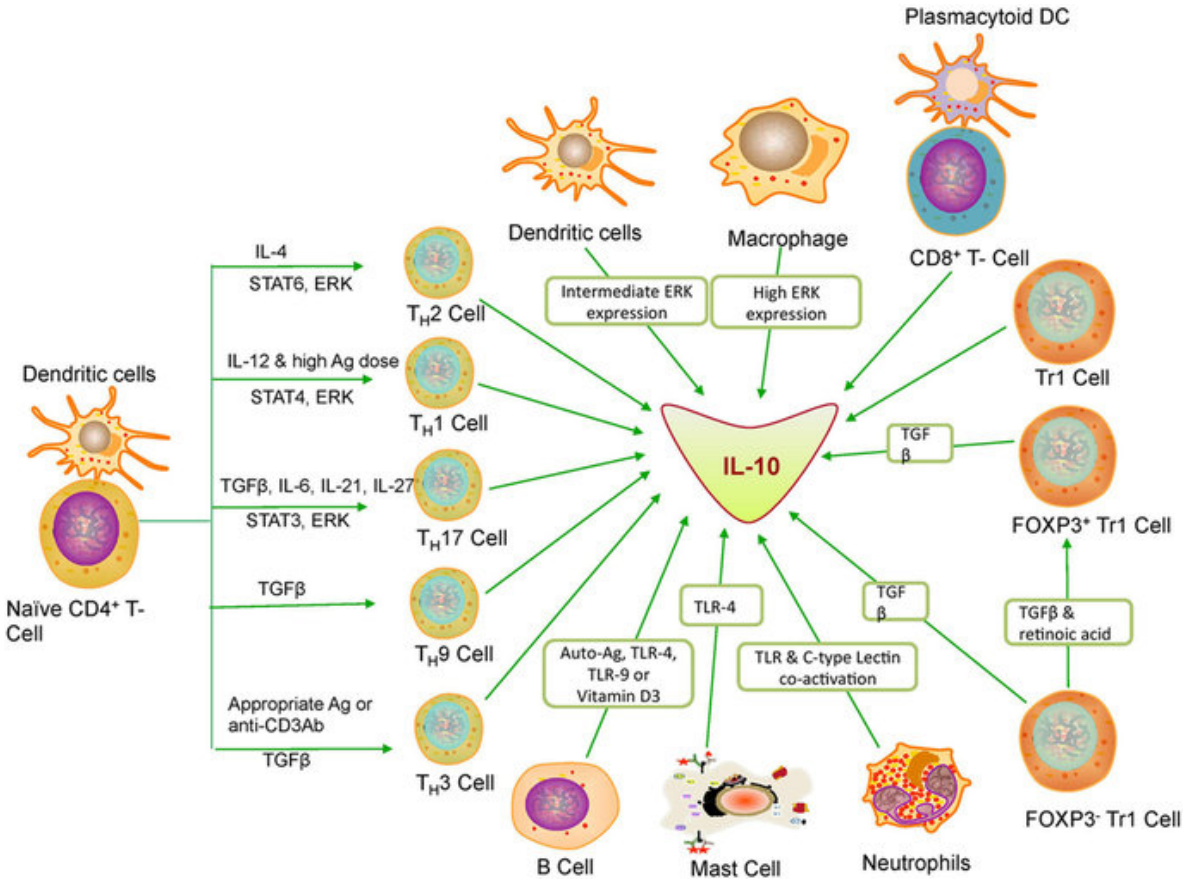

Figure 15) The types of cells that secrete interleukin-10 and the factors required to activate secretion from each of the cells (Nagpal *et al.*, 2017)

The gene that encodes the IL-10 protein spans approximately 4.7 kilobases on chromosome 1 21-32 (Iyer & Cheng, 2012). The gene coding IL-10 contains 5 exons and 4 introns (Iyer & Cheng, 2012). This gene encodes a biologically active form of IL-10, which is 36 kiloDaltons homodimer, composed of two non-covalently bonded monomers each made up of 160 amino acid chains (Verma *et al*., 2016). Two disulphide bridges link the two monomers, allowing the molecule to maintain its biological and structural activity (Verma *et al*., 2016).

Due to being derived from numerous different cell types, IL-10 has effector functions in numerous different cell types including, mast cells, eosinophils, dendritic cells, T-helper cells and IL-10 secreting regulatory T cells (Hawrylowicz & O'Garra, 2005). IL-10 also plays a critical role as a feedback regulator of diverse immune responses (Ouyang *et al*., 2011; Saraiva & O'Garra, 2010).

Interleukin-10 invokes its anti-inflammatory effects through its effect on antigen presenting cells (Figure 16). This prevents the production of Th-1 associated cytokines such as IL-2 and interferon gamma, and the Th-2 cytokines, IL-4 and IL-5 (Mosser & Zhang, 2008). The other effect of IL-10 is the inhibition of pro-inflammatory cytokine and mediator production from macrophages and dendritic cells (Sabat *et al*., 2010). This dramatically represses IL-1, IL-6, IL-12 and TNFα, following exposure to IL-10 (Keel *et al*., 1997; Sabat *et al*., 2010).

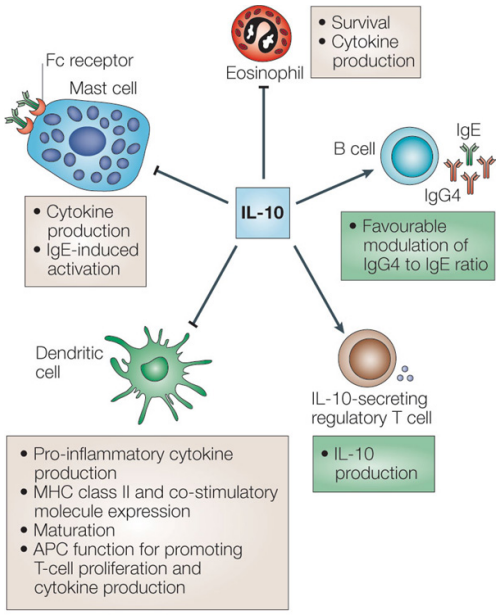

Figure 16) The main functions of interleukin-10 and the cells on which it acts (Hawrylowicz & O'Garra, 2005).

Not all IL-10 activity is immunosuppressive. IL-10 can stimulate B-cell activation, prolong B- cell survival and contribute to inducing antibody class switching in B-cells (Moore *et al*., 2001). It can also stimulate natural killer cells proliferation and cytokine production (Mosser & Zhang, 2008).

Interelukin-10 has also been shown to enhance the differentiation of IL-10 secreting T-regulatory cells (Treg), thus providing a positive regulatory feedback loop for the induction of IL-10 (Saraiva & O'Garra, 2010). However, *in vivo*, Tregs must receive signals to induce IL-10 secretion. In vitro analysis has shown that IL-2, IL-4 and TGFβ have been shown to induce IL-10 expression (Saraiva & O'Garra, 2010).

To invoke these immunomodulatory and anti-inflammatory effects, IL-10 needs to trigger signalling pathways within cells (Figure 17). This occurs through the binding of IL-10 to a two-receptor complex, consisting of interleukin-10 receptor 1 (IL-10R1) and interleukin-10 receptor 2 (IL-10R2) (Mosser & Zhang, 2010). IL-10R1 binds to IL-10 with a high affinity and causes a conformational change that leads to the recruitment and oligomerisation of IL-10R2 to the receptor (Hutchins *et al*., 2013). The binding of IL-10R2 and formation of the IL-10 receptor complex enables signal transduction following the binding of the IL-10 ligand (Verma *et al*., 2016).

Ligation of IL-10 to the receptor complex, activates the Janus-associated kinase (JAK)/signal transducer and activator of transcription (STAT) pathway, leading to large-scale changes in transcription and expression of immunomodulatory genes (Murray, 2006). This ligation causes phosphorylation of the receptor-associated JAK. This leads to phosphorylation of tyrosine residues on the intracellular domain of IL-10R1 (Verma *et al*., 2016). This phosphorylation allows STAT 3 to bind to IL-10R1 and itself become phosphorylated. The phosphorylated STAT 3 molecules can then dimerise to one another, allowing it to translocate through the nuclear membrane (Verma *et al*., 2016). Once in the nucleus, STAT 3 dimers can bind to STAT 3 binding elements in the promoters of various IL-10 responsive genes (Verma *et al*., 2016).

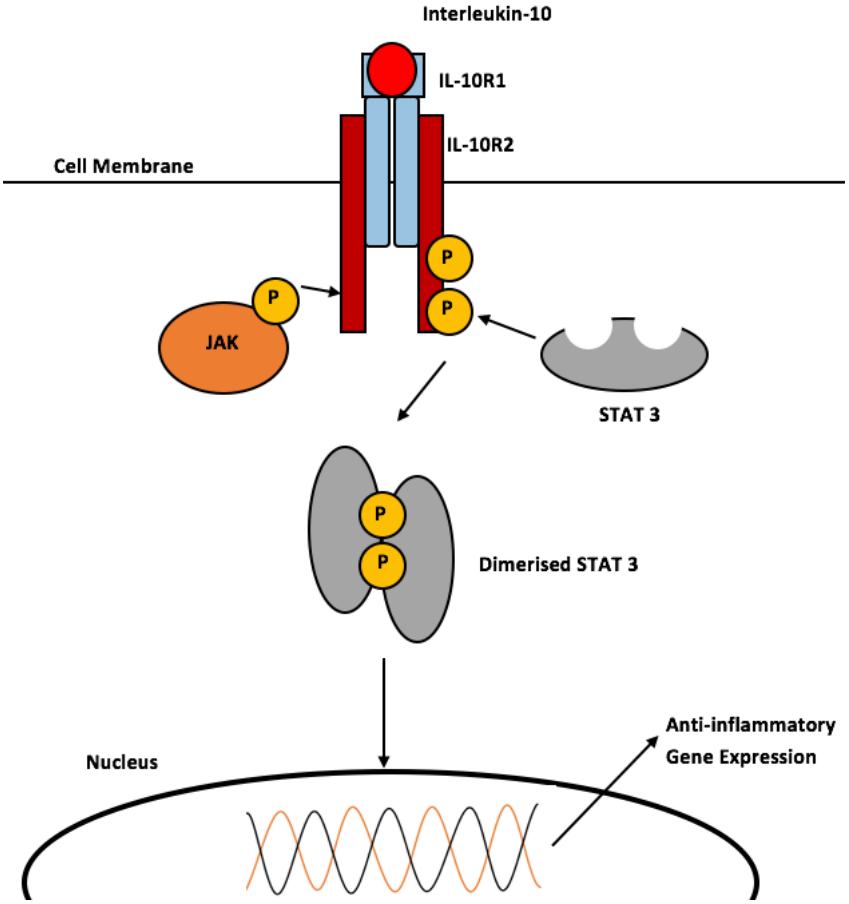

Figure 17) The Interleukin-10 signalling pathway. The binding of interleukin-10 to the IL-10R1 receptor, triggers the recruitment of IL-10R2, causing the downstream signalling through the JAK/STAT pathway. This ultimately results in changes in gene expression, enhancing the expression of anti-inflammatory genes.

The binding of STAT 3 dimers induces transcription of target genes. The activation and transcription of these genes invoke the immunomodulatory and anti-inflammatory effects associated with IL-10 (Mosser & Zhang, 2008). These effects are caused by inducing downregulation of pro-inflammatory cytokine gene expression or by increasing the expression of pro-inflammatory cytokine "mopping" molecules (Saraiva & O'Garra, 2010). The binding of STAT 3 can also induce the expression of the suppressor of cytokine signalling 3 (SOCS 3) (Iyer & Cheng, 2012). This molecule inhibits pathogen and damage recognition receptors, such as Toll-like receptors, ultimately leading to decreased expression of pro-inflammatory cytokines, including TNFα, IL-6 and IL-1β (Ouyang *et al.*, 2011).

Despite the ability of interleukin-10 to decrease pro-inflammatory responses, if the production of interleukin-10 is too strong, immunodeficiency can occur (Taylor *et al*., 2006). It has been shown that overexpression of IL-10 results in significant elevation in bacteraemia (Dolgachev *et al*., 2014). Experiments have also shown that elevated levels of interleukin-10 in trauma patients appears to be a predictor of poor prognosis, as it is associated with immunosuppressive complications, resulting in higher risk of infection, the development of multi-organ failure and death (Schneider *et al*., 2004).

Interleukin-10 is a key anti-inflammatory cytokine, that plays an important role in preventing an overactive pro-inflammatory response. However, if the anti-inflammatory response is too strong, it can lead to immune suppression and the development of adverse complications.

1.3.6 - Tumour Necrosis Factor alpha

Tumour Necrosis Factor alpha (TNF α), is a multifunctional cytokine playing a key role in apoptosis and cell survival as well as in inflammation and immunity (van Horssen, ten Hagen & Eggermont, 2006). TNFα is best known as an inflammatory cytokine produced predominantly by macrophages/monocytes, but can also be produced CD4+ lymphocytes, NK cells, neutrophils, mast cells, eosinophils, and neurones during acute inflammation and is responsible for a diverse range of signalling events within cells (Idriss & Naismith, 2000). Despite being predominantly a pro-inflammatory cytokine, TNFα has an optional capacity to induce apoptosis (Marques-Fernandez *et al*., 2013). Under pathophysiological conditions, TNFα shows dual functions, being strongly engaged in both tissue regeneration/expansion and destruction (Wajant, Pfizenmaier & Scheurich, 2003).

TNFα interacts with cells through two main receptors TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2) (MacEwan, 2002). TNFR1 is constitutively expressed in most tissues, where TNFR2 expression is highly regulated and is typically located on cells of the immune system (Wajant, Pfizenmaier & Scheurich, 2003). TNFα induces the NF-κβ signalling pathway. NF-κβ is a global trans-activator of numerous pro-inflammatory cytokines (IL-1, IL-2, IL-6, IL-8, IL-12 and TNFα), chemokines, adhesion molecules and cell survival molecules (Sedger & McDermott, 2014; Bradley, 2008).

If TNFα is produced in high amounts and present in systemic circulation, it can stimulate the production of pro-inflammatory cytokines IL-1 and IL-6 by leukocytes (Cereda *et al*., 2012). It also results in the synthesis of acute phase proteins in the liver, causing an acute phase response and intracellular clotting (Cereda *et al*., 2012). Following traumatic injury, TNFα has been shown to become elevated in the initial period after the injury (Spielmann *et al*., 2001; Namas *et al*., 2009). It has also been described that an elevated concentration of TNFα has protective properties, as it has been shown to be elevated in survivors of major trauma compared to non-survivors (Surbatovic *et al*., 2013).

1.3.7 - Methods of cytokine detection

The measurement of proteins within biological fluids is vitally important within scientific research. It plays a major role in the research of a variety of diseases ranging from cancer to inflammation, to vaccine development (Noble & Bailey, 2009). Methods used to measure the proteins include Western blots, Enzyme-linked immunosorbent assays (ELISA) and modern flow cytometry based cytometric bead array.

1.3.7.1 - Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assays (ELISA's) are a commonly used method in the scientific research field. It was first developed in 1971, following the modification of the radioimmunoassay. It uses the basic immunological concept of antigens binding to specific antibodies to detect molecules within a sample (Gan & Patel, 2013).

Several variations of the ELISA method have been developed since its creation in 1971, these mainly involve the use of different reporter molecules, or the position in the method where the antibody is added to the sample, either before or after the addition of the primary antibody. The main variations of the ELISA method are A direct ELISA, an indirect ELISA, sandwich ELISA's and competitive ELISA's (Figure 18).

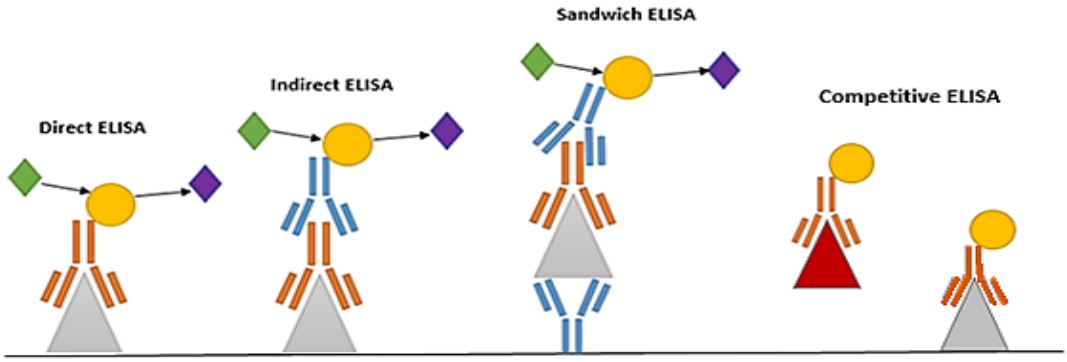

Figure 18) The four main types of ELISA commonly used in research. Primary antibody (orange); Secondary antibody (blue); reporter molecule (yellow); Substrate pre-reaction (green); Substrate post-reaction (purple); Red triangle represents incorrect antigen bound to antibody.

ELISA methods are similar throughout the variations that have arisen in the years since the development of the method. They all involve the initial binding of the desired antigen to a primary antibody before a secondary antibody is added that either binds to the primary antibody or the antigen (Bidwell *et al.*, 1976). The secondary antibodies are commonly linked to a reporter molecule, which exhibits a significant change when a substrate or the correct wavelength of light is added. The labels linked to antibodies are enzymes or fluorescent molecules that when provoked yield a visible colour change following the addition of a chromogenic substrate or the appropriate wavelength of light to stimulate the fluorescent molecule (Bidwell *et al.*, 1976). The amount of change that occurs can be correlated to the amount of antigen within a sample, with qualitative or quantitative measures can be assessed based upon the colorimetric reading (Gan & Patel, 2013).

The primary antibody can be directly conjugated with a reporter molecule, however this method is less reliable and more expensive than the use of the secondary antibody, as the reporter must be conjugated directly to a specific antibody for the antigen, rather than against a primary antibody, which can be used in the detection of several antigens that are bound to the same type of antibody.

The ELISA method has both its positives and negatives, which impact its current use in diagnostics and research. The ELISA assay shows both good reproducibility, along with good specificity and high sensitivity. Another benefit is that the method has been used in a vast amount of research journals and publications, meaning that both methods and results are available to review and compare. Due to this vast array of research a wide variety of ELISA kits are available for most molecules, and if kits are unavailable, primary antibodies can be created using animal models.

The ELISA method does have its drawbacks, mainly in the fact that it uses a relatively large volume of the sample compared to other methods available, this may prevent analysis being conducted if the sample size is small. Another negative to the ELISA method is that it is labour intensive and time consuming compared to other methods, as it requires multiple washing steps and incubation times. These washing steps can also lead to the development

of false positives arising as the excess antibodies are not correctly removed or false negatives through over-washing.

1.3.7.2 - Flow cytometry and the cytometric bead array

Flow cytometry is described as the measurement of physical and/or chemical characteristics of cells as they travel through a liquid stream (Shapirio, 2005). This liquid stream forces the cells into a single file line before they are passed through a light source, i.e. a laser. By passing through the source of light a cells size, granularity, roughness and internal structures can be analysed (Shapirio, 2005).

When cells pass through the light source, they cause the light to scatter, which can be measured by detectors (Figure 19). This scattering can be either forward scattering or side scattering. The greater the amount of light side scatter produced, the more granular a cell is, while the forward scatter of light relates to the size of the cell. The detectors that record changes in side scatter, forward scatter and fluorescence, relay the changes back to a computer, which analyses and visualises the data (Givan, 2013).

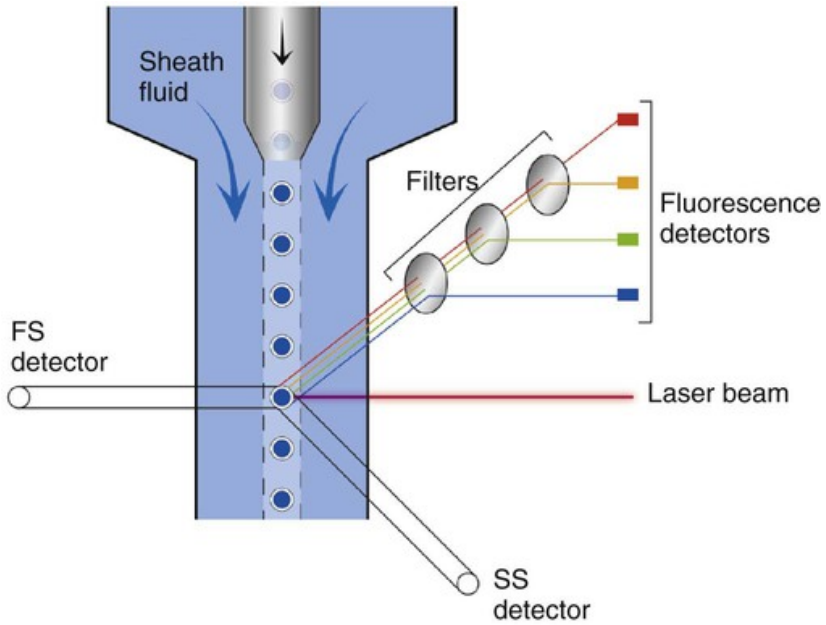

Figure 19) The process of measuring cell size and granularity using flow cytometry. Fluorescence of cellular structures can also be measured using specific monoclonal antibody fluorescent dye conjugates.

Both internal and external cellular components can be stained using monoclonal antibodies, conjugated with fluorescent dyes. This allows the presence of internal cellular components to be identified and quantified within a cell (Givan, 2013). This ability to measure the internal cellular structures provides a variety of uses such as measuring cell death, the effectiveness of cancer treatments, quantification of cellular structures or receptors and the measurement of proteins and other molecules within biological fluids.

Flow cytometry based methods are advancing each year, with the invention of more stable staining antibodies, better fluorophores and increasing the power of the flow cytometry software, allowing it to more accurately distinguish cells and measure changes in cellular fluorescence. Further inventions, such as novel approaches for the measurement of protein concentration in biological fluids, have allowed the measurement of proteins in biological fluids to continue to advance. One of these approaches is the cytometric bead array.

Cytometric bead arrays are a flow cytometry based method that allows multiple different analytes, namely proteins and cytokines, within a sample to be analysed simultaneously during a single test. Cytometric bead arrays use the broad range of fluorescence available using flow cytometry and antibody coated beads, to determine the concentration of analytes within a sample. Each analyte has its own antibody-coated bead, with its own unique fluorescence intensity allowing several different antibody coated beads to be analysed simultaneously in a single tube, termed a multiplex assay.

The cytometric bead array method employs the use of antibody coated beads to capture the chosen analytes (Figure 20-1 and Figure 20-2). During a multiplex assay, each type of capture bead will have its own light scatter characteristics allowing it to be differentiated from capture beads for a different analyte. Once the analytes have been captured, a fluorescent conjugate is added (Figure 20-3), which allows detection of the captured analytes during analysis using flow cytometry (Morgan *et al.*, 2004).

As the conjugated beads pass through the flow cytometer laser, set to the excitation wavelength of the fluorophore attached to the conjugate, the fluorophore is excited by the laser and emits a signal, which is detected by the flow cytometer and recorded (Figure 20-4). The amount of excitation that occurs, correlates with the concentration of the analytes within the sample, based on the results of known concentrations and the development of a standard curve.

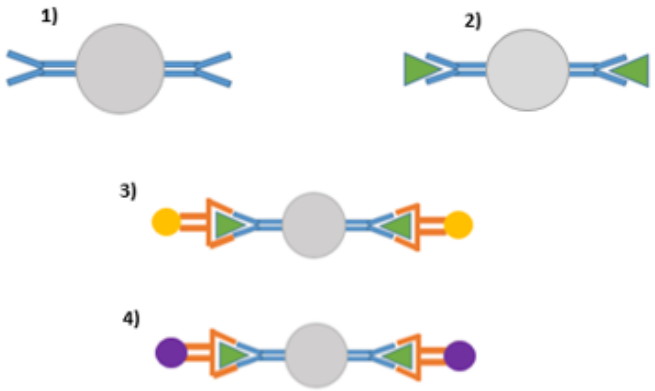

Figure 20) The cytometric bead array method for the detection of a specific analyte.

The ability to multiplex the capture beads allows the simplification of panel assays, as only a single sample is required to detect and quantify numerous analytes within a sample (Morgan *et al*., 2004). This is beneficial when sample volume is limited, for example during analysis of paediatric samples, Clinical studies and studies involving animal models. Cytometric bead arrays also save significant time compared to conventional methods due to the ability to analyse multiple analytes at once.

The cytometric bead array provides a highly sensitive way of detecting a large number cytokines found at low concentrations in pathogenic conditions (Jimenez *et al.*, 2005). It also is an attractive method to study immune response and biomarkers in large-scale studies due to the requirement of reduced sample volume and time-saving benefits for the researcher (Moncunill *et al.*, 2013).

However, the initial investment in equipment, namely the flow cytometer, to conduct the cytometric bead array method is significantly more expensive than the instruments required to conduct the ELISA method. Therefore, this may act as a barrier to entry for institutions, organisations and researchers to use the cytometric bead array method over the ELISA method.

1.4 - Clinical response to trauma

The clinical response to trauma involves several organ and metabolic systems including inflammatory, cytokine, coagulation and cellular responses. These responses are invoked by the initial injury and further exacerbated by secondary insults in the form of a biphasic response to trauma (Butt & Shrestha, 2008).

The first trauma impact determines the severity of the initial injury. This invokes the primary response, termed the first hit and acts to prime the immune system (Godinho *et al*., 2015). Further exogenous and endogenous factors, such as surgery, bacterial infection, respiratory distress and ischemia, can cause secondary activation of the immune response. The reactivation of the immune system invokes a significantly stronger response, compared to the response following the initial response (Harwood *et al*., 2005). This secondary activation of the immune system plays a key role in the initiation and severity of post traumatic complications (Keel & Trentz, 2005).

During trauma, the balance of the inflammatory response plays a key role in the ability of the body to recover following a traumatic insult. The initial immune response to trauma is to initiate a pro-inflammatory response at the focus of the injury. However, in major trauma, this pro-inflammatory response can occur systemically and induce a vast pro-inflammatory response throughout the whole body. If unregulated, this response can invoke damage to the body and induce early multi-organ failure.

The pro-inflammatory response is downregulated by an anti-inflammatory response. This response is the body attempting to maintain homoeostasis and prevent damage from an overactive pro-inflammatory response. But this anti-inflammatory response can cause damage to the body if it becomes overactive and dominates the inflammatory response to trauma. This overactive anti-inflammatory response can cause the body to develop a state of immunosuppression, leading to the susceptibility to nosocomial infections and the risk of developing systemic infections in the form of septic complications and late stage organ failure (Figure 21).

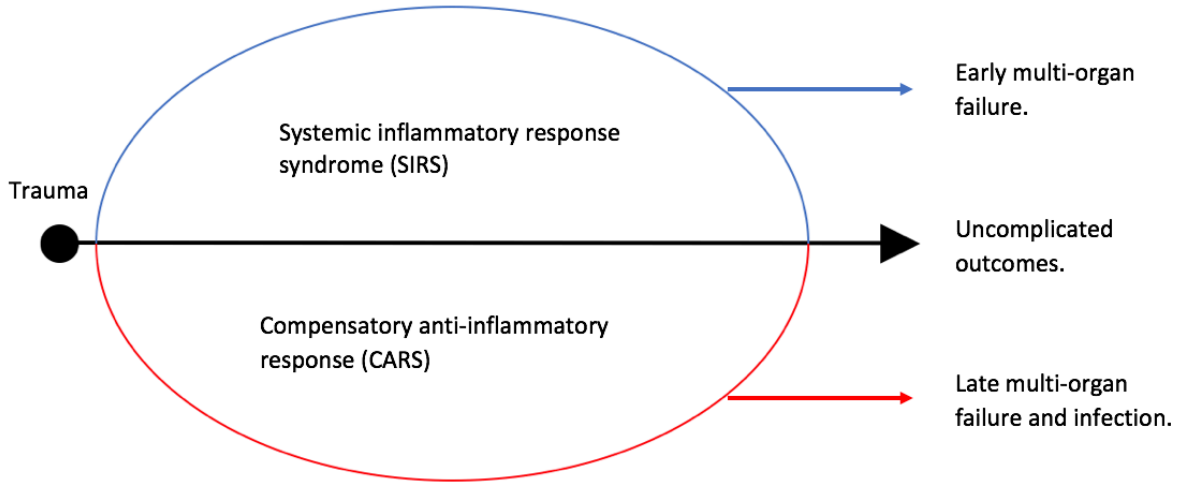

Figure 21) The balance between the systemic inflammatory response syndrome and the compensatory anti-inflammatory response, with the complications that arise of one response becomes dominant over the other.

In trauma, the inflammatory response to trauma is split into two main responses, the systemic inflammatory response syndrome (SIRS) and the compensatory pro-inflammatory response syndrome (CARS). These responses aim to balance each other to maintain homoeostasis, prevent complications and allow the initiation of optimised wound healing (Figure 21). It has long been thought that CARS follow SIRS, however, it has now been identified that both responses occur simultaneously (Rosenthal & Moore, 2016).

1.4.1 - Systemic inflammatory response syndrome

The concept of the systemic inflammatory response syndrome was first developed in 1991 at the sepsis definitions consensus conference (Bone *et al*., 1992). This concept was used to describe the complex chain of events involved in the response to numerous different pathologic disorders including, infection, traumatic injury, burns and pancreatitis (Balk, 2014).

Systemic inflammatory response syndrome is a result of systemic activation of the innate immune system, regardless of the causative agent (Hoesel & Ward, 2004). This response is differentiated from the response involved in sepsis and septic shock, by the need for an infectious stimulus to be present in sepsis and septic shock (Hoesel & Ward, 2004).
Many factors have been postulated to trigger SIRS, including products released from bacteria in the form of PAMPS, as well as intracellular products released during necrotic cell damage, induced following traumatic injury (Bosmann & Ward, 2013).

Systemic inflammatory response syndrome manifests itself as two of the following criteria in a clinical setting (Table 1). SIRS can clinically manifest itself as, hyperthermia or hypothermia, tachycardia, tachypnea or hyperventilation, or leukocytosis or leukopenia (Robertson & Coopersmith, 2006).

Table 1) The definitions for systemic inflammatory response syndrome and sepsis.

Systemic inflammatory response syndrome	
Temperature	>38^{o}C or 36^{o}C
Heart rate	>90 beats per minute
Respiratory rate	>20 breaths per minute
White blood cell count	>12x10^{9} or <4x10^{9}
Sepsis	
	SIRS plus evidence of infection

These criteria used to describe SIRS are general, due to it requiring clinical criteria that are commonly abnormal in a variety of different pathological conditions (Robertson & Coopersmith, 2006). Due to this the criteria for describing SIRS has been widely criticised due to it being too non-specific for widespread use.

Due to these criteria, the incidence of SIRS is extremely high within hospitals. With 33% of all hospital admissions meeting the criteria of undergoing SIRS. This rises even further in ICU patients (50%), and up to 80% of surgical ICU patients (Brun-Buisson, 2000).

The inducement of the systemic inflammatory response stimulates a variety of changes to immune cells. The hyper-inflammation associated with SIRS causes the enhanced expression of cellular adhesion molecules on blood leukocytes and endothelial cells, allowing the migration of monocytes and neutrophils into inflamed tissue (van der Poll & Meijers, 2010; Bosmann & Ward, 2013). This inflammatory response also activates neutrophils and macrophages, which generate abnormally high amounts of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Van Berlo *et al*., 2010; Bosmann & Ward, 2013). The greater the amount of these reactive oxygen and nitrogen species, the more extensive the chemical changes that can occur to cells at the site of inflammation (Storr *et al*., 2013). These reactive species can induce DNA damage and ultimately DNA fragmentation. This fragmentation causes cell death in cells exposed to high levels of reactive oxygen and nitrogen species (Storr *et al*., 2013; Bosmann & Ward, 2013). Cell death induced by reactive species, potentially further amplifies cell death following traumatic injury, causing additional damage to tissue and organs, rather than initiating the repair process following traumatic injury.

During the systemic pro-inflammatory response, there is a fierce interplay between cytokines. In cases where pro-inflammatory cytokines are rapidly produced with no or a delayed compensatory mechanism, can induce the development of a pro-inflammatory cytokine storm (Matsuda & Hattori, 2006). Cytokine storms have potential to do significant damage to body tissues and organs. If this occurs in the lungs, for example, it causes fluids and immune cells to accumulate within the organ, this can result in the blockage of airways and organ failure because of the adaptions induced by the cytokine storm.
These pro-inflammatory cytokines also activate the recruitment of phagocytic cells to the site of inflammation (Tisoncik *et al*., 2013). These cells release proteases and free oxygen radicals, which further increase the tissue damage induced by the traumatic injury.

A compensatory anti-inflammatory response commonly occurs immediately following the initiation of a systemic inflammatory response, to avoid the potential complications associated with an overactive pro-inflammatory response with no opposing response.

1.4.2 - Compensatory anti-inflammatory response syndrome

The compensatory anti-inflammatory response is the mechanism used to dampen the pro-inflammatory response induced by traumatic injury. This allows the body to return to normal levels of inflammation and maintain homoeostasis. The compensatory anti-inflammatory response was first described in 1996, by the presence of anti-inflammatory cytokines and pro-inflammatory antagonist receptors (Bone, 1996; Gentile *et al*., 2012).

Compensatory anti-inflammatory response induces a state, characterised by altered cytokine production, decreased antigen presentation, decreased lymphocyte proliferation and increased immune cell apoptosis (Zarjou & Agarwal, 2011). An exaggerated anti-inflammatory response can make individuals susceptible to nosocomial infection or secondary infection, which can initiate the septic cascade (Balk, 2014). The compensatory anti-inflammatory response involves cellular adaptions, characterised by lymphocyte dysfunction, including reduced proliferation and Th-1 cytokine production in response to antigens. Other responses involve lymphocyte apoptosis, downregulation of monocyte human leukocyte antigen receptors and monocyte deactivation, reduced pro-inflammatory cytokine production in response to stimuli, and increased interleukin-10 production (Ward, Casserly & Ayala, 2008).

The global down-regulation of the immune system has been observed through the down-regulation of both Th-1 and Th-2 responses in patients with severe burns and major trauma (Puyana *et al*., 1998). Lymphocytes from trauma and burns patients have reduced levels of Th1 cytokines but elevated Th-2 cytokines (IL-4 and IL-10). The reversal of this response has been shown to increase patient survival in patients who developed sepsis (Ward, Casserly & Ayala, 2008; O'Sullivan *et al*., 1995).

Genome-wide expression of blood leukocytes from severe blunt trauma patients identified the down-regulation of genes involved in T cell responses (Xiao *et al*., 2011). However, it also showed that antigen presentation and the upregulation of anti-inflammatory gene expression increased in parallel with the elevation in pro-inflammatory genes (Xiao *et al*., 2011; Gentile *et al*., 2012). This research confirmed that the systemic inflammatory response and compensatory anti-inflammatory response occur simultaneously, and the balance between these two responses is a key factor in optimal recovery following trauma.

The host defence response tries to strike a balance between SIRS and CARS to induce reparative mechanisms and limit the entry and potential overload of microbes (Keel & Trentz, 2005). This balance avoids a potential autoimmune inflammatory response, whilst also avoiding anti-inflammatory immunosuppression (Keel & Trentz, 2005).

1.5 - Complications of traumatic injury

The complications of traumatic injury have a major impact on patients in the period following trauma. There are two main peaks in trauma related deaths, the first, which occurs due to damage caused by the injury and a second peak of trauma related deaths, which happens because of conditions that arise in the period following injury. If the patient survives the initial traumatic insult, they are still at risk of developing complications, because of the inflammatory response that occurs to repair damage caused by the injury. This inflammation can become overactive and result in damage to tissue vascularity, resulting in ischemia and organ damage. Alternatively, the anti-inflammatory response can dominate and lead to immunosuppression. Anti-inflammatory immunosuppression causes the body to become susceptible to nosocomial infection, and potentially result in septic complications. These complications result in increased length of stay in the ICU and have major financial impacts on health services worldwide. Therefore, the ability to predict patients likely to develop these complications is of great importance.

1.5.1 - Acute respiratory distress syndrome

Since its first description in 1967, acute respiratory distress syndrome (ARDS) has been widely recognised as a major clinical problem worldwide, due to its high morbidity and mortality burden on the population (Ashbaugh *et al*., 1967; Confalonieri *et al*., 2017). The development of ARDS is commonly associated with blunt chest trauma (Salim *et al*., 2006), septic complications, aspiration of gastric contents and complications of long bone fractures.

The estimated incidence of ARDS ranges from 17-64 cases per 100,000 of the population per year (Monahan, 2013) (Blank & Napolitino, 2011). The associated mortality of ARDS is high, ranging from 11% to 68% worldwide. However, many of the patients included in these statistics die from multi-organ dysfunction opposed to directly for ARDS induced hypoxemia (Young & O'Sullivan, 2016). The development of ARDS is a major burden on health services, through the additional cost of treating the complication alongside the main cause of injury and the increased length of stay in the hospital to manage and treat the ARDS symptoms (Salim *et al*., 2006).

The extent to which ARDS has developed was originally measured using the American-European consensus conference (AECC) definition of ARDS. The definition, first described in 1994 states defines ARDS as; the acute onset of hypoxemia (Arterial pressure of oxygen to fraction of inspired oxygen [PaO^2/FiO^2] <200mmHg), with bilateral, infiltrates on frontal chest radiographs, with no evidence of hypertension (Table 2) (Force, 2012).

Table 2) The American-European Consensus Criteria for defining ARDS

The American-European Consensus Criteria
All the following features must be present. 1. Acute onset (<7 days) 2. Hypoxemia PaO^2/FiO^2 <200 mmHg 3. Diffuse bilateral pulmonary infiltrates on frontal chest radiograph, consistent pulmonary oedema; infiltrates can be patchy and/or asymmetric. 4. The absence of left atrial hypertension based upon clinical assessment or PCWP < 18mmHg if measured.
Abbreviations: PaO^2, partial pressure of oxygen; FiO^2, fraction of inspired oxygen; mmHg, millimetres of Mercury; PCWP, pulmonary capillary wedge pressure.

This definition of ARDS has received criticism, due to it not considering the relevance of precipitating conditions to prognosis, no uniform system of interpreting the radiograph results and no standardisation of mechanical ventilation support strategies to be used when hypoxemia is quantified (Leaver & Evans, 2007). These criticisms led to a modified version of the AECC criteria to be developed. Termed the Berlin definition of ARDS, this addressed most of the criticisms that the AECC definition received. This definition (Table 3), is now commonly used to identify the development of ARDS in patients who have suffered an injury to their chest cavity or lung tissue.

Acute respiratory distress syndrome is a syndrome of inflammation and increased the permeability of pulmonary tissue, associated with the development of clinical and physiological abnormalities (Sharma, 2010). The pathophysiology of ARDS progresses through three main stages, an acute stage, a proliferative stage and a fibrotic stage (Mackay & Al-Haddad, 2009).

Table 3) The Berlin definition of defining ARDS

The Berlin Definition	
Timing	Within one week of a known clinical insult or new or worsening respiratory symptoms.
Chest imaging	Bilateral opacities – not fully explained by effusions, lobar/lung collapse, or nodules.
Origin of oedema	Respiratory failure not fully explained by cardiac failure of fluid overload. Need objective assessment (e.g., echocardiogram) to exclude hydrostatic oedema if no risk factor present.
Oxygenation	
Mild	200 mmHg < PaO^2/FiO^2, <300 mmHg with PEEP or CPAP > 5cmH_2O
Moderate	100 mmHg < PaO^2/FiO^2, <200 mmHg with PEEP
Severe	PaO^2/FiO^2 < 100 mmHg with PEEP

Abbreviations: CPAP, continuous positive airway pressure; PaO^2, partial pressure of oxygen; FiO^2, fraction of inspired oxygen; PEEP, positive end-expiratory pressure.

The acute or exudative phase of ARDS is characterised by the rapid onset of acute respiratory failure within the first week following injury (Villar, 2011). The pathogenesis of this stage is a result of a combination of factors including damage from the initial injury and the activation of both local and systemic inflammatory responses (Villar, 2011). The initial insult triggers a series of cell-mediated responses, resulting in damage to the capillary endothelium and impaired fluid removal from the alveolar space (Villar, 2011). The pro-inflammatory response is invoked by the release of numerous pro-inflammatory cytokines including, TNFα, IL-1, and IL-6. This results in an escalation of neutrophil response, the accumulation of leukocytes and erythrocytes, along with platelet activation (Figure 22) (Bhatia & Moochhala, 2004).

These factors all result in increasing of vascular permeability, expansion of gaps in the alveolar epithelial membrane and triggering the necrosis of type I and II pneumocytes (Pierrakos *et al*., 2012; Monahan, 2013). This allows fluid to pass through into the lungs, leading to pulmonary oedema (Figure 22). Pulmonary oedema and damage to the alveolar membrane, cause the main symptoms of acute phase ARDS, including hypoxemia, decreased lung compliance, poor air exchange and pulmonary hypertension (Ware, Matthay & Zimmerman, 2012; Pierrakos *et al*., 2012; Monahan, 2013).

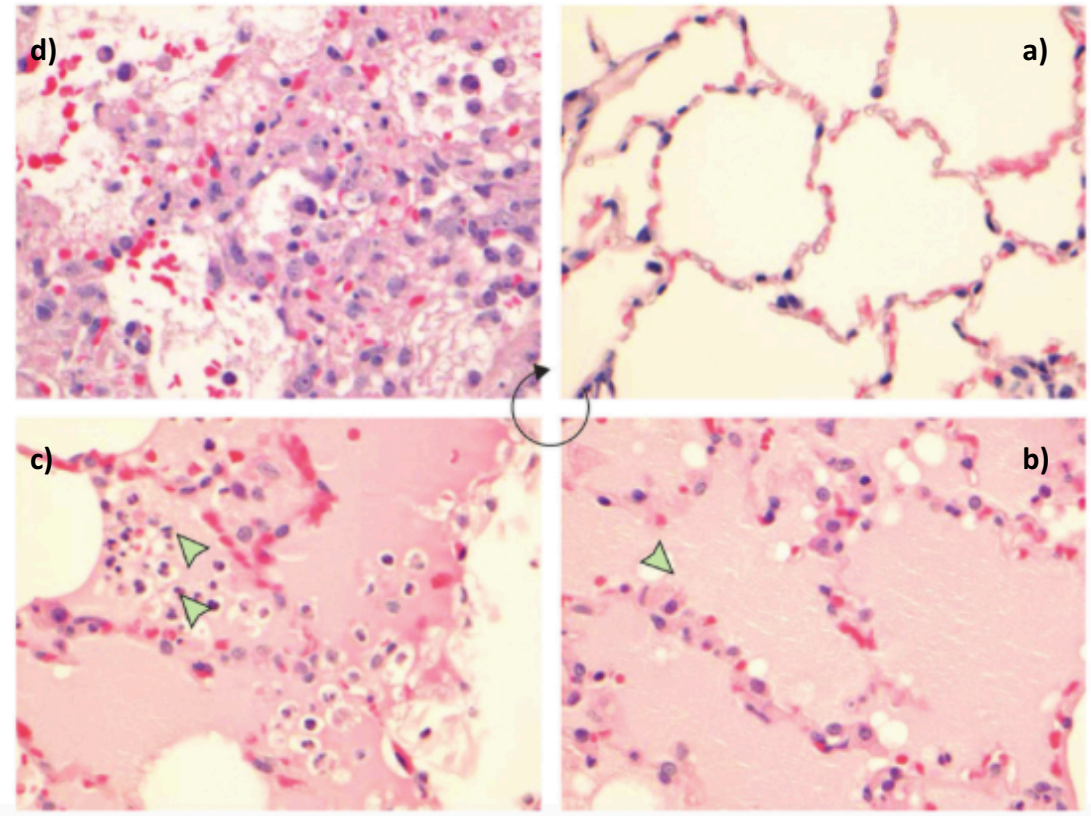

Figure 22) The cellular pathology cycle of ARDS. a) Normal alveolar tissue, filled with air. b) The alveoli begin to fill with proteinaceous fluid, shown by the green arrow. c) Leukocytes, mainly neutrophils, begin to infiltrate the alveoli, shown by the green arrow. d) Macrophages, erythrocytes and fibrin strands are now found in the alveoli. Adapted from Short *et al.*, 2013.

Although most survivors of ARDS return to normal lung function within 6-12 months, following the proliferative phase (Ware, Matthay & Zimmerman, 2012). Proliferation allows pneumocytes and pulmonary epithelial cells to replenish following the damage caused during the acute phase. This resolution of tissue damage causes complications during the period where the tissue recovers. Symptoms of lung tissue resolution involve, exercise intolerance, muscle weakness and diminished mental function following prolonged hypoxemia (Monahan, 2013).

However, if the lungs cannot resolve the damage, fibrotic methods are used to repair damage (Ware, Matthay & Zimmerman, 2012). Patients undergoing fibrotic repair exhibit persistent hypoxemia, and a further decrease in pulmonary compliance (Herridge *et al.*, 2011; Monahan, 2013). They become reliant on external mechanical ventilation and have significant levels of disability and mortality compared to ARDS patients who successfully resolve the damage caused by ARDS (Herridge *et al.*, 2011).

1.5.2 - Multiple Organ Dysfunction and Multiple Organ Failure

Multiple organ dysfunction syndrome (MODS) is defined as the development of potentially reversible physiological derangements, involving two or more organ systems, which are not directly involved in the admission to the intensive care unit and arise in the wake of a potentially life threatening insult (Holzheimer & Mannick, 2001). This organ dysfunction ultimately leads to organ failure and the development of multiple organ failure (MOF), if two or more organ systems fail.

The incidence of MODS ranges anywhere from 11% to 40% in patients admitted to the ICU (Ramirez, 2013; Osterbur *et al*., 2014). This is the most common cause of death in the ICU, being responsible for 80% of all intensive care deaths (Osterbur *et al*., 2014). Multiple organ dysfunction is also the leading cause of mortality in trauma patients, who survive the initial traumatic injury (El-Menyar *et al*., 2012; Maier *et al*., 2007). The mortality rate of intensive care related MODS increases based on a number of organ systems which are undergoing dysfunction or have failed (Table 4) (Balk & Goyette, 2002). The ICU mortality rate is lowest in patients who have no organ dysfunction (9%), increasing progressively with the number of organs which have developed dysfunction and begun to fail (Balk & Goyette, 2002). The failure of two organ systems to meet the criteria of MOF has an associated mortality rate of 38% (Balk & Goyette, 2002). This increases to a mortality rate of 83% in patients who develop organ failure in four or more organ systems (Balk & Goyette, 2002).

Patients who develop MODS or MOF commonly develop pulmonary MODS before any other organ system, with this occurring in 99% of all MODS ICU patients (Ceisla *et al*., 2004; Dewar *et al*., 2009). This is due to capillary leakage, resulting in alveolar flooding and deactivation of pulmonary surfactant production (Ramirez, 2013).

Table 4) The Multiple organ dysfunction score criteria (Marshall *et al*., 1995)

Organ system	Score points				
	0	**1**	**2**	**3**	**4**
Respiratory (PaO_2/FiO_2)	>300	226-300	151-225	76-150	<75
Renal (Serum Creatinine) (umol/L)	<100	101-200	201-350	351-500	>500
Hepatic (Serum Bilirubin) (umol/L)	<20	21-60	61-120	121-240	>240
Cardiovascular (PAR) (HRxCVP/MAP)	<10	10.1-15	15.1-20	20.1-30	>30
Haematological (Platelet count) ($x10^3$/ml)	>120	81-120	51-80	21-50	<20
Neurological (Glasgow Coma Scale)	15	13-14	10-12	7-9	<6

Abbreviations: PaO^2, partial pressure of oxygen; FiO^2, fraction of inspired oxygen; µmol/L, micromole/litre; HR, heart rate; CVP, central venous pressure; MAP, mean arterial pressure.

Multiple organ failure can occur during two main periods following traumatic injury. It can be classed as either early MOF where it occurs within 48 hours of injury, or be termed late MOF, which occurs after 72 hours from the time of injury (Dewar *et al*., 2011; Maier *et al*., 2007). Early organ failure is commonly a direct result of the traumatic injury, which has led to ischemic and necrotic damage to the organ, triggering the organ to fail (Dewar *et al*., 2011). Late organ failure is a consequence of a secondary inflammatory insult, such as infective complications, which cause the body to invoke a rapid and strong inflammatory response. This strong inflammatory response is associated with the dysfunction of the immune system, caused by an excessive pro-inflammatory response with normal or decreased compensatory anti-inflammatory response (Ramirez, 2013). This leads to the loss of homoeostasis between the pro and anti-inflammatory responses (Ramirez, 2013).

The loss of homoeostasis is a common result of the two-hit hypothesis of inflammation, meaning that the second hit, which triggers the loss of homoeostasis in the ICU. This can be caused by an otherwise innocuous nosocomial infection such as pneumonia (Jin *et al*., 2016). This immune dysregulation can result in hyper-inflammation, causing damage surrounding microvessels resulting in ischemia to regions of the organ/tissue (Rendy, Sapan & Kalesaran, 2017).

The reperfusion of these ischemic regions to re-oxygenate the tissue can have a major impact on the development of multiple organ failure, through the flushing of pro-inflammatory mediators, cellular micro-aggregates and reactive oxygen species (Baue, 2000; Dewar *et al*., 2009; Balk & Goyette, 2002). The release of pro-inflammatory mediators, following ischemia, can invoke an inflammatory response within tissues and organs away from the initial site of ischemia. This results in the expansion of the ischemia, allowing the generation of further inflammatory mediators, which can be released following further tissue reperfusion (Balk & Goyette, 2002). These inflammatory mediators induce endothelial damage and increase vascular permeability, allowing the leakage of fluid out of the blood vessels and enter tissues, resulting in oedema.

Cellular micro-aggregates are commonly composed of neutrophils, platelets, red blood cells and fibrin (Sibinga & Cash, 2001). These aggregates are often seen following the transfusion of blood into trauma patients (Sibinga & Cash, 2001). These can impair the blood flow in capillaries and post-capillary venules resulting in tissue hypoxia and ischemia (Balk & Goyette, 2002). This ischemic damage persists after reperfusion, resulting in tissue/organ damage and dysfunction (Balk & Goyette, 2002). This damage, following prolonged ischemia, may ultimately result in organ failure.

Reactive oxygen species are released from damaged tissues, with unregulated elevations in ROS concentrations causing damage to lipids, proteins and DNA (Scheiber & Chandel, 2014). These elevations have been shown to increase pro-inflammatory cytokine production (Kong *et al*., 2011). This exacerbates the innate immune response during inflammation to pathogens and DAMP's following trauma, resulting in impaired clearance of pathogens and the enhancement of septic complications (Thimmulappa *et al*., 2006; Scheiber & Chandel, 2014). Each of these substances that are washed through the body can act as a secondary hit inflammatory insult. However, if all the substances released at once from ischemic tissue, it can trigger a massive systemic inflammatory insult that can impact multiple organs throughout the body simultaneously. Despite the incidence of MODS and MOF decreasing in recent years due to the advancement of ICU care methods and techniques (Nost-Kolb *et al*., 2001), it remains a major burden of the world's healthcare systems, due to it increasing patient's length of stay, hospital resource use and ICU mortality rate.

1.5.3 - Infection and sepsis

Sepsis is a life-threatening condition that arises when the body's response to the presence of infection causes injury to its own tissues and organs. It is defined by the presence (confirmed or unconfirmed) of infection that can result in acute organ dysfunction, hypotension and ultimately death (Dellinger *et al.*, 2013). Sepsis is described as an illness caused by the presence of infection in a normally sterile area of the body (Lever & Mackenzie, 2007). This description is commonly used to differentiate conditions that share identical symptoms to sepsis, which may arise from non-microbial conditions such as pancreatitis (Lever & Mackenzie, 2007).

Sepsis is the leading cause of complication in critically ill patients worldwide, this is despite continual advances in antibiotics and resuscitation therapies (Pierrakos & Vincent, 2010). Sepsis affects 3 in 1000 people per year, accounting for over 750,000 cases per year in the United States and is responsible for more than 65,000 admissions annually in the United Kingdom (Angus *et al.*, 2001; Gupta & Jonas, 2006). In the United Kingdom sepsis causes 36,800 deaths per year, making it the second highest cause of death in the UK behind coronary heart disease (Griffiths & Anderson, 2009).

Sepsis has become a major financial burden on health systems worldwide (Prucha, Bellingan & Zazula, 2015), due to longer lengths of stay in the hospital, increased antibiotic usage, and the necessity for patients to be placed in intensive care units (ICU's). There are varying degrees of severity for sepsis conditions, including sepsis, severe sepsis and septic shock. These are differentiated based on the type of symptoms exhibited by the patient. Sepsis is the presence of a microorganism within the blood; it is the most common type of sepsis along with having the lowest mortality rate (Singer *et al.*, 2016).

Severe sepsis occurs when a septic patient develops evidence of hypoperfusion or the dysfunction of at least one organ (Singer *et al.*, 2016). This condition is more severe than sepsis and has a higher mortality rate. Septic shock is the most severe form of sepsis and occurs when the patient suffers from hypotension or requires vasopressors, along with fluid resuscitation having no or minimal effect (Lever & Mackenzie, 2007).

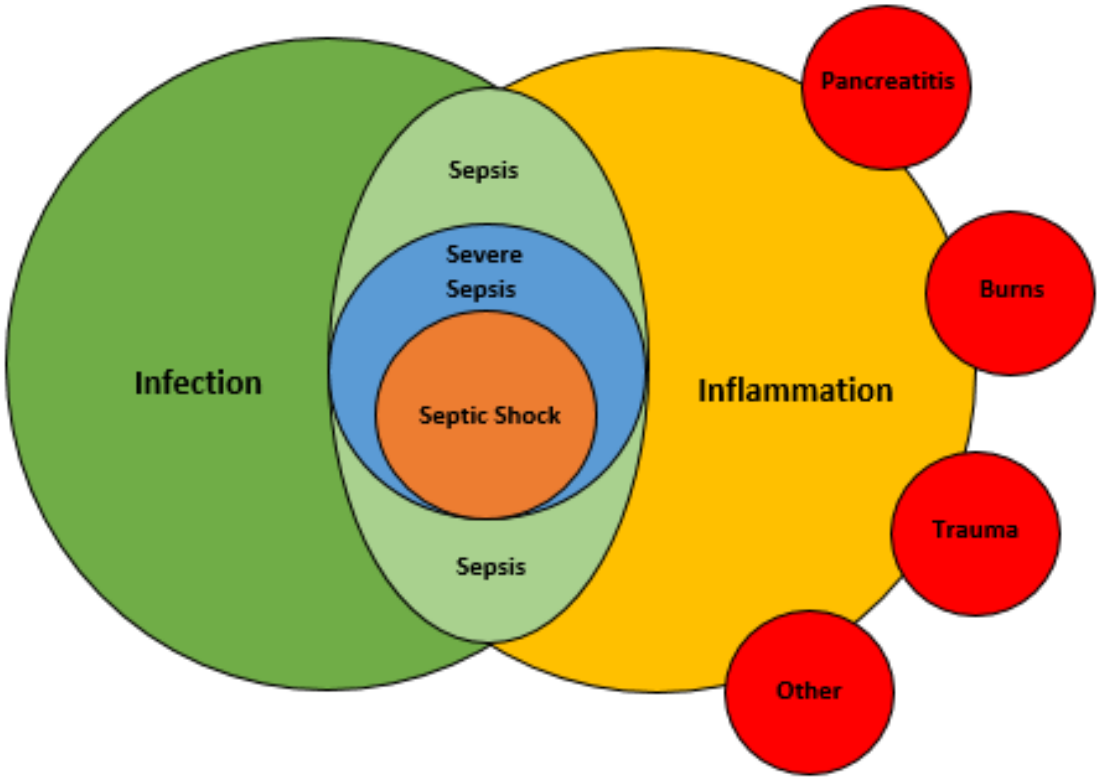

Figure 23) The link between Infection and Inflammation that leads to the development of sepsis.

Sepsis commonly originates following a breach in the integrity of a host barrier of protection, such as the skin during surgery (Lever & Mackenzie, 2007). The entrance of a microbe following the loss of barrier integrity causes an initial host response to try to clear the organism. However, in sepsis cases this response becomes amplified and dysregulated, resulting in the symptoms associated with sepsis (Cohen, 2002).

The septic response is a complex chain of events involving inflammatory processes, humoral and cellular abnormalities along with circulatory aberrations (Figure 23) (Pierrakos & Vincent, 2010). These events result in tissue hypoperfusion and hypoxia, which are dominant factors in the development of organ failure during sepsis (Cohen, 2002).

1.5.4 - Coagulation disorders following traumatic injury

The human body possesses intrinsic mechanisms to regulate haemostasis, allowing the balance of clot formation and breakdown to be maintained. However, an imbalance in these mechanisms can result in the development of coagulation disorders. Coagulation disorders are present in most trauma patients in the form of haemorrhagic disorders, thrombosis and in the case of disseminated intravascular coagulation (Miniello *et al*., 2004). Coagulation abnormalities occur quickly after trauma with fibrinogen, reaching critical levels, with coagulation abnormalities being present within 25 minutes of injury (Maegele, Schochl & Cohen, 2014; Floccard *et al*., 2012). Coagulopathies are encountered in 25-30% of trauma patients and are associated with a worse outcome (Rugeri *et al*., 2007). These haemostatic manifestations are associated with increased need for blood transfusion, a longer length of hospital stay and a higher mortality rate (Martini, 2016).

In cases of trauma death following massive haemorrhage, commonly due to non-surgical bleeding or trauma related coagulopathy (MacLeod, 2008). Trauma induced coagulopathy (TIC) is a complex disorder and is not fully understood (White, 2013). It involves several key processes including dysfunction of natural anticoagulant mechanisms, platelet dysfunction, fibrinogen consumption and hyper-fibrinolysis, which have all been identified as potential causative factors of TIC (White, 2013).

In the immediate period following traumatic injury, the injury exposes tissue factor the circulation, initiating thrombin generation and clot formation. Platelets also become activated through cellular signalling mechanisms including collagen, glycoprotein VI, von Willebrand Factor (vWF) and glycoprotein Ib. The activation of platelets amplifies thrombin generation and the clotting process, causing the consumption of coagulation factors. Patients who develop massive haemorrhage due to major trauma, often develop metabolic failure, commonly referred to as “the triad of death” (Figure 24) (Maani, DeSocio & Holcomb, 2009). This triad is composed of hypothermia, acidosis and coagulopathy (MacLeod, 2008).

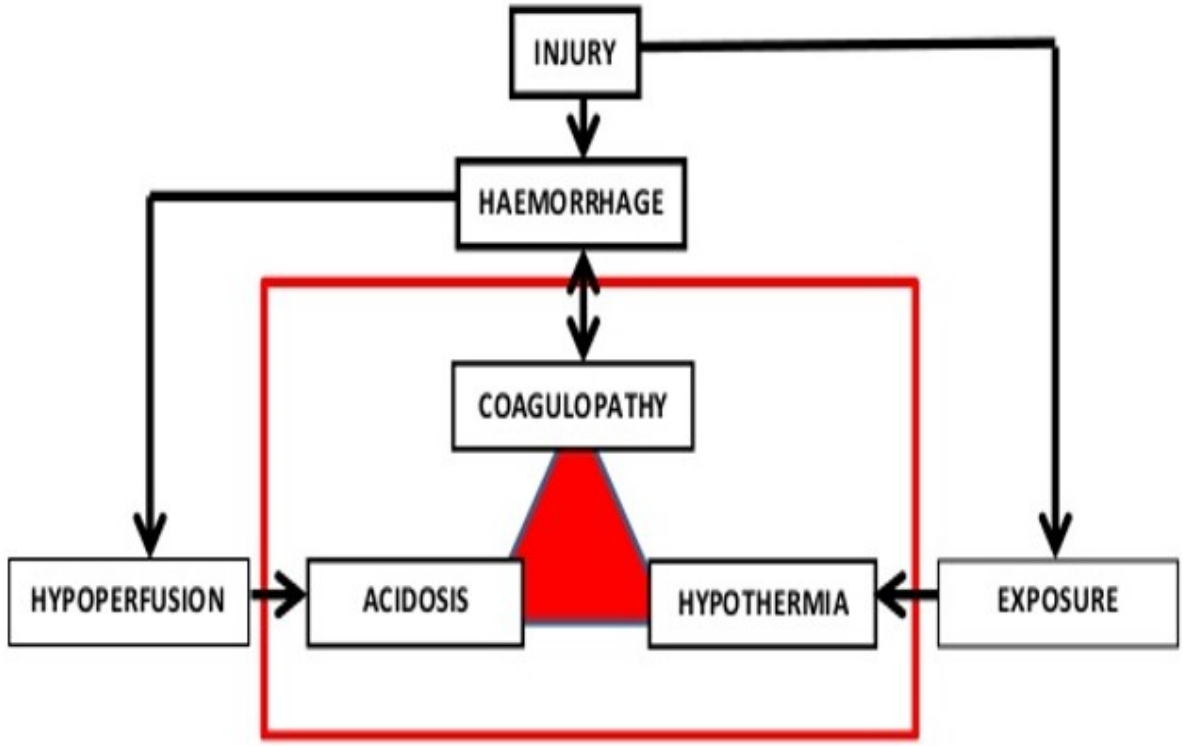

Figure 24) The lethal triad of coagulopathy disorders following traumatic injury.

Hypothermia is a decrease in core body temperature, which is caused by blood loss or exposure at the point of traumatic injury. Hypothermia can result in impaired stability of fibrin clots, as well as slowing the initiation and propagation of the coagulation cascade (Dirkmann *et al*., 2008). The hypothermic insult is associated with a decrease in thrombin generation, as well as compromised formation of platelet plugs and fibrin clots (Maani, DeSocio & Holcomb, 2009). Johnston, Chen & Reed, 1994, observed that a decrease in body temperature caused a decrease in all coagulation factors, lowering the temperatures further reduced the amount of functional clotting factors.

Acidosis is the decrease in blood pH caused by an increase in metabolic acids due to hypoxemia and hypo-perfusion. Acidosis can worsen existing coagulopathies by inhibiting enzyme complexes, which are vital to clot formation (Dirkmann *et al*., 2008). This inhibition of enzyme complexes by blocking the binding enzyme complexes to positively charged lipid membranes (Tieu, Holcomb & Schreiber, 2007).

Acidosis was found to have a profound inhibitory effect on the thrombin generation rate; this was further exacerbated when combined with hypothermia (Martini *et al*., 2005). A reduction of blood pH from 7.4 to 7.0 decreased the activity of Factor VIIa by 90%, and the rate of prothrombin activation by 70% (Meng *et al*., 2003). In a clinical setting, significant bleeding was found to occur in patients with acidosis and hypothermic complications, despite adequate blood, platelet and plasma replacement (Tieu, Holcomb & Schreiber, 2007).

Coagulation factors can be depleted or diluted, resulting in an impaired clotting response. The direct loss of coagulation factors through haemorrhage can quickly deplete the bodies stores of fibrinogen and platelets (Martini *et al*., 2005). As a part of the response to traumatic haemorrhage clinicians and paramedics often give patients intravenous fluids to maintain blood volume (Iijima *et al*., 2013). These intravenous fluids dilute the remaining blood components including clotting factors decreasing the effectiveness of the clotting cascade and resulting in impaired coagulation (Maegele, Schochl & Cohen, 2014).

As a part of the response to traumatic injury, an inflammatory response occurs. This inflammatory response can increase procoagulant activity (Pierce & Pitlet, 2014). Inflammatory cytokines may also activate platelets and increase their expression of procoagulants (MacLeod, 2008). This reaction along with suppression of anticoagulant activity and fibrinolysis results in the development of disseminated intravascular coagulopathy (DIC) (ten Cate, 2000).

The development of DIC is partly explained by the early activation of protein C, resulting in its depletion (Brohi, Cohen & Davenport, 2007). As protein C is re-synthesised, it returns to normal concentrations, during which a hypercoagulable state exists (Brohi, Cohen & Davenport, 2007). Several studies have identified a late hypercoagulable state, increasing the risk of thromboembolic complications (Shreiber *et al*., 2005; Engelman *et al*., 1996). These complications can trigger the blockage of post capillary venules, resulting in hypoxia to organs and tissues, which may lead to multiple organ failure (Kidokoro, Iba and Hong, 1999).

1.6 - Clinical measurements of traumatic injury severity

Trauma centres around the world use a variety of different systems to identify and stratify patients based upon the types of injury that they receive during trauma. These scoring systems are used to identify patients who have the most severe injuries and provide insight into potential therapeutic interventions that are required to treat them or identify those patients who are at the greatest risk of developing complications in the days and weeks after the traumatic injury.

The most commonly used scoring systems used in trauma are, the Acute Physiology and Chronic Health Evaluation (APACHE) system, Injury severity score (ISS), Sequential Organ Failure Assessment (SOFA) score and the Glasgow Coma Scale (GCS). Each of these systems has its own positives and negatives as they each identify factors that other systems do not. Each scoring system will be further explained within this section.

1.6.1 - Acute Physiology and Chronic Health Evaluation score

The APACHE scoring system, first developed in 1985, is used to predict mortality and measure injury severity in critically ill patients who have been admitted to the Intensive Care Unit of a hospital (Knaus *et al*., 1985). The patient's APACHE score is calculated within 24 hours of admission to the ICU, with the score being calculated based upon 13 physiological factors measured within the 24 hours following admission. The 13 factors used in the APACHE calculation are PaO2, temperature, mean arterial pressure, arterial pH, heart rate, respiratory rate, serum sodium concentration, serum potassium concentration, creatinine, haematocrit, white blood cell count and the Glasgow Coma Scale score (Knaus *et al*., 1985). From these physiological variables, an overall score ranging from 0 to 71 is calculated (Figure 25).

Patients who score higher on the APACHE scale, have more severe injuries and a greater risk of in-hospital mortality due to their injuries (Rapsang & Shyam, 2014). APACHE scores have been shown to inform on patient prognosis and assist the investigator in comparing and evaluating different forms of treatment or therapeutic options (Rapsang & Shyam, 2014).

Physiologic Variable	+4	+3	+2	+1	0	+1	+2	+3	+4
Rectal temperature (C)	≥ 41	39-40.9		38-38.9	36-38.4	34-35.9	32-33.9	30-31.9	≤ 29.9
Mean arterial pressure (mm Hg)	≥ 160	130-159	110-129		70-109		50-69		≤ 49
Heart rate (bpm)	≥ 180	140-179	110-139		70-109		55-69	40-54	≤ 39
Respiratory rate (bpm)	≥ 50	35-49		25-34	12-24	10-11	6-9		≤ 5
Oxygen delivery (mL/min) OR PaO_2 (mm Hg)	≥ 500	350-499	200-349		< 200 > 70	61-70		55-60	< 55
Arterial pH	≥ 7.7	7.6-7.69		7.5-7.59	7.33-7.49		7.25-7.32	7.15-7.24	< 7.15
Serum sodium (mmol/L)	≥ 180	160-179	155-159	150-154	130-149		120-129	111-119	≤ 110
Serum potassium (mmol/L)	≥ 7	6-6.9		5.5-5.9	3.5-5.4	3-3.4	2.5-2.9		< 2.5
Serum creatinine (mg/dL)	≥ 3.5	2-3.4	1.5-1.9		0.6-1.4		< 0.6		
Hematocrit (%)	≥ 60		50-59.9	46-49.9	30-45.9		20-29.9		< 20
White cell count (10^3/mL)	≥ 40		20-39.9	15-19.9	3-14.9		1-2.9		< 1

Age Points

Age	Points
≤ 44	0
45-54	2
55-64	3
65-74	5
≥75	6

Chronic Health Points

History of Severe Organ Insufficiency	Points
Nonoperative patients	5
Emergency postoperative patients	5
Elective postoperative patients	2

Figure 25) The scoring criteria used to generate the APACHE II score. It is a measure of 11 physiological criteria, along with age and patient chronic health status. The points for each of these categories are added together to generate the APACHE II score.

The APACHE score has shown a good calibration and discriminatory value across a range of disease processes and remains a commonly used international severity scoring system worldwide (Bouch & Thompson, 2008). Several variants of the APACHE scoring system have been developed since its inception, to enhance the predictive and diagnostic power of the scoring system. Overall, the APACHE scoring system provides a robust predictor of patient outcomes, due to the use of a high number of physiological variables and the identification of patient's co-morbidity status to determine the risk of complications or mortality to the patient.

1.6.2 - Injury Severity Score

The Injury severity score (ISS) is a method of scoring the severity of injury in polytrauma patients. It has become the most common and widely used scoring system worldwide since its creation in 1976 (Baker *et al*., 1976). The score is calculated based upon the description of six regions of the body, namely: a) External, skin injuries; b) Limbs; Abdomen, including organs and lumbar spine; c) Chest, including organs, diaphragm, rib cage, thoracic spine; Face; Head and neck, including the brain and cervical spine (Restrepo-Álvarez *et al.*, 2016).

The injury severity score is calculated from the sum of the squares of the single highest Abbreviated Injury Scale (AIS) score for each of the three most severely injured body regions (Deng *et al.*, 2016). The injury severity is purely based on the anatomical findings defined by the AIS (Paffrath *et al*.,2014). The most recent version of the AIS, adapted in 2008, lists approximately 2000 different injuries to nine regions of the body (Lossius *et al*., 2012).

The severity of each injury is graded on a scale from 1 to 6 (Table 5), where a score of 1 describes a minor injury, while a score of 6 is given for untreatable, mostly lethal injuries (Paffrath *et al*., 2014). Most clinical studies describe patients who are critically ill trauma patients as having an ISS of >16, these patients had an associated 10% increase in mortality rate (Champion *et al*., 1990).

Table 5) The Abbreviated Injury Scale injury scoring table, used to enumerate the severity of trauma injuries and calculate trauma patient's injury severity score.

Injury severity	Abbreviated injury score
Minor	1
Moderate	2
Serious	3
Severe	4
Critical	5
Probably lethal	6

The ISS is limited in the fact that it is calculated based on the squared values of three body regions. This means that if a person suffers multiple injuries but they all occur in one region of the body, then the ISS will be significantly lower and not accurately reflect the severity of the person's injuries (Osler *et al*., 1997).

Since the inception of the ISS, modifications have been made to make the method to be more accurate in predicting patient mortality following trauma. These modifications have resulted in a new scale called the new injury severity score (NISS), which uses the three most injured body parts regardless of body region (Deng *et al.*, 2016). This modification to the ISS allows a more accurate representation of a person's injuries, allowing a more precise prediction of patient outcomes, thus allowing optimised treatment strategies to be adopted and utilised.

Table 6) The difference between the Injury severity score and the New Injury Severity Score for a patient with multiple injuries following trauma.

Organ system	Injury description	AIS score	ISS score	NISS score
Head/neck	Cerebral Contusion	3	9	9
Face	None	0		
Chest	Flail chest	4		16
	Haemothorax	3		
	Pericardial injury	4	16	16
Abdomen	Minor splenic contusion	2	4	
Extremities	None	0		
External	None	0		
	Sum of top 3 AIS scores		**29**	**41**

Despite the example patient **(**Table 6), having identical injuries for each scoring system, the scores from each ISS based system are significantly different. This is because the NISS considers the three highest scoring injuries, irrespective of the organ system involved. This allows a greater representation of overall injury severity, as the most severe injuries are accounted for, rather than using a relatively minor injury because it occurs in a different organ system.

1.6.3 - Sequential Organ Failure Assessment score

The SOFA scoring system was developed in 1994 and analyses six organ systems (respiratory, cardiovascular, renal, hepatic, central nervous system and coagulation) and scores them based on their function. The function of these organ systems is scored on a scale of 0, which is normal function up to 4, which indicates mostly abnormal function. The SOFA score uses physiological and laboratory variables; however, it does not consider: age, ethnicity and co-morbidity disease, which are key drivers in the mortality related to sepsis (Macdonald *et al.*, 2014). The specific criteria used to develop the overall SOFA score are detailed in Table 7.

Table 7) The criteria used to develop an overall SOFA score for trauma patients based upon the function of six organ systems.

System	Score				
	0	**1**	**2**	**3**	**4**
Respiration					
PaO_2/FiO_2, mmHg (kPa)	>400 (53.3)	<400 (53.3)	<300 (40)	<200 (26.7) with respiratory support	<100 (13.3) with respiratory support
Coagulation					
Platelets ($x10^3/\mu l$)	>150	<150	<100	<50	<20
Hepatic					
Bilirubin, mg/dL (µmol/L)	<1.2 (20)	1.2-1.9 (20-32)	2.0-5.9 (33-101)	6.0-11.9 (102-204)	>12.0 (204)
Cardiovascular					
	MAP >70 mm Hg	MAP <70 mm Hg	Dopamine <5 or any dose dobutamine	Dopamine 5.1-15 Or epinephrine <0.1 Or norepinephrine <0.1	Dopamine >15 Or epinephrine >0.1 Or norepinephrine >0.1
Central nervous system					
Glasgow coma scale score	15	13-14	10-12	6-9	<6
Renal					
Creatinine, mg/dL (umol/L)	<1.2 (110)	1.2-1.9 (110-170)	2.0-3.4 (171-299)	3.5-4.9 (300-440)	>5.0 (440)
Urine output, ml/d				<500	<200

Abbreviations: FiO_2, Fraction of Inspired Oxygen; MAP, Mean Arterial Pressure; PaO_2, Partial pressure of oxygen.

Table 8) The criteria required to calculate the qSOFA score and quickly identify patients at higher risk of developing an infection and further complications while in the hospital.

Criteria	Measurement
Respiratory rate	>22/min
Systolic blood pressure	<100 mg Hg
Mentation	Abnormal

An alternative to the SOFA scoring method has been developed to allow an initial assessment of overall SOFA score to be conducted at the patient's bedside as soon as they are admitted to hospital. The system termed quick Sequential Organ Failure Assessment (qSOFA), is comprised of only three factors that are used to rapidly evaluate the risk of a patient having more severe outcomes before a complete SOFA score can be calculated (Table 8) (Singer *et al.*, 2016).

These criteria can be determined within minutes of admission and may direct initial treatments by permitting the identification of patients at high risk of infection, while a complete SOFA score is calculated as this requires more complex tests and further scoring systems such as the Glasgow Coma Scale and a full calculation of the injury severity score (Singer *et al.*, 2016).

1.6.4 - Glasgow Coma Scale

The Glasgow Coma Scale is a system of scoring the severity of trauma. First described in 1974, the system is used to quantify the severity of head injury and measures several responses to stimuli to determine the level of consciousness (Restrepo-Álvarez *et al.*, 2016). It evaluates a patient's neurological status by recording eye opening, verbal response and motor response to standardised verbal and physical stimuli, to indicate the severity of head trauma (Smith & Smith, 2017).

The Glasgow Coma Scale is scored up to a maximum combined score of 15. A score of 15 indicated a fully awake person and a low score of 3 signifying a deep coma, (Table 9) (Teasdale *et al*., 2014). A score of less than or equal to 8, is the traditionally used value for differentiating between severe and moderate to mild head injury for management and treatment purposes (Balestreri *et al.*, 2004).

Table 9) The scoring criteria used to develop a Glasgow Coma Scale using eye opening response, verbal response and motor response, to measure the cognitive function of trauma patients.

Eye opening response	Verbal response	Motor response
4 - Spontaneous	5 - Orientated	6 - Obeys commands
3 - To verbal stimuli	4 - Confused	5 - Localises pain
2 - To pain	3 - Inappropriate words	4 - Withdraws from pain
1 - None	2 - Incoherent	3 - Flexion to pain or decorticate
	1 - None	2 - Extension to pain or decorticate
		1 - None

The GCS was originally developed to gauge a patient's neurological deterioration or impairment, as well as act as a predictor for patient outcome. However, it is now commonly used as a clinical indicator, and a tool to manage patients (Sternbach, 2000). The main limitation of using the Glasgow Coma Scale is that a patient's verbal response is unable to be determined in a patient who has been intubated (Smith & Smith, 2017). Thus, a complete GCS cannot be calculated or the value is significantly lower than if the patient was not intubated (Smith & Smith, 2017). Despite attempts to modify the GCS methodology to offer improvements and eliminate limitations in the method, the criteria are still the same as when the method was first developed in 1974.

1.7 - The role of omics in biomarker development

Omics technologies are a component of the evolving field of systems biology, with the aim of providing a complete insight into highly complex models of disease (Farid & Morris-Stiff, 2015). They are termed omics technologies, related to the suffix -ome, which is defined as "all constituents considered collectively" (Rotoff & Motsinger-Reif, 2016). Omics technologies are used in the detection of genes (genomics), messenger ribonucleic acid (mRNA) (transcriptomics), proteins (Proteomics) and metabolites (metabolomics), in a biological sample (Wheelock *et al*., 2013; Horgan & Kenny, 2011). These are conducted in a non-targeted and non-biased manner (Kell, 2007; Horgan & Kenny, 2011).

The basic aspect of these approaches is that a complex system can be understood more thoroughly if all considered together (Horgan & Kenny, 2011). Omics technologies are commonly used in scientific research to generate hypotheses, as large outputs of data can be mined to identify potential molecules to further analyse and generate a hypothesis around (Horgan & Kenny, 2011; Brown *et al*., 2005). Omics has already utilised in key areas of basic science, including drug and biomarker discovery (Matthews, Hanison & Nirmalan, 2016).

Advancements in biotechnology and computational performance in recent years have allowed omics technologies to rapidly develop alongside these advancements (Farid & Morris-Stiff, 2015). This has permitted vast amounts of information to be collected and analysed simultaneously, allowing a complete disease profile to be developed. This data analysis is conducted through a process known as bioinformatics, which compares the results of scientific research analysis to a computer database, to determine the identity of molecules in biological samples (Wheelock *et al*., 2013; Krug *et al*., 2012).

Since the development of genomics, the completion of the human genome (Venter *et al*., 2001; Lander *et al*., 2001) and the rapid advancement in technology and medical research that has been a result, advancements have begun into developing an understanding of humans at a proteomic and metabolomic level, to ascertain the importance of these systems on biological functions and in disease states, and to provide a complete representation of human beings as a biological system.

1.7.1 - Proteomics

The proteome consists of all proteins expressed by a certain species (Lovric, 2011). Proteomics follows on from the genomic and transcriptomic systems, as proteins are created from the transcription of mRNA. However, all individuals of a species do not have identical proteomes, due to internal and external factors, such as post-transcriptional modification, health conditions and environmental factors (Lovric, 2011; Graves & Haystead, 2002).

Proteomic analysis can be classified into three main groups, expression proteomics, structural proteomics and functional proteomics (Chandrasekhar *et al*., 2014). Expression analysis is used to quantify expression of proteins under two conditions, normally healthy against disease states (Wright *et al*., 2012). Structural analysis aims to identify the three-dimensional structure of proteins, using x-ray crystallography, allowing the complexities of functional proteins to be determined (Manjasetty *et al*., 2012). Functional proteomics develops knowledge of protein function and the molecular mechanism of protein interaction, allowing the role of proteins in disease conditions to be determined (Monti *et al*., 2005).

Numerous different methods can be used to undertake proteomic analysis. Initially, separation based proteomics is to identify the different proteins within a sample based on their molecular weight (Abdallah *et al*., 2012). Electrophoresis based methods can be used to analyse expression and qualitative expression (Yan *et al*., 2002). However, these methods do not provide high enough resolution to conduct a complete proteomic analysis of biological samples (Wright *et al*., 2012). Higher resolution analysis can be achieved by employing alternative methods, such as mass spectrometry.

1.7.2 - Metabolomics

Metabolomics is the study of the metabolites within a biological system (Horgan & Kenny, 2011). Metabolites are the substrates and products of essential cellular functions, such as energy production and storage, signal transduction and apoptosis (Johnson, Ivanisevic & Siuzdak, 2016).

Metabolites can also be derived from exogenous substances including microorganism, xenobiotic and dietary metabolites (Johnson, Ivanisevic & Siuzdak, 2016). Metabolites have been described as proximal reporters of disease because of their abundance within biological fluids under pathological conditions (Gerszten & Wang, 2008; Clish, 2015). During biomarker discovery studies involving metabolomic analysis, there are commonly two main stages, a discovery stage, in which a group of novel biomarker candidates are identified, and a validation phase, where potential biomarkers are validated in a different patient cohort or using an alternative methodology (Barderas *et al*., 2011).

Metabolites can be identified within many biological fluids or tissues, with blood plasma and urine the most commonly used biological matrices, due to their relatively uncomplicated availability and high relevance within a clinical setting (Barderas *et al*., 2011; Darde, Barderas & Vivanco, 2007). There are two main methods of generating metabolomics data; these are nuclear magnetic resonance (NMR) and mass spectrometry (MS) (Alonso, Marsal & Julià, 2015). Mass spectrometry is commonly preferred over NMR for metabolomic analysis of biological fluids, due to its sensitivity and its ability identify thousands of metabolites in a single analysis (Vuckovic, Issaq & Veenstra, 2013; Jardine, 2012).

A greater knowledge of metabolic pathways also offers the ability to develop new biomarkers for disease states, by identifying changes between healthy and disease states. This allows diseases to be identified earlier, providing quicker, specific therapeutic intervention to provided, before the disease can progress.

1.7.3 - Mass spectrometry and bioinformatics

Mass spectrometry (MS) is an analytical technique that acquires spectral data based on the mass to charge ratio and relative intensity of molecules (Alonso, Marsal & Julià, 2015). It involves several steps including ionisation, transportation, deflection and detection. However, before MS analysis can be conducted the biological sample needs to be separated using chromatography based methods (Alonso, Marsal & Julia, 2015). These methods involve the interaction of molecules within a sample to an adsorbent material within a separation column (François, Sandra & Sandra, 2009).

Following separation, the compounds and molecules need to be ionised to give them a positive charge and convert them to the gaseous phase without causing degradation to the analytes (Yates, Ruse & Nakorchevsky, 2009).

The choice of ion source used to ionise molecules is of importance when conducting MS analysis. This is due to different ionisation techniques fragmenting molecules to different extents (Lössl, van de Waterbeemd & Heck, 2016). Several ionisation methods are available to generate the positively charged fragments. These methods can be conducted in a vacuum, such as electron impact ionisation (EI) and matrix-assisted laser desorption ionisation (MALDI) (Yates, Ruse & Nakorchevsky, 2009), or at atmospheric pressure through chemical ionisation and electro spray ionisation (ESI) (Mumtaz *et al*., 2017).

Ions are transported through the spectrometer; they pass through a strong magnetic or electrical field (Hoffmann & Stroobant, 2007). These fields are used to separate and analyse ions based on their mass. This involves the amount of deflection caused by the field or the time taken to reach the detector (time of flight instruments) (Domon & Aebersold, 2006). Ions with lower masses will reach the detector first, and as the strength of the field is increased higher mass ions can be detected (Burinsky, 2006).

The detection of ions involves the measurement of charged ions when they hit the surface of the detector (Yerlekar & Kshirsagar, 2014). This charge is then converted by a computer to develop the mass to charge ratio and abundance of an ion. The mass to charge ratio can then be compared to those of known molecules using bioinformatics analysis. This determines the identity of analytes and their function within a biological system.

Bioinformatics analysis is commonly compared to publically available databases such as the Research Collaboratory for Structural Bioinformatics protein database and the National Center for Biotechnology Information protein database.

1.8 - Aims of the study

The study was designed for a cohort of 200 patients, to meet the statistical powering target. This MSc by research position acted as a 50-patient pilot study to determine if interleukin-6 and interleukin-10 showed positive trends in predicting patient outcome and could be used to stratify patients.

The main aim of this pilot study is to identify if interleukin-6 and interleukin-10 concentrations play a key role and be utilised to predict the development of poor patient outcome, following traumatic injury requiring immediate surgery or direct admission to the ICU. From this main overall aim, several sub-aims can be generated:

- To optimise the cytometric bead array to simultaneously detect interleukin-6 and interleukin-10 in trauma patient serum samples.
- The measurement of both interleukin-6 and interleukin-10 in a large patient cohort to identify the changes in concentration over a 5-day period following traumatic injury.
- To identify if the concentrations of interleukin-6 and interleukin-10 differ in patient populations with a poor outcome compared to a good outcome.

These aims will be met using a variety of different methods and techniques including, flow cytometry based cytometric bead arrays, metabolomics analysis and clinical data management and examination.

Chapter 2 - Methods

2.1 - Study design

The overall study is designed to identify the interplay between immune and metabolic systems, to identify a panel of markers that can predict patient outcome, in a large cohort of patients. The study was granted UKCRN-NIHR Portfolio status (**BIT 19377**), which supported research nurse funding for clinical activities in Manchester Royal Infirmary (MRI) and Salford Royal Hospital (SR).

As a part of study development, a statistical power calculation was conducted by statisticians at Manchester Royal Infirmary to identify the number of patients required for statistically significant results to be achieved. This was based on a power calculation was based on 90% confidence of falsely rejecting the null hypothesis. This calculation identified the need for 200 patients, accounting for withdrawals. For the period of the MSc by research position, an aim of analysing approximately 50 patient samples for interleukin-6 and interleukin-10.

2.1.1 - Ethical considerations

The description of the project required ethical approval to be required from Local Ethics Commit Manchester, NHS/HSC Research and Development offices (**IRAS ID 172620**) and the ethical committee at the University of Salford, under ethics code **ST1617-17**.

2.1.2 - Patient recruitment

Patients were recruited from MRI and SR. Patients who were suitable to participate in the study were identified using the criteria in Table 10.

Table 10) The criteria used to identify suitable patients for the study. Patients are required to meet all three primary criteria and be either directly admitted to the intensive care unit or require immediate surgical intervention.

Requirement	Yes/No
Age >16 and <90	Yes
Male and Female	Yes
ISS >15	Yes
Immediate surgical intervention	
ICU admission	Yes

Abbreviations: ISS, Injury Severity Score; ICU, Intensive Care Unit.

Suitable patients were required to consent as soon as possible before taking and storage of samples using the consent form (Appendix 1). Once recruited, 20mls of blood was taken from patients at three time points. These samples were taken on day 1 (within 24 hours of admission), day 3 (third day after admission) and day 5 (the fifth day after admission) after the traumatic injury (Table 11).

Table 11) The time points at which blood samples and clinical data were collected.

	D1	D2	D3	D4	D5	D6	D7	D8
Blood sample	X		X		X			
Data collection	X		X		X			X

Alongside blood sampling, a series of clinical information was collected (Table 12).

Table 12) The clinical data collection sheet used for each of the four clinical data collection points.

Day			Date		
HR		BP	___/__	Temp	_____°C
Hb		WCC		PLT	
eGFR		Creatinine		Bilirubin	
PT		Intubated	Y / N	NIV/CPAP	Y/N
FiO2	____%	P/F Ratio	_____kPA	Lactate	___mmol/L
Noradrenaline	___µg/kg/min	CRP	_____mg/L	CVVH/IHD	Y/N
Sedated		GCS	____/N/A		
Treated with antibiotics	Y / N	Source of sepsis		_____/empirical/unknown	

Abbreviations: HR, Heart rate; Hb, Heamoglobin; eGFR, Estimated glomerular filtration rate; FiO^2, Fraction of Inspired Oxygen; BP, Blood pressure; WCC, White cell count; CRP, C Reactive Protein; GCS, Glasgow Coma Score; PLT, Platelet count; CVVH, Continuous Veno-Venous Heamofiltration.

This clinical data was then used to calculate patient SOFA scores, which would be used as a predictor of patient outcome. These criteria and clinical scoring systems were then compared to IL-6 and IL-10 concentration determined using methods 2.3 and 2.4. From this comparison, it identified if patient's outcome and injury severity correlated with IL-6 and IL-10 concentration.

2.1.3 - Evaluation of good and poor outcome

Patients were grouped into those with a good and poor outcome following traumatic injury. To distinguish between good and poor outcome patients the criteria in Table 13 was used.

Table 13) The definitions of good and poor outcome for trauma patients used in this study.

Good Patient Outcome
• A SOFA score of <3 5 days after traumatic injury.
Poor Patient Outcome
• A SOFA score of >3 5 days after traumatic injury. • The patient remaining hospitalised 8 days after injury.

Abbreviations: SOFA, Sequential organ failure assessment.

These criteria were then compared to interleukin-6 and interleukin-10 concentration, to analyse if the concentration of these cytokines correlated with a good or poor outcome following traumatic injury.

2.2 - Sample preparation

Initially, 10mls of blood was removed from blood containers and added to two 15ml centrifuge tubes, which were then centrifuged at 644xg (2000rpm) for 5 minutes. Once centrifuged, the top layer of serum was removed and 1ml aliquots transferred to cryovials, before storage at -80^{o}C for later analysis.

A further 10mls of patient blood was carefully layered over Ficoll Histopaque, before being centrifuged for 20minutes at 522xg (1800rpm). Following centrifugation, the serum was extracted and transferred to cryovials and stored at -80^{o}C.

Peripheral Blood Mononuclear Cells (PBMC's) were also extracted from the Ficoll Histopaque and washed in Phosphate Buffered Saline (PBS) by centrifuging at 272xg (1300rpm) for 10 minutes. After washing, the supernatant was discarded and the cell pellet was re-suspended using 1ml of freezing media (90% Fetal Bovine Serum (FBS) and 10% Dimethyl sulfoxide (DMSO)). The mixture was then transferred to cryovials and stored in the 'Mr Frosty' freezing container for 24 hours, before being moved to long-term sample storage.

2.3 - Cytometric bead array for standard, known concentrations

Before the cytometric bead array assay was conducted three different reagents were required to be prepared. These reagents were the standard concentrations, the mixed capture beads and the PE detection reagent.

The preparation of known standard concentrations was then used to create a standard curve, which was used to determine the concentration of Interleukin-6 and Interleukin-10 in unknown samples. The standards were prepared by opening phials of lypholysed standard spheres from each BD Bioscience cytometric bead array flex sets to be used in the analysis and pooling them in one tube. The standard spheres were then reconstituted in 4mls of assay diluent and left to equilibrate at room temperature for 15 minutes.

During this period, nine additional tubes were filled with 500µl of assay diluent and labelled with the standard concentrations, shown in Table 10). After this period, the standard spheres were mixed with a pipette and a serial dilution was then conducted in the pattern shown in Table 13). A negative control was also prepared, which was filled with just assay diluent, created independently from the serial dilution.

Table 14) Standard concentrations created following serial dilution of standard spheres from the BD Bioscience Interleukin-6 and Interleukin-10 Flex sets.

Tube label	Standard Dilution	Concentration (pg/ml)
1 (Top Standard)	1:1	2500
2	1:2	1250
3	1:4	625
4	1:8	312
5	1:16	156
6	1:32	80
7	1:64	40
8	1:128	20
9	1:256	10
10 (Negative control)	No Dilution	0

Subsequently, the preparation of the standard concentrations, the capture beads were prepared. This was done by initially calculating the number of tests to be conducted as this was used to determine the volume of capture beads required, as 1 test equals 1µl of capture bead stock required.

Once calculated, the phial of capture bead stock was vortexed for 15 seconds to re-suspend the beads and the required volume of capture beads was pipetted into a tube. The capture beads were then washed, by adding 500µl of wash buffer into the tube and centrifuging for 5 minutes at 200xg.

After washing, the supernatant was aspirated and discarded, with the pellet being resuspended in capture bead diluent to a concentration of 50µl per test. Before the assay procedure was done, the capture beads were vortexed and incubated for 15 minutes.
In the preparation of the PE detection reagent, the stock PE detection reagent was diluted using detection reagent diluent to a 1 in 50 dilutions, therefore to a concentration of 1µl of detection reagent with 49µl of detection reagent diluent. Once prepared, the diluted PE detection reagent was stored at 4^{o}C, protected from light.

Once the standard concentrations and reagents were made 50µl of each standard concentration was placed into individual Eppendorf tubes. 50µl of the mixed capture beads were then added to each tube containing the serum sample, the contents of the tubes were then mixed and left to incubate at room temperature for one hour.

Following the one hour incubation, 50µl of the diluted PE detection reagent was added to each tube and left to incubate at room temperature in the dark, for two hours. After two hours, 1ml of wash buffer was placed into each tube before they were centrifuged for 5 minutes at 200xg. After centrifugation, the supernatant from each tube was discarded, being careful not to disturb the pellet and the pellet was re-suspended in 300µl of wash buffer before analysis by flow cytometry.

2.4 - The cytometric bead array for the analysis of interleukin-6 and interleukin-10 in unknown patient samples

Patient serum samples were removed from the -80^{o}C freezer and defrosted at room temperature. Once defrosted, 50µl of the serum was aliquoted into triplicate Eppendorf tubes. Following this, 50µl of prepared mixed capture beads (See Page 62) were added to the patient serum samples and mixed by gentle pipetting, before being incubated at room temperature for one hour.

After one hour, 50µl of the prepared PE detection reagent (See Page 62) was added to each sample containing tube, before being incubated in the dark, at room temperature, for two hours. Subsequently, 1ml of wash buffer was added to each tube and centrifuged at 200xg for five minutes. The supernatant was then discarded and the pellet was re-suspended using 300µl of wash buffer, and then analysed using flow cytometry.

2.5 - Flow cytometry analysis of the cytometric bead array

Once prepared, the cytometric bead array was analysed using flow cytometry. During this analysis, the different capture beads for IL-6 and IL-10 were distinguished from the general bead population and the median PE fluorescence for each capture bead population was measured.

The median fluorescence of PE was then converted to the concentration of IL-6 and IL-10 using the standard curves generated during optimisation of the cytometric bead array method (See Chapter 2.8 for further information). The concentrations were then compared to patient clinical parameters, to determine if they have potential to predict patient outcome and the development of complications.

2.6 - Methods for metabolomics analysis in trauma patient samples

Metabolomics analysis was conducted by Waters Corporation, grouped using interleukin-10 concentrations generated using methods 2.4 – 2.5, using the following method. An initial cohort of 35 patients was selected at presentation to hospital based on injury severity scoring obtained in theatre or during ICU care. The metabolome and lipidome profiles of the 35-patient cohort were developed using the following liquid chromatography, mass spectrometry approach.

Patient serum samples were subjected to a methonal based extraction process to extract metabolites and lipids from the samples. The metabolites were then separated using an Acquity UPLC system. Data was then acquired using a VION (IMS-Q-ToF) mass spectrometer operating in positive and negative resolution modes, offering independent analysis. Each sample was analysed in triplicate using this method. Metabolite data obtained following mass spectrometry analysis were searched against the human metabolite database and quantified using Progenesis QI v2.3 LC/MS analysis software. Any metabolites of statistical significance (CV <30%; ANOVA (p) <0.05) were considered for further investigation.

These patients were then grouped based upon their cytokine profile, examined using a cytometric bead array method described in chapter 2.4. The patient's metabolomic and lipidomic profiles were then grouped based on patients with a day 1 interleukin-10 concentration of >12 pg/ml and those with a concentration of <12 pg/ml. Metabolomic data generated by Waters Corporation was then analysed at the University of Salford to identify metabolites with significantly differentiated expression between the two patient groups.

Chapter 3 - Results

3.1 - Optimisation of the cytometric bead array

The optimisation of the cytometric bead array for interleukin-6 and interleukin-10 involved several experiments to set up the correct flow cytometry gating for the capture beads to differentiate each population of capture bead and develop the standard curves used to calculate the concentration of IL-6 and IL-10 within patient serum samples.

3.1.1 - Optimisation of the interleukin-6 cytometric bead array

Firstly, interleukin-6 capture beads were analysed independently, to optimise the gating for this population of capture bead. Gates were added around the general bead population before identifying the IL-6 capture beads (Figure 26).

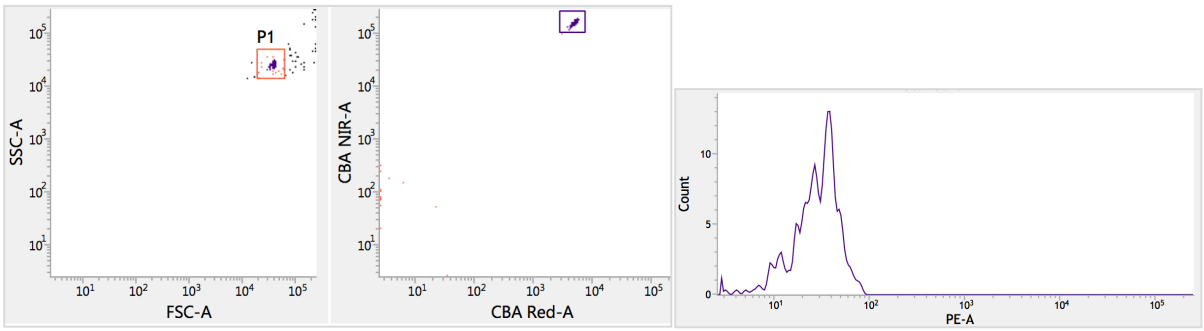

Figure 26) The flow cytometry gating used to identify the interleukin-6 capture bead population used to measure the median PE fluorescence.

Once the gating was optimised for interleukin-6, a standard curve was generated using standard concentrations of IL-6 (See Chapter 2.3 for the full method) (Figure 27).

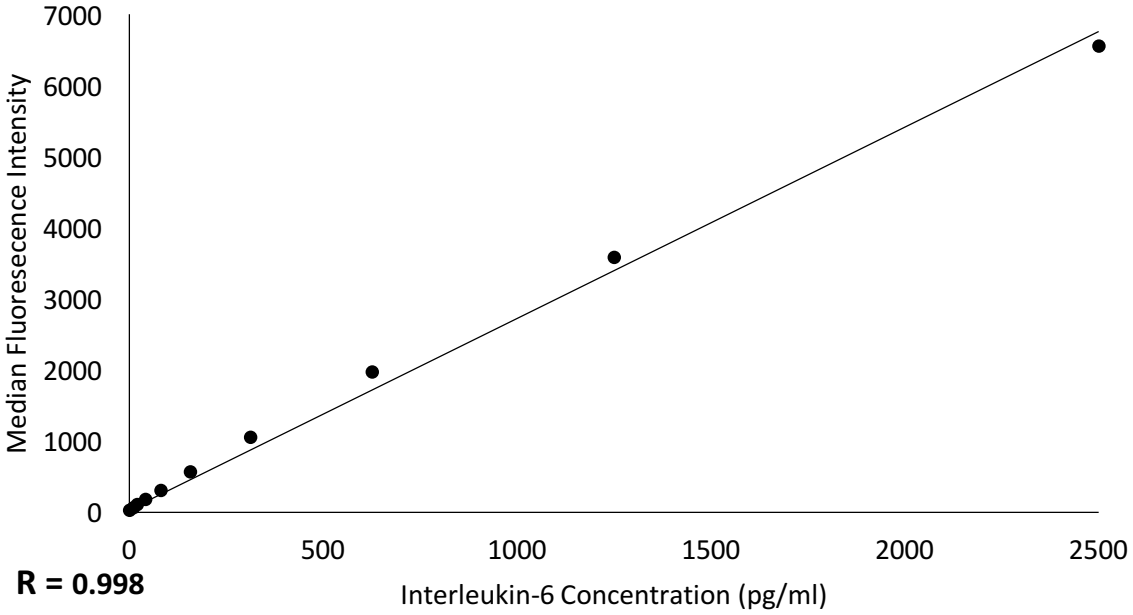

Figure 27) The standard curve generated for interleukin-6 from a single capture bead population.

3.1.2 - Optimisation of the interleukin-10 cytometric bead array

Following the generation of a standard curve for interleukin-6, this method was repeated for interleukin-10 cytometric bead arrays (Figure 28-29).

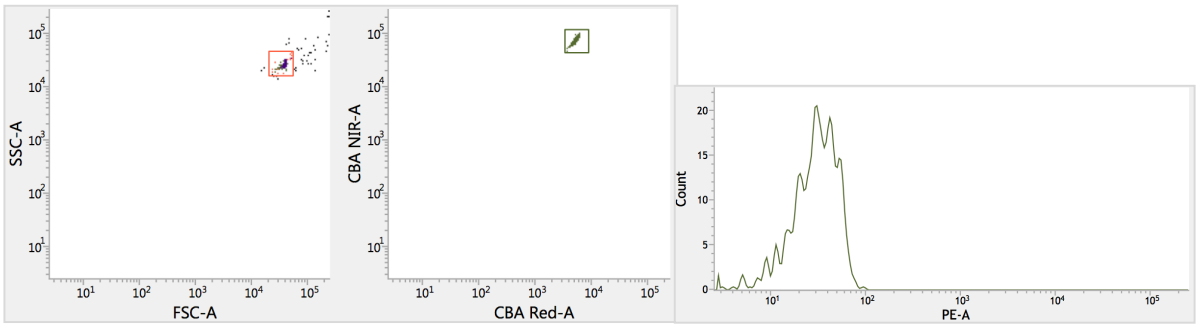

Figure 28) The flow cytometry gating used to identify the interleukin-10 capture bead population used to measure the median PE fluorescence.

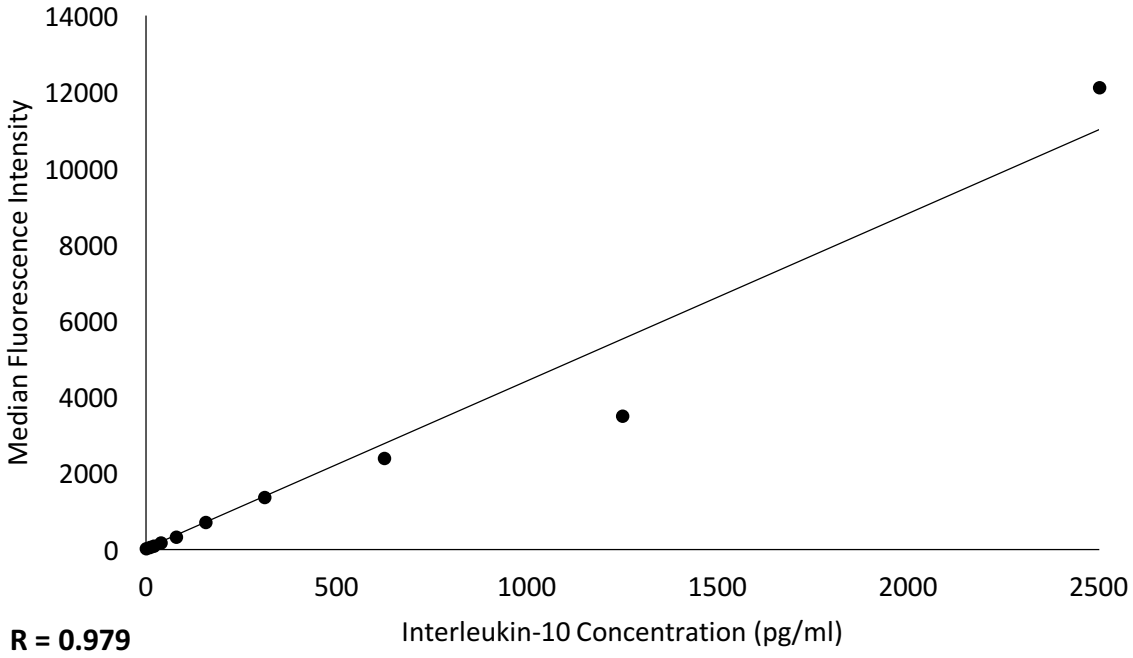

Figure 29) The standard curve generated for interleukin-10 from a single capture bead population.

3.1.3 - Optimisation of the multiplexed interleukin-6 and interleukin-10 cytometric bead array

After both interleukin-6 and interleukin-10 had been individually optimised, a multiplex assay containing both types of capture beads was conducted. This allowed gating of both cytokine capture beads when simultaneously analysed using flow cytometry (Figure 30).

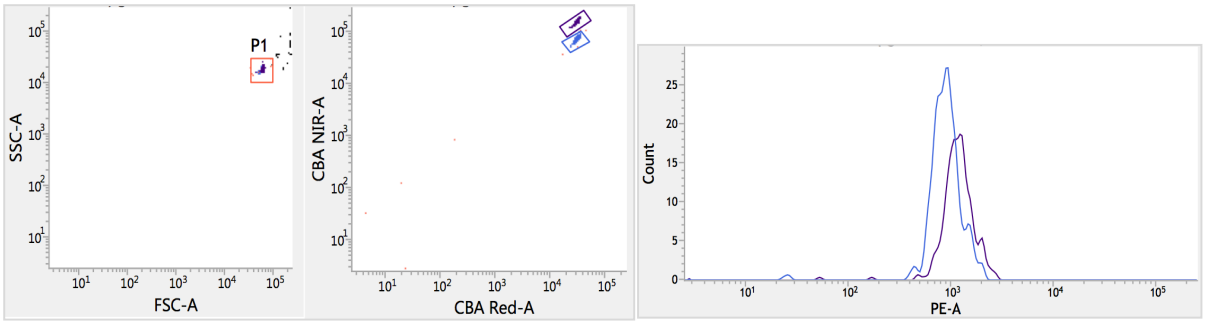

Figure 30) The flow cytometry gating used to identify interleukin-6 and interleukin-10 capture bead populations during the multiplex assay. The individual median PE fluorescence for each capture bead population can be measured.

Following the optimisation of the multiplex assay gating, standard curves for IL-6 and IL-10 were then generated using this multiplex assay method (Figure 31-32).

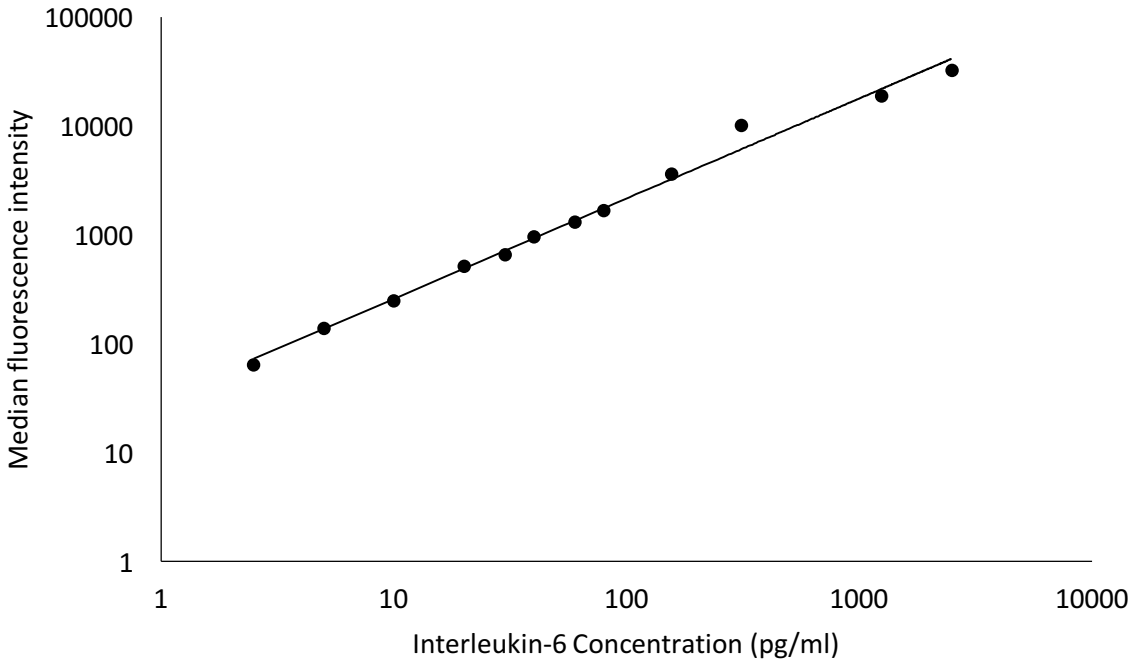

Figure 31) The interleukin-6 standard curve generated during a multiplex assay containing both interleukin-6 and interleukin-10 capture beads.

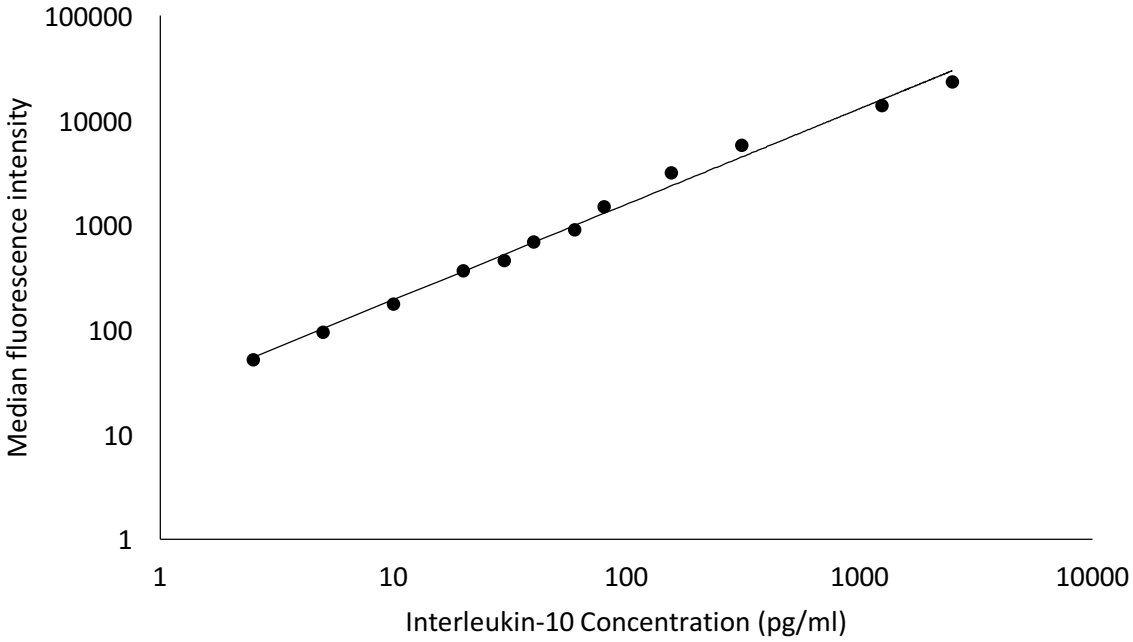

Figure 32) The interleukin-10 standard curve generated during a multiplex assay containing both interleukin-6 and interleukin-10 capture beads.

These standard curves were then used to interpolate the concentration of interleukin-6 and interleukin-10 in trauma patient samples.

3.2 - Analysis of the patient cohort

Patient samples were received from Manchester Royal Infirmary and Salford Royal Hospital. As of the 15th June 2017, the study has received samples from 110 patients, with 69 remaining in hospital to provide all three samples. The patients who did not provide all three samples were discharged, refused to consent or were transferred to a different hospital (Figure 33).

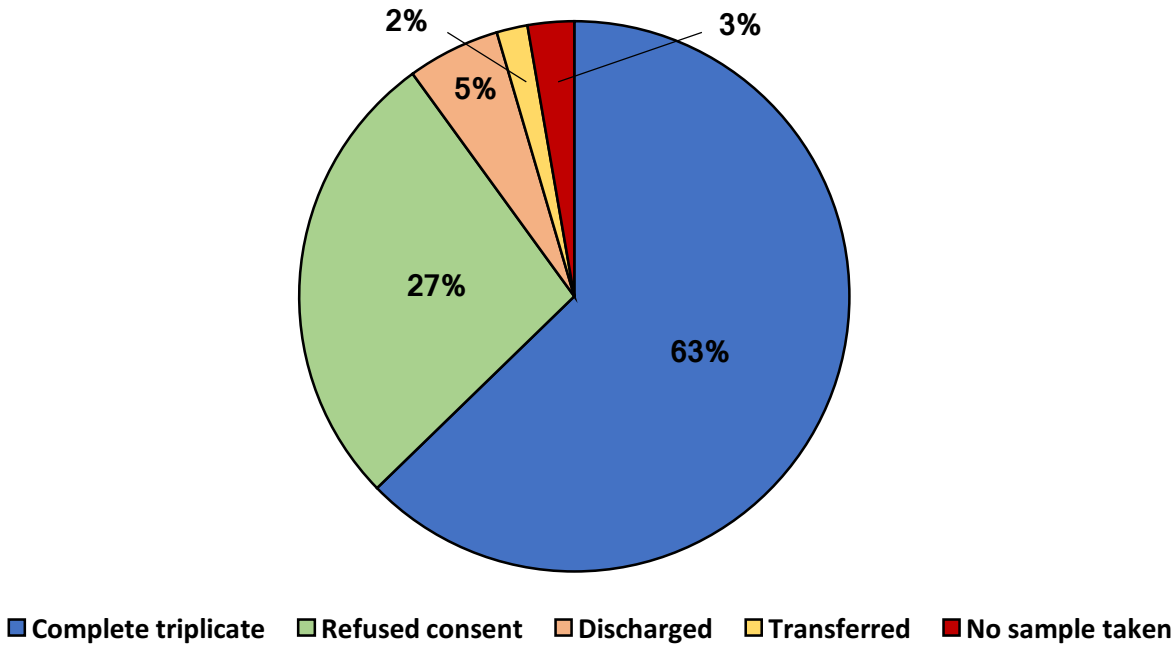

Figure 33) Analysis of patient samples received from Manchester Royal Infirmary and Salford Royal Hospitals to determine the percentage of patients who completed all three days' samples and those who did not, with the reasons why.

Following receipt of patient's day 5 sample, the patients were grouped based on whether they had a good or a poor outcome following traumatic injury. These groupings were decided based upon the patient's day 5 SOFA score and if they remained in hospital past day 8 following traumatic injury. The criteria used to distinguish good and poor outcome patients are shown in Table 13).

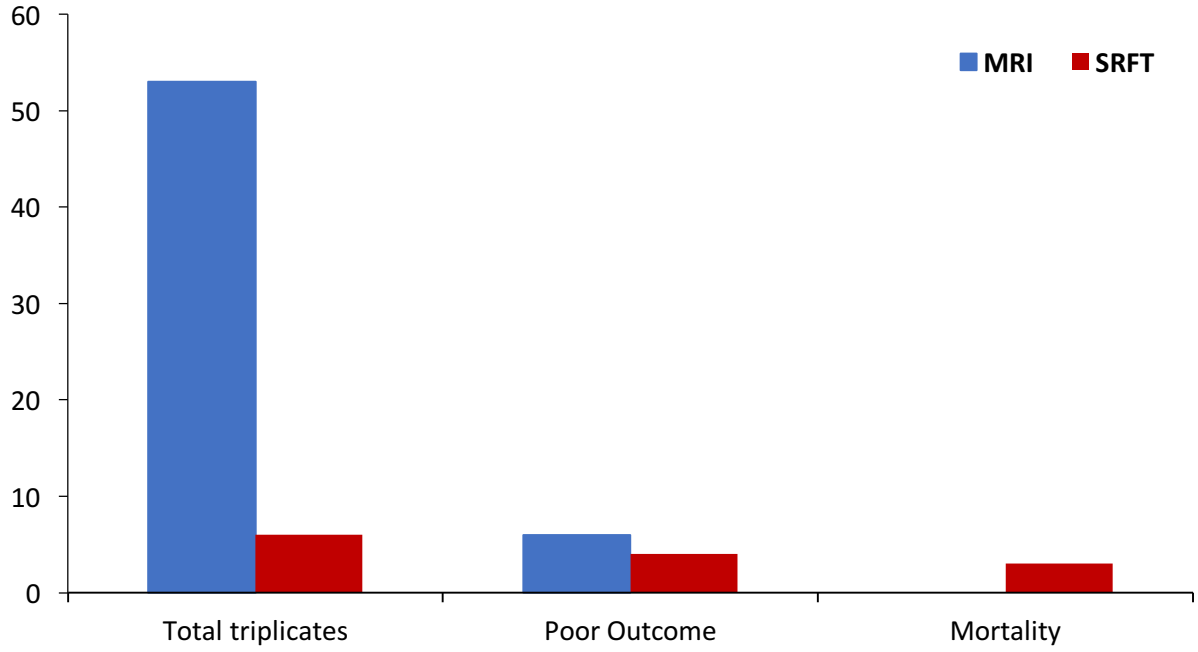

Figure 34) The number of patients who had a poor outcome from complete triplicate samples received from Manchester Royal Infirmary (MRI) and Salford Royal Hospital (SRFT).

Using the criteria in table 13, the patient cohort was further analysed to compare good and poor outcome for all triplicate samples received from both Manchester Royal Infirmary and Salford Royal Hospital. The results of this analysis are shown in Figures 34 -36).

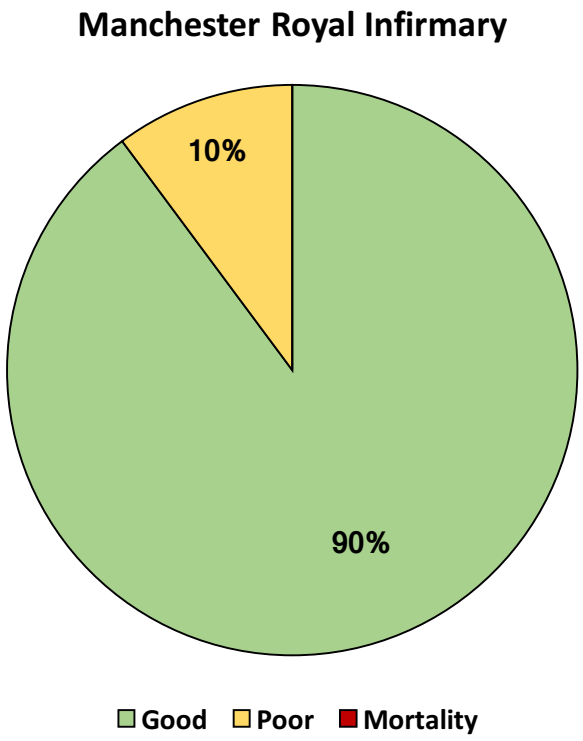

Figure 35) A comparison of good and poor outcome patients from Manchester Royal Infirmary.

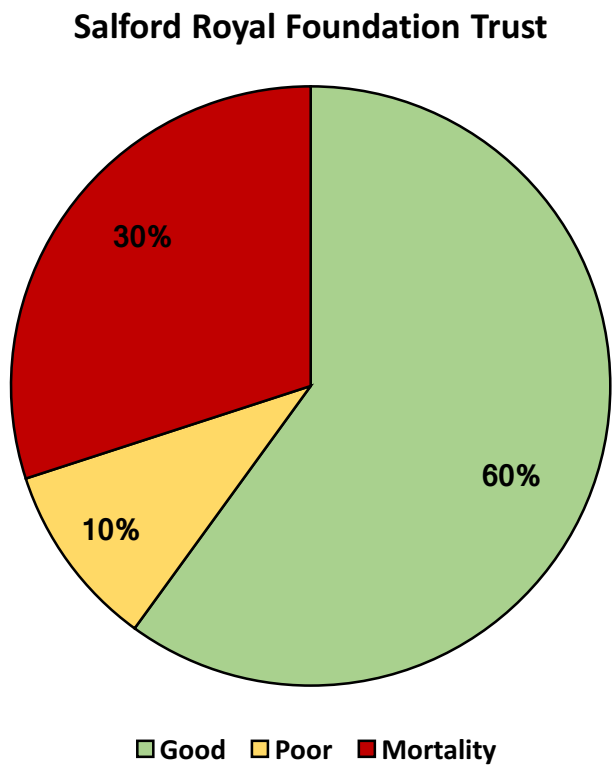

Figure 36) A comparison of good and poor outcome patients from Salford Royal Hospital.

Of the 69 sample triplicates received from Manchester Royal infirmary and Salford Royal Hospital, the first 50 triplicate samples were analysed for their Interleukin-6 and Interleukin-10 concentrations as a part of the pilot study. These concentrations were then compared to clinical scoring methods and commonly measured biological molecules, to determine if interleukin-6 and interleukin-10 could be used as biomarkers for predicting patient outcome and the development of complications, before they arise.

3.3 - Analysis of trauma patient's clinical data

Patient clinical data was provided on each selected time point during the 8-day period (Table 11) following traumatic injury, by the research teams at MRI and SR (Appendices 2 - 5). This was used to calculate patient SOFA scores and analyse commonly used markers for inflammation and the development of sepsis. The markers for inflammation and sepsis development that were used for comparison was C Reactive Protein (Figure 37) and Lactate (Figure 38).

Day 1 C Reactive Protein was found to have an average concentration of 50.92 ± 73.06 mg/L (Figure 39), with the concentrations ranging between 1 mg/L and 345 mg/L. On average C Reactive Protein was found to be at its highest concentration in day 3 following traumatic injury at 201.52 ± 108.29 mg/L (Figure 39). In day 3 concentrations ranged between 15 mg/L and 438 mg/L. In day 5, C Reactive Protein was found at an average concentration of 149 ± 101.22 mg/L (Figure 39), with concentrations ranging between 3 mg/L and 350 mg/L. Day 8 trauma patient samples were found to contain an average of 162.15 ± 148.75 mg/L (Figure 39) of C Reactive Protein. The day 8 concentrations ranged between 1 mg/L and 563 mg/L.

The lactate concentration peaked in day 1 was found at an average of 2.72 ± 1.78 mM/L (Figure 40), with concentrations ranging between 0.7 and 7.7 mM/L. The lactate concentration then decreased in day 3 to an average concentration of 1.14 ± 0.44 mM/L (Figure 40), with day 3 concentrations ranging between 0.5 and 2.1 mM/L. The concentration further decreased to an average of 1.01 ± 0.30 mM/L (Figure 40), with concentrations between 0.5 and 1.4 mM/L. The average day 8 lactate concentration increased slightly from that of day 5 to a concentration of 1.14 ± 0.34 mM/L (Figure 40). In day 8, lactate concentrations ranged between 0.6 and 1.6 mM/L.

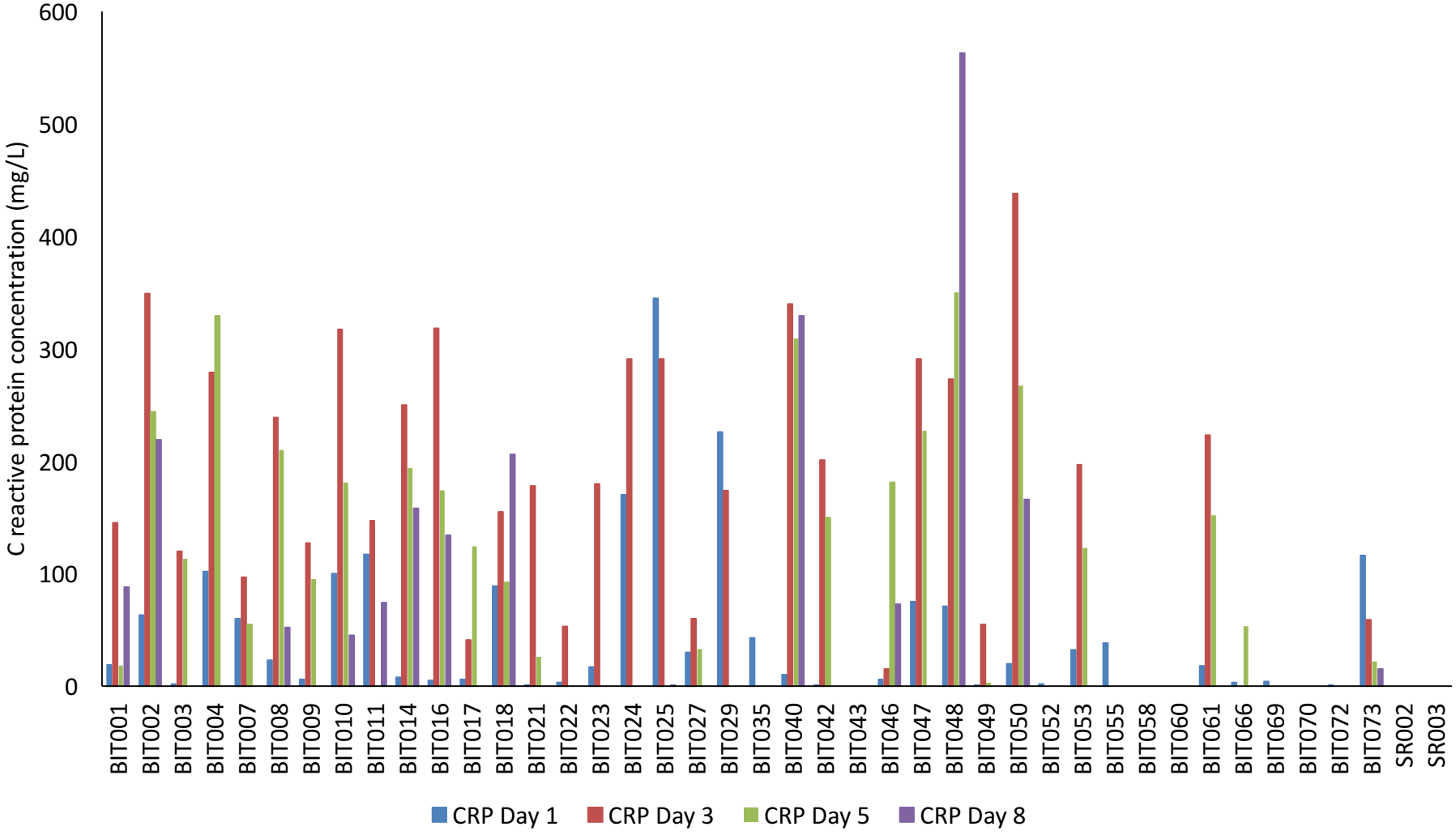

Figure 37) The C Reactive Protein concentration for all samples (n=50) analysed during the pilot study.

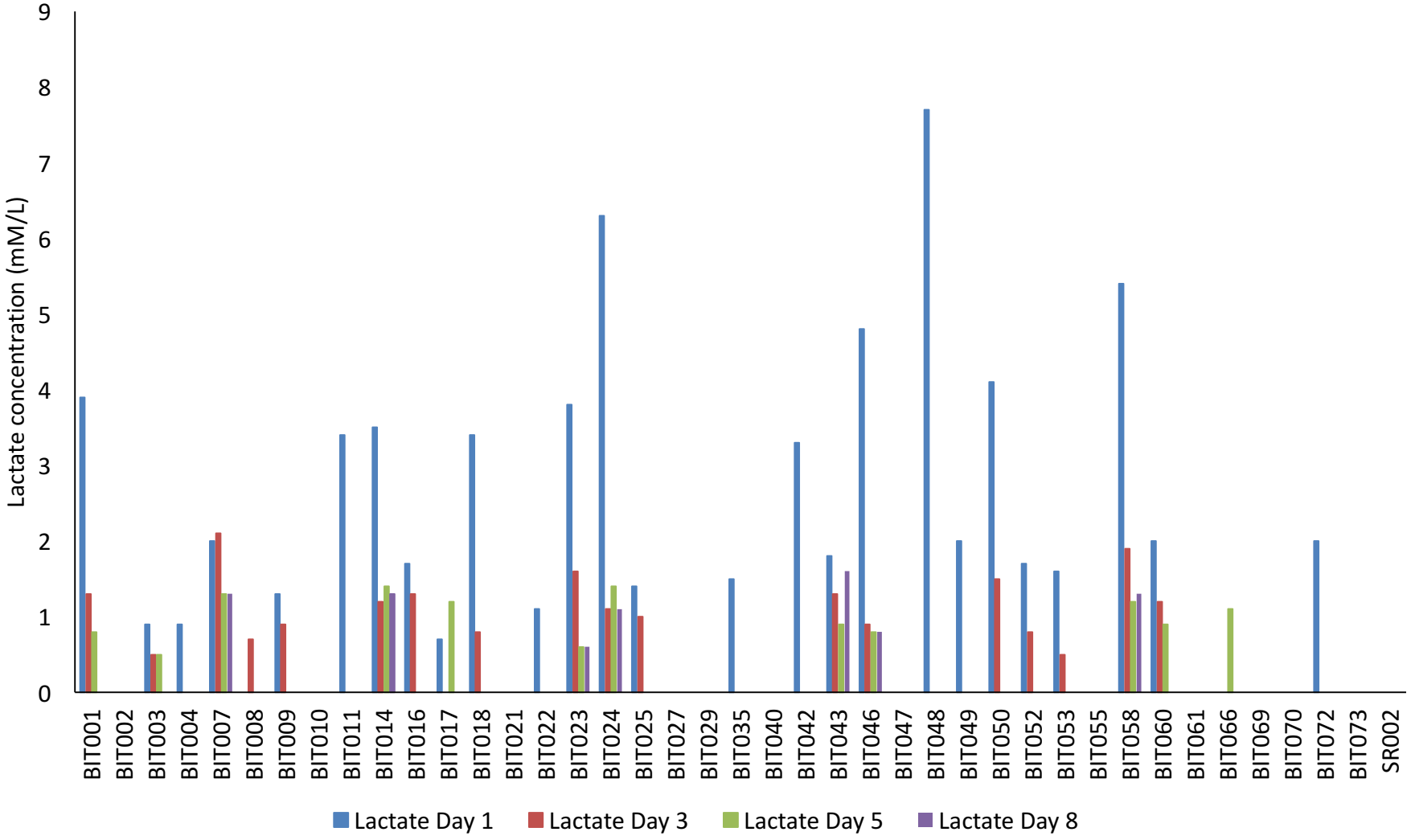

Figure 38) The concentration of lactate for all samples (n=50) analysed during the pilot study.

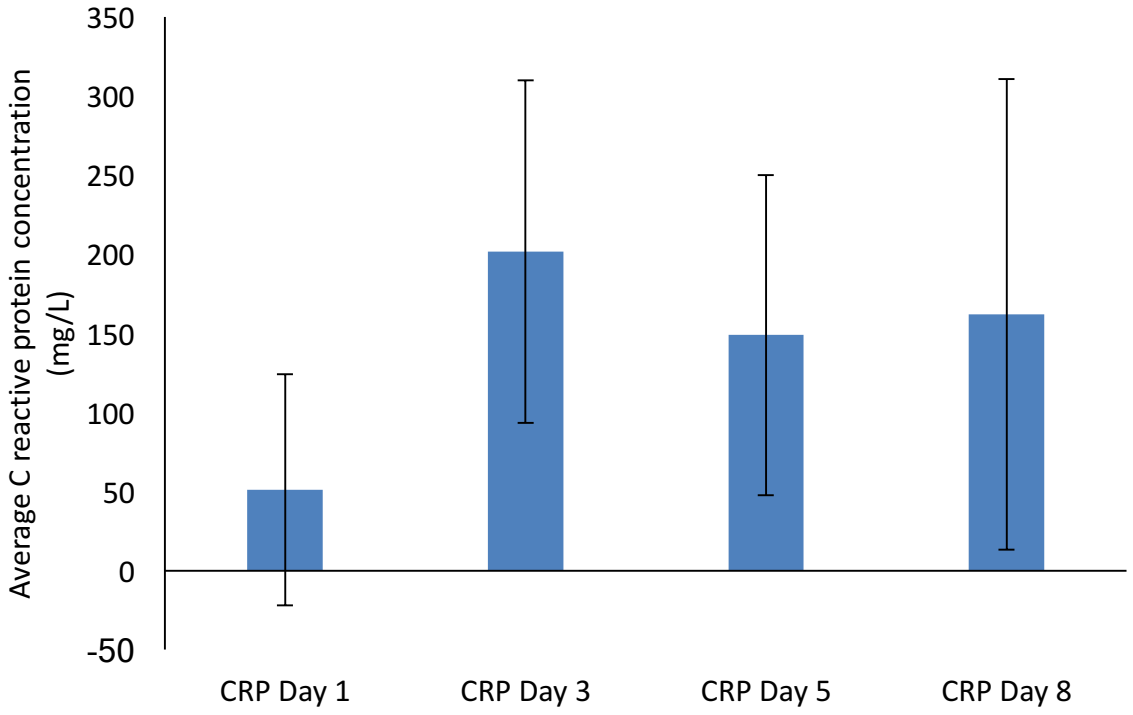

Figure 39) The average C Reactive Protein concentration for all samples (n=50), at each time point following traumatic injury.

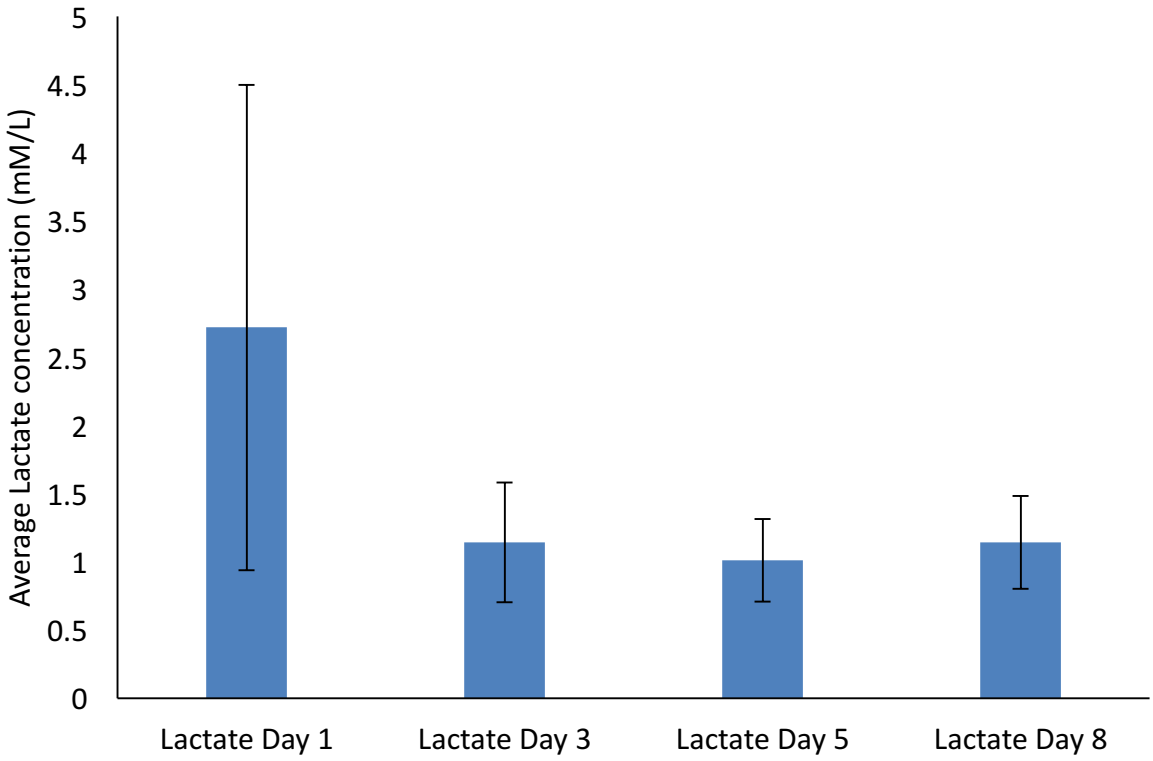

Figure 40) The average concentration of lactate for all samples (n=50), at each time point following traumatic injury.

3.4 - Interleukin-6 as a predictor of patient outcome

3.4.1 - Interleukin-6 concentrations in trauma patient serum samples

The pro-inflammatory cytokine, Interleukin-6 was measured in patient serum samples using the cytometric bead array. This was conducted in triplicate, for all analysed patient day 1, day 3 and day 5 samples (Figure 41).

Interleukin-6 concentration in the 46 triplicate samples varied from day 1 to day 5 (Appendix 2). The day 1 samples ranged from 1188.87 pg/ml to 0.83 pg/ml with a median value of 54.28 pg/ml and a mean value of 113.51 pg/ml. The day 3 samples ranged from 421.36 pg/ml to 0.25 pg/ml with a median value of 29.43 pg/ml and a mean value of 48.32 pg/ml. The day 5 samples ranged from 4635.45 pg/ml to 0.26 pg/ml with a median value of 10.90 pg/ml and a mean value of 128.48 pg/ml.

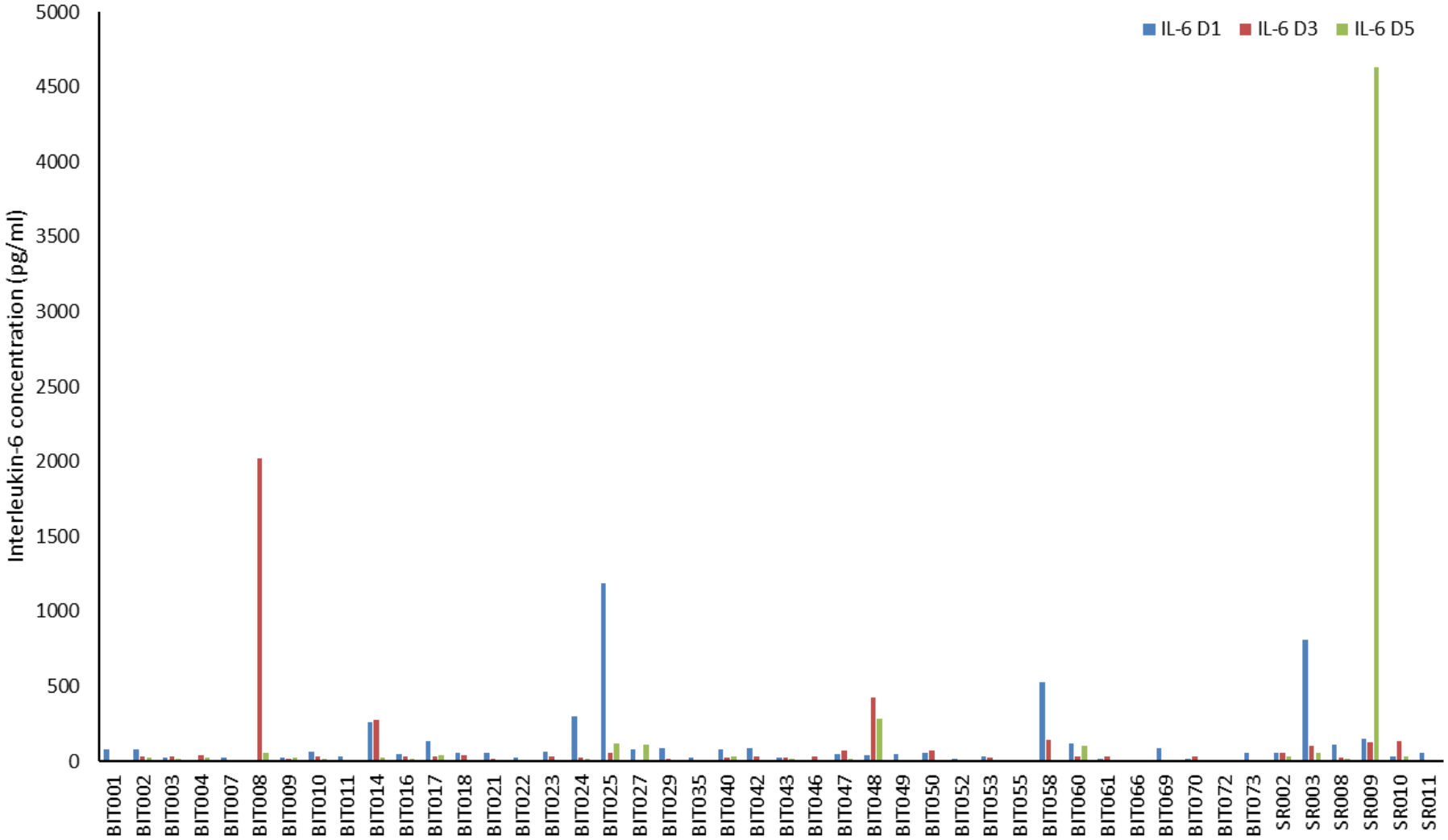

Figure 41) The interleukin-6 concentration of all analysed patient samples (n=50), showing the change over the 5-day period.

3.4.2 - A comparison between interleukin-6 concentrations and patient SOFA score

The interleukin-6 concentrations of patient samples were then compared to SOFA score (Appendix 3). Patients were grouped based on their SOFA score for each day and the average interleukin-6 concentration for each group was compared to the SOFA score (Figures 42 - 44).

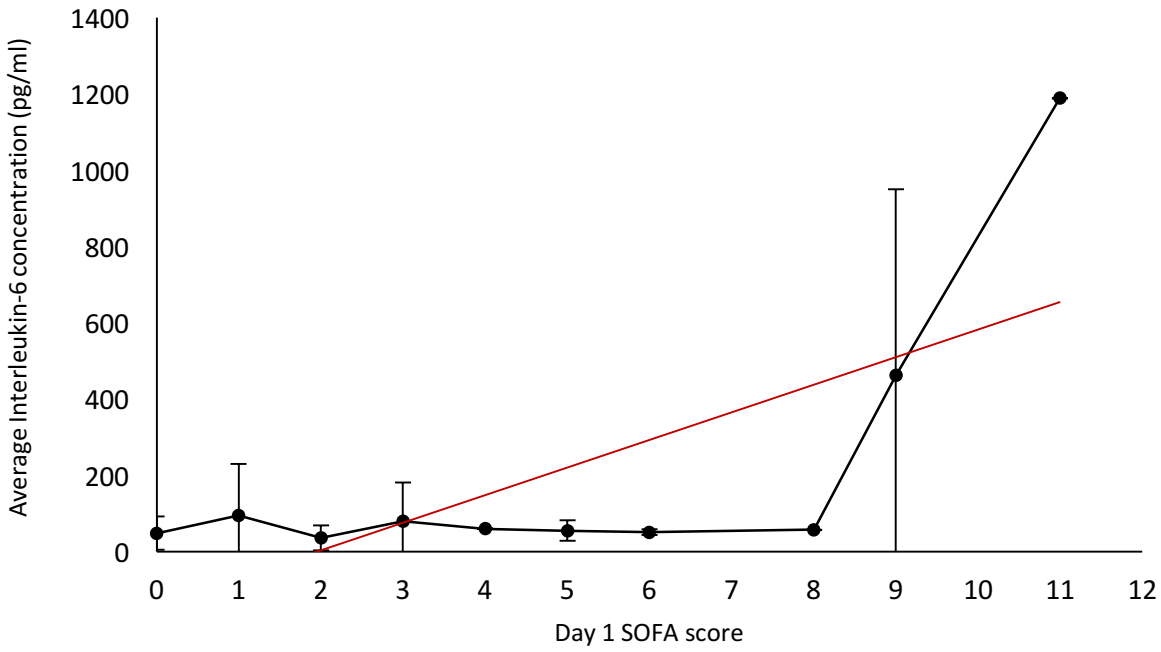

Figure 42) A comparison of averaged interleukin-6 against day 1 SOFA score, in patients grouped based upon day 1 SOFA score.

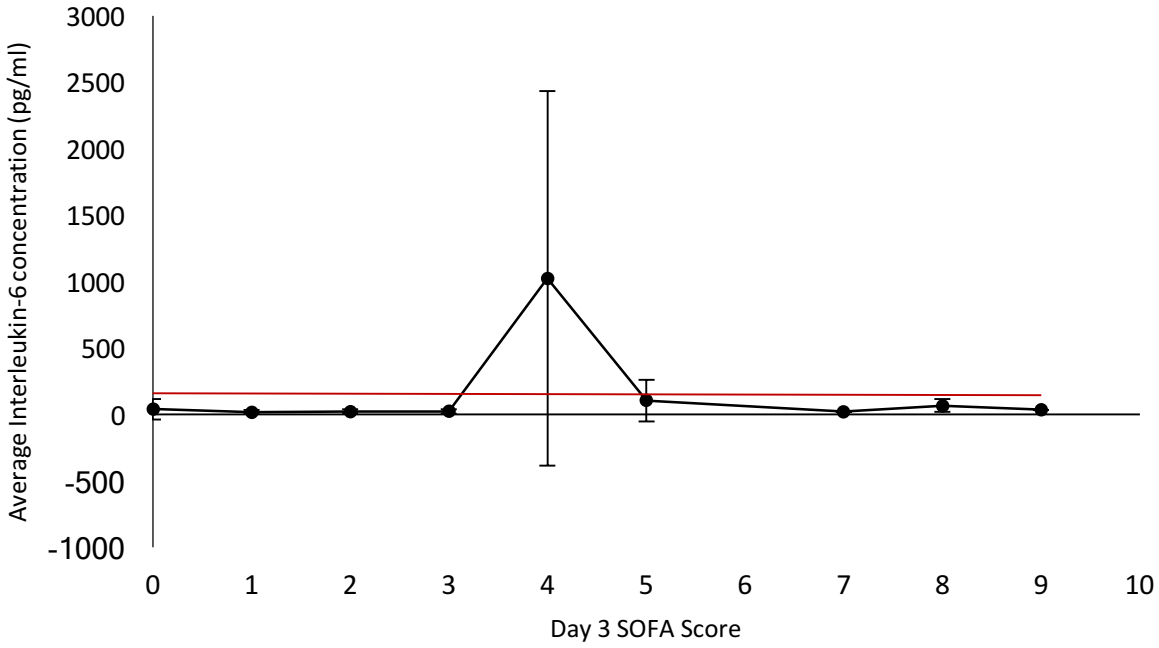

Figure 43) A comparison of averaged interleukin-6 against day 3 SOFA score, in patients grouped based upon day 3 SOFA score.

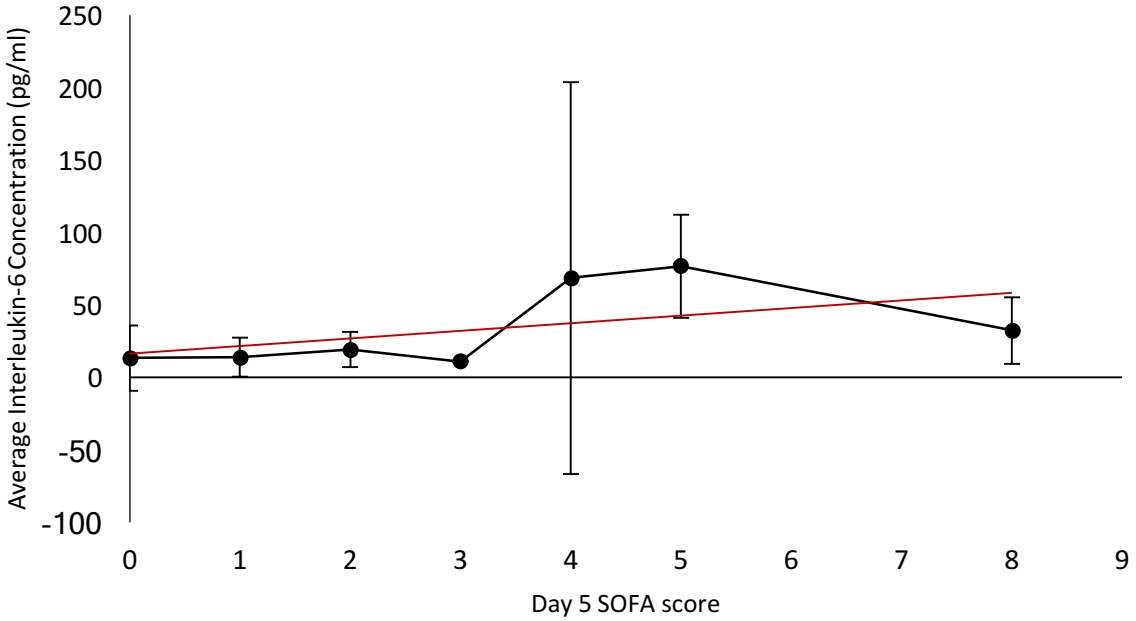

Figure 44) A comparison of averaged interleukin-6 against day 5 SOFA score, in patients grouped based upon day 5 SOFA score.

Day 1 averaged interleukin-6 concentrations (Figure 42) was found to plateau between SOFA scores 0 to 8, as the concentrations fluctuate between 48.39 pg/ml and 95.15 pg/ml. The average concentration then rapidly increased to 462.39 pg/ml in SOFA score of 9 and increased further in the patients with a SOFA score of 11 to 1188.87 pg/ml.

Day 3 interleukin-6 concentrations (Figure 43) spiked in patients with a SOFA score of 4 as it rises from 22.90 pg/ml in SOFA score of 3 to 1023.84 pg/ml in patients with a SOFA score of 4. The concentration then falls to 105.87 pg/ml in patients with SOFA scores of 5. Interleukin-6 concentration then fluctuates between 67.31 pg/ml and 21.47 pg/ml, in SOFA scores 7, 8 and 9.

Day 5 interleukin-6 concentrations (Figure 44) ranged between 10.90 pg/ml and 19.08 pg/ml in patients with SOFA scores of 0 and 3. The concentration then increased to 68.49 pg/ml in patients with a SOFA score of 4 and increased further to 76.70 pg/ml for SOFA scores of 5. The concentration then decreased to 32.33 pg/ml in patients with a day 5 SOFA score of 8.

3.4.3 - Interleukin-6 as a predictor of patient outcome

Patients were grouped further into good and poor outcomes using the criteria in Table 13). Good and poor outcome patient groups interleukin-6 concentrations were then averaged for each of the three sample time points and the good outcome patients were then compared to those with a poor outcome (Figure 45).

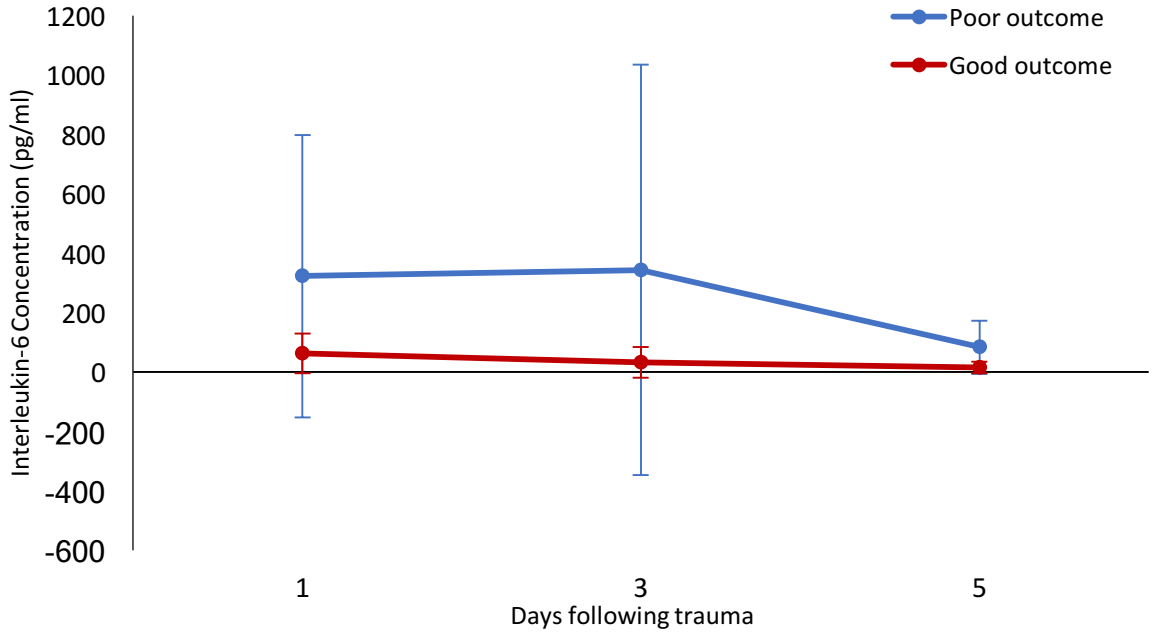

Figure 45) A comparison between the interleukin-6 concentrations of good outcome patients (n= 42) and poor outcome patients (n= 8) on day 1 (Good = 62.63±66.89) (Poor = 323.45±474.94), day 3 (Good = 33.03±50.99) (Poor = 334.41±689.98) and day 5 (Good = 15.71±19.97) (Poor = 84.16±89.99) following traumatic injury.

The results of good versus poor outcome patients (Figure 45), shows a substantial variation between poor outcome patients and good outcome patients. In Day 1 the average concentration of interleukin-6 in poor outcome patients was 323.45 pg/ml compared to 62.63 pg/ml in good outcome patients. The difference increased in day 3 as the average interleukin-6 concentration of poor outcome patients increased to 344.41 pg/ml, while the good outcome patients average interlekin-6 concentration decreased to 33.03 pg/ml. The day 5 average interleukin-6 concentrations for both good and poor outcome patients decreased to 84.16 pg/ml in poor outcome patients and 15.71 pg/ml in patients with a good outcome.

3.5 - Interleukin-10 as a predictor of patient outcome

3.5.1 - Interleukin-10 concentrations in trauma patient serum samples

The anti-inflammatory cytokine, Interleukin-10 concentration was measured in triplicate, using cytometric bead arrays for all sample time points (Appendix 4). Once measured, comparisons were made between patient SOFA scores and, good and poor outcome following traumatic injury. The results of interleukin-10 concentrations for all patient samples are shown in Figure 46.

The day 1 interleukin-10 concentration for all measured samples ranged from 128.64 pg/ml to 1.48 pg/ml, with a median value of 5.87 pg/ml and a mean concentration of 12.39 pg/ml. The day 3 samples ranged from 10.72 pg/ml to 0.70 pg/ml, with a median value of 2.59 pg/ml and a mean concentration of 3.25 pg/ml. Day 5 samples ranged from 57.58 pg/ml to 0.31 pg/ml, with a median value of 1.99 pg/ml and a mean concentration of 4.32 pg/ml.

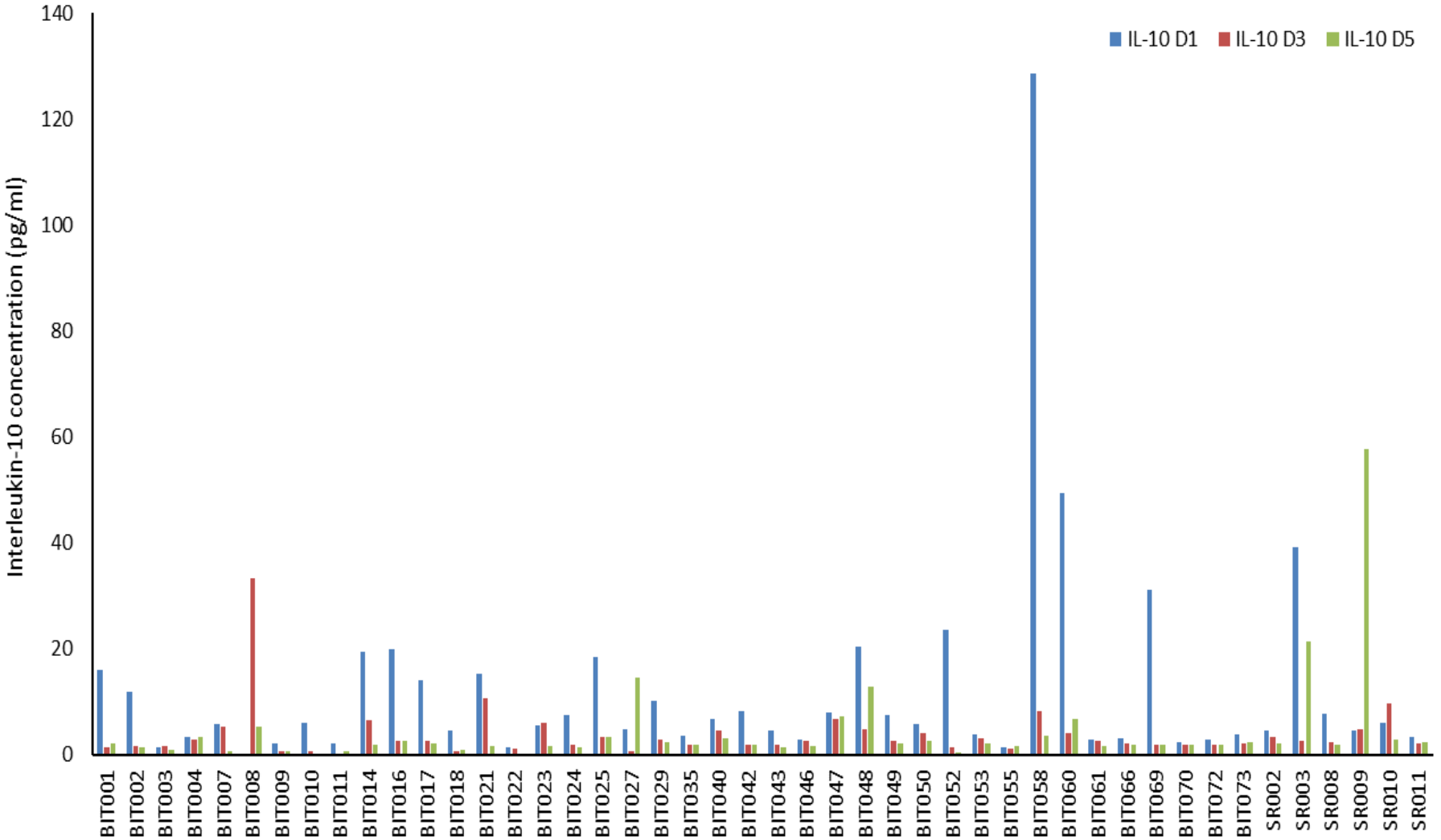

Figure 46) The interleukin-10 concentration of all analysed patient samples (n= 50), showing the change in concentration over the 5-day

3.5.2 - A comparison between interleukin-10 concentrations and patient SOFA scores

The interleukin-10 concentrations of patients were compared to SOFA scores. This was done by grouping patients based upon SOFA score and averaging the interleukin-10 concentration for each SOFA score group. These were then compared to determine if there was a correlation between interleukin-10 concentration and patient SOFA scores (Figures 47-49).

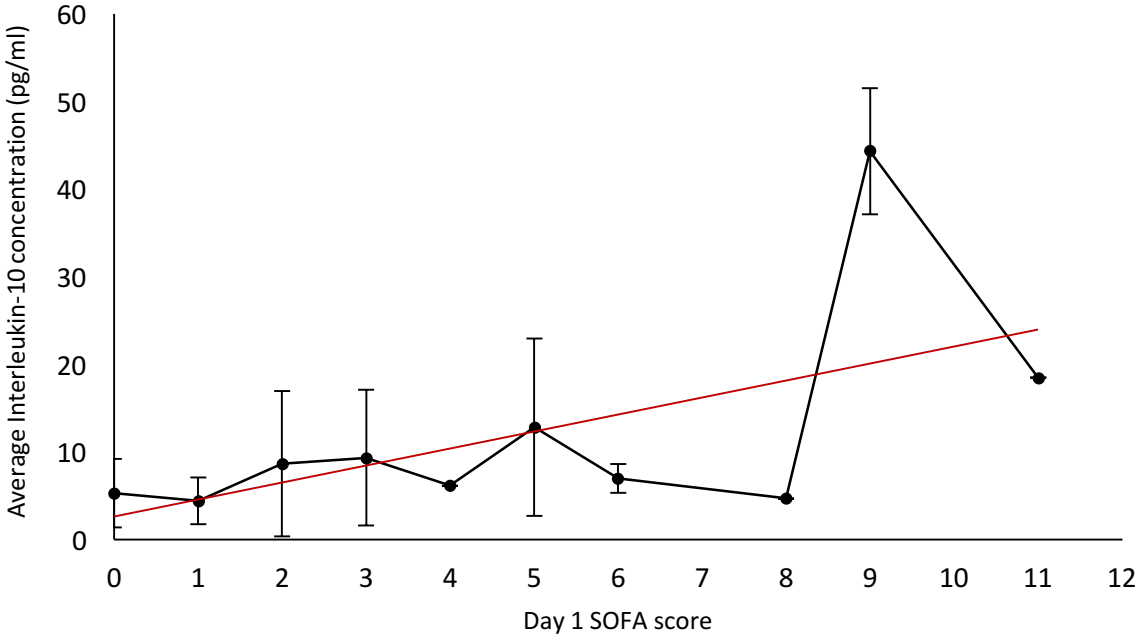

Figure 47) A comparison of averaged interleukin-10 against day 1 SOFA score, in patients grouped based upon day 1 SOFA score.

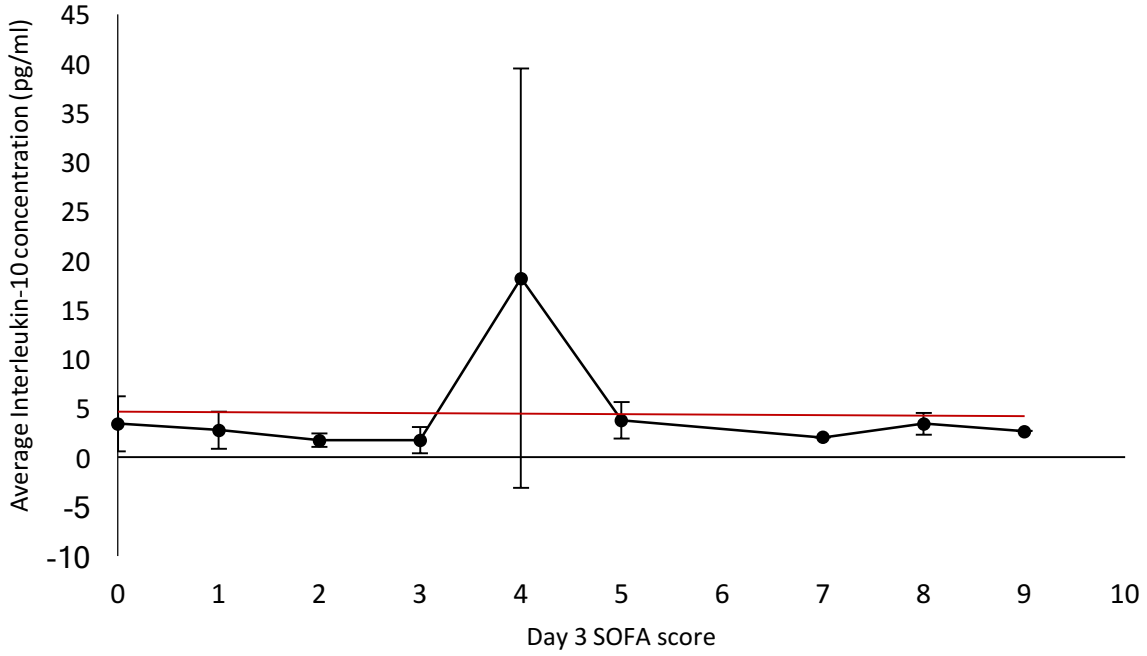

Figure 48) A comparison of averaged interleukin-10 against day 3 SOFA score, in patients grouped based upon day 3 SOFA score.

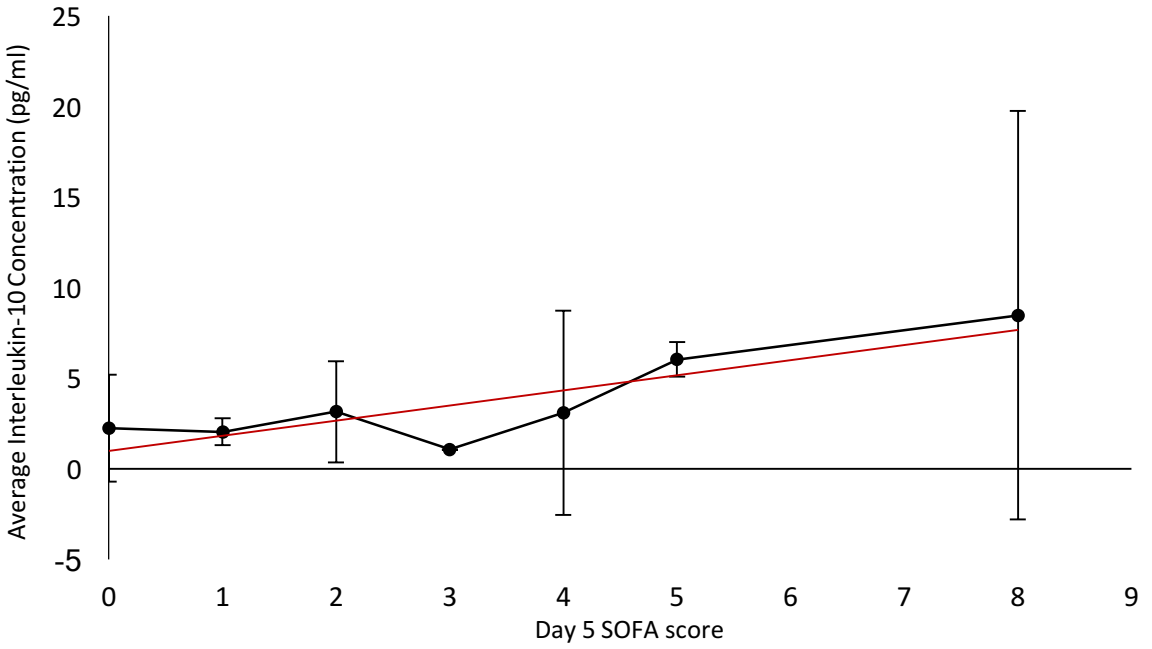

Figure 49) A comparison of averaged interleukin-10 against day 5 SOFA score, in patients grouped based upon day 5 SOFA score.

The day 1 interleukin-10 concentration (Figure 47) fluctuated between 4.45 pg/ml and 12.83 pg/ml between SOFA scores 0 and 8, before sharply rising to 44.32 pg/ml in patients with a SOFA score of 9. It then decreased to 18.49 pg/ml in patients with a SOFA score of 11
In day 3 interleukin-10 concentrations (Figure 48) ranged between 1.74 pg/ml and 3.41 pg/ml for SOFA scores of 0 and 3. In patients with a SOFA score of 4, the interleukin-10 concentration increased to 18.21 pg/ml, before dropping down to between 3.75 pg/ml and 2.02 pg/ml between SOFA scores 5 and 9. In day 5 samples, the interleukin-10 concentration (Figure 49) increased alongside SOFA score. It increased from 2.24 pg/ml in patients with a SOFA score of 0 to a concentration of 8.47 pg/ml.

3.5.3 - Interleukin-10 as a predictor of patient outcome

The interleukin-10 concentration of patient serum samples was compared to their patient outcome. The patients were grouped into good and poor patient outcomes using the criteria shown in Table 13), and the average interleukin-10 concentration for each group was evaluated over the three sample time points (Figure 50).

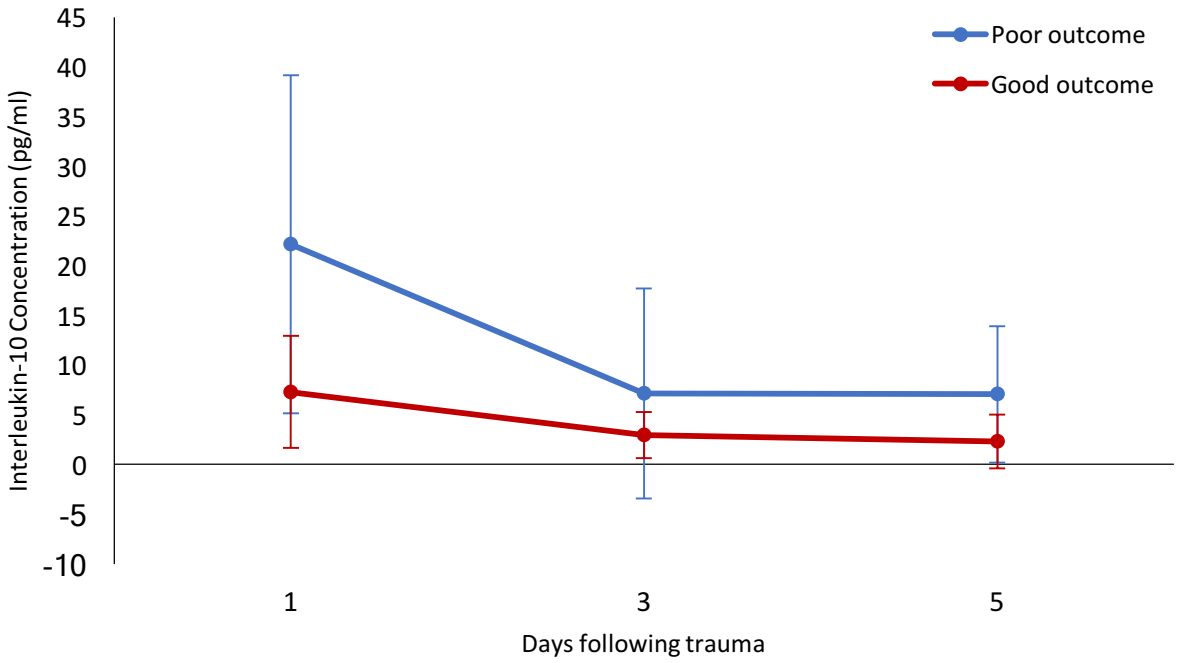

Figure 50) A comparison of interleukin-10 in good outcome patients (n= 42) and poor outcome patients (n= 8) on day 1 (Good = 7.30±5.64) (Poor = 22.16±16.99), day 3 (Good = 2.98±2.32) (Poor = 7.16±10.57) and day 5 (Good = 2.29±2.71) (Poor = 7.06±6.85) following traumatic injury.

Interleukin-10 concentration differed significantly between good and poor outcome patients. In day 1, patients with a good outcome had an average interleukin-10 concentration of 7.30 pg/ml compared to 22.16 pg/ml in patients with a poor outcome. Both good and poor outcome patients decreased on day 3, with poor outcome patients remaining higher than in good outcome patients. In patients with a good outcome, the interleukin-10 concentration decreased to 2.98 pg/ml, with poor outcome patients decreasing to 7.16 pg/ml. In day 5, both patient groups slightly decreased. Poor outcome patients decreased to 7.06 pg/ml, while good outcome patients decreased to 2.30 pg/ml.

3.6 - Interleukin-6/interleukin-10 ratio as a predictor of patient outcome

3.6.1 - Interleukin-6/interleukin-10 concentrations in trauma patient serum samples

Once interleukin-6 and interleukin-10 were analysed, a ratio between the pro and anti-inflammatory cytokines was developed. Once developed, comparisons were made between patient SOFA scores and, good and poor patient (Figure 51).

The day 1 interleukin-6/interleukin-10 ratio ranged from a high of 64.30:1 to a low 0.28:1. The day 1 median ratio was 7.86:1 with a mean ratio of 10.59:1. In day 3 the maximum ratio was 85.12:1 with a minimum of 0.13:1. The day 3 median ratio was 10.72:1 with a mean ratio of 15.54:1. In the day 5 samples, the maximum interleukin-6/interleukin-10 ratio was 80.51:1 with a minimum ratio of 0.13:1. The day 5 median ratio was 6.83:1, with a mean value of 10.69:1 .

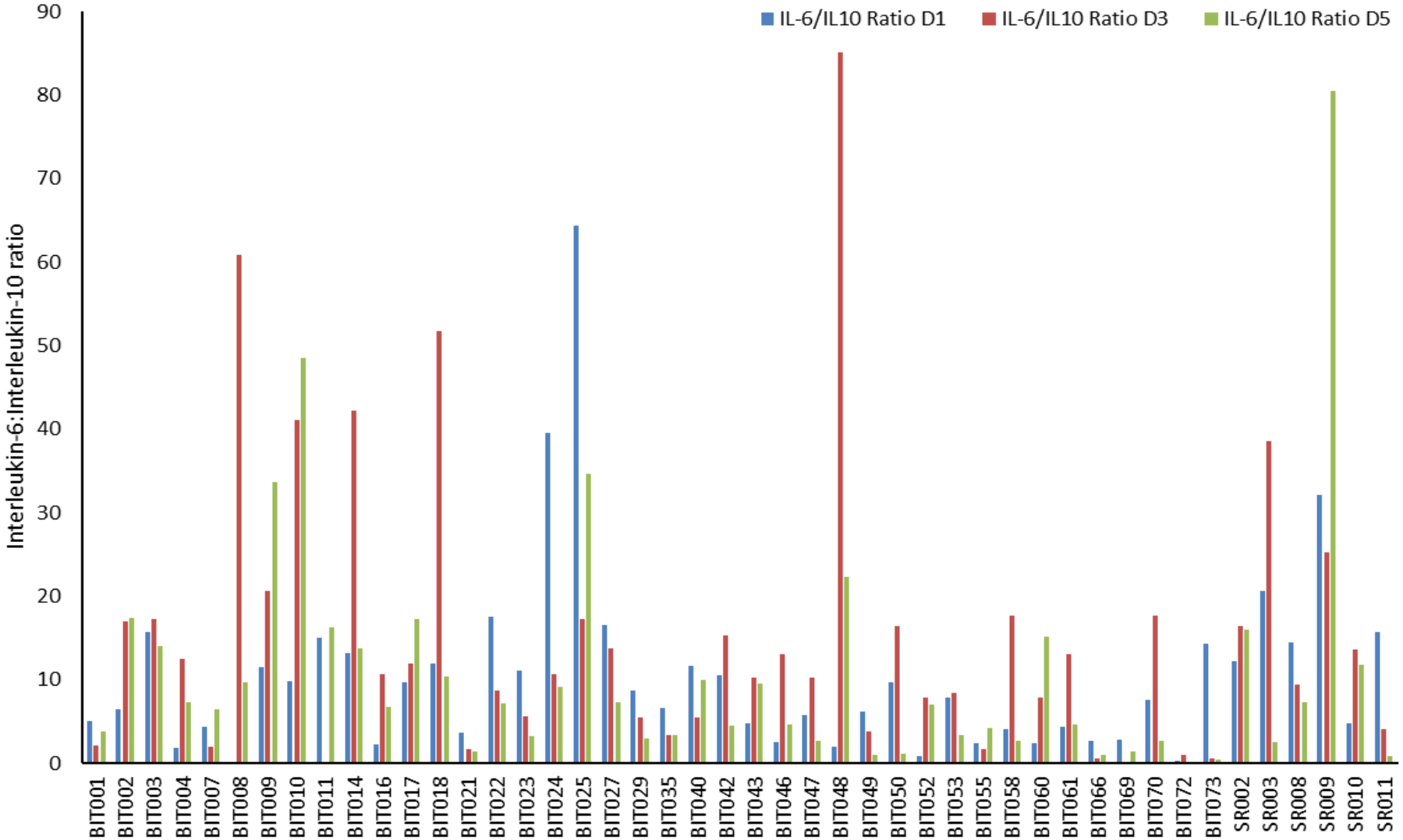

Figure 51) The interleukin-6/Interleukin-10 ratio for all analysed patient samples (n= 50), showing the change in ratio over the 5-day period.

3.6.2 - A comparison between interleukin-6/interleukin-10 ratio and patient SOFA scores

Patients were then grouped based on their SOFA scores and compared to the interleukin-6/interleukin-10 ratio. These were then compared to determine if there was a correlation between interleukin-10 concentration and patient SOFA scores (Figures 52 - 54).

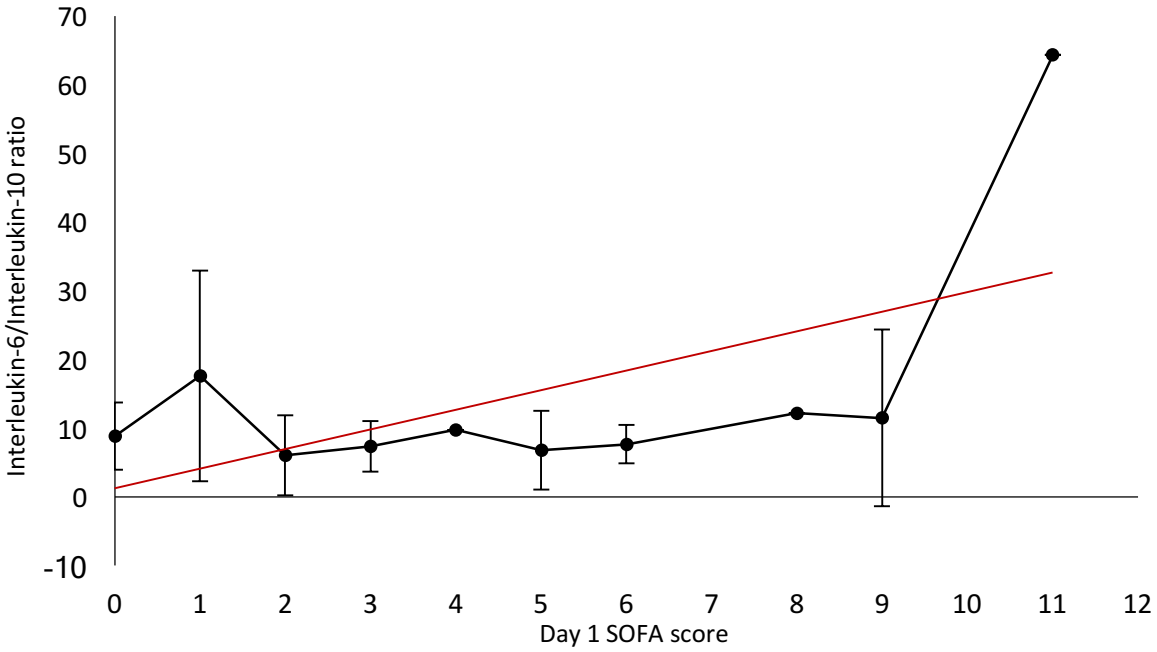

Figure 52) A comparison of averaged interleukin-6/interleukin-10 ratio against day 1 SOFA score, in patients grouped based upon day 1 SOFA score.

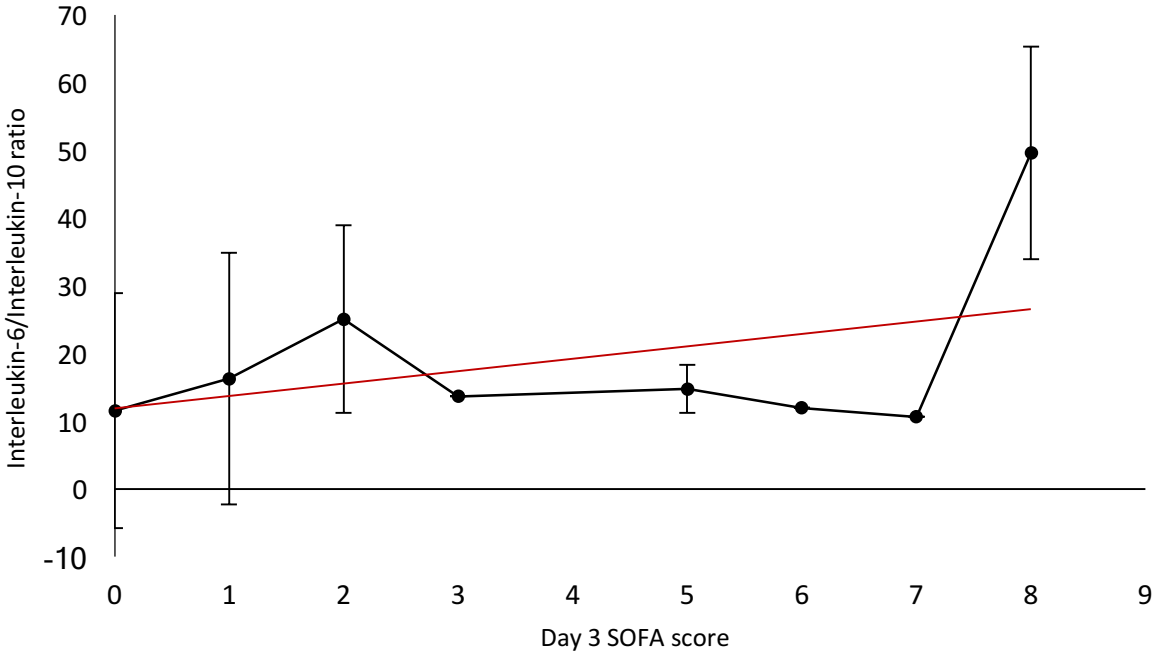

Figure 53) A comparison of averaged interleukin-6/interleukin-10 ratio against day 3 SOFA score, in patients grouped based upon day 3 SOFA score.

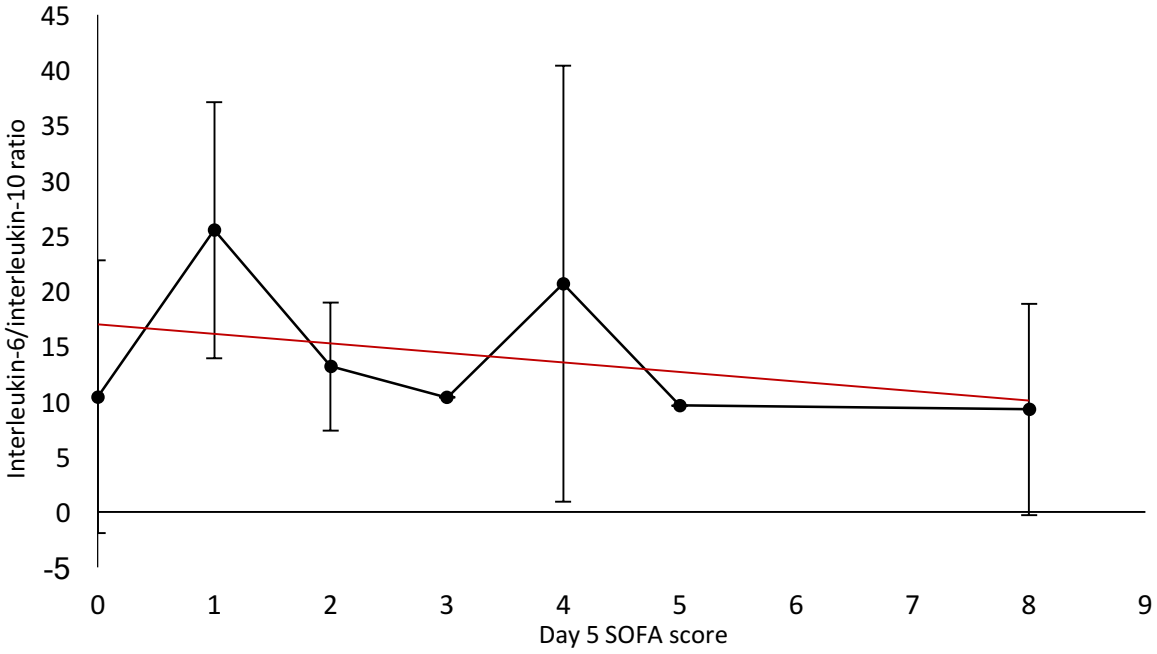

Figure 54) A comparison of averaged interleukin-6/interleukin-10 ratio against day 5 SOFA score, in patients grouped based upon day 5 SOFA score.

The interleukin-6/interleukin-10 ratio was found to be elevated on day 1 in patients with higher SOFA scores (Figure 52). With patients with a SOFA score of 11 having a ratio of 64.30:1 compared to between 6.08:1 and 17.60:1 in patients with SOFA scores below 9. In day 3 (Figure 53) patient ratios ranged from 10.65:1 to 49.67:1. Patients with a SOFA score of 2 had a high ratio at 25.09:1, while Patients with a SOFA score of 8 had the highest ratio at 49.67:1. In day 5 (Figure 54), interleukin-6/interleukin-10 ratio ranged between 25.50:1 and 9.27:1. The ratio decreased from patients with a SOFA score of 0 to those with a SOFA score of 8, with peaks occurring at SOFA scores of 1 and 4, with ratios of 25.50:1 and 20.66:1 respectively.

3.6.3 - Interleukin-6/interleukin-10 ratio as a predictor of patient outcome

The Interleukin-6/interleukin-10 ratio was compared to their patient outcome. The patients were grouped into good and poor patient outcomes using the criteria shown in Table 13), and the average interleukin-10 concentration for each group was evaluated over the three sample time points (Figure 55).

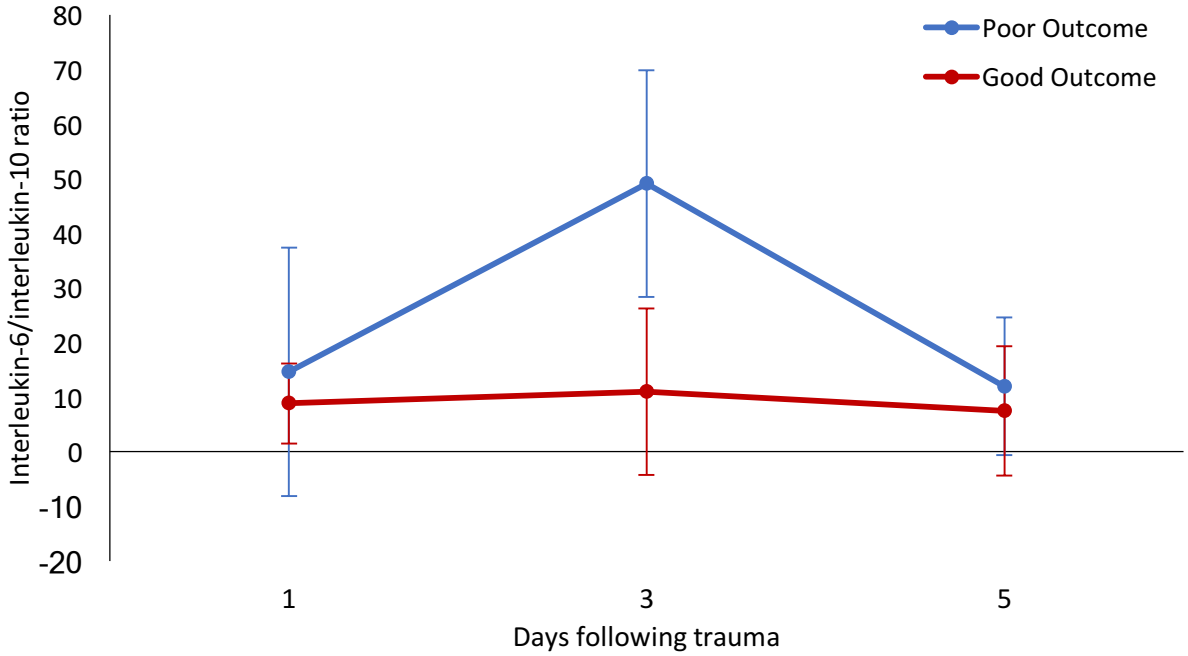

Figure 55) A comparison of averaged interleukin-6/interleukin-10 ratio between good outcome patients (n= 42) and poor outcome patients (n= 8) on day 1 (Good = 8.85±7.34) (Poor = 14.68±22.78), day 3 (Good = 11.00±15.22) (Poor = 49.14±20.81) and day 5 (Good = 12.00±12.57) (Poor = 7.50±11.88) following traumatic injury.

Figure 55 demonstrates that the interleukin-6/interleukin-10 ratio was higher in patients with a poor outcome compared to those with a good outcome. The ratio was slightly higher poor outcome patients on days 1 and 5, whilst being considerably elevated on day 3 compared to good outcome patients.

3.7 - Metabolomics analysis of trauma patient serum samples

Further analysis was conducted to identify significantly differentially expressed metabolites based on the day 1 interleukin-10 concentration. Patients were grouped based on day 1 interleukin-10 concentration >12 and <12 pg/ml.

Metabolomics analysis using LC/MS identified 9249 individual compounds in the Progenesis QI v2.3 analysis of the positive ion dataset. Their reproducibility and biological variability were evaluated through replicate injections by Progenesis QI v2.3, with further data analysis being conducted at the University of Salford to identify differentially regulated metabolites.

Following patient grouping based on their day 1 interleukin-10 concentration, 611 metabolites were found to be differentially regulated day 1, day 3 and day 5 trauma patient serum samples (Appendices 5-7). Metabolites were further narrowed to those with a fold change of >2, to identify those with a greater potential for further analysis.

The day 1 metabolite profile (Figure 56) identified 22 metabolites. Two of these metabolites were found to be meaningfully elevated above the rest of the metabolites identified in day 1. These metabolites are Dioxin B and Sulfoglycolithocholic acid.

The day 3 metabolite profile (Figure 57) identified 49 metabolites. Eight of the 49 metabolites were discovered to be elevated higher than the rest of the metabolites. These metabolites are cholic acid, ergosta-5,7,22,24(28)-tetraen-3beta-yl acetate, PG, Chenodeoxy cholic acid, glycochenodeoxycholic acid 7-sulfate, geissospermine and PI.

The Day 5 metabolite profile (Figure 58) identified 28 metabolites. Two of these metabolites were found to be notably elevated above the rest of the metabolites identified in day 5. These metabolites are Amoxicillin and Geissospermine.

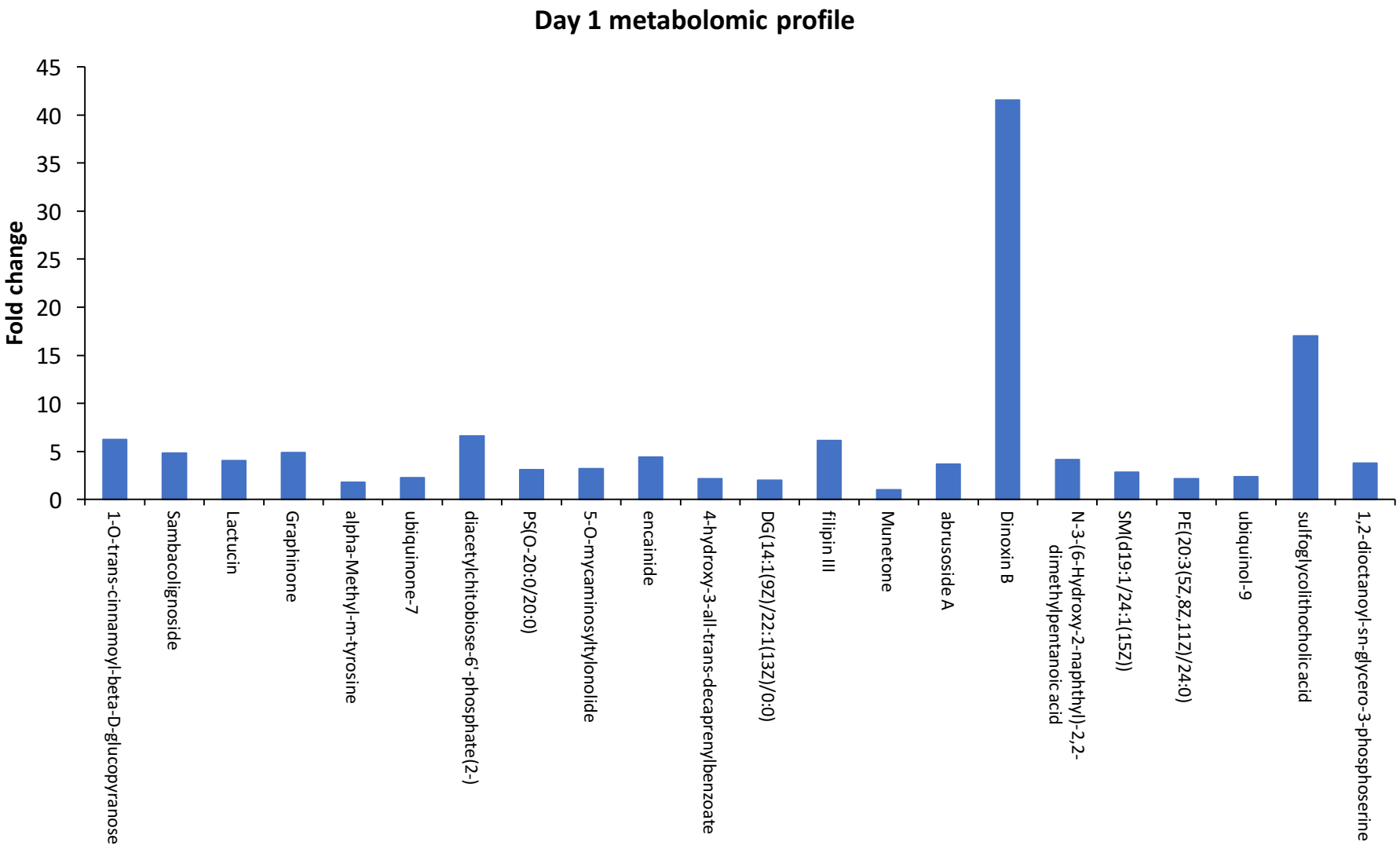

Figure 56) The significantly expressed Day 1 metabolites (n= 22) identified in patients with interleukin-10 concentrations of >12 pg/ml

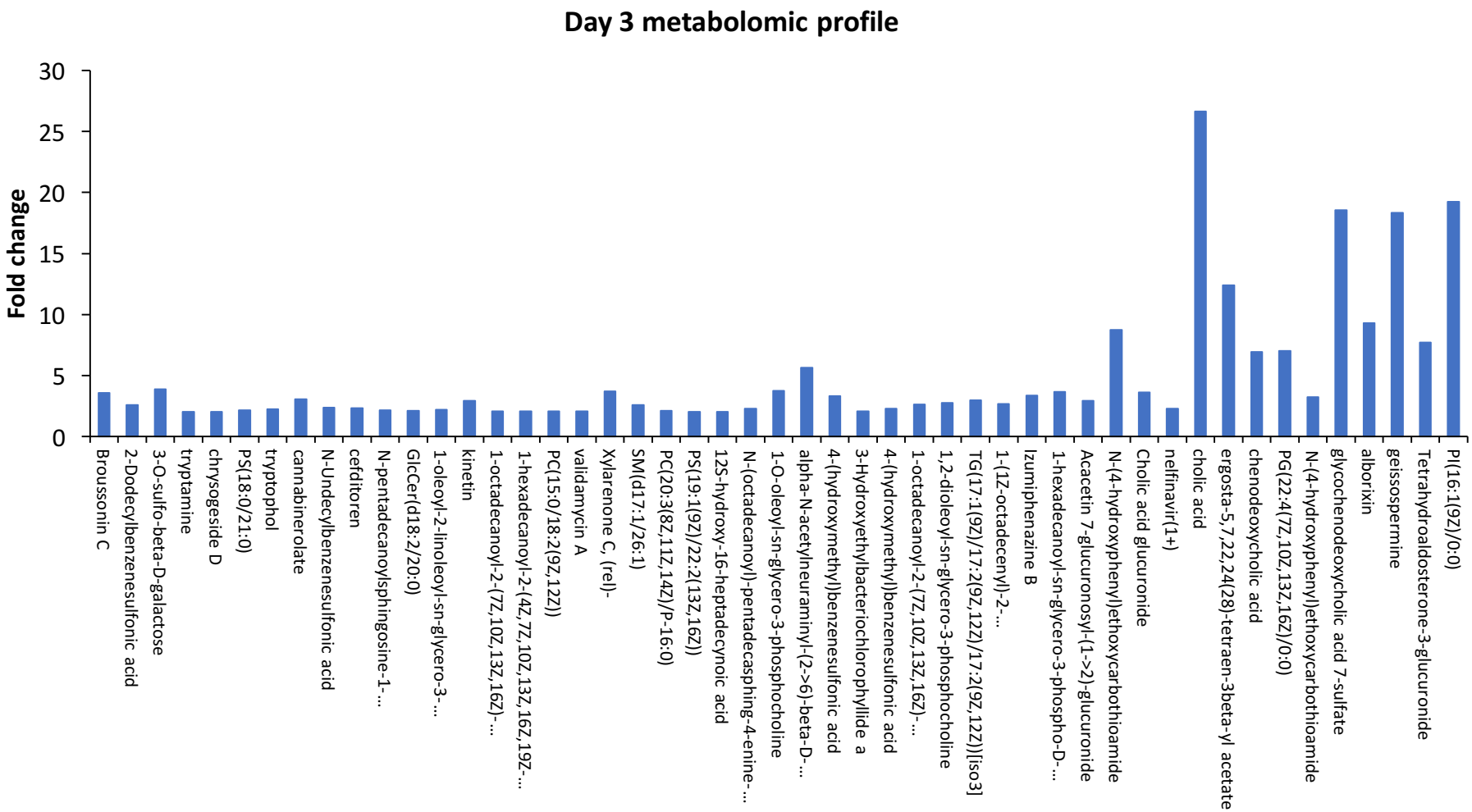

Figure 57) The significantly expressed Day 3 metabolites (n= 49) identified in patients with interleukin-10 concentrations of >12 pg/ml

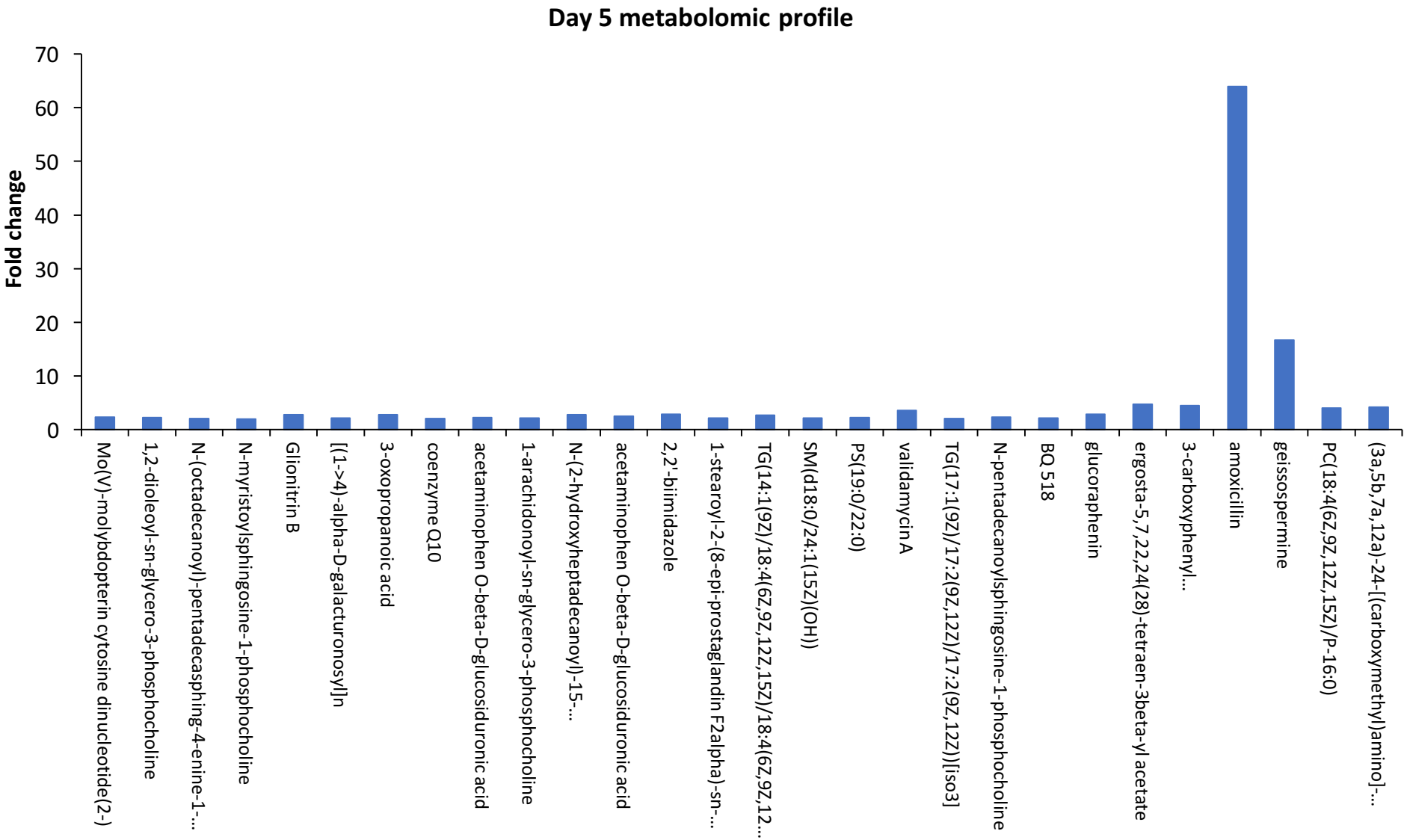

Figure 58) The significantly expressed Day 5 metabolites (n= 28) identified in patients with interleukin-10 concentrations of >12 pg/ml.

Chapter 4 - Discussion

Trauma is a major health burden, affecting both high and low-income countries and is considered one of the main causes of death and disability worldwide (World Health Organisation, 2012). This is due to trauma being the main cause of death in adults under the age of 40 and the incidence of trauma increasing in the elderly population in developed countries, due to the rapidly growing ageing population in developed countries (Kehoe *et al*., 2015). This changing trend in the age of trauma patients has resulted in falls from a height of <2m becoming the main cause of traumatic injury in the United Kingdom. The increasing numbers of elderly patients suffering trauma, places an increasing burden health services due to these patients having significant levels of comorbidities, which may exacerbate or impair the recovery following traumatic injury.

Trauma related injuries are often complex in nature with patients required to stay in the hospital for extended periods of time, often involving specialist care within an intensive care unit (ICU). These complex care needs result in a high cost to health services. This has been shown in the UK National Health Service, where trauma has an annual estimated cost of £1.6 billion, accounting for 7% of the total NHS budget as of 2008 (Christensen *et al*., 2008).

It has been shown that inflammation following traumatic injury is a key component in the recovery of traumatic injury. However, this response has been shown to be linked to the development of the systemic inflammatory response syndrome and septic complications. As both interleukin-6 and interleukin-10 have been demonstrated to play major roles in the inflammatory response. This project aims to determine if the concentrations of these two cytokines can be used as biological markers to predict patient outcome and the development of complications. The concentration of these cytokines would then be used to group patient metabolite data to identify significant metabolites, which would be further examined determine if they had any diagnostic or prognostic value for trauma patients. Additional analysis of identified metabolites along with interleukin-6 and interleukin-10 would be conducted, to determine if they could be used to stratify patient cohorts, and identify those at risk of developing complications quickly following admission. This stratification would allow aggressive treatment to be provided rapidly, offering patients the best opportunity for recovery and preventing the development of complications.

Patient recruitment analysis

This pilot study encompasses a variety of different methods to identify the variations in interleukin-6 and interleukin-10 concentration, along with analysis of metabolomics profiles for trauma patients. Initially, the patient cohort was analysed to identify and group patients based upon if they had a good outcome or a poor outcome following trauma. From the cohort who provided samples for all three time points (n=69), 50 patients were analysed to identify their outcome using the criteria described in Table 13). Of the 50 patients, 42 had a good outcome, whilst 88 patients had a poor outcome per the distinguishing criteria. Of the 8 poor outcome patients, three patients died in the period following their injury.

When the analysed patients were categorised based on the hospital from which they were admitted, it identified a difference between the two hospitals. The results identified that 10% of the trauma patients from Manchester Royal Infirmary developed a poor outcome following trauma, compared to 40% of the patients recruited from Salford Royal Hospital. This difference is due to Salford Royal being a major trauma centre for cranial based injuries, indicating that the patients from Salford Royal may have significantly more severe injuries than those admitted to Manchester Royal Infirmary. The difference may also be a result of the significantly smaller patient cohort recruited from Salford Royal Hospital compared to Manchester Royal Infirmary. From the 69 triplicate samples received, 50 triplicate samples were analysed to determine their interleukin-6 and interleukin-10 concentrations in days 1, 3 and 5 following their admission to hospital, following traumatic injury.

Cytokine profile of trauma patient serum samples

The analysis of interleukin-6 concentration in trauma patient samples identified the highest concentration to be found in day 1 following trauma, with this concentration decreasing in day 3 and decreasing further in day 5. This shows that the pro-inflammatory response following trauma is at its peak in the period shortly after the traumatic injury before decreasing in the following days, as the patients begin to recover from their injuries.
This pattern of interleukin-6 has previously been shown in research by Mörs *et al*., 2016, Gebhard *et al*., 2000, and Seekamp *et al*., 2002. Gebhard *et al*., 2000 observed the peak concentration to occur at 12 hours after injury, increasing from the site of injury to 12 hours, before decreasing for the 7 days after the injury.

Interleukin-10 concentration was found to be elevated at all sample time-points with the highest concentration on day 1, with it decreasing in days 3 and 5. The elevated interleukin-10 concentration following major trauma has been observed in other studies looking at major trauma and following major surgery (Neidhardt *et al*., 1997; Giannoudis *et al*., 2000; Smith *et al*., 2000; Easton & Balogh, 2014). The change in interleukin-10 concentration over a 5-day period matches that shown in Sun *et al*., 2011, which showed interleukin-10 to peak 24 hours after the initial injury.

Following the measurement of interleukin-6 and interleukin-10 concentrations, patients were grouped based on their SOFA scores for each of the day's samples were taken. The patient SOFA score was used to assess the likelihood of patients developing organ failure and further complications. When patients in this study were grouped using their SOFA scores their interleukin-6 concentration on day 1 was found to be the greatest in patients with a SOFA score of 11, which was the highest observed in the patient cohort. These results are like those of Sousa *et al*., 2015, which observed a correlation between clinical scoring systems and interleukin-6 concentration in the development of MODS and ultimately death, with patients with higher interleukin-6 concentrations having a greater risk of developing complications following trauma.

When interleukin-10 was compared to patient SOFA scores was found to peak in SOFA score 9 on day 1 with a lower concentration in SOFA scores 0-8 and a decrease in patients with a SOFA score of 11. In day 3 the interleukin-10 concentration was found to peak in patients with a SOFA score of 4 before decreasing in higher SOFA scores. Day 5 interleukin-10 concentration was found to be the highest at higher SOFA scores. This showed a strong positive correlation between interleukin-10 and patient SOFA scores. These results differ from those written in Liu *et al*., 2017, which showed a positive correlation between interlukinin-10 and SOFA score on day 1 after trauma. Patients were further grouped into good and poor outcome based on criteria including SOFA score on day 5 after injury and length of ICU stay. The interleukin-6 concentrations of good outcome patients were notably lower than those in poor outcome patients for the three time points collected. This indicates an elevated interleukin-6 concentration may be linked to the development of a poor outcome in trauma patients.

The results of this study resemble those observed in Gebhard *et al*., 2000, which identified an elevated interleukin-6 concentration in more severely injured patients compared to ones with less severe injuries. This idea was further supported by Stensballe *et al*., 2009, which noted that serum interleukin-6 concentration was higher in patients who did not survive 30 days following injury. They also identified that the interleukin-6 concentration of non-survivors was up to 6 times higher than those observed in surviving patients, which are comparable to that observed in our study when the poor outcome groups was compared to the good outcome group of patients.

In interleukin-10 analysis for patients grouped based on their outcome. The analysis showed significant differences between poor outcome and good outcome patients. In day 1, the interleukin-10 concentration was found to be 3 times higher in poor outcome patients than in patients who had a good outcome. The higher interleukin-10 concentration in poor outcome patients has been similarly observed in Mijatovic *et al*., 2015, in which non-survivors having a higher interleukin-10 concentration at each time point measured compared to good outcome patients.

The measurement of interleukin-6 and interleukin-10 allowed a ratio between the two cytokines to be measured. This ratio was found to be elevated in poor outcome patients in day 3 following traumatic injury. It was also shown to decrease in day 5 in patients grouped on their SOFA score. These results differ from those in Sousa *et al*., 2015, which observed a low interleukin-6/interleukin-10 ratio at 24 and 72 hours following traumatic injury correlated with negative outcome, namely organ dysfunction, organ failure and death.

In general, these results determine that both interleukin-6 and interleukin-10 play key roles in the inflammatory response to traumatic injury and that the changes in both cytokines occur in parallel rather than the previously thought pro-inflammatory response followed by a counter anti-inflammatory response. It also identifies that both interleukin-6 and interleukin-10 can offer advances in the ability to predict patient outcome, through their correlation with commonly used clinical scoring systems, calculated after patient discharge or at a point when the modification of treatments too late to benefit the patient.

Metabolomic analysis of trauma patient serum samples

Patient serum samples were further analysed to identify changes in the metabolite profile of trauma patients. It also compared the changes in these metabolite profiles over the 5-day period following trauma to identify modifications in the profile, which may act as a diagnostic biomarker for the prediction of patient outcome after traumatic injury. This analysis identified 611 metabolites in trauma patient serum samples grouped based on day 1 interleukin-10 concentration. Of these metabolites, twelve key molecules were identified as potential markers, based on their elevated fold change in the metabolite within the day 1, 3 and 5 samples. One of these main molecules identified in the day 5 trauma patient samples with a day 1 interleukin-10 concentration of >12 pg/ml is amoxicillin.
This is a penicillin based antibiotic, used in the treatment of various types of bacterial based infection. The presence of amoxicillin in the day 5 metabolite profile, in patients with higher day 1 interleukin-10 concentrations, indicates that these patients have prolonged antibiotic usage. The potential ability of interleukin-10 concentrations to detect the need for prolonged antibiotic usage may act as an early signpost for patients with a greater risk of bacterial infection and therefore act as a potential indicator for the development of septic complications, allowing treatment to be provided earlier following admission for those at greater risk and limit usage to those patients who are unlikely to acquire infective complications.

Metabolomic analysis provided a novel approach to the identification of altered molecules in trauma patient serum samples. The identified molecules can be further examined to classify if they can be effectively measured in patient biological samples and if they accurately and precisely offer predictive value for patient outcome and the development of complications.

Limitations of the study

This study had factors which lead to limitations in the effectiveness of the study. One of these limitations was due to the study using a heterogeneous patient cohort containing the diverse population found in the Greater Manchester region, resulting in a large range in serum cytokine levels identified in the patient cohort. This range in serum cytokine concentration is a result of patients, gender, ethnicity, type of injury, the number of transfusions given, genetic polymorphisms and the co-morbidity status.

These potential factors could be controlled by increasing the size of the patient cohort to allow potential outlying patients to be identified and further examined to identify the cause of the outlying result. Patients could also be additionally grouped based on their gender and co-morbidities, to identify if they have an impact on the serum levels of interleukin-6 and interleukin-10.

The results of the pilot study have identified that interleukin-6 and interleukin-10 can be used to identify patient outcome early after admission and that correlations are observed in the comparison between interleukin-10 concentration on day 5 and patient's day 5 SOFA score. However, the heterogeneity of the population along with the sample size being below the target for statistical powering resulted in substantial error bars, indicating an uncertainty in the data points. Further data collection up to the target of 200 patients, would decrease the uncertainty in the results and allow statistical analysis to be conducted, to validate any differences that were identified during this pilot study.

The size of the patient cohort used for the pilot study may have been a limiting factor in this study, due to the study being statistically powered for a cohort of 200 patients. Therefore, statistical analysis cannot be conducted as the cohort used for the pilot study is not large enough to represent the whole patient cohort and statistical analysis would not identify significant differences between variables until the target of 200 patients was reached. Consequently, the results of the study so far can only offer trends for the data analysed during the pilot study and not be representative of a full cohort of 200 patients.

The retrieval of clinical data used to determine if patients had a good or a poor outcome following traumatic injury takes a prolonged time to organise and collate. This delays the ability to identify poor outcome patients, following the completion of sample retrieval from the patients. This is a result of the large volume of work conducted by research nurses and clinicians, preventing them from collating all the information required to generate patient SOFA scores, ISS and length of hospital stay. The clinical data also did not provide a description of the type of injury, either penetrating or blunt. Without this information, it cannot be determined if the patient has had significant blood loss due to their injuries. This lack of knowledge regarding blood loss, may play a major role in the concentration of cytokines within the patient's serum. Significant blood loss at the initial point of injury, may require the use of intravenous fluids or blood transfusion to maintain an adequate volume of blood in the patient. These interventions may dilute the concentrations of cytokines and metabolites within the serum, causing results that are lower than expected based on the severity of their injuries. This may account for anomalously low interleukin-6 and interleukin-10 concentrations identified in this study, but further clinical information is required to confirm this assumption.

Future Research and potential applications of the study

To continue this study, further research would have to be conducted. The first piece of research to be conducted would be to continue to recruit patients and process these patients triplicate samples. Once the statistical target of 200 patient triplicates is reached the samples will be analysed for interleukin-6 and interleukin-10. The continuation of the current study will allow the trends observed in this pilot study to be statistically validated in the complete cohort of 200 patients.

During this study, samples were taken at time intervals of the first 24 hours after injury (day 1), 72 hours after injury (day 3) and 120 hours (day 5). In future studies these time points could be adjusted to add more samples to the earlier time periods following trauma, to identify the exact point at which the concentrations of interleukin-6 and interleukin-10 peak following traumatic injury.

This will identify the promptness of the initiation of the inflammatory response in patients following trauma and identify if a delay in the commencement of the inflammatory response, leaves patients at risk of debilitating complications. The time-points for sample collection could also be expanded beyond the day 5 collection point to analyse if there is a secondary immune response as described to occur in septic complications following traumatic injury. Samples could be collected on day 8, day 10 and day 14 after initial injury and hospital admission. However, collection of samples at these time-points may be difficult for many patients included in the study as they are commonly discharged within seven days of hospital admission, leaving only patients with major or life threatening injuries for sampling, limiting the size of patient cohort if the sample collection points were extended.

Further to this, the panel of cytokines will be expanded to generate a more expansive and detailed cytokine panel. This panel will be made up of additional inflammatory cytokine that have been shown to play key roles in the inflammatory response. This panel will include cytokines such as TGFβ, IL-1, IL-2, IL-4, IL-8, TNFα and IFNγ. This panel will also be expanded to include other molecules that have been found to have modified concentrations during an inflammatory response including HMGB1, heat shock proteins, chemokines and cellular adhesion molecules involved in the transmigration of leukocytes during the inflammatory response.

Metabolomic analysis could also be continued for the full cohort of 200 patients to identify any additional pathways involved in the response to trauma that were not identified in the initial 35 triplicate samples analysed during the pilot study. Further examination of the metabolomic data will identify additional metabolites, which were not initially identified and remove any outlying data observed in the pilot study patient cohort. This additional analysis will also validate the presence of the penicillin based antibiotic metabolites, when patients were grouped using day 1 interleukin-10 concentrations, in a larger cohort.

Further applications of this research may involve using molecules identified through metabolomic analysis to compare trauma patients to healthy individuals. This research will allow the identification of modified pathways involved in traumatic injury. The identification of these modified metabolic pathways has the potential to identify therapeutic targets to potentially decrease the impact of trauma and increase the effectiveness of currently used therapeutic options. These metabolites can also be used as biomarkers, to stratify patients into groups based on the likelihood of them developing complications. Stratification allows aggressive treatment to be provided at the earliest opportunity, giving the patient the greatest opportunity of an uncomplicated recovery from traumatic injury.

The findings of the pilot study could be applied in the development of an assay, to rapidly and cost effectively measure the concentration of both interleukin-6 and interleukin-10 at the point of admission. This would allow a specific and selective test, to predict if the patient was going to have a poor outcome because of their injuries, and allow clinicians to provide optimal care and treatment strategies early, to prevent patients developing complications and allow them the best chance at a full recovery from their injuries.

The ultimate long term goal of the study would be to develop a panel of molecules with known concentrations, to differentiate between good and poor outcome in trauma patients. The biomarker panel could be used to identify the patient cohorts with poor outcome as early as possible following the traumatic injury. This would allow clinicians to provide aggressive treatment options earlier, allowing the patient to begin the recovery process quicker and avoid the development of late stage complications following trauma. The ability to rapidly identify patients who are likely to have complications following trauma will enhance patient's quality of care during and after their hospitalisation.

Conclusion

In conclusion, this research identifies interleukin-6 and interleukin-10 as potential biomarkers for the prediction of patient outcome following traumatic injury. It showed that interleukin-10 concentration can be used to identify patient outcome at an early stage in the period following traumatic injury, through its strong correlation with patient SOFA scores and the significantly elevated penicillin based antibiotic metabolites identified in day 5 samples, using day 1 interleukin-10 concentrations. The results generated so far, identify key trends in the data, with a larger patient cohort required to develop a statistically validated interpretation of the results.

Chapter 5 - References

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Chapter 6 - Appendices

Appendix 1 - Patient recruitment consent form

Central Manchester University Hospitals NHS

NHS Foundation Trust

Investigator: Prof Kevin Mackway-Jones and Dr Richard Body

Patient Information Sheet

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish.
This sheet tells you the purpose of this study, what will happen to you if you take part and provides more detailed information about how the study will be carried out. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. Thank you for reading this.

What is the purpose of the study?
We are investigating a condition called major trauma. This is a process whereby a person becomes severely injured. It is known that, after a major injury, the body activates inflammatory mechanisms that are designed to promote healing. This inflammatory process and other mechanisms that reverse this process can become exaggerated after a significant injury. This can result in the person developing further illness after the initial injury.

We are investigating the levels of inflammation proteins called cytokines and cells in the immune system called T-regulatory cells. It is hoped that these markers in the blood can be used to predict whether someone will survive after a major trauma and be of use in targeting treatments in the future for patients.

Why have I been chosen?
You have been asked to take part in this study as you have suffered a major injury requiring hospitalisation. We are planning to study 200 patients in total, admitted to Manchester Royal Infirmary.

Do I have to take part?
It is up to you to decide whether or not to take part. If you do decide to take part in the study, you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part, you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part at all, will not affect the standard of care you receive.

What will happen to me if I take part?
You will be asked to provide a 20ml blood sample on the day of your injury and on the third and fifth days afterwards. The blood samples will be sent to a laboratory to estimate the levels of inflammatory cytokines and T regulatory cells. We request that blood samples are treated as a gift and are able to be stored at the University of Salford after the completion of this study in order to perform further analysis at a later date.
All samples will be coded and not contain any personal identifying information. These samples will initially be stored at this hospital and then be sent to the University of Salford for storage and analysis. Samples will be stored beyond the end of this study in accordance with the Human Tissue Act.

What do I have to do?
You will not have to do anything different if you decide to take part. The medical and nursing staff will take the blood samples while in the emergency department and on the ward. We will continue to collect daily clinical information from your medical notes relating to your condition throughout your stay in hospital.
With your consent, we will share your name, postcode and date of birth with the Health and Social Care Information Centre. This will enable the Health and Social Care Information Centre and other central UK NHS bodies to provide us with information about your health status after hospital discharge for up to 6 months.
If you do not wish to be part of this study, no further information will be collected about you for the trial and the doctors will continue to provide you with whatever medical treatment is needed.

Will this affect the way I am treated in hospital?
No. Inclusion in the study will not change the care that you receive and the doctors and nurses caring for you will not be aware of the results of the tests in the study.

What are the possible benefits of taking part?
This study will improve our understanding of why some people survive major trauma and others do not and hopefully improve our care for people with major trauma in the future. However, this study will not have any direct benefits to your health.

What are the possible disadvantages and risks of taking part?
Blood samples will need to be collected. This will usually be done from existing lines, but it might be necessary to collect a sample from a new needle, which might result in some minor discomfort during collection and possibly a small bruise.

Will information from this study be kept confidential?
All information, including personal information, which is collected about you during the course of the research will be kept password protected and strictly confidential. Any information about you which leaves the hospital will have your name, hospital number and address removed and will be identified only by your Trial subject number, date of birth and initials, so that you cannot be recognised from it. This is with the exception of information obtained from The Health and Social Care Information Centre as described earlier. Only the researchers and representatives of regulatory authorities and research ethics committees may have direct access to it. Other doctors in this hospital treating you will be told of your participation in this study.

What will happen to the results of the research study?
The results of this study will be presented at medical meetings and published in scientific journals. Only group information and no personal information will be presented. If you are interested in the results you will be able to contact the investigators for further information.

Who is organising and funding the research?
This study is being organised by doctors and scientists at Manchester Royal Infirmary and the University of Salford. It is funded by the University of Salford.

Who has reviewed the study?
All research in the NHS is looked at by an independent group of people called a Research Ethics Committee.

Who can I contact for independent research information?
If you have any questions about being in a research study, you can contact the Trust's Patient Advice Liaison Service (PALS). They will give you advice about who you can talk to for independent advice.

Further information
Thank you for considering participation in this study. If you have any questions about this research, the local study staff will be more than happy to answer them. Their contact details are:

Study Investigators Contact details

Study Investigator	Prof Kevin Mackway-Jones and Dr Richard Body
Study Nurse	Richard Clark
Day time Telephone	0161 276 6777
Emergency Telephone	0161 276 4712

CONSENT FORM FOR PATIENTS ABLE TO GIVE CONSENT

Patient #		Site #	
Name of Research Doctor			

Please initial each box if you agree with the following:

☐ I, *(forename and surname*)…… freely agree to take part in the study.

☐ I confirm that I have read and understood the patient information sheet dated January 2015 Version 1.0 for the above study and have been able to ask questions which have been answered fully.

☐ I understand that my participation is voluntary and I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.

☐ I understand my identity will never be disclosed and any information collected will remain confidential.

☐ I agree that my medical records and other personal data generated during the study may be examined by the research team and by representatives of Regulatory authorities, where it is relevant to my taking part in this research.

☐ I agree that I will not seek to restrict the use to which the results of the study may be put.

☐ I agree to gift my samples to a tissue bank for future scientific study.

☐ I understand that information held and managed by The Health and Social Care Information Centre and other central UK NHS bodies may be used in order to provide information about my health status. To do this, I understand that my name, postcode and date of birth will be shared with The Health and Social Care Information Centre.

Patient	Person responsible for collecting the informed consent
Date:	*Date:*
Signature:	*Signature:*
Printed Name:	*Printed Name:*

Appendix 2 - Day 1 clinical data for trauma patients selected for the pilot study

	HR	Sys BP	Dia BP	MAP	Temp	Hb	WCC	PLT	eGFR	Creat	Bili	PT	Intubated	NIV / CPAP	FiO2	P/F
BIT001	115	110	60	76.66667	37.8	134	31.6	356	42	132	34	15.6	N	N	0.21	N
BIT002	125	122	84	96.66667	38	122	18.6	142	50	134	28	16.6	N	N	0.28	38.2
BIT003	94	70	45	53.33333	36	123	11.5	180	55	116	6	15.4	N	N	0.3	N
BIT004	129	101	61	74.33333	36.5	112	16.2	213	76	85	7	14.4	N	N	0.21	63.1
BIT007	110	126	66	86	37.5	140	19	298	90	76	12	14.7	N	N	0.35	48.3
BIT008	110	85	60	68.33333	37.7	99	5.9	152	65	69	13	20.8	Y	N	0.21	58
BIT009	90	102	55	70.66667	37.9	108	22	232	81	101	15	16.3	N	N	0.28	N
BIT010	116	99	35	56.33333	37.8	130	9.9	286	80	68	7	13.7	N	N	0.3	21.6
BIT011	90	103	71	81.66667	36.4	114	15.4	318	27	158	8	19.7	N	N	0.4	N
BIT014	112	102	56	71.33333	36.2	78	16.6	129	78	88		16.3	Y	N	0.4	29.2
BIT016	117	72	48	56	36.3	115	26.6	150	50	126	9	15.7	N	N	0.35	34.7
BIT017	127	94	63	73.33333	36.2	120	10.9	222	72	83	6	13.7	Y	N	0.6	71.9
BIT018	109	175	90	118.3333	35.4	146	16.8	260	90	49	6	11.3	N	N	1	N
BIT021	146	90	70	76.66667	35.2	109	33.5	235	50	146	10	14.2	Y	N	0.3	50
BIT022	97	117	75	89	35.6	128	27.1	408	90	79	5	10.8	N	N	0.28	N
BIT023	98	88	65	72.66667	35.7	112	22.1	156	83	91	18	12.3	N	N	0.32	43.6
BIT024	115	74	40	51.33333	38.2	83	16.4	155	90	56	8	11.2	Y	N	0.6	62.3
BIT025	122	85	45	58.33333	33.9	97	16.1	130	31	241	20	11.9	N	Y	0.35	9.34
BIT027	129	163	65	97.66667	38	100	28.8	203		111	61	10.7	Y	N	1	27.6
BIT029	94	159	84	109	35.5	119	6.8	132	90	74	28	11.2	N	N	1	N
BIT035	89	168	93	118	36.2	139	14	295	67	83	15	11.2	N	N	0.85	N
BIT040	110	109	88	95	36.1	131	33.2	226	80	99	11	11.4	N	N	0.6	61.6
BIT042	99	135	67	89.66667	38.4	118	19.5	213	N	93	5	11.9	N	N	0.21	N
BIT043	109	79	30	46.33333	37.7	117	19	194	81	101	9	12	N	N	0.35	33.4
BIT046	92	80	50	60	35.5	73	29.6	78	59	81	16	13.4	N	N	0.85	31.4
BIT047	160	77	56	63	35	147	28.7	248	67	114	12	12.4	Y	N	0.7	41.9
BIT048				0		145	27.1	287	81	91	12	12.1	y	n		
BIT049	123	177	77		35.1	121	16.4	129	89	102	27	12	n	n	0.32	39.2
BIT050	97	123	59		35/6	149	8.7	359	64	116	10	11.5	y	n	0.28	0
BIT052	112	151	76		36.5	94	13.3	161	66	88	12	13	y	n	0.35	40.3
BIT053	85	103	52	69	34.6	117	8.1	154	62	77	7	11.5	n	n	0.85	30.6
BIT055	88	165	75	105	38.2	105	8.9	146	82	81	18	12.3	n	n	0.28	49.8
BIT060	130	70	40	50	35.7	80	13.9	101	45	146	25	12.7	Y	N	0.95	27.7
BIT061	116	180	105	130	38.1	128	14.4	220	89	63	10.9	11	y	n	0.8	19.9
BIT066	22?	162	85	110.6667	35	138	13.2	178	81	83	8	11.9	n	n	0.28	n
BIT067	82	146	81	102.6667	36.9	124	12.5	166	99	84	15	11.4	n	n	0.35	n
BIT069	112	95	55	68.33333	35.5	104	19.7	257	79	104	15	11.9	y	n	0.85	
BIT070	110	178	117	137.3333	35.8	143	26.9	339	89	88	11	11.8	y	n		
BIT072	53	138	72	94	35.6	130	6.8	242	n	76	20	12.6	n	n	0.21	n
BIT073	53	138	72	94	35.6	129	16	210	56	87	32	11.7	n	n	0.7	9.79

	Lactate	Norad	CRP	CVVH/HD	Sedated	GCS	Antibiotics	Septic source	MAP SOFA	PLT SOFA	CREAT SOFA	BILI SOFA	RESP SOFA	GCS SOFA	SOFA DAY 1
BIT001	1.2	0	19	N	N	15	Y		0	0	1	2	0	0	3
BIT002	3.9	0	63	N	N	15	Y		0	1	1	1	2	0	5
BIT003	N	0	2	N	N	15	Y		1	0	1	0	0	0	2
BIT004	0.9	0	102	N	N	15	N		0	0	0	0	0	0	0
BIT007	0.9	0	60	N	N	15	Y		0	0	0	0	1	0	1
BIT008	2	0	23	N	Y	N	Y		1	0	0	0	0	0	1
BIT009	N	0	6	N	N	14	Y		0	0	0	0	0	1	1
BIT010	1.3	0	100	N	N	15	N		1	0	0	0	3	0	4
BIT011	N	0	117	N	N	15	N		0	0	1	0	0	0	1
BIT014	3.4	0	8	N	Y	N	Y	empirical	0	1	0	0	2	0	3
BIT016	3.5	0	5	N	N	15	Y		1	1	1	0	2	0	5
BIT017	1.7	0	6	N	Y	N	Y	empirical	0	0	0	0	0	0	0
BIT018	0.7	0	89	N	N	15	Y	empirical	0	0	0	0	0	0	0
BIT021	3.4	0	1	N	N	15	Y	empirical	0	0	1	0	1	0	2
BIT022	N	0	3	N	N	15	Y	empirical	0	0	0	0	0	0	0
BIT023	1.1	0	17	N	N	14	Y	empirical	0	0	0	0	1	1	2
BIT024	3.8	0	170	N	Y	N	Y		1	0	0	0	0	0	1
BIT025	6.3	0.07	345	N	N	15	Y	empirical	3	1	2	1	4	0	11
BIT027	1.4	0	30	N	Y	N	Y	empirical	0	0	1	2	2	0	5
BIT029	N	0	226	N	N	15	Y	empirical	0	1	0	1	0	0	2
BIT035	N	0	43	N	N	15	N		0	0	0	0	0	0	0
BIT040	1.5	0	10	N	N	15	Y	empirical	0	0	0	0	0	0	0
BIT042	N	0	1	N	N	15	Y	empirical	0	0	0	0	0	0	0
BIT043	3.3	0		N	N	15	N		1	0	0	0	2	0	3
BIT046	1.8	0	6	N	N	15	Y	empirical	1	2	0	0	2	0	5
BIT047	4.8	1.01	75	N	Y	N	Y	empirical	4	0	1	0	1	0	6
BIT048			71	n	y	N	y	e	1	0	0	0	4	0	5
BIT049	7.7	0	1	n	n	y15	y	empirical	1	1	0	1	2	0	5
BIT050	2	0	20	n	y	N	y	empirical	1	0	1	0	4	0	6
BIT052	4.1	0	2	n	y	N	y	empirical	1	0	0	0	1	0	2
BIT053	1.7	0	32	n	n	15	y	empirical	1	0	0	0	2	0	3
BIT055	1.6	0	38	n	n	15	y	empirical	0	1	0	0	1	0	2
BIT060	5.4	0.19		N	Y	N	Y	empirical	4	1	1	1	2	0	9
BIT061	2	0	18	n	y	N	y	empirical	0	0	0	0	3	0	3
BIT066	n	0	3	n	n	15	y	empirical	0	0	0	0	0	0	0
BIT067		0	13	n	n	15	y	empirical	0	0	0	0	0	0	0
BIT069		0	4	n	y	N	y	empirical	1	0	0	0	4	0	5
BIT070		0	n	n	y	N	y	empirical	0	0	0	0	4	0	4
BIT072	n	0	1	n	n	14	y	eye	0	0	0	1	0	1	2
BIT073	2	0	116		n	15	y	empirical	0	0	0	1	4	0	5

Appendix 3 - Day 3 clinical data for trauma patients selected for the pilot study

	HR	Sys BP	Dia BP	MAP	Temp	Hb	WCC	PLT	eGFR	Creat	Bili	PT	Intubated	NIV / CPAP	FiO2	P/F
BIT001	98	124	91	102	37.5	77	18.1	194	90	78	9	16.7	N	N	0.21	N
BIT002	126	136	76	96	36.2	96	10.8	157	75	94	12	15.7	N	N	0.28	33.3
BIT003	115	89	55	66.33333	36.4	70	5.4	135	64	101	7	15.2	N	N	0.21	N
BIT004	123	92	80	84	38.1	79	15.8	152	90	54	9	15	N	N	0.21	51.2
BIT007	87	125	70	88.33333	37.6	112	6.8	144	90	52	16	13.1	N	N	0.21	N
BIT008	130	140	90	106.6667	36.9	96	20.6	147	89	65	26	14.4	Y	N	0.21	31
BIT009	82	112	43	66	36.8	80	8.2	156	79	104	11	15.7	N	N	0.24	N
BIT010	133	136	78	97.33333	38.2	109	8.1	236	90	57	6	14	N	N	0.5	32.4
BIT011	91	113	49	70.33333	38.2	78	13.5	190	36	137	11	17.9	N	N	0.4	N
BIT014	112	103	60	74.33333	36.4	112	21	163	77	101		15	N	N		N
BIT016	95	109	82	91	36.5	86	11.5	96	45	137	11	18	N	N	0.3	28.3
BIT017	130	170	90	116.6667	36.1	124	10.1	116	85	72	31	15.6	Y	N	1	55.4
BIT018	100	142	81	101.3333	38	100	5.6	133	90	46	9	11.3	N	N	0.4	N
BIT021	109	141	86	104.3333	36	94	13.1	154	90	80	16	10.9	N	N	0.28	N
BIT022	87	136	82	100	37.2	97	8.6	278	90	62	6	11.4	N	N	0.21	N
BIT023	90	100	56	70.66667	36.8	89	10	131	90	79	8	11.4	N	N	0.33	N
BIT024	99	96	50	65.33333	37.3	69	15	166	90	147	3	10.2	Y	N	0.4	47.2
BIT025	170	100	35	56.66667	36.9	97	17.2	142	63	100		10.4	N	Y	0.6	18.24
BIT027	72	149	69	95.66667	37.5	94	13.3	138	90	73	6	10.2	N	N	0.21	31.2
BIT029	98	107	69	81.66667	35.4	108	5.7	142	90	66	17	10.1	N	N	0.24	N
BIT035	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
BIT040	107	131	85	100.3333	37.3	96	10.7	350	90	80	14	N	N	N	0.28	N
BIT042	126	132	85	100.6667	38.4	103	11.1	177	N	73	9	12.3	N	N	0.21	N
BIT043	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
BIT046	105	110	62	78	35.5	75	32.3	78	51	92	15	13	N	N	0.32	25.8
BIT047	125	107	49	68.33333	38.4	83	6.8	97	90	89	20	11.5	N	N	0.28	40.6
BIT048				0		74	6.9	101	14	427	8	11.2	y	n		n
BIT049	99	146	76	99.33333	37.4	111	7.9	111	90	63	12	11.1	n	n	0.32	n
BIT050	118	110	56	74	39	118	7	200	90	76	14	n	n	n	0.28	n
BIT052	110	151	100	117	37.4	84	6.4	110	90	66	10	11.3	n	n	0.24	n
BIT053	103	160	88	112	37.6	107	6.4	116	90	47	17	11.9	n	n	0.55	21.9
BIT055	87	137	62	87	37.7	105	5.9	113	90	72	114	12.3	n	n	0.21	42.5
BIT060	87	100	50	66.66667	37.8	70	7.9	92	70	99	8	10.9	Y	N	0.5	18.4
BIT061	100	85	64	71	38.2	91	11	164	90	48	12	11.6	y	n	0.4	22.6
BIT066	87	147	77	100.3333	36.1	132	8.8	233	90	66	n	n	n	n	0.21	n
BIT067	76	135	76	95.66667	36.6	n	n	n	n	n	n	n	n	n	0.21	n
BIT069	125	145	80	101.6667	37.1	125	8.2	192	90	65	23	11	n	n	0.24	58.2
BIT070	54	139	63	88.33333	37.3	n	n	n	n	n	n	n	n	n	0.21	n
BIT072	98	153	70	97.66667	37	n	n	n	n	n	n	n	n	n	0.21	n
BIT073	98	153	70		37	122	4.5	208	75	68	n	11.9	n	n	0.21	n

	Lactate	Norad	CRP	CVVH/HD	Sedated	GCS	Antibiotics	Septic source	Steroids	CAM +ve	MAP SOFA	PLT SOFA	CREAT SOFA	BILI SOFA	RESP SOFA	GCS SOFA	SOFA DAY 3
BIT001		0	145	N	N	15	Y		N	N	0	0	0	0	0	0	0
BIT002	1.3	0	349	N	N	15	Y		N	N	0	0	0	0	2	0	2
BIT003		0	120	N	N	15	Y		N	N	1	1	0	0	0	0	2
BIT004	0.5	0	279	N	N	15	N		N	N	0	0	0	0	1	0	1
BIT007		0	97	N	N	15	N		N	N	0	1	0	0	0	0	1
BIT008	2.1	0	239	N	Y	N	Y		Y		0	1	0	1	2	0	4
BIT009	0.7	0	127	N	N	15	Y		N	N	1	0	0	0	0	0	1
BIT010	0.9	0	317	N	N	15	N		N	N	0	0	0	0	2	0	2
BIT011		0	147	N	N	15	N		N	N	0	0	1	0	0	0	1
BIT014		0	250	N	N	15	Y	empirical	Y	N	0	0	0	0	0	0	0
BIT016	1.2	0	318	N	N	15	Y		N	N	0	2	1	0	2	0	5
BIT017	1.3	0	41	N	Y	N	Y	empirical	N		0	1	0	1	0	0	2
BIT018		0	155	N	N	15	N		N	N	0	1	0	0	0	0	1
BIT021	0.8	0	178	N	N	15	N		N	N	0	0	0	0	0	0	0
BIT022		0	53	N	N	15	N		N	N	0	0	0	0	0	0	0
BIT023		0	180	N	N	15	Y	empirical	N	N	0	1	0	0	0	0	1
BIT024	1.6	0	291	N	Y		Y		Y	N	1	0	1	0	1	4	7
BIT025	1.1	0	291	N	N	15	Y	empirical	N	N	1	1	0	0	3	0	5
BIT027	1	0	60	N	N	15	Y	empirical	Y	N	0	1	0	0	2	0	3
BIT029	N	0	174	N	N	15	Y	empirical	N	N	0	1	0	0	0	0	1
BIT035	N	0	N	N	N	N	N		N	N	0	0	0	0	0	0	0
BIT040	N	0	340	N	N	15	Y	empirical	N	N	0	0	0	0	0	0	0
BIT042	N	0	201	N	N	15	N		N	N	0	0	0	0	0	0	0
BIT043	N	0	N	N	N	N	N	N	N	N	0	0	0	0	0	0	0
BIT046	1.3	Norad	15	N	N	15	Y	empirical	N	N	N	2	0	0	3	0	5
BIT047	0.9	0	291	N	N	15	Y	empirical	N	N	1	2	0	1	1	0	5
BIT048			273	y	y	N	y	e	y		1	1	3	0	0	0	5
BIT049	n	0	55	n	n	15	n		n	n	0	1	0	0	0	0	1
BIT050	n	0	438	n	n	15	n		n	n	0	0	0	0	0	0	0
BIT052	1.5	0	n	n	n	15	y	empirical	n	n	0	1	0	0	0	0	1
BIT053	0.8	0	197	n	n	15	n		n	n	0	1	0	0	3	0	4
BIT055	0.5	0	n	n	n	15	y	empirical	n	n	0	1	0	3	1	0	5
BIT060	1.9	0.05		N	Y	N	Y	empirical	N	N	3	2	0	0	3	0	8
BIT061	1.2	0	223	n	y	N	y	empirical	n	n	0	0	0	0	3	0	3
BIT066	n	0	n	n	n	15	n		n	n	0	0	0	0	0	0	0
BIT067	n	n0	n	n	n	15	n		n	n	4	0	0	0	0	0	4
BIT069	0.7	0	n	n	n	14	y	empirical	n	n	0	0	0	1	0	1	2
BIT070	n	0	n	n	n	15	y	empirical	n	n	0	0	0	0	0	0	0
BIT072	n	0	n	n	n	15	y	eye	y	n	0	0	0	0	0	0	0
BIT073	n	0	59	n	n	15	y	empirical	y	n	1	0	0	0	0	0	1

Appendix 4 - Day 5 clinical data for trauma patients selected for the pilot study

	HR	Sys BP	Dia BP	MAP	Temp	Hb	WCC	PLT	eGFR	Creat	Bili	PT	Intubated	NIV / CPAP	FiO2	P/F
BIT001	94	111	78	89	37.2	102	10.1	356	90	84	N	N	N	N	0.21	N
BIT002	107	131	79	96.33333	36.6	91	7.5	191	90	65	18	16.9	N	N	0.28	45.2
BIT003	110	85	65	71.66667	36.6	83	6.3	211	86	78	13	14	N	N	0.21	N
BIT004	117	92	76	81.33333	37.3	102	12	221	90	45	17	15	N	N	0.21	53.6
BIT007	85	115	85	95	36.5	122	7.7	217	90	61	10	13.1	N	N	0.21	N
BIT008	108	102	62	75.33333	35.7	85	20.3	199	90	48	22	12.5	Y	N	0.28	
BIT009	80	115	47	69.66667	37.1	83	7.6	233	90	91	15	14.7	N	N	0.21	N
BIT010	124	126	94	104.6667	36.2	114	6.7	270	90	38	7	13.6	N	N	0.4	N
BIT011	98	115	60	78.33333	36.9	N	N	N	N	N	N	N	N	N	0.32	N
BIT014	103	130	64	86	35.9	91	10.6	357	90	78	N	N	N	N	N	N
BIT016	100	113	70	84.33333	36.4	83	10	125	58	111	11	14.1	N	N	0.4	29.8
BIT017	112	140	65	90	37.7	82	6.2	85	90	60	8	13.6	N	N	N	N
BIT018	113	176	96	122.6667	37.6	111	8.5	228	90	53	13	10.7	Y	N	0.4	25.8
BIT021	98	133	78	96.33333	36	109	9.7	339	90	70	9	10.4	N	N	0.21	N
BIT022	54	130	75	93.33333	36.1	N	N	N	N	N	N	N	N	N	N	N
BIT023	80	111	58	75.66667	37.1	N	N	N	N	N	N	N	N	N	0.21	N
BIT024	95	105	50	68.33333	36.7	82	9	198	90	52	6	9.9	Y	N	0.3	45.4
BIT025	75	96	46	62.66667	34.7	92	14.9	166	83	79	16	11.1	N	N	0.6	22.1
BIT027	66	116	80	92	36.4	118	11.9	350	90	75	N	N	N	N	0.21	N
BIT029	86	107	69	81.66667	35.4	N	N	N	N	N	N	N	N	N	0.21	N
BIT035	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
BIT040	117	134	82	99.33333	38.5	103	12.2	409	90	86	21	N	N	N	0.24	N
BIT042	106	127	73	91	37.4	101	8.6	239	N	64	8	11.3	N	N	0.21	N
BIT043	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
BIT046	110	86	37	53.33333	35.8	80	48.7	84	83	60	24	13.4	N	N	0.5	23.5
BIT047	112	139	65	89.66667	38.2	86	7.1	179	90	76	22	11.4	N	N	0.28	43.9
BIT048				0		74	12.7	245	19	321	12	11	y	n		n
BIT049	97	147	81		37.2	123	8.2	248	90	71	8	n	n	n	0.21	n
BIT050	98	115	74	87.66667	36.1	119	7.2	339	90	69	11	n	n	n	0.28	n
BIT052	98	132	87	102	35.9	n	n	n	n	n	n	n	n	n	0.21	n
BIT053	105	155	72		37.5	102	6.2	207	90	48	n	11	n	n	0.32	n
BIT055	90	98	63	74.66667	37.5	n	n	n	n	n	n	n	n	n	0.21	n
BIT060	125	105	50	68.33333	37.7	77	6.5	165	75	94	15	10.8	Y	N	0.6	11.3
BIT061	97	140	55	83.33333	35.8	82	8.8	213	90	38	14	11.1	n	n	0.35	27.4
BIT066	87	145	72		35.7	129	9.9	261	90	62	13	n	n	n	0.21	n
BIT067	Discharged			0												
BIT069	110	149	88	108.3333	37.1	125	8.4	242	90	67	7	11.1	n	n	0.28	63.7
BIT070	56	145	63	90.33333	36	123	10.6	349	78	98	n	n	n	n	0.21	n
BIT072	104	136	71	92.66667	35.7	n	n	n	n	n	n	n	n	n	n	n
BIT073	83	131	83		36.9	127	5.6	254	76	67	7	n	n	n	0.28	n

	Lactate	Norad	CRP	CVVH/HD	Sedated	GCS	Antibiotics	Septic source	Steroids	CAM +ve	MAP SOFA	PLT SOFA	CREAT SOFA	BILI SOFA	RESP SOFA	GCS SOFA	SOFA DAY 5
BIT001	N	0	18	N	N	15	Y		N	N	0	0	0	0	0	0	0
BIT002	0.8	0	244	N	N	15	Y		N	N	0	0	0	0	1	0	1
BIT003	N	0	113	N	N	15	Y		N	N	0	0	0	0	0	0	0
BIT004	0.5	0	329	N	N	15	N		N	N	0	0	0	0	0	0	0
BIT007	N	0	55	N	N	15	N		N	N	0	0	0	0	0	0	0
BIT008	1.3	0	210	N	Y	N	Y	Abdomen	Y	N	0	0	0	1	4	0	5
BIT009	N	0	95	N	N	15	Y		N	N	1	0	0	0	0	0	1
BIT010	N	0	181	N	N	15	Y	Chest	N	N	0	0	0	0	0	0	0
BIT011	N	0		N	N	15	N		N	N	0	0	0	0	0	0	0
BIT014	N	0	194	N	N	15	N		N	N	0	0	0	0	0	0	0
BIT016	1.4	0	174	N	N	15	Y		N	N	0	1	1	0	2	0	4
BIT017	N	0	124	N	N	15	N		N	N	0	2	0	0	0	0	2
BIT018	1.2	0	92	N	Y	N	Y	empirical	N	N	0	0	0	0	3	0	3
BIT021	N	0	26	N	N	15	N		N	N	0	0	0	0	0	0	0
BIT022	N	0		N	N	15	N		N	N	0	0	0	0	0	0	0
BIT023	N	0		N	N	15	N		N	N	0	0	0	0	0	0	0
BIT024	0.6	0		N	Y	N	Y		N	Y	1	0	0	0	1	0	2
BIT025	1.4	0		N	N	15	N		N	N	1	0	0	0	3	0	4
BIT027	N	0	33	N	N	15	Y	POST OP	N	N	0	0	0	0	0	0	0
BIT029	N	0	N	N	N	15	N		N	N	0	0	0	0	0	0	0
BIT035	N	0	N	N	N	N	N	N	N	N	0	0	0	0	0	0	0
BIT040	N	0	309	N	N	15	Y	empirical	N	N	0	0	0	1	0	0	1
BIT042	N	0	150	N	N	15	N		N	N	0	0	0	0	0	0	0
BIT043	N	0	N	N	N	N	N	N	N	N	0	0	0	0	0	0	0
BIT046	0.9	0	181	N	N	13	N	empirical	N	N	1	2	0	1	3	1	8
BIT047	0.8	0	227	N	N	15	N		N	N	0	0	0	1	1	0	2
BIT048			350	n	y	N	y	empirical	n	n	1	0	3	0	0	0	4
BIT049	n	0	3	n	n	15	n		n	n	1	0	0	0	0	0	1
BIT050	n	0	267	n	n	15	n		n	n	0	0	0	0	0	0	0
BIT052	n	0	n	n	n	15	n		n	n	0	0	0	0	0	0	0
BIT053	n	0	123	n	n	15	n		n	n	1	0	0	0	0	0	1
BIT055	n	0	n	n	n	15	n		n	n	0	0	0	0	0	0	0
BIT060	1.2	0		N	Y	N	Y	empirical	Y	Y	1	0	0	0	4	0	5
BIT061	0.9	0	152	n	n	15	y	empirical	n	n	0	0	0	0	2	0	2
BIT066	n	0	53	n	n	n	n		n	n	1	0	0	0	0	0	1
BIT067											1	0	0	0	0	0	1
BIT069	1.1	0	n	n	n	15	n		n	n	0	0	0	0	0	0	0
BIT070	n	0	n	n	n	15	y	empirical	n	n	0	0	0	0	0	0	0
BIT072	n	0	n	n	n	15	y	eye	y	n	0	0	0	0	0	0	0
BIT073	n	0	22	n	y	15	y	empirical	y	n	1	0	0	0	0	0	1

Appendix 5 - Day 8 clinical data for trauma patients selected for the pilot study

	HR	Sys BP	Dia BP	MAP	Temp	Hb	WCC	PLT	eGFR	Creat	Bili	PT	Intubated	NIV / CPAP	FiO2	P/F
BIT001	80	117	78	91	36.9	108	27.5	460	90	86	N	N	N	N	0.21	N
BIT002	111	134	95	108	36.6	91	12.5	184	90	64	29	16.9	N	N	0.35	N
BIT003	89	93	53	66.33333	37.1	N	N	N	N	N	N	N	N	N	0.21	N
BIT004	92	114	64	80.66667	36.6	N	N	N	N	N	N	N	N	N	0.21	N
BIT007	75	134	65	88	36.3	N	N	N	N	N	N	N	N	N	0.21	N
BIT008	113	121	68	85.66667	36	94	29.5	241	90	36	23	13.8	Y	N	0.4	N
BIT009	80	119	63	81.66667	36.9	96	10.5	337	90	79	19	15.2	N	N	0.21	N
BIT010	125	134	100	111.3333	37.3	121	9.6	546	90	44	6	14.2	N	N	0.35	N
BIT011	91	121	68	85.66667	36.9	73	15.8	123	59	79		15.1	N	N	0.21	N
BIT014	100	112	72	85.33333	36.2	101	15.2	481	88	90	N	N	N	N	N	N
BIT016	93	97	66	76.33333	35.9	105	11.9	233	64	101	16	14.4	N	N	0.28	43.3
BIT017	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
BIT018	105	151	94	113	35.7	94	6.4	193	90	41	9	11.4	N	N	0.28	N
BIT021	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
BIT022	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
BIT023	90	105	57	73	37.8	N	N	N	N	N	N	N	N	N	21	N
BIT024	99	110	65	80	37.3	107	N	N	N	N	N	N	N	N	0.21	N
BIT025	87	83	45	57.66667	35.3	80	14.2	329	75	86	9	10.7	N	N	8	19.2
BIT027	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
BIT029	N	104	68	80	36	N	N	N	N	N	N	N	N	N	0.21	N
BIT035	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
BIT040	112	149	73	98.33333	39	100	17.3	708	90	82	26	N	N	N	0.28	N
BIT042	DISCHARGED			0												
BIT043	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
BIT046	154	86	55	65.33333	35.9	77	44	96	90	54	96	15	N	N	0.45	20.7
BIT047	120	150	62	91.33333	38.8	72	8.7	302	90	68	40	11.5	N	N	0.28	52.1
BIT048				0		84	20.4	270	12	470	18	10.9	y	n		
BIT049	76	126	63	84	36.2	n	n	n	n	n	n	n	n	n	0.21	n
BIT050	86	101	57	71.66667	36.3	125	7.1	351	90	64	13	n	n	n	0.21	n
BIT052	104	139	74	95.66667	36.2	n	n	n	n	n	n	n	n	n	0.21	n
BIT053	95	160	95	116.6667	37	101	3.6	100	n	n	n	n	n	n	0.32	n
BIT055	71	80	51	60.66667	37.4	n	n	n	n	n	n	n	n	n	0.21	n
BIT060	118	106	46	66	37.3	92	14.6	317	90	80	17	10.6	Y	Y	0.65	12.7
BIT061	99	140	77	98	35.5	n	n	n	n	n	n	n	n	n	0.21	n
BIT066	61	140	65	90	35.6	n	n	n	n	n	n	n	n	n	0.21	n
BIT067	DISCHARGED			0												
BIT069	56	120	69		36.5	n	n	n	n	n	n	n	n	n	0.21	n
BIT070	81	132	73		35.5	n	n	n	n	n	n	n	n	n	0.21	n
BIT072	DISCHARGED			0												
BIT073	n	n	n	0	n	126	5.9	271	68	74	n	11.4	n	n	0.21	n

	Lactate	Norad	CRP	CVVH/HD	Sedated	GCS	Antibiotics	Septic source	Steroids	CAM +ve	Tranexamic acid	Transfusion	MAP SOFA	PLT SOFA	CREAT SOFA	BILI SOFA	RESP SOFA	GCS SOFA	SOFA DAY 8
BIT001	N	0	88	N	N	15	N	N	N	N	N	N	0	0	0	0	0	0	0
BIT002	N	0	219	N	N	15	Y	N	N	N	N	N	0	0	0	1	0	0	1
BIT003	N	0	N	N	N	15	N	N	N	N	N	Y	1	0	0	0	0	0	1
BIT004	N	0	N	N	N	15	N	N	N	N	Y	Y	0	0	0	0	0	0	0
BIT007	N	0	N	N	N	15	N	N	N	N	N	N	0	0	0	0	0	0	0
BIT008	1.3	0	52	N	Y	N	Y	Abdomen	Y	N	N	Y	0	0	0	1	0	0	1
BIT009	N	0		N	N	15	N	N	N	N	N	N	0	0	0	0	0	0	0
BIT010	N	0	45	N	N	15	Y	Chest	N	N	N	N	0	0	0	0	0	0	0
BIT011	N	0	74	N	N	15	N	N	N	N	N	Y	0	1	0	0	0	0	1
BIT014	N	0	158	N	N	15	N	N	N	N	Y	Y	0	0	0	0	0	0	0
BIT016	1.3	0	134	N	N	15	N	N	N	N	N	N	0	0	0	0	1	0	1
BIT017	N	0	N	N	N	N	N	N	N	N	N	N	0	0	0	0	0	0	0
BIT018	N	0	206	N	N	15	Y	empirical	N	N	N	N	0	0	0	0	0	0	0
BIT021	N	0	N	N	N	N	N	N	N	N	N	N	0	0	0	0	0	0	0
BIT022	N	0	N	N	N	N	N	N	N	N	N	N	0	0	0	0	0	0	0
BIT023	N	0		N	N	15	N	N	N	N	Y	N	0	0	0	0	0	0	0
BIT024	0.6	0		N	N	14	Y	N	N	N	N	Y	0	0	0	0	0	1	1
BIT025	1.1	0	1	N	N	15	Y	Chest	N	N	N	N	1	0	0	0	3	0	4
BIT027	N	0	N	N	N	N	N	N	N	N	N	N	0	0	0	0	0	0	0
BIT029	N	0	N	N	N	15	N	N	N	N	N	N	0	0	0	0	0	0	0
BIT035	N	0	N	N	N	N	N	N	N	N	N	N	0	0	0	0	0	0	0
BIT040	N	0	329	N	N	15	Y	empirical	N	N	N	N	0	0	0	1	0	0	1
BIT042											Y	N	0	0	0	0	0	0	0
BIT043	N	0	N	N	N	N	N	N	N	N	N	N	0	0	0	0	0	0	0
BIT046	1.6	0	73	N	N	14	Y	empirical	N	N	Y	Y	1	2	0	2	3	1	9
BIT047	0.8	0	N	N	N	15	N		Y	N	Y	Y	0	0	0	2	1	0	3
BIT048			563	y	y		y	empirical	y	n	y	y	0	0	0	0	0	0	0
BIT049	n	0	n	n	n	15	n		n	n	y	n	0	0	0	0	0	0	0
BIT050	n	0	166	n	n	15	n		n	n	y	n	0	0	0	0	0	0	0
BIT052	n	0	n	n	n	15	n		n	n	y	y	0	0	0	0	0	0	0
BIT053	n	0	n	n	n	15	n		n	n	y	y	0	1	0	0	0	0	1
BIT055	n	0	n	n	n	15	n		n	n	y	y	1	0	0	0	0	0	1
BIT060	1.3	0.07		N	Y	12	N		Y	Y	Y	Y	3	0	0	0	4	2	9
BIT061	n	0	n	n	n	15	n		n	n	y	y	0	0	0	0	0	0	0
BIT066	n	0	n	n	n	15	n		n	n	n	n	0	0	0	0	0	0	0
BIT067											y	n	0	0	0	0	0	0	0
BIT069	n	0	n	n	n	15	n		n	n	y	y	1	0	0	0	0	0	1
BIT070	n	0	n	n	n	15	y	empirical	n	n	y	n	1	0	0	0	0	0	1
BIT072							y	eye	y	n	n	n	0	0	0	0	0	0	0
BIT073	n	0	15	n	n	15	y	empirical	y	n	y	n	0	0	0	0	0	0	0

Appendix 6 - Mean Interleukin-6 concentrations for triplicate patient serum samples with standard deviation for each triplicate

Patient ID	IL-6 D1	D1 SD	IL-6 D3	D3 SD	IL-6 D5	D5 SD
BIT001	11.3283	0.9530	2.4228	0.0292	3.0025	0.0723
BIT002	10.7934	0.3000	5.4975	0.3256	5.1244	0.3172
BIT003	4.7401	0.3551	5.5119	0.1875	3.7344	0.2526
BIT004	2.7799	0.0147	6.3927	0.2931	4.9771	0.1508
BIT007	25.3024	2.0796	10.5158	0.8736	4.4768	0.5955
BIT008	No Sample	No Sample	2020.8664	170.5098	51.5865	1.2747
BIT009	25.9519	1.1096	14.4543	0.1097	26.3803	0.6223
BIT010	60.3894	3.5942	32.1705	1.9517	14.8551	0.2760
BIT011	32.3502	0.6154	No Sample	No Sample	11.3450	0.2522
BIT014	255.4891	18.2785	278.6363	15.1042	27.0436	2.0413
BIT016	7.1854	0.1036	5.5007	0.2906	4.1860	0.1874
BIT017	136.0772	6.4167	32.2258	5.6547	35.8879	0.2194
BIT018	54.2813	2.2867	39.4671	2.3461	10.9028	1.0234
BIT021	55.0690	5.8551	18.5171	0.9390	2.4316	0.2073
BIT022	25.9795	0.8281	10.9857	1.5302	2.4178	0.0863
BIT023	62.0062	3.6937	33.9118	1.0813	5.7620	0.0957
BIT024	296.9882	7.7382	21.4745	1.1173	12.8513	0.3852
BIT025	1188.8666	27.6828	57.2524	2.3106	118.5821	2.8819
BIT027	82.1823	1.9754	10.8751	0.6663	106.6008	4.7322
BIT029	88.4424	3.4160	16.2232	0.4194	7.2821	0.1807
BIT035	23.3124	1.4037	6.7432	0.0415	6.5359	0.5026
BIT040	77.3317	4.0353	25.3715	2.4820	31.5763	1.6702
BIT042	85.3883	1.2650	29.1441	1.5558	8.4706	0.2941
BIT043	21.3224	0.5117	20.7973	0.2719	13.6390	4.6577
BIT046	7.3927	0.6112	43.3226	1.8412	12.6163	0.3056
BIT047	46.1694	1.8038	68.1972	1.2747	19.2772	0.3526
BIT048	41.5123	0.4173	421.3611	18.4044	285.8775	14.0998
BIT049	46.5563	1.9535	9.7419	0.4153	2.1967	0.0479

BIT050	56.1745	2.6663	68.8191	6.3337	2.7771	0.2127
BIT052	19.0008	0.7671	11.3726	0.3526	3.8550	0.2045
BIT053	30.8301	3.9548	26.8225	0.9143	7.4618	0.2283
BIT055	3.6339	0.1658	2.1690	0.2760	7.0334	0.2719
BIT058	525.9173	8.7858	143.7607	3.8314	9.9492	1.8210
BIT060	117.5733	4.7740	32.8753	3.8878	101.8194	0.6333
BIT061	12.4505	0.2760	34.9344	1.0626	8.3047	0.2533
BIT066	8.5811	0.3830	1.2017	0.4998	1.8650	0.4981
BIT069	88.1245	1.0414	0.2481	0.5266	2.5560	0.1436
BIT070	18.2546	0.4194	32.6542	1.6453	5.1401	0.1570
BIT072	0.8285	0.0957	1.9617	2.5641	0.2620	0.1333
BIT073	55.5665	1.7929	1.0773	0.2912	0.9944	1.3938
SR002	57.3768	3.2343	56.5062	2.7127	34.4645	0.4069
SR003	807.1246	39.8579	101.7365	2.7136	54.2260	3.3252
SR008	110.8641	1.0260	22.8287	0.4781	13.8048	0.3526
SR009	146.0823	27.4667	122.8661	0.9562	4635.4485	365.5366
SR010	28.0939	0.7678	132.3460	4.9082	34.0638	0.3590
SR011	53.9082	1.6397	8.8299	0.5598	2.0999	0.1658

Appendix 7 - Patient SOFA scores over a 5-day period

Patient ID	SOFA D1	SOFA D3	SOFA D5
BIT001	3	0	0
BIT002	5	2	1
BIT003	2	2	0
BIT004	0	1	0
BIT007	1	1	0
BIT008	1	4	5
BIT009	1	1	1
BIT010	4	2	0
BIT011	1	1	0
BIT014	3	0	0
BIT016	5	5	4
BIT017	0	2	2
BIT018	0	1	3
BIT021	2	0	0
BIT022	0	0	0
BIT023	2	1	0
BIT024	1	7	2
BIT025	11	5	4
BIT027	5	3	0
BIT029	2	1	0
BIT035	0	0	0
BIT040	0	0	1
BIT042	0	0	0
BIT043	3	0	0
BIT046	5	9	8
BIT047	6	5	2
BIT048	5	5	4
BIT049	5	1	1
BIT050	6	0	0

BIT052	2	1	0
BIT053	3	4	1
BIT055	2	5	0
BIT058	N/A	N/A	N/A
BIT060	9	8	5
BIT061	3	3	2
BIT066	0	0	1
BIT069	5	2	0
BIT070	0	0	0
BIT072	2	0	0
BIT073	5	1	1
SR002	8	5	8
SR003	9	8	8
SR008	6	1	1
SR009	N/A	N/A	N/A
SR010	N/A	N/A	N/A
SR011	N/A	N/A	N/A

Abbreviations: N/A, Patient SOFA score unavailable at the time of writing.

Appendix 8 - Interleukin-10 concentrations for triplicate patient serum samples

Patient ID	IL-10 D1	SD	IL-10 D3	SD	IL-10 D5	SD
BIT001	5.2654	0.3725	2.5059	0.0569	2.6244	0.0137
BIT002	4.4673	0.0958	2.5513	0.0205	2.5078	0.0712
BIT003	2.4980	0.0272	2.5454	0.0237	2.4130	0.0395
BIT004	2.8831	0.0214	2.7883	0.0330	2.8713	0.0718
BIT007	5.8730	0.4245	5.4169	0.3020	0.6899	0.3604
BIT008	No Sample	No Sample	33.2499	4.0467	5.3547	0.1534
BIT009	2.2656	0.1402	0.7003	0.0933	0.7832	0.1565
BIT010	6.1736	0.3110	0.7832	0.1402	0.3064	0.1257
BIT011	2.1515	0.2250	No Sample	No Sample	1.0631	0.0718
BIT014	19.3904	0.5502	6.6090	0.1244	1.9753	0.2177
BIT016	20.0091	0.8500	2.7745	0.0239	2.7370	0.0224
BIT017	14.1037	0.7567	2.6906	0.2657	2.0790	0.0783
BIT018	4.5358	0.2939	0.7625	0.0823	1.0527	0.0359
BIT021	15.2025	1.0566	10.7243	0.4141	1.7162	0.0647
BIT022	1.4881	0.0180	1.2704	0.8803	0.3375	0.0647
BIT023	5.5827	0.0440	6.0596	0.2375	1.7680	0.0180
BIT024	7.5005	0.3052	2.0168	0.1402	1.4155	0.0933
BIT025	18.4886	0.5138	3.3229	0.2608	3.4266	0.1295
BIT027	4.9608	0.1731	0.7936	0.1425	14.6324	0.8856
BIT029	10.2371	0.3577	2.9808	0.2023	2.4729	0.1866
BIT035	3.5095	0.1177	2.0375	0.0539	1.9442	0.0933
BIT040	6.6815	0.1768	4.6809	0.4581	3.1674	0.1402
BIT042	8.1432	0.4357	1.9028	0.1822	1.8717	0.0783
BIT043	4.5047	0.2375	2.0272	0.1177	1.4363	0.2375
BIT046	3.6339	0.0950	3.0638	0.0622	2.5558	0.2071
BIT047	8.1224	0.1257	6.6815	0.1000	7.3139	0.2645
BIT048	20.4374	0.7066	4.9504	0.0647	12.8079	1.4752
BIT049	7.5419	0.3156	2.5351	0.0622	2.1101	0.0475
BIT050	5.8315	0.0539	4.2040	0.2292	2.5351	0.0311

BIT052	23.6924	1.0136	1.4363	0.1177	0.5552	0.0950
BIT053	3.9242	0.3938	3.1674	0.0718	2.1723	0.1295
BIT055	1.4985	0.0359	1.3015	0.1092	1.6540	0.0647
BIT058	128.6391	2.6091	8.1639	0.2967	3.7376	0.0950
BIT060	49.3900	1.5541	4.2144	0.2850	6.7126	0.4679
BIT061	2.8564	0.0950	2.6802	0.1470	1.7680	0.0180
BIT066	3.1985	0.1900	2.0790	0.1565	1.9961	0.0950
BIT069	31.1767	0.8420	1.9028	0.0359	1.8820	0.1077
BIT070	2.4003	0.1295	1.8509	0.0933	1.9028	0.1768
BIT072	3.0119	0.0783	2.0168	0.0475	1.9857	0.1092
BIT073	3.8827	0.0950	2.1101	0.0180	2.4936	0.3115
SR002	4.7120	0.0311	3.4473	0.0647	2.1515	0.1713
SR003	39.2416	2.3795	2.6388	0.1257	21.4844	1.0890
SR008	7.6819	0.0660	2.4211	0.0718	1.9028	0.0647
SR009	4.5565	0.1077	4.8571	0.1257	57.5793	3.2396
SR010	5.9766	0.3888	9.7603	0.3068	2.8979	0.1534
SR011	3.4473	0.0359	2.1826	0.2023	2.5040	0.0311

Appendix 9 - Significantly modified metabolites in Day 1 trauma patient serum samples

Table 15) The D1 metabolites identified in patients with interleukin-10 concentrations of >12 pg/ml.

Description	Max Fold Change	m/z
Munetone	1.025472303	455.1220115
alpha-Methyl-m-tyrosine	1.795774295	178.085297
DG(14:1(9Z)/22:1(13Z)/0:0)	1.978404598	643.5264083
4-hydroxy-3-all-trans-decaprenylbenzoate	2.178383295	840.645032
PE(20:3(5Z,8Z,11Z)/24:0)	2.181271853	834.6383569
ubiquinone-7	2.266962038	659.4991604
ubiquinol-9	2.382961134	795.6366729
SM(d19:1/24:1(15Z))	2.835119335	862.6708856
PS(O-20:0/20:0)	3.093220862	816.6469225
5-O-mycaminosyltylonolide	3.218512167	615.3864577
abrusoside A	3.667869723	681.3436326
1,2-dioctanoyl-sn-glycero-3-phosphoserine	3.778979068	510.251131
Lactucin	4.042674261	277.1056635
N-3-(6-Hydroxy-2-naphthyl)-2,2-dimethylpentanoic acid	4.149461227	543.2796911
encainide	4.401388367	335.215046
Sambacolignoside	4.832954226	462.1641061
Graphinone	4.898774186	319.1537541
filipin III	6.145829424	637.3954667
1-O-trans-cinnamoyl-beta-D-glucopyranose	6.243818991	328.1378124
diacetylchitobiose-6'-phosphate(2-)	6.628058432	485.115622
sulfoglycolithocholic acid	16.98931479	512.2670485
Dinoxin B	41.54089278	613.2998733

Appendix 10 - Significantly modified metabolites in Day 3 trauma patient serum samples

Table 16) The D3 metabolites identified in patients with interleukin-10 concentrations of >12 pg/ml.

Description	Max Fold Change	m/z
1-O-palmitoyl-2-O-[1-(14)C]-linoleoyl-sn-glycero-3-phosphocholine	1.161735132	760.586052
PC(15:0/19:1(9Z))	1.161735132	760.586052
Indoleacrylic acid	1.186425062	188.0708579
1,2-dihexadecanoyl-sn-glycero-3-phosphocholine	1.198420784	734.5698887
sphingosine 1-phosphate	1.209928541	380.2554797
1-palmitoyl-2-(9Z-heptadecenoyl)-sn-glycero-3-phosphocholine	1.224626223	763.5942226
withalongolide H	1.269517773	761.3798447
mastoparan-A	1.304697126	812.5351886
N-(2-hydroxyhenicosanoyl)-1-O-beta-D-glucosyl-15-methylhexadecasphing-4-enine	1.333966577	794.6056798
PS(17:0/22:4(7Z,10Z,13Z,16Z))	1.343204887	413.7860746
PS(15:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	1.402896048	397.754044
PC(15:0/18:1(11Z))	1.403677367	746.5693917
chrysogeside D	2.024084196	788.5434407
12S-hydroxy-16-heptadecynoic acid	2.028355776	563.4292865
PS(19:1(9Z)/22:2(13Z,16Z))	2.029918818	836.5803482
tryptamine	2.040537793	159.0927115
3-Hydroxyethylbacteriochlorophyllide a	2.057699249	615.243979
validamycin A	2.063994793	478.1974203
1-octadecanoyl-2-(7Z,10Z,13Z,16Z)-docosatetraenoyl-sn-glycero-3-phosphoethanolamine	2.06736313	776.5590549
PC(15:0/18:2(9Z,12Z))	2.072554764	724.5267455

1-hexadecanoyl-2-(4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoyl)-sn-glycero-3-phosphocholine	2.076690637	840.5302249
PC(20:3(8Z,11Z,14Z)/P-16:0)	2.11123002	802.5585318
GlcCer(d18:2/20:0)	2.119893	788.5789628
PS(18:0/21:0)	2.161728031	814.5963463
N-pentadecanoylsphingosine-1-phosphocholine	2.171551685	723.5197242
1-oleoyl-2-linoleoyl-sn-glycero-3-phosphocholine	2.213813018	818.5540813
tryptophol	2.255029639	142.0660967
N-(octadecanoyl)-pentadecasphing-4-enine-1-phosphoethanolamine	2.279921018	645.4961903
4-(hydroxymethyl)benzenesulfonic acid	2.310644245	187.0070987
nelfinavir(1+)	2.311460013	567.3160352
cefditoren	2.335407119	541.0226888
N-Undecylbenzenesulfonic acid	2.388269828	311.1682475
SM(d17:1/26:1)	2.602691124	861.6604733
2-Dodecylbenzenesulfonic acid	2.606535581	325.1837676
1-octadecanoyl-2-(7Z,10Z,13Z,16Z)-docosatetraenoyl-sn-glycero-3-phosphoethanolamine	2.639937684	776.5542718
1-(1Z-octadecenyl)-2-(4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoyl)-sn-glycero-3-phosphoethanolamine zwitterion	2.666503752	774.5425822
1,2-dioleoyl-sn-glycero-3-phosphocholine	2.786108562	820.5687263
Acacetin 7-glucuronosyl-(1->2)-glucuronide	2.94520252	671.102883
kinetin	2.945917708	429.1538366
TG(17:1(9Z)/17:2(9Z,12Z)/17:2(9Z,12Z))[iso3]	3.000104573	873.6682825
cannabinerolate	3.075178428	340.2014055
N-(4-hydroxyphenyl)ethoxycarbothioamide	3.253366949	196.0431535
4-(hydroxymethyl)benzenesulfonic acid	3.338710567	187.0071651
Izumiphenazine B	3.358396783	503.0746343
Broussonin C	3.601232625	311.1682809

Cholic acid glucuronide	3.615522134	619.2867178
1-hexadecanoyl-sn-glycero-3-phospho-D-myo-inositol	3.683851138	571.2874536
Xylarenone C, (rel)-	3.709455172	863.5619864
1-O-oleoyl-sn-glycero-3-phosphocholine	3.766520274	556.3223247
3-O-sulfo-beta-D-galactose	3.899418025	259.0121958
alpha-N-acetylneuraminyl-(2->6)-beta-D-galactosyl-(1->4)-N-acetyl-beta-D-glucosamine	5.639433664	655.2240267
chenodeoxycholic acid	6.93414554	391.2844859
PG(22:4(7Z,10Z,13Z,16Z)/0:0)	7.010237177	595.2860054
Tetrahydroaldosterone-3-glucuronide	7.702602288	539.2490686
N-(4-hydroxyphenyl)ethoxycarbothioamide	8.758731038	196.0434759
alborixin	9.307318298	865.5662862
ergosta-5,7,22,24(28)-tetraen-3beta-yl acetate	12.42471216	871.6529564
geissospermine	18.33675809	613.3574145
glycochenodeoxycholic acid 7-sulfate	18.56891078	510.255934
PI(16:1(9Z)/0:0)	19.22760442	569.271834
cholic acid	26.66142448	389.2680757

Appendix 11 - Significantly modified metabolites in Day 5 trauma patient serum samples

Table 17) The D5 metabolites identified in patients with interleukin-10 concentrations of >12 pg/ml.

Description	Max Fold Change	m/z
1-hexadecanoyl-sn-glycero-3-phosphocholine	1.162691341	496.3402
1-oleoyl-2-linoleoyl-sn-glycero-3-phosphocholine	1.190290142	784.58572
N-tetracosanoylsphingosine	1.210806551	632.63381
SM(d17:1/24:1)	1.224592717	799.66925
Adouetine X	1.269009665	523.3243
1-linoleoyl-sn-glycero-3-phosphocholine	1.272602568	520.34056
PC(16:0/18:1(11Z))	1.287975062	782.57025
TG(15:0/18:4(6Z,9Z,12Z,15Z)/20:5(5Z,8Z,11Z,14Z,17Z))	1.336606421	897.63545
1-elaidoyl-sn-glycero-3-phosphocholine	1.383934792	522.3563
SM(d18:2/24:1)	1.464140515	833.65021
undecaprenyl dihydrogen phosphate	1.481116677	885.6354
1,2-dilinoleoyl-sn-glycero-3-phosphocholine	1.577396877	804.54926
PS(17:1(9Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	1.688191802	410.76215
N-myristoylsphingosine-1-phosphocholine	2.038679988	673.52874
coenzyme Q10	2.054926276	843.65754
N-(octadecanoyl)-pentadecasphing-4-enine-1-phosphoethanolamine	2.056076132	645.49619
TG(17:1(9Z)/17:2(9Z,12Z)/17:2(9Z,12Z))[iso3]	2.080584041	873.66828
SM(d18:0/24:1(15Z)(OH))	2.138313768	809.65406
BQ 518	2.167508224	631.24081
1-stearoyl-2-(8-epi-prostaglandin F2alpha)-sn-glycero-3-phosphocholine	2.189330969	840.5765
1-arachidonoyl-sn-glycero-3-phosphocholine	2.212555681	578.30011
[(1->4)-alpha-D-galacturonosyl]n	2.214400532	175.02458
PS(19:0/22:0)	2.225594435	842.63427
acetaminophen O-beta-D-glucosiduronic acid	2.226640792	326.08765
1,2-dioleoyl-sn-glycero-3-phosphocholine	2.296154359	820.56873

N-pentadecanoylsphingosine-1-phosphocholine	2.33879719	723.51972
Mo(V)-molybdopterin cytosine dinucleotide(2-)	2.389107471	843.92679
acetaminophen O-beta-D-glucosiduronic acid	2.548877944	326.08766
TG(14:1(9Z)/18:4(6Z,9Z,12Z,15Z)/18:4(6Z,9Z,12Z,15Z))[iso 3]	2.723249213	815.62437
3-oxopropanoic acid	2.76362566	175.02463
N-(2-hydroxyheptadecanoyl)-15-methylhexadecasphing-4-enine-1-phosphocholine	2.777370868	699.54655
Glionitrin B	2.83105144	364.04121
2,2'-biimidazole	2.873456213	113.02494
glucoraphenin	2.921141098	470.00011
validamycin A	3.56627774	478.19742
PC(18:4(6Z,9Z,12Z,15Z)/P-16:0)	4.066336488	736.52729
(3a,5b,7a,12a)-24-[(carboxymethyl)amino]-1,12-dihydroxy-24-oxocholan-3-yl-b-D-Glucopyranosiduronic acid	4.211322656	622.32165
3-carboxyphenyl phenylacetamidomethylphosphonate	4.462697571	384.03944
ergosta-5,7,22,24(28)-tetraen-3beta-yl acetate	4.795272992	871.65296
geissospermine	16.71930533	613.35741
amoxicillin	63.91268563	364.09636