INVESTIGATING CYTOKINE BIOMARKERS AS EARLY PREDICTORS OF POOR CLINICAL OUTCOME FOLLOWING MAJOR TRAUMA

RENATA GEORGETA APREUTESEI

Master of Science by Research

Science, Engineering and Environment School

University of Salford

2019

TABLE OF CONTENTS

List of Figures

List of Tables

Acknowledgements

Firstly, I would like to express my sincere gratitude to my supervisor Prof. Niroshini Nirmalan for the professional guidance and support for my MSc Res study and related research, for the patience, motivation, and immense knowledge. Her guidance helped me in all the time of research and life in general. I could not have imagined having a better supervisor and mentor for my MSc Res study.

I would also like to acknowledge the invaluable contribution of the Clinical and academic teams, nursing teams and student cohorts involved in the trauma biomarker study. I am especially grateful to my co-students in the trauma team, Dr Basmah Allarakia and Matthew Jones for guiding and supporting me with their knowledge and patience during my research and for their contributions towards accumulating the annotated Major Trauma Biobank at the University of Salford, Manchester, UK.

My thanks also go out to the team of research nurses and clinicians involved in the study at Central Manchester Foundation Trust and Salford Royal Foundation Trust for recruiting the patients and providing the clinical data required for the study.

Abbreviations

AIS - Abbreviated Injury Scale

APP- Acute phase protein

ARDS - Acute respiratory distress syndrome

CARS - Compensatory anti-inflammatory response syndrome

CBA- Cytometric bead array

CMFT- Central Manchester Foundation Trust

CRP - C reactive protein

DAMPs - Damage associated molecular patterns

DIC - Disseminated intravascular coagulopathy

DMSO- Dimethyl sulfonate

eGFR - Estimated Glomerular Filtration Rate

FBS - Foetal Bovine serum

FSC – forward scatter

FiO2 - Fraction of Inspired Oxygen

GCS – Glasgow coma scale

GM-CSF- Granulocyte-macrophage colony stimulating factor

HMGB-1 - High mobility group box 1

ICAM - Intercellular adhesion molecule

ISS - Injury Severity Score

LFA-1 – Lymphocyte-function-associated antigen-1

MODS - Multi-organ dysfunction syndrome

MOF - Multiple organ failure

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

NK – Natural killer cells

PAMPs - Pathogen associated molecular patterns

PaO_2 - Partial pressure of oxygen

PBMCs - Peripheral blood mononuclear cells

PECAM - Platelet/endothelial cell adhesion molecule 1

PG-E2 – Prostaglandin-E2

PK – prekallikrein

POCT – Point of care testing

PRR - Pathogen recognition receptor

ROS - Reactive oxygen species

sIL-6R - Soluble Interleukin-6 receptor

SIRS - Systemic inflammatory response syndrome

SOFA - Sequential Organ Failure Assessment

SRFT - Salford Royal Foundation Trust

TARN – The trauma audit and research network

T-bet – T-box transcription factor

TGF - Transforming growth factor

TIC - Trauma induced coagulopathy

TLR – Toll-like receptor

TNF-α - Tumour necrosis factor-alpha

T-reg – T regulatory cells

TxA2 – Thromboxane A2

VLA-4 – leucocyte integrins

VCAM - Vascular cell adhesion protein

ABSTRACT

Despite recent advances in critical care, trauma still continues to be the leading cause of morbidity and mortality among individuals under 40 years of age and the third main cause of death throughout the world, carrying a substantial economic burden. Globally, the most common traumatic injuries are acquired through penetrating wounds, blunt injuries or burn mechanisms. Recent reports from the UK suggest a significant change in the demographics of major trauma. The typical major trauma patient is older, with a slight predominance of males, and with traumatic injuries resulted from falls from less than 2m in height.

Mortality due to major trauma follows a biphasic pattern. Early fatalities occur due to haemorrhagic shock or brain injuries, whereas the late deaths (>5 days post-trauma) are attributed to progressive multiple organ failure (MOF) and sepsis postulated to result from dysfunctions of the immune system. Depending on the severity of trauma, the immune response can be over-active with uncontrolled release of pro and anti-inflammatory mediators causing multiple organ failure.

This study focusses on the second-phase mortality induced due to major trauma. The hypothesis tested is that the late mortality and morbidity results from an imbalance of the pro and anti-inflammatory immune responses (SIRS and CARS) triggered after major trauma. While a balanced response of these two arms of the immune system result in homeostasis, the hyperactivity of the compensatory anti-inflammatory response (CARS) results in immunosuppression and increased risk of nosocomial infections and late onset MOF.

The study is designed to investigate the role of IL-6, IL-10, IL-12 and IL-4 in the development of the late onset MOF with a view to evaluating their use as a predictive biomarker of poor clinical outcome. The data from the analysis of 80 major trauma patients show averaged IL-6

and IL-10 levels to decrease in major trauma patients from Day 1 to Day 5 post-admission (IL-6 p=0.000; IL-10 p=0.000), while the trends for IL-4 and IL-12 increased over the same period for the cohort (IL-4 p=0.031; IL-12 p<0.001). When patients were clustered on the basis of Sequential organ failure assessment scores (SOFA) on Day 5 (cut off $\geq$3), statistically significant differences were observed in the Day 1 IL-10 and IL-6 levels at the cut off $\geq$3 for the respective SOFA score (IL-10 p=0.023, IL -6 p=0.015) indicating a strong potential for their use as early biomarkers of poor clinical outcomes. The trends in Day 1IL - 10 and IL- 6 concentration were maintained when the SOFA cluster cut offs were increased to $\geq$ 6 (IL-10 p=0.037; IL-6 p<0.001). When similar analyses were carried out with IL-12 data, a reverse trend was observed with higher Day 1 IL-12 levels being observed in the $\leq$3 Day 5 SOFA cluster, suggesting that high levels of IL-12 on Day 1 post-trauma is associated with good clinical outcomes downstream. Although the IL-12 data failed to show statistical significance at a SOFA cut off of 3 (p=0.268), when the data was clustered more stringently on a SOFA cut off of 6, the differences were statistically significant (p= 0.023). Similar trends were maintained for SOFA outcomes on Day 8. Similar to IL-6 and IL-10 patterns, high IL-4 levels on Day 1 post admission showed a significant association with poor outcome patient clusters on Day 5 (SOFA cut off 6; p=0.025)

When cytokine levels were defined using Day 5 serum C-reactive protein concentration as a measure of poor outcome (rather than SOFA scores), similar trends were observed. However, the data was not statistically significant, possibly due to confounding factors resulting from a lower cohort size (n =30) where CRP records were available. In conclusion, a multiplex panel of these 4 cytokines may be of good predictive value for the early stratification of major trauma patients for focussed clinical intervention and improvement of clinical outcome.

CHAPTER 1: INTRODUCTION

1.1 DEFINITION AND INCIDENCE OF TRAUMA: AN OVERVIEW

Traumatic injury represents physical injuries of sudden onset which require immediate medical attention (Madar et al., 2018). Worldwide, there are approximatively 16,000 deaths per day resulting from traumatic injuries, with many more patients left with moderate to severe disabilities. Traumatic injury accounts for more deaths that malaria, tuberculosis and HIV combined (Lendrum & Lockey, 2012). Traumatic injuries resulted from road traffic accidents (RTAs) cost most countries 3% of the gross domestic product, with 93% of fatalities occurring in low and middle-income countries. Although traumatic injuries affect people of all ages, most at risk are young males in the 15-29-year-old group (Lendrum & Lockey, 2012; World health organisation, 2018).

Every year in the United Kingdom, 16,000 fatalities occur due to traumatic injury. There are many more non-fatal injuries and an un-estimated number of people left with permanent disabilities requiring long-term rehabilitation following trauma (The trauma audit and research network report, TARN, 2018). The annual NHS costs for treating traumatic injuries is estimated at £ 1.6 billion (Lendrum & Lockey, 2012; The Royal College of Surgeons of England,2009).

In a bid to improve trauma care services in the UK, the Department of Health in 2008 published a report entitled “High quality care for all”, which resulted in the creation of specialised regional major trauma centres in the UK with responsibility for coordinating the management of patients with traumatic injuries in a particular healthcare region (Lendrum & Lockey, 2012). The programme aims to deliver treatment for everyone, around the patients’ needs, with 24-hour trauma teams available for pre-hospital intervention, emergency

department services and transfer to specialist surgical facilities. The regional trauma system oversees public health and injury prevention aspects, emergency medical services, major trauma centres, research and education with the aim of reducing death and disability following traumatic injury (The Royal College of surgeons of England,2009).

The data collected regarding patient demographics, type of injury, interventions, observations, surgical procedures etc., are collated in a national website registry "Trauma audit and research network" (TARN) which is available for access through NHS trusts across England and Wales. TARN creates monthly and quarterly reports regarding the epidemiology and the level of trauma care within the hospitals, providing long-term metrics for trauma audit and research (The Royal College of surgeons of England,2009).

Recent publications based on data from TARN, reported a major demographic change in the patterns of traumatic injury in the UK (Kehoe et al., 2015). While traditionally, major trauma was known to involve young males and road traffic injuries, the study reports that the typical major trauma patient is now older with a slight predominance in males and with the mechanism precipitating major trauma resulting from falls from < 2m in height. While road traffic collision injuries are related to younger patients, adults aged 60 and older account for more than 50% of severely injured patients (Kehoe et al., 2015, Trauma audit & research network, 2017). The predicted expansion in the ageing population and the existence of multiple co-morbidities in this cohort is likely to result in poorer outcomes and increased hospital stays with accompanying implications of increase cost burdens. An urgent review of the trauma care practices is therefore required to accommodate the changing demographics of major trauma in the UK.

1.2 PATHOPHYSIOLOGY OF TRAUMA

Traumatic injuries can result from blunt, penetrating and burn mechanisms. They are caused by road traffic accidents, falls, occupational hazards, knife and gun injuries, sport injuries, natural disasters and many other physical injuries which require immediate care. The severity of the traumatic incident is clinically assessed using the Injury Severity Score (ISS) which combines the anatomical scores (Abbreviated Injury Scale) from multiple injuries in six allocated body regions (head and neck, face, chest, abdomen, extremity and external). The ISS is an internationally recognised metric which correlated to measure of severity including mortality, morbidity and hospitalization time (Lecky, Bouamra & Woodford, 2017).

Following a traumatic event, the response of the body to the insult is to induce local and systemic inflammatory responses in a bid to restore homeostasis and to stimulate the repair mechanisms. A trimodal model distribution is recognised for mortality due to major trauma; in the early phase, the death occurs at the scene of trauma or shortly after arriving at hospital. Deaths due to major trauma occur in three phases, a) Immediate (45%) due to severe destructive injury, b) The second peak of early deaths can occur 24-48 hours after traumatic injury and accounts for about 10% of the death due to major trauma (Saadia & Schein, 1999; Sobrino & Shafi, 2013). c) Late phase death (45%), in the days or weeks that follow, primarily due to multiple organ failure MOF (Shafi et al., 2012).

Advanced haemorrhage control and early resuscitation have significantly impacted on increasing survival rates in the early post-traumatic phase. Despite the recent developments in the fields of molecular medicine and Omics-based sciences significant gaps exist in our knowledge of posttraumatic molecular and immunological changes, we have yet to harness this knowledge in terms of applying it to impact patient management or improve patient

outcomes. Therefore, while advances in emergency care management have impacted favourably in reducing the first two phases, the third late phase peak continues unabated.

Severe traumatic injuries can lead to acute traumatic ischemia of tissues. This is a consequence of blood loss due to injury to blood vessels as in open wounds, or at microcirculation levels due to oedema, as in blunt injuries or burns (Kemmer, 2006). The haemorrhage results in a lack of blood supply to organs with a redistribution of the blood to the heart and brain. Insufficient oxygen delivered to the tissues leads hypoxia-induced to cell dysfunction and cell death (Michaels, 2004).Advances in the early trauma management protocols are directed at delivering rapid and effective resuscitation and fluid administration, with a view to improving the blood volumes, blood pressure and cardiac output (Goettler, Giannoudis & Rotondo, 2017). However, there is increasing evidence to suggest that reperfusion, through the release of free radicals and pro-inflammatory mediators, can result in further damage to the ischemic tissue, a phenomenon described as ischemic-reperfusion injury (Dorweiler et al, 2007).

If ischemic-reperfusion injury is severe, the cells might undergo apoptosis or necrosis which will drive inflammation and the development of multiple organ failure, resulting in the late peak in mortality, the so-called “two-hit” hypothesis (Rostein, 2003). Ischemic reperfusion injury is an important clinical problem which can affect any organ system such as the brain, heart or skeletal muscle. Reactive oxygen species (ROS) and activated neutrophils are responsible for local and systemic damage caused by ischemic reperfusion which will manifest as systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome (MODS) (Dorweiler et al, 2007; Gillani et al., 2011).

This process results in massive physiological changes, but the most important changes occur in the immune system. The first step of an inflammatory reaction to traumatic injury involves fluid phase mediators (cytokines, chemokines, coagulation and complement activation products, oxygen radicals, eicosanoids) and cellular effectors (neutrophils, monocytes/macrophages, and endothelial cells) that translate the trauma-induced signals into cellular responses (Huber-Lang & Gebhard, 2016).

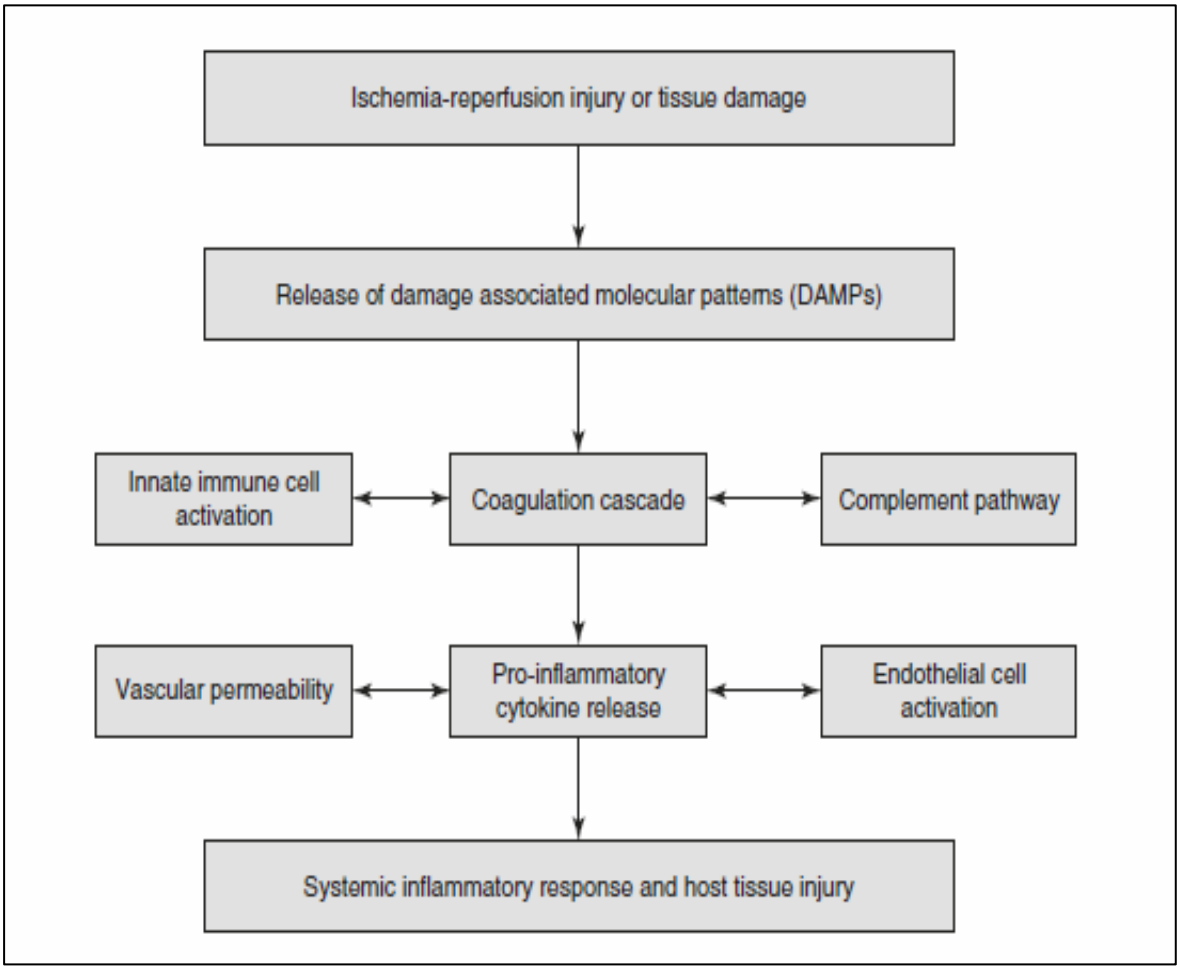


Figure 1: The response to traumatic injury (Costantini & Coimbra, 2017)

Traumatic injuries cause physiological inflammatory reactions which activate neutrophils and macrophages through interaction with cytokines, chemokines and complement. Local inflammatory response, ischemic-reperfusion injury and tissue damage can induce a

systemic inflammatory response, even in the absence of invading pathogens (Fig 1). The systemic inflammatory immune response (SIRS) is triggered by the release of damage associated molecular patterns (DAMPs), coagulation and complement cascade, and by the innate immune cells activated by host tissue injury (Costantini & Coimbra, 2017; Hietbrink et al., 2016).

1.2.1 Danger associated molecular pattern (DAMPs)

The danger concept was first acknowledged by Matzinger in 1994, who stated that "the role of the immune system role is to detect and protect against danger and to discriminate between self and non-self. The way a cell dies dictates the activation or not of an immune response" (Matzinger, 1994). Endogenous molecules released by cells undergoing stress are referred to as alarmins and can be sensed by dendritic cells as danger signals and can trigger innate and adaptive responses (Fig 2). The alarmins are triggered either by cellular damage (damage-associated molecular patterns (DAMPs)), or conserved features of molecules found on bacteria or viruses (pathogen-associated molecular patterns (PAMPs)). Dendritic cells recognise foreign molecules through pattern recognition receptors (PRRs). Therefore, they recognise molecular features present on pathogens (PAMPs) as well as specific molecules generated as a response to trauma/stress or sterile injury (DAMPs) (Galucci, 2016).

Tissue trauma, microbial toxins, excessive heat or radiation, ischemia, as well as diverse chemical stimuli can cause rupture of cells and release DAMPs acting as initiators of inflammation. As Figure 2 shows, DAMPs can activate tissue-resident macrophages, induce maturation of immature dendritic cells, and trigger production of cytokines, chemokines, and other factors responsible for neutrophils and monocyte/macrophages recruitment from peripheral blood (Martin, 2016).

DAMPs molecules are present intracellularly, therefore, the innate immune system cannot recognize them as they are sequestrated inside the cell. They can be released only when the cell undergo necrosis and intracellular content is released, signalling the death to innate immune system (Kono et Rock, 2008). The leaking of the intracellular proteins from the dead cells initiates an inflammatory response. They bind to host inflammatory cells via pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) and activate the complement cascade. (Rider et al., 2017).

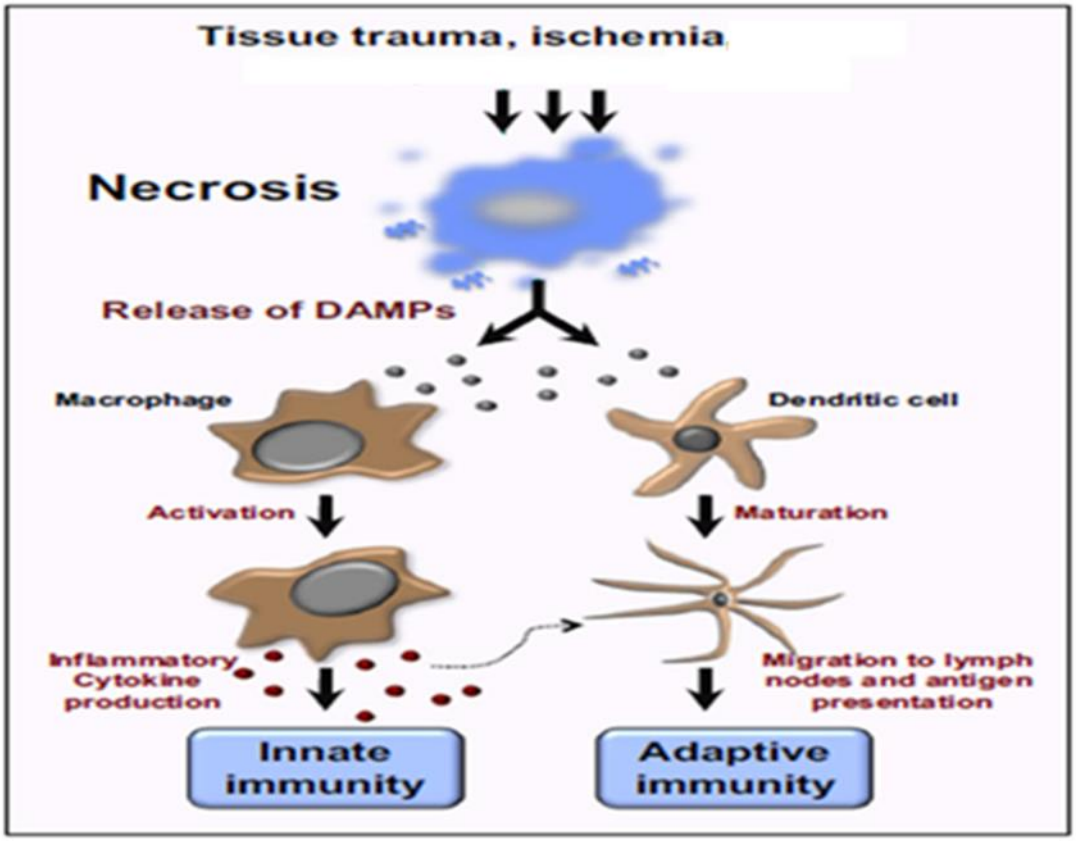


Figure 2: The immune system activation (Martin, 2016)

DAMPs include protein like heat shock proteins (HSP), high-mobility group box1 (HMGB1), monosodium urate (UA), Hyaluronan, adenosine, RNA/DNA, mitochondrial DAMPs, S100 proteins interleukin-1α (IL-1 α) and interleukin-33 (IL-33), histones (Hirsinger et al, 2012). High mobility group box 1 protein (HMGB1), IL-1 α, IL-33 and S100 proteins have been studied extensively. They have dual-functions, both intracellular and extracellular and are

associated with beneficial housekeeping roles and tissue repair, being able to trigger a rapid inflammatory response (Berthelot & Latz, 2017).

1.2.1.1 Heat-shock proteins (HSPs)

HSP is one of the first endogenous molecules described to stimulate an immune response during sterile injury. HSPs play a role in intracellular trafficking, protein folding and maintenance of protein integrity. It is released from a variety of cell types, is present on the cell surface and can be found in serum (Feng et al, 2003). Purified HSPs have shown to trigger dendritic cell (DC) maturation and to stimulate their migration to lymphoid organs (Kono & Rock, 2008). However, Broere et al., (2011) considered that HSPs are not DAMPs as they are freely present in the extracellular fluid, as opposed to being released from the intracellular habitat in response to necrotic conditions. Moreover, HSPs seem to have a dampening effect on immune activation. The other school of thought supports the view that HSPs were mis-classified as DAMPs in earlier studies, due to contamination of recombinant HSP in lipopolysaccharides (Broere et al., (2011).

1.2.1.2 High mobility group box 1 protein (HMGB1)

HMGB1 is a DNA-binding nuclear protein with a role in modulating gene expression. HMGB1 plays an important part in response to sterile injuries, such as haemorrhagic shock and ischemic/reperfusion injury. HMGB1 is released upon cell death, but it has also been shown that immune cells such macrophages and monocytes secrete HMGB1 in response to stimulation by cytokines or lipopolysaccharides (Hirsiger et al., 2012). Monocytes can also secrete HMGB1 due to complement activation product C5a, which plays an important role in sepsis and also in sterile injury. In severe trauma, the level of HMGB1 is proportional to the level of complement activation (Hirsiger et al., 2012). During apoptotic cell death, nuclear

HMGB1 is tightly attached to chromatin and is not released into the extracellular matrix, preventing an inflammatory response (Voll et al., 2008). However, upon tissue injury or cell necrosis, HMGB1 is released into extracellular space stimulating the production of tumour necrosis factor-α (TNF- α) (Kono & Rock, 2008). HMGB1 is considered a cytokine as it stimulates pro-inflammatory responses from monocytes/ macrophages. HMGB1 appears in the extracellular matrix 8-12 hours after the macrophage response to a pro-inflammatory stimulus (Andersson & Tracey, 2003), and can sustain the inflammation for several days. However, in sterile damage and tissue death, HMGB1 mediates early inflammation responses that only last hours (Bertheloot & Latz, 2017).

1.2.1.3 Interleukin-1α (IL-1α)

IL-1α is a dual-function cytokine that presents both nuclear and extracellular functions. Extracellular IL-1α has been shown to have an important role in sterile inflammatory diseases and in cancer (Borthwick, 2016). In resting cells, IL-1α is found in the nucleus and has a role in gene expression, acting as a transcription factor. It is continually expressed in epithelial cells, keratinocytes and fibroblasts, but is only released after loss of cellular integrity, like in necrosis. In non-sterile pathogen-associated cell damage, upon stimulation with LPS or TNF-α, IL-1α can also promote the expression of inflammatory cytokines IL-6 and IL-8 (Werman et al., 2004). IL-1 activates lymphocytes, monocytes and macrophages by inducing the production of inflammatory mediators. Studies on mice show that antibodies to IL-1α inhibited the immune response in sterile inflammation, sustaining the role of IL - 1α as a DAMP (Hirsinger et al., 2012).

1.2.1.4 Interleukin-33

IL-33 is similar to IL-1α and HMGB1 regarding nuclear tissue expression and being released in extracellular matrix after loss of cellular integrity. It can also act as a cytokine and nuclear factor. IL-33 is expressed in structural and lining cells such as endothelial cells and the fibroblastic reticular cells of lymphoid tissues; it is also expressed by epithelial cells of tissues exposed to the environment (Moussion, Ortega & Girard, 2008). Similar to IL-1α and HMGB1, IL-33 may be released from dead or dying cells during trauma or infection and act as an alarmin to alert the immune system about cell or tissue damage. Mast cells, which are positioned close to endothelial cells of blood vessels and epithelial surfaces exposed to the environment, are the cellular target of IL-33 and may play an important role as an activator of mast cells during innate immune response to pathogens. The level of IL-33 in the serum increases during sepsis or trauma, acute myocardial infarction, idiopathic pulmonary fibrosis or asthma, (Moussion, Ortega & Girard, 2008).

1.2.1.5 S100 proteins

S100 proteins comprise 25 members with a large variety of functions. The role of S100 proteins inside the cell is to regulate cell proliferation, differentiation, migration. It is also involved in Ca_2^+ homeostasis, inflammation and cell death. When released in the extracellular space, some S100 proteins act as DAMPs, initiating immune responses, cell migration, chemotaxis, but also tissue development and repair (Bertheloot & Latz, 2017).

1.2.1.6 Mitochondrial DAMPs

Mitochondrial DAMPs are made up of circular DNA strands containing CpG DNA repeats and were found to be elevated in severely injured patients (Hirsinger et al., 2012). Mitochondrial DNA (mtDNA) retain molecular patterns similar to bacterial DNA, and when released into

circulation after traumatic injury, directly activate neutrophils *via* p38 MAPK pathway inducing an inflammatory response (Zhang, Itaqaki & Hauser, 2010).

1.2.2 Haemorrhage and coagulopathies in trauma

The most common cause of death after trauma is due to traumatic brain injury and/or haemorrhage. Coagulopathies following trauma are common and affect one in three severely injured patients; the bleeding is accountable for poor clinical outcomes and increased mortality (Cohen & Christie, 2016). Trauma induced coagulopathy (TIC) represents a serious secondary consequence of a traumatic injury. TIC can start as a consequence of tissue hypo-perfusion, showing that resuscitation after blood loss can have a significant adverse role in clotting (Tarmey & Kirkman, 2016).

After an injury and haemorrhage, the body tries to achieve homeostasis. It involves three stages comprised of vasoconstriction, platelet activation and blood clot formation (Palta, Saroua & Palta, 2014). Vasoconstriction is achieved through contraction of smooth muscles in the wall of the blood vessels with a role in narrowing the damaged blood vessels to reduce blood loss. One of the reactions of the body to blood loss in maintaining the homeostasis after cellular and vascular damage, is the production of thrombin. Thrombin is produced in less than 5 minutes after vascular damage and is the key to activating platelet aggregation, clot-formation, fibrin activation and activation of factor XIII, all essential processes in ensuring blood clot stability (Mann & Lorand, 1993). The activated platelets stick to each other and to collagen fibres from blood vessels and to Von Willebrand factor (vWF), forming a temporary blood plug. Platelets further release thromboxane A2 (TxA2) for platelet accumulation and vasoconstriction (Eisinger et al., 2018). The homeostasis is reached when

the blood clot containing fibres which trap platelets is formed, with the haemostatic plug acting to limit the leakage (Luyendyk et al., 2018).

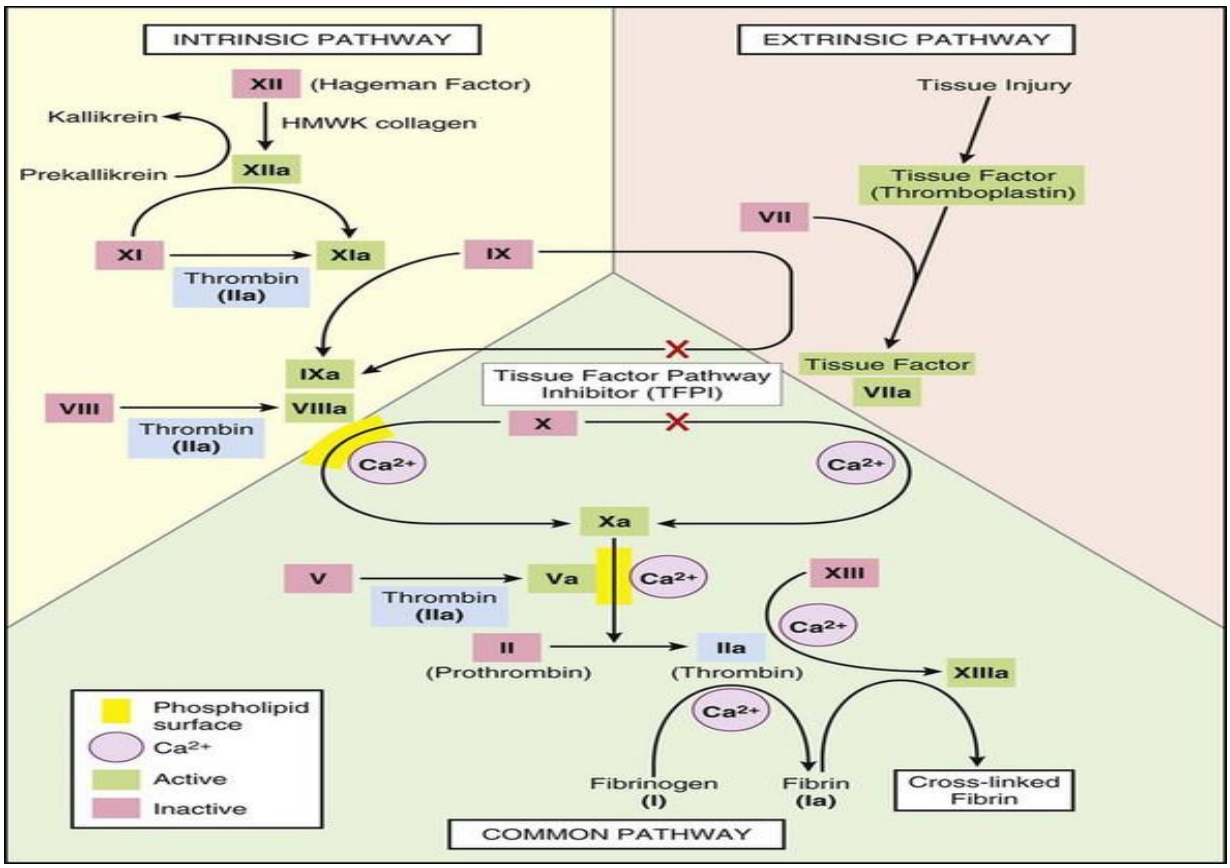


Figure 3: The coagulation cascade (Russel & Suarez, 2015)

Thirteen coagulation factors are involved in three coagulation pathways, depending on the initial event which triggered the cascade (Figure 3). The extrinsic pathway starts when a vascular injury occurs, and tissue factor is released. The intrinsic pathway starts when damaged endothelium comes into contact with circulating blood, triggering a cascade of coagulation factors (XII, XI, IX and VIII). Both pathways converge in a common pathway where prothrombin and fibrinogen become activated and result in clot formation. Figure 3 depicts the extrinsic, intrinsic and common pathways involved in the coagulation cascade (Russel & Suarez, 2015). In tissue injury, the cascade is initiated with the activation of factor VII(VIIa) by tissue factor (TF) from the cell membrane, as shown in Figure 3.

The clotting factors are precursors of proteolytic enzymes, zymogens, found in inactive form. Activated factor VIIa mediates the conversion of factor X to factor Xa. The intrinsic pathway is initiated when Factor XII auto activates and form F XIIa which activates prekallikrein (PK) liberating Kinins. Kinins are peptides which bind to receptors on many cell types and induce inflammation, vasodilation and vascular permeability. F XIIa and thrombin activate F XIa which activates factor IX, which further, together with cofactor VIII activate factor X. The common pathway beginning with factor X and V and calcium, converts prothrombin to thrombin, which will cleave circulating fibrinogen to fibrin, activating factor XIII, resulting in crosslinking fibrin polymers in the platelet plug (Palta, Saroa & Palta, 2014; Grant & Aird, 2016).

Patients suffering from blunt trauma and massive tissue injury have an increased thrombin generation, and soon after trauma many procoagulant particles are observed in the circulation (Hayakawa, 2017). In major trauma, trauma-induced coagulopathies can result in, hyper-fibrinogenolysis and consumption coagulopathy, characterised by disseminated intravascular coagulation (DIC). In severe trauma, the anti-coagulant pathway is impaired, plasma protein-C decreases, resulting in dysregulation of thrombin generation (Martini, 2016). Hyper-fibrinogenolysis is controlled by enzymes t-PA which catalyse plasminogen to plasmin, which initiate fibrinogen degradation. Under normal physiological states, the blood coagulation is a dynamic process with clot formation, anticoagulation and fibrinolysis (Martini, 2016). In severe shock, endothelial cells will increase t-PA production, called “acute release of t-PA”. However, in traumatic injury, the activation of platelets with thrombin generation and clotting processes causes the consumption of the coagulation factors, leading to hypo-coagulation and hyperfibrinolysis. Another hypothesis is that activated protein-C is responsible for hypo-coagulation and hyperfibrinolysis observed in some trauma

patients (Martini, 2016). However, it was also observed that transfusions can lead to more dilution of fibrinogen leading to massive bleeding (Hayakawa, 2017).

As shown before, major haemorrhage and coagulation abnormalities are of great concern regarding death in the first 24-48 hours after trauma. Furthermore, it has also been shown that coagulation and inflammation are inter-connected and can potential impact on each other (Tsao et al., 2015). The PAMPs appear to impact on coagulation. Upregulation of plasminogen activator inhibitor (PAI) trigger TLR-2, 2, 4 and 9 activations, can also result in increase of inflammatory cytokines production. Platelets are also known to be mediators of immune responses, as they express TLRs involved in immune response recognising PAMPs and DAMPs from damaged cells (Semeraro et al., 2011).

DAMPs like extracellular histones have also been shown to have a massive pro-thrombotic effect, with fibrin and platelet deposition in lung alveoli and intra-alveolar haemorrhage. It is likely that they contribute to late multiple organ dysfunction in trauma. Platelets are also known to be mediators of immune responses, as they express TLRs involved in immune response recognising PAMPs and DAMPs from damaged cells (Semeraro et al., 2011).

1.2.3 Complement activation

The complement system is a component of innate immunity and consists of a complex of approximatively 30 plasma proteins and cell surface proteins in inactive form. They are mostly produced by the liver and can be activated through pattern recognition or through pathogen-bound antibodies. Complement activation leads to activation of an enzyme cascade (Huber-Lang & Gebhard, 2016). The complement proteins are proteases activated by cleavage of its zymogen precursor, which then cleaves its substrate (Janeway et al., 2001).

The complement system can be activated through four pathways: the lectin pathway, classical pathway, alternative pathway, and coagulation pathway (Amara et al., 2008).

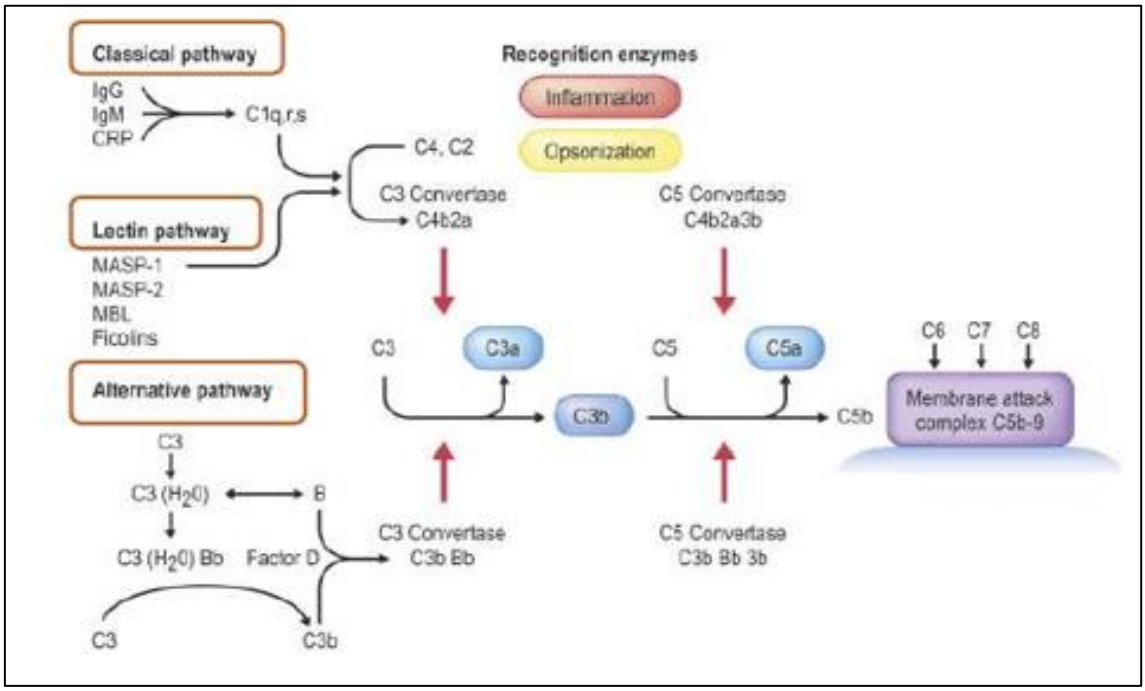


Figure 4: Complement activation pathway (Clos & Mold, 2008)

1.2.3.1 Lectin pathway

The lectin pathway is initiated by soluble carbohydrate-binding proteins such as mannose-binding lectin (MBL) and ficolins which recognize and bind to carbohydrates on microbial surfaces such as PAMPs (Figure 4). MBL is present in low concentrations in plasma, but its production is increased during the acute-phase response, whereas ficolins are synthesized in the liver and circulate in the blood or are synthesized and secreted by lung and blood cells. These proteins are activated through cleavage by zymogens upon binding to pathogen surfaces, which further cleaves complement C4, releasing C4a and cause a conformational change in C4b. C4b then binds to C2, which is further cleaved producing C2a and will remain bound to C4b forming the complex C4b2a. C4b2a will cleave the molecule C3 in C3a and C3b. One molecule of C4b2a can cleave up to thousand molecules of C3. C3b binds covalently to

pathogen surface, whereas C3a initiates a local inflammatory response (Beltrame et al., 2014).

1.2.3.2 Classical pathway

Classical pathway is initiated *via* a pathogen sensor known as the C1 complex. C1 can interact with the pathogens, but also with antibodies. Because of this peculiarity, the classical pathway functions in both innate and adaptive immunity. The C1 complex is comprised of C1q, as shown in Figure 4, acting as microbial sensor and of C1r an C1s proteases in an inactive form. The activation of complex starts when C1q interacts with the pathogen, causing a conformational change in C1r-C1s complex, leading to activation of C1r which cleaves C1s, generating a serine protease. Following this step, activated C4 and C2 form the active C3 convertase C4b2a, similar to the lectin pathway (Zhi & Skare, 2018). C1q can attach to the surface of pathogens and bind to C-reactive protein, however it mainly binds to Fc region of antibodies bound to pathogens, particularly IgMs-called natural antibodies. IgM binds C1q immediately after infection clearing bacteria before they become dangerous. Lectin and classical pathways result in opsonisation of the pathogens which make them easier to be detected by phagocytes (Murphy & Weaver, 2017).

1.2.3.3 Alternative pathway

The alternative pathway can get spontaneously activated by C3 convertase as shown in Figure 4, not the same as C4b2a convertase, shown in the previous pathways, but a C3bBb convertase which produce C3b. C3b can further generate more of itself, which together with the C3b generated by the previous pathways result in a large amount of C3b. The effect of complement activation is to generate and deposit large amount of C3b on the surface of the pathogens and trigger phagocytosis (Harboe & Mollnes, 2008).

For phagocytosis to take place, complement protein C5 needs to be activated. C5 is activated by all 3 complement pathways producing C5a and C5b molecules. C5a complement fragments bind to C5a receptors on phagocytes and trigger the phagocytosis of the C3b coated pathogens. On the other hand, C5a and C3a initiate local inflammatory response by binding to specific receptors on endothelial cells and mast cells (Lachmann, 2018). The generation of large amount of these 2 complement fragments have shown to be connected to anaphylactic shock. Association of C5a and C3a leads to activation of mast cells to release histamines and TNF-α, and also can induce contraction of smooth muscles, vascular permeability inducing extravasation of immunoglobulins, complement molecules and phagocytic cells, initiating the adaptive immune response (Kemp & Lockey, 2002).

Another effect of complement activation is the formation of the membrane-attack complex (MAC) that punches holes in the pathogen's cell membrane destroying the pathogen, as shown in Figure 4. The formation of the complex starts with the release of C5b, which together with C6, C7, C8 and C9 form a pore in the pathogen membrane (Clos & Mold, 2008). This pore formation is used only for killing of a restricted number of pathogens, making the opsonization and the triggering of inflammation the most important role of the complement activation (Murphy & Weaver, 2017).

1.2.3.4 Complement system activation by the coagulation pathway

Activation of the complement system takes place simultaneously with the coagulation response, one responsible for the innate immunity and the other to maintain the homeostasis in case of injury or pathogens invasion (Choi et al, 2006). Studies have shown that a new complement pathway has been found and is activated in absence of C3 molecule, with thrombin having C5 convertase activity and generating C5a. These studies show that

there is a direct linkage between the complement and clotting pathways (Huber-Lang et al., 2006).

Most recent studies show that exposure to thrombin can also generate C3a as well as C5a. Excessive production of these complement molecules during severe tissue injury or systemic inflammation may contribute to attraction of phagocytes to the site of inflammation and release ROS, protease and cytokines which can have negative effects during systemic infection. A recent study by Amara et al., has found that there is an extensive cross-talk between the coagulation cascade and complement cascades. The coagulation cascade and complement system show to be simultaneously activated by severe tissue injury, acute trauma or during systemic inflammation, causing excessive generation of C3a and C5a. The formation of thrombin at the site of injury resulted from the activation of coagulation cascade, shows to trigger the complement system as well, with essential role for the protection against invading pathogens (Amara et al., 2010).

1.2.4 Pattern recognition receptors (PRRs)

Inflammation is the response of the body to eliminate harmful agents and to promote the regeneration of damaged tissue. The phagocytes are a front-line defence strategy, detecting damaging substances released by microbes or dying cells through pattern recognition receptors (PRRs) such as toll-like receptors (TLRs) (Eom et al, 2018). DAMPs and PAMPs are released from stressed or infected cells and bind to specific receptors which will lead to initiation of a series of processes like cell differentiation, cell death or secretion of pro-inflammatory cytokines.

PRRs activate downstream signalling pathways that lead to the induction of innate immune responses by producing inflammatory cytokines like Type I interferon (IFN) and other

mediators which induce the maturation of dendritic cells. These processes trigger immediate inflammation as the host's defensive response, but also initiate antigen-specific adaptive immune responses. These responses are crucial for the clearance of infecting microbes as well providing consequent instruction of antigen-specific adaptive immune responses (Kawasaki & Kawai, 2014).

Toll-like receptors (TLRs), shown in Figure 5, are the first PRRs identified. They are able to recognise a large variety of PAMPs and DAMPs. TLRs are expressed on the cell surface or as intracellular vesicles or endosomes (Kumar et al, 2009). They are type I trans-membrane proteins with an ectodomain. It contains leucine-rich repeats that are involved in PAMP recognition, a trans-membrane region and cytosolic Toll-IL-1 receptor (TIR) domain that activates downstream signalling pathways, regulating the innate immunity and initiate adaptive immune responses. There are 10-12 types of TLRs, able to detect specific PAMPs from bacteria, viruses, mycobacteria, fungi and parasites (Kawai et Akira, 2011).

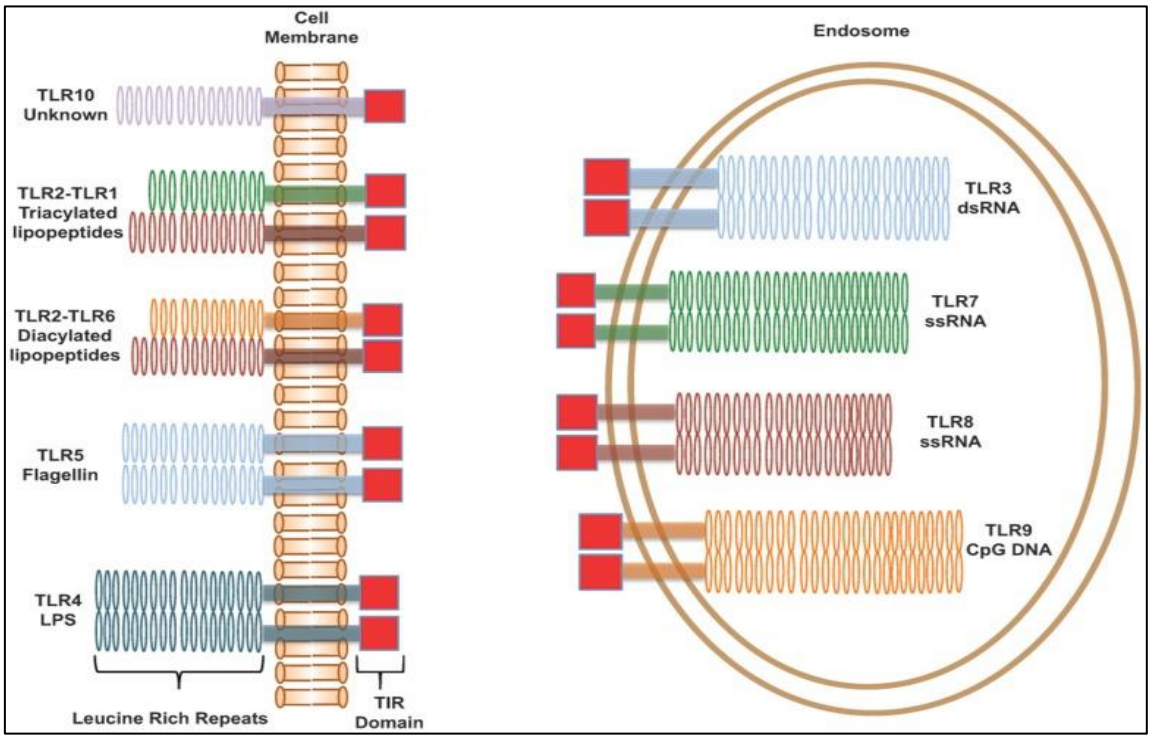


Figure 5: Toll-like receptors (Godfrey et al, 2012)

TLR -2, -4, -5 and -10 are expressed on the surface of antigen presenting cells (APC) like macrophages and dendritic cells, whereas TLR-3, -7, -8 and -9 are present in endosomes (Liu, Zhang & Zhao, 2009). Receptors for advanced glycation end-products (RAGE), are pattern recognition of endogenous molecules which area multi-ligand receptors of the immunoglobulin family and play a critical role in innate immunity system. S100 protein family are ligands for RAGEs and are involved in chronic inflammation, neuro-generative disorders, tumour growth (Leclerc et al., 2009). HMGB1 is a ligand for TLR and RAG, whereas TLR2 and TLR4 are the major mediators for HMGB1 resulting in inflammatory cytokines secretion and production of nitric oxide (Komai et al., 2016).

1.2.5 The inflammatory response to traumatic events

Inflammation is a physiological process that is necessary for the tissue repair after traumatic injury. Inflammation is controlled by pro and anti-inflammatory processes, modulating or dampening down its effects in order achieve homeostasis (Choilean & Redmond, 2006). An uncontrolled pro-inflammation spread systemically resulting in systemic inflammation and resulting in tissues damage and organ failure. On the other hand, an over-active anti-inflammatory response can take over resulting in a compensatory anti-inflammatory response (CARS). In this case the immune-paresis results system, dampening the ability of the immune system to react to a new danger signal. The result is hyper-susceptibility to microbial infections resulting in very serious complication like sepsis and multiple organ failure (Kimura et al., 2010).

1.2.5.1 Innate immune cell activation

In the first 10-15 minutes after injury, the initial vasoconstriction is followed by inflammation which manifests with erythema, heat and vasodilation. Inflammation is mediated by

endothelial mediators, leukotrienes, prostaglandins and histamines. Oedema is a sign of inflammation and is caused by leaking of plasma from the intravascular compartment into the interstitial compartment under the action of histamines, bradykinins, leukotrienes, complement, and platelet-activating factors. The increased vascular permeability has a positive role in facilitating the cells and inflammatory mediators to move easily to the site of injury (Hietbrink et al., 2006).

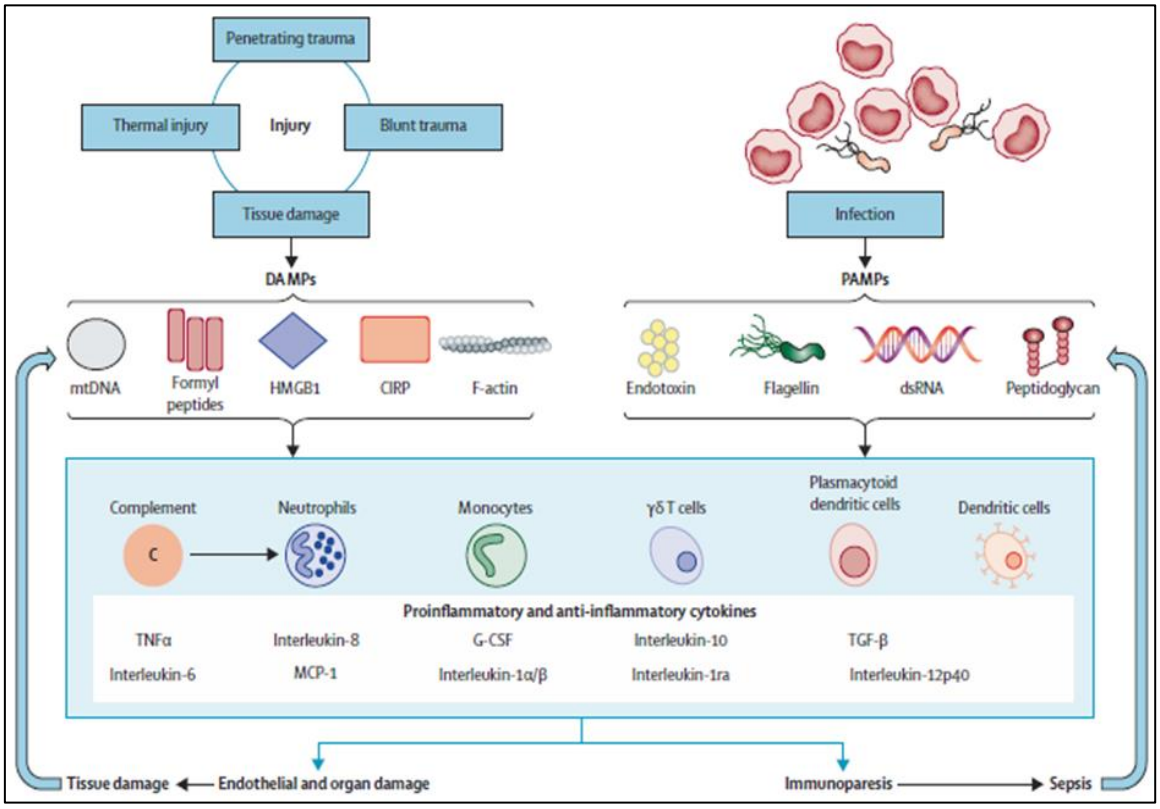


Figure 6: Innate immune cell activation by tissue damage-generating DAMPs and infection-generating PAMPs (Lord et al., 2014)

Infectious agents and traumatic injury can both trigger the initiation of an immune response. DAMPs can trigger a sterile inflammatory response, as in traumatic injuries, whereas PAMPs are triggered by exposure to exogen molecules (Figure 6). Both DAMPs and PAMPs are shown to stimulate complement activation, tissue factor expression on monocytes and activate innate immune cells leading to endothelial and organ damage or immuno-

suppression, with release of more DAMPs in a vicious cycle of tissue damage and sepsis (Ito, 2014).

The innate immune system represents the first line of defence of the body triggered by exposure of neutrophils, monocytes, macrophages and dendritic cells to any exogenous or endogenous molecules (PAMPs and DAMPs). These host cells present on the surface protein receptors for PAMPs and DAMPs, known as pattern recognition receptors (PRRs) with role in initiation of inflammatory processes (Morgan, 2017).

1.2.5.2 Neutrophils

Neutrophils are the most abundant leukocytes in the human blood with a short life in a healthy individual. The main role of neutrophils is as host defence against rapidly dividing bacteria, fungi and yeast. Neutrophils are equipped with microbicidal defensive strategies, such as reactive oxygen species (ROS) production, degranulation, phagocytosis and neutrophil extracellular traps (NET) (McCracken & Allen, 2014). Tissue damage and haemorrhagic shock which takes place in traumatic injuries, induces ischemia and changes in metabolism. During reperfusion stage, the ischemic area is replenished with oxygen leading to radical oxygen species (ROS) formation**.** ROS have role of chemoattractant and activator of neutrophils and other polymorphonuclear granulocytes, which will be active in the area in the first 3 days after trauma (Kovtun, 2018). Neutrophils express on the surface pattern recognition receptors (PRRs); TLRs expressed on the surface are able to bind PAMPs and endogenous DAMPs resulting in neutrophil activation. HMGB1 can activate neutrophils by binding to their TLR2, TLR4 and RAGE receptors, leading to upregulation of pro-inflammatory cytokines, such as IL-1β, IL-8 and TNF-α. Similarly, mitochondrial DAMPs released in circulation during trauma, increased the adherence of neutrophils to endothelial cells,

through upregulation of adhesion molecule I-CAM1 and E-selectin on endothelial cells, and through L-selectin and CD18 on neutrophils surface, contributing to increased endothelial permeability present in SIRS (Hazeldine, Hampton & Lord, 2014).

The first impact of neutrophils with tumour necrosis factor alpha (TNF-α) and granulocyte macrophage colony stimulating factors (GM-CSF) present in the peripheral blood of injured patients results in a pre-activation of neutrophils with enhanced functions like in chemotaxis, adhesion, rolling, diapedesis, extravasation and oxidative burst. Enhanced oxidative burst make neutrophils to have an increased cytotoxic potential, which might lead to incontrollable cytotoxicity and result in SIRS or multiple organ failure (MOF). The most elevated values of oxidative burst were found to be between day 2 and 5 after trauma, however, sometimes the values remain elevated until day 13 (Hietbrink et al., 2006).

1.2.5.2 Monocytes

Blood monocytes are precursors of macrophages, dendritic cells and osteoclasts (Figure 7). Monocytes originate from bone marrow and continuously enter the blood circulation. During injury or inflammation, the monocytes invade the inflamed tissue and participate in immunological response removing the microorganisms, the debris and dead cells, and participate in tissue healing.

The differentiation of monocytes in scavenging macrophages or antigen presenting dendritic cells is dictated by the environmental signals at the site of inflammation (Spangers, de Vries et Everts, 2016; Ingersoll et al., 2011). Interferon γ (INF- γ) and Interleukin-6 (IL-6) stimulate monocytes to differentiate into macrophages, whereas tumour necrosis factor (TNF) presence induces monocyte differentiation in dendritic cells (DC) (Delneste et al, 2003; Chomarat et al, 2003).

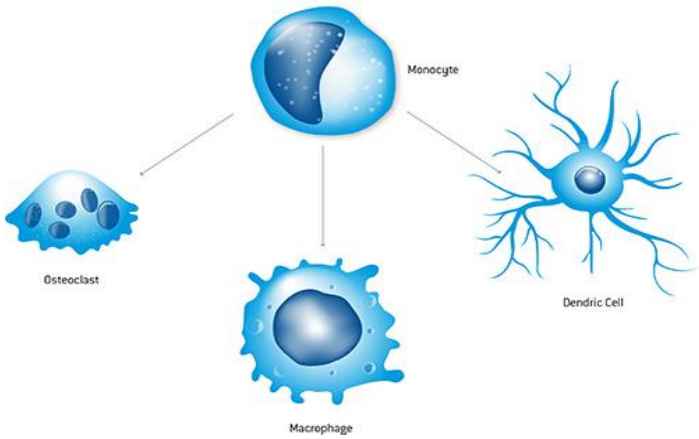


Figure 7: Monocyte differentiation. (www.lonza.com, 2018)

The monocytes secrete several inflammatory mediators, such as TNF-α, IL-1β, IL-6 and IL-8 which are used in many studies as biomarkers for early detection of organ dysfunction in patients who suffered severe traumatic injury. Studies have found that after traumatic injury the monocytes are hyper-activated, but this state is rapidly replaced by a paralysis in cell

function which alter cytokines production with potential fatal immunologic outcome. (Bhardwaj et al, 2018; Kirchhoff et al, 2009).

Macrophages produce a large amount of pro-inflammatory cytokines in response to danger signals, which in some situations can trigger a cascade of inflammatory mediators, leading to tissue destruction. Mainly, macrophages synthesise TNF, Il-6, IL-12 and interferons (Hamidzadeh et al., 2017).

Dendritic cells (DC) are the key for linking the innate immune response to adaptive immune system. They initiate the antigen-specific immune response generating specific T-cells as response to pathogens. Dendritic cells recognize the pathogens due to receptors present on their membrane; they bind peptides belongings to degraded pathogens and present them to T-cells to activate them and induce their proliferation (Collin & Bigley, 2018). In addition, dendritic cells also secrete pro-inflammatory cytokines, such as IL-12, fundamental for T-cell stimulation and differentiation. Mature DC enter the lymph nodes and starts production of antigen-specific memory T-cells. Apart from conventional DCs, another type of DCs are identified and known as plasmacytoid DCs; they respond to virus infection and secrete type I interferons (Mellman, 2013; Collin & Bigley, 2018).

1.2.5.3 Leukocytes transendothelial migration

To initiate an immune response, either from the innate or from adaptive immune response, the leukocytes need to cross the blood vessels. The extravasation of leukocytes takes place through an elaborate mechanism which includes adhesion between leukocytes and endothelium, diapedesis and traverse the endothelium, followed by migration towards the site of damage. A large array of molecules is involved in this process. The inflammatory response starts at the site of injury with in haemodynamic changes in post capillary venules

resulting in reduced blood flow rate (Schnoor et al., 2015). This event facilitates the leukocytes to make a close contact with the endothelial cells. All leukocytes require the activation of LFA-1 leukocyte integrins; neutrophils and monocytes require Mac-1 activation; monocytes, eosinophils and effector T and B cells requires activation of VLA-4 integrins (Nourshargh & Alon, 2014). Neutrophil extravasation will be taken as example for all leukocytes transendothelial migration, as shown in Figure 8.

Normally, neutrophils make only temporary and reversible contact with the endothelium at a velocity of 40 μm/s through endothelial P-selectins and neutrophils' receptors, as seen in Figure 8. Histamines promote the translocation of P-selectin from Wiebel-Palade bodies in the luminal side of endothelial cells interacting with the counter-receptors on leukocytes (Hyun, Lefor & Kim,2009) . The on-off binding process is very rapid in a rolling-like manner. Due to inflammatory signals, the endothelial cell will express more surface adhesion molecules E-selectins providing more binding sites for neutrophil receptors; now, the speed of the neutrophils is decreased to 5 μm/s. E-selectin helps to bring the neutrophils closer to endothelial cells where they will enter in contact with other chemokines, inducing a conformational change in β2-integrin LFa-1 on neutrophils (Hyun, Lefor & Kim, 2009; Muller, 2013).

Integrins are adhesion receptors found in resting state in inactive conformation at low affinity state. They are inside-out signalling molecules stimulated by chemokines. Leukocytes' integrins consist of β2-subunits coupled with several α- subunits. The integrins bind to endothelial ICAM-1 and VCAM-1 resulting in a strong and firm arrest of the neutrophils at endothelium surface (Figure 8, point A - adhesion). Mac-1 integrins on neutrophil surface binds to endothelium PECAM-1 and traverse through the endothelial cells

(Figure 8, point B – transmigration) travelling to the site of inflammation (Futosi, Fodor & Mocsai, 2013). Out of the blood vessels, neutrophils bind to extracellular matrix receptors inducing activation of intracellular signalling pathways, resulting in increased chemotaxis and chemokinesis (Miralda, Uriate & McLeish, 2017).

Neutrophils migrate towards the site of interest following the concentration gradient of chemoattractant such as bacterial peptides, anaphylatoxin complement C5a, platelet activation factor (PAF), IL-8 or leukotriene B4 (LTB4) or other inflammatory molecules. IL-8 might contribute to mobilization of immature and less deformable neutrophils from the bone marrow which can create side effects by getting trapped in distal organs.

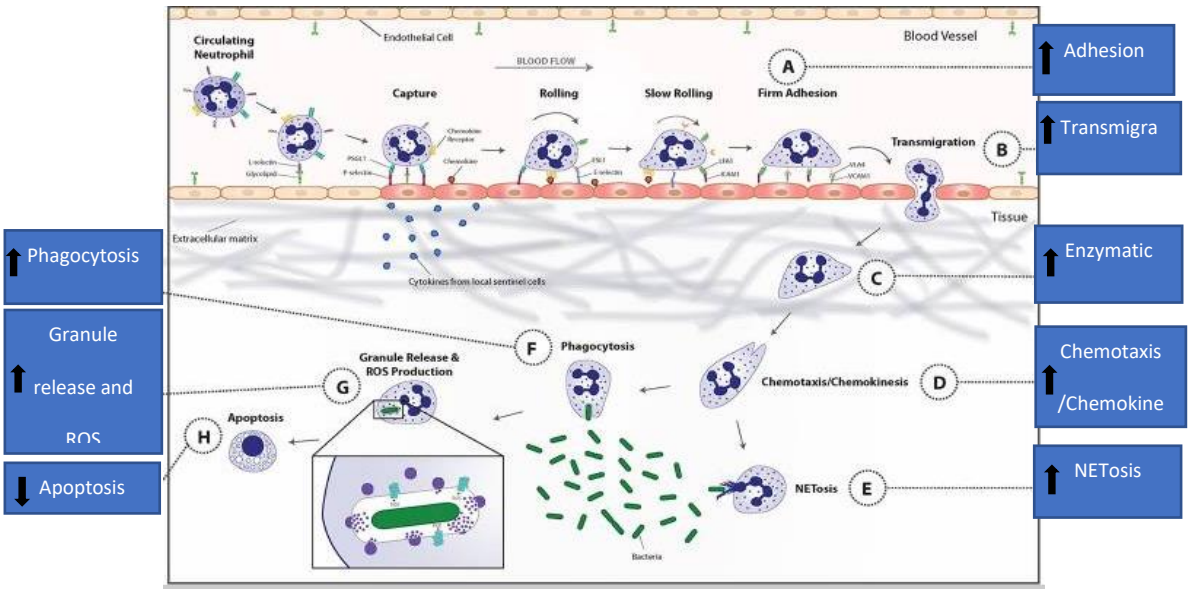


Figure 8: Neutrophil migration (Miralda, Uriate & McLeish, 2017)

The next step is the binding of neutrophils to opsonised pathogens, then phagocytosis and degranulation of antimicrobial factors. Phagocytosis and formation of the phagosome trigger intra-phagosomal release of antibacterial peptides, proteases myeloperoxidase and superoxide anion (O_2). Superoxide anion initiate the generation of reactive oxygen species,

called respiratory burst, with result in annihilation of bacteria trapped in the phagosome (Figure 8, point F and G), followed by apoptosis of neutrophils (El-Benna et al., 2016).

Another defence mechanism of neutrophils is the formation of a web-like structure called neutrophils extracellular traps (NETs), known as NETosis (Figure 8, point E) made up of chromatin, HMGBI1, histones, and antimicrobial proteins (Lipinska-Gediga, 2017). McIlroy et al (2014); Hamaguchi et al (2013) show that NETs formation is characteristic to critically ill patients and develop after major trauma in patients with systemic inflammatory response syndrome (SIRS).

1.2.5.4 Vascular changes that allow the migration of the lymphocytes

The transmigration of lymphocytes from vascular lumen require interaction with the endothelial cells (EC), pericytes and basement membrane (BM). This process takes approximatively 20-45 minutes. The transmigration generally starts with a pore formation within endothelial cells junctions, followed by sub-endothelial crawling along pericytes until they reach a zone of low matrix proteins (i.e. Laminin 10 and collagen type IV) in venular basal membrane, known as low-expression region (LER). They present as gaps between adjacent pericytes which are used as gates to penetrate the vessel wall and to migrate at the site of inflammation (Wang et al., 2006; Voisin & Nourshargh, 2013). The gap formation between pericytes are induced by pro inflammatory cytokines such as TNF-α and IL-1β through interaction with their corresponding receptors expressed on the surface of the pericytes (Proebstl et al., 2012).

1.2.6 Acute phase response (APR)

APR is part of the innate immune response against potential pathogens, however can be also induced by various physiological changes resulted from trauma or stress. APR initiate an

inflammatory process comprised of pyrexia, leucocytosis and hormone alteration, with role in enhancing the healing process and establishing homeostasis. (Cray, Zaias & Altman ,2009). During the APR, the liver secretes plasma proteins called acute phase protein (APP) as shown in Figure 9. However, recent studies have found that APP can be expressed also by other tissues and secreted into interstitial fluids (Schrodl et al., 2016).

The production of APP from hepatocytes is induced by systemic release of inflammatory cytokines such as IL-1, IL-6 and TNF-α from macrophages and monocytes at the site of injury or infection. However, IL-6 is the major mediator responsible for hepatocytic secretion of most of APPs (Jain, Gautam et Naseem,2011). The peak of concentration is reached within 24-48 hour after injury and decline when the infection recedes. Acute phase proteins are used in diagnostic medicine and prognosis of cardiovascular diseases, organ transplant or autoimmunity. Upon stimulation from cytokines, the hepatocytes increase the synthesis and release of positive acute-phase proteins, including C-Reactive protein (CRP) (Figure 9). IL-6 is the major cytokine stimulus for CRP production. (Hengst, 2003). CRP was the first APP to be described and it is used as a marker for infection, trauma, autoimmune diseases or malignancies.

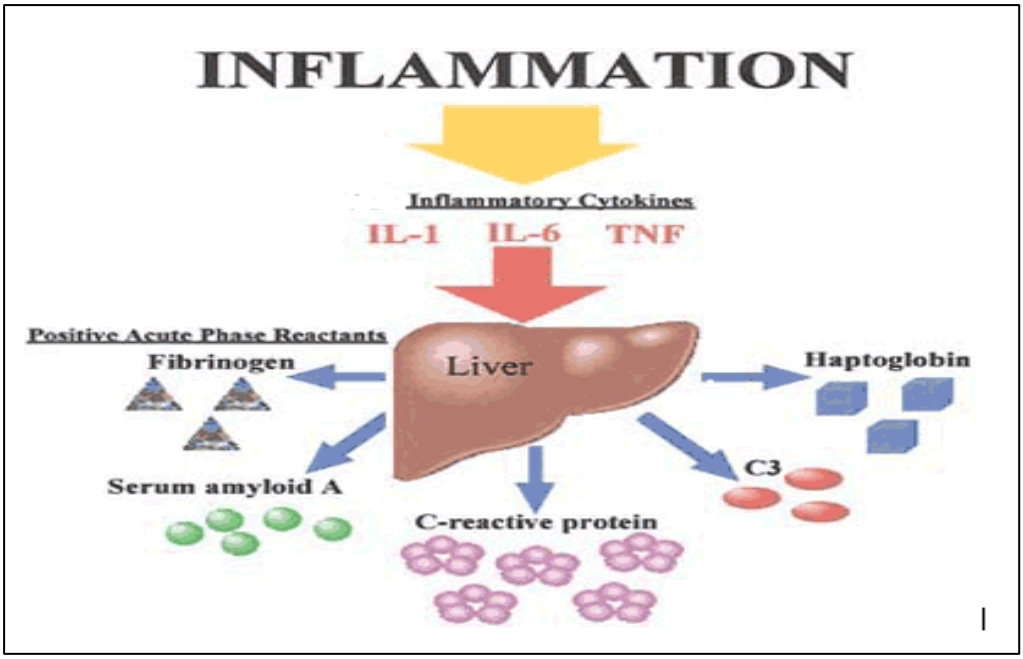


Figure 9: Stimulation and synthesis of positive acute-phase reactants during inflammation (Hengst, 2003).

Other APPs are serum amyloid A (SAA) and serum amyloid P (SAP). They are considered as major APP, and each one has a distinct physiological function for the immune system (Gruys et al., 2005).

CRP can bind to polysaccharides on bacteria or parasites and activate complement system and phagocytosis; moreover, CRP upregulate or modulate secretion of cytokines and chemokines. In addition to CRP, SAA has role in chemotaxis of monocytes, PMCs and T-cells and down-regulate the inflammatory process. (Cray, Zaias & Altman ,2009). Other APPs are coagulation proteins (fibrinogen), haptoglobin which is a transport protein with bacteriostatic effect and inhibitor of neutrophils respiratory burst activity, and complement protein(C3) (Jain, Gautam & Naseem, 2011).

C-reactive protein (CRP) is an acute-phase protein which increases rapidly in response to a traumatic event. Generally, the levels of CRP range between 0.1 and 10 mg/l. The levels of CRP increase very fast in response to a traumatic event, infection or inflammation (Lee et

al.,2005). Levels higher than 90 mg/l were found to align with surgical complications (Neumaier & Scherer, 2008), whereas CRP levels higher than 100mg/l were correlated with sepsis (Povoa et al., 2005). CRP is known to have a short life, meanings that higher levels of CRP maintained for days after a traumatic event may show an ongoing inflammation which can lead to sepsis. (Lee et al.,2005).

1.3 THE ADAPTIVE IMMUNE RESPONSE

The innate immune system provides the first line of defence against various microorganisms or foreign molecules. However, the innate immune system cannot eliminate all pathogens and foreign invaders. In this situation, the adaptive immune system gets activated and provide more protection against these threats. The major components of adaptive immune system are B-lymphocytes (B-cells) and T lymphocytes (T-cells), the first is responsible for humoral immunity represented by B-cells, whereas T-cells mediate cell-mediated immunity (Schenten & Medzhitov, 2011).

The bigger player responsible for the connection between the innate immune system to adaptive immune system is represented by dendritic cells (DCs) which carry pathogen antigens-peptide on MHC class I or II (Figure 10), then present them to T lymphocytes (Janeway, Travers & Walport, 2001). The role of the DC is crucial, and the efficiency of the immune response depends on pathogen recognition and presentation to other cells of the immune system (McCullough, Ruggli & Summerfield, 2009).

After DCs activation they migrate to lymph nodes presenting their antigen to T-cells and initiating an adaptive immune response. Once the T-cells are activated they differentiate in effector cells. Factors such as adhesion molecules and cytokines produced by DCs dictate which type of effector cells they become.

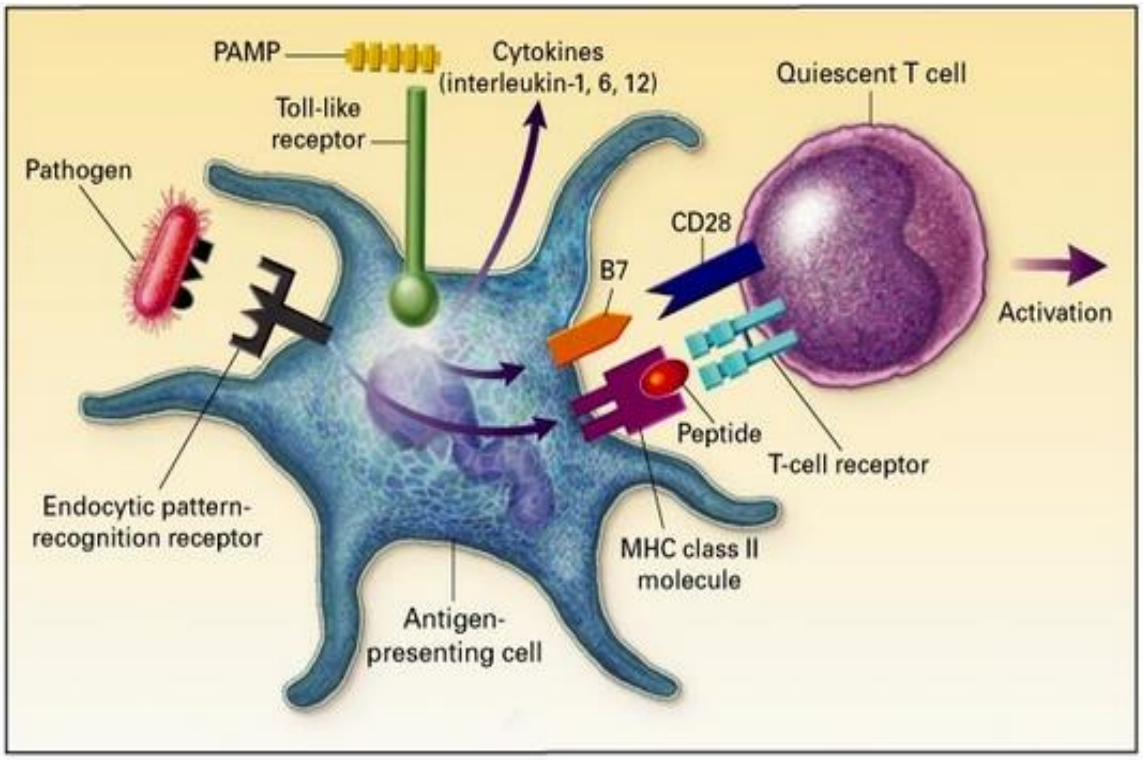


Figure 10: Initiation of the adaptive immune response by antigen presenting-dendritic cells (Medzhitov & Janeway Jr, 2000).

DCs can express IL - 12, IL - 1, IL - 6 or IL - 18 and interferon-gamma (IFNy). They are known to induce naïve T cells to express T- bet and STAT 4 and STAT 1 and to differentiate towards T helper 1 (Th1) cells as shown in Figure 11. Viruses or bacteria can induce a Th1 response by activating natural killer cells.

In contrast, allergens and parasites induce T helper 2 (Th2) responses via the release of IL - 4 with possible involvement of eosinophils and mast cells; it also induces naïve T cells to express STAT 6 and GATA3 and to differentiate in Th2 cells (Lee &Iwasaki, 2007; Zang et al., 2014).

T-box transcription factor (T-bet) is the master regulator of Th1 differentiation through activation of a set genes which promotes only the differentiation towards Th1 phenotype

and suppress the development of Th2 phenotype, impairing the function of transcription factor GATA3 (Luckheeram et al., 2012).

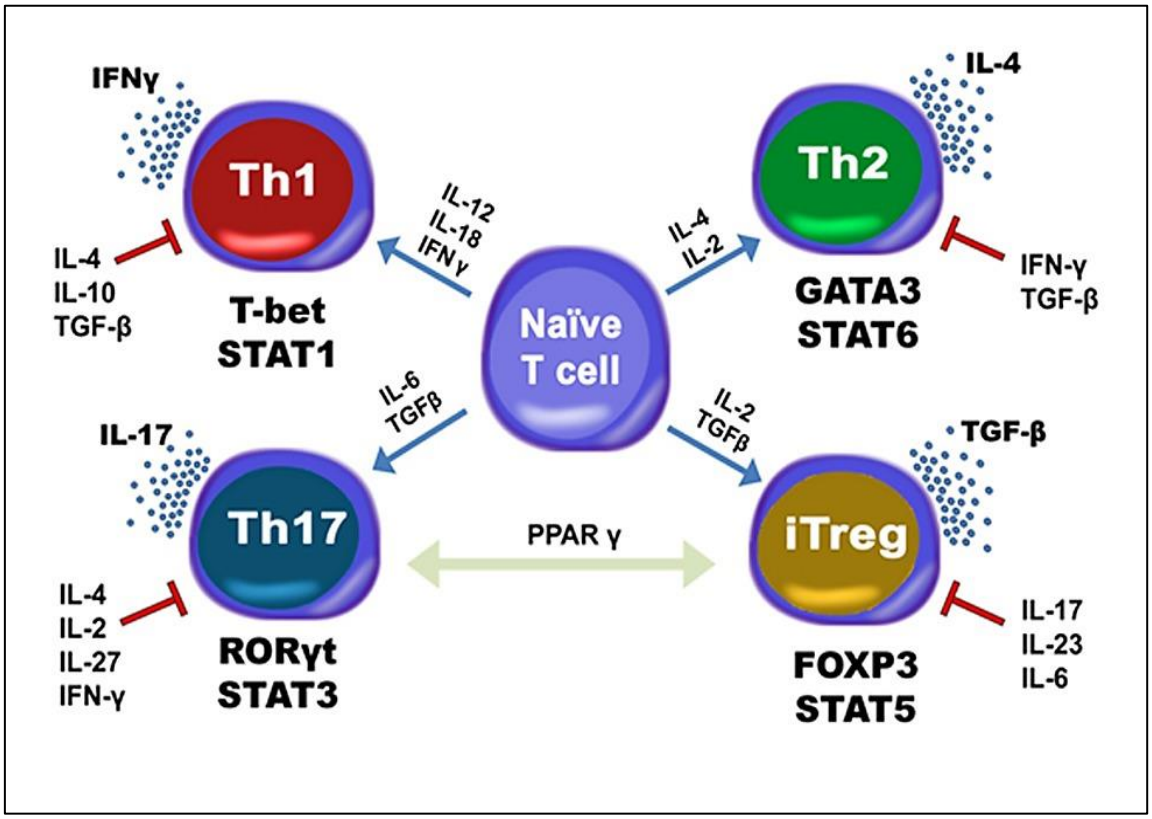


Figure 11: Th1 and Th2 differentiation (Carbo et al, 2013)

Th1 and Th2 cells undergo differentiation through help derived from cytokines, DC, macrophages, and other cell types. Th1 cells are activated by IL - 12 and IFNy and inhibited by IL - 4, whilst for Th2 cells the reverse is true. (O'Garra & Arai, 2000).

In addition to Th1 and Th2 cells, and depending of environmental factors, naïve T cells can differentiate in other subsets of T cells, such as Th17 and T regulatory (Treg) cells, and also in Th9 and T follicular-helper cells. The cytokines present in the environment are the major inducers of differentiation in different subsets. As already shown in Figure 11, Th1 differentiation is coordinated mainly by IL - 12 expressed by macrophages and DC, whereas

Th2 differentiation is driven by IL - 4 presence. Th2 cells express themselves IL - 4 and IL - 10 which antagonize Th1 development (O'Garra & Arai, 2000).

T helper cells are seen as the most prolific cytokine producers. Th1 cells express cytokines such as IFN-y which promotes pro-inflammatory responses. IFN-y enhances IL-12 production directly and further, more Th1 cells generation. On the other hand, Th2 cells express cytokines such as IL - 10 and IL - 4, which modulate pro-inflammatory response, and are known as anti-inflammatory cytokines (Berger, 2000; Osugi et al., 1997).

1.3.1 What are the cytokines?

Cytokines are hormone-like polypeptides comprising of a family of soluble proteins with a fundamental role in facilitating communication between cells and external environment. Under the term of cytokines comes lymphokines, monokines, interleukins (IL), interferons (IFN), tumor necrosis factor (TNF), chemokines and colony stimulating factors (Zhang & An, 2009). They are secreted mainly by white blood cells; however, it was found that fibroblasts, epithelial cells or endothelial cells can also express a variety of cytokines.

The most prolific producers of cytokines are T helper cells (Th) and macrophages They regulate the immune system through immune cell differentiation and proliferation, but also have role in the regulation of inflammation and in haematopoiesis. The cytokines bind to specific receptors on the cells, inducing signal transduction and initiating gene transcription which results in translation of proteins which can change cell's function or characteristics (McInnes, 2017). The cytokines are pleiotropic, redundant and multifunctional. They regulate the response to infection, inflammation, or trauma. There are proinflammatory cytokines and anti-inflammatory cytokines. The anti-inflammatory cytokines antagonize pro-inflammatory types by reducing inflammation and promoting healing (Dinarello, 2000).

The pleiotropic function of cytokines can be observed in figure 12, showing that one single cytokine can affect the activity of multiple cell types. The redundancy characteristic of cytokines means that multiple cytokines can have similar effects on one type of cell. These functions are possible due to the ability of certain cytokines to bind to multiple types of receptors or by the ability of many types of cytokines to bind to same receptor (Nicola, 1994; Ozaki & Leonard, 2002).

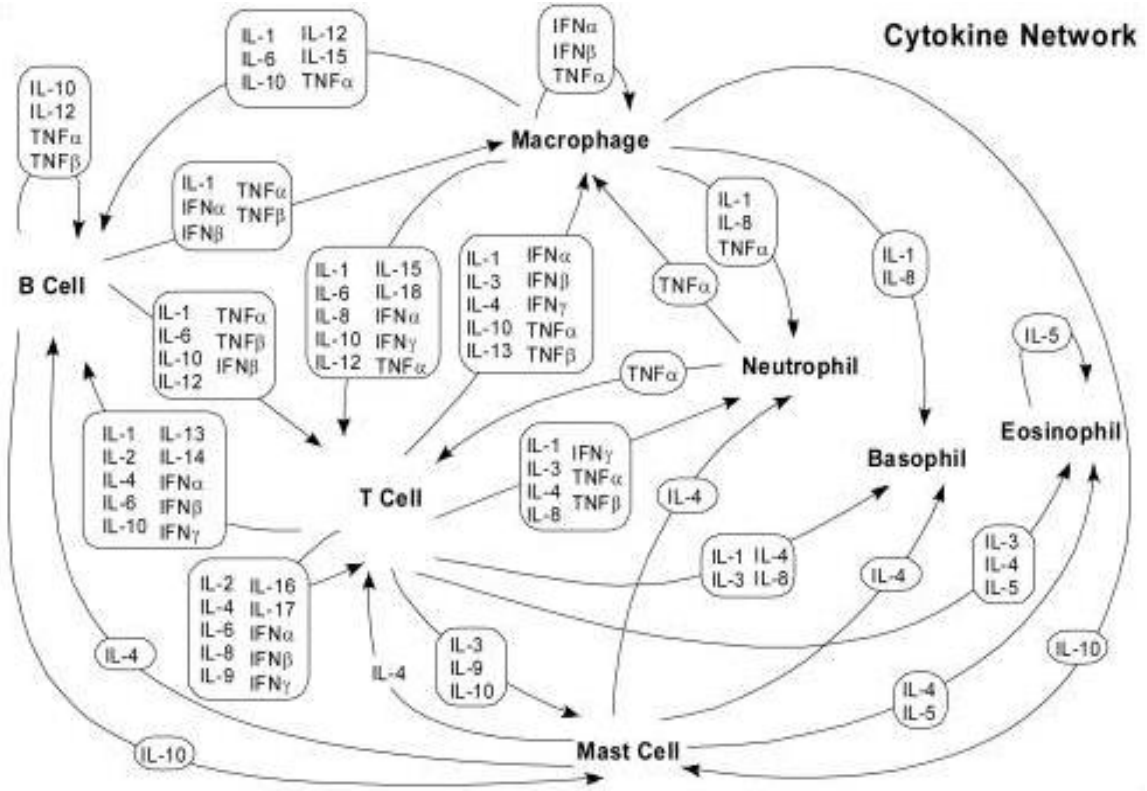


Figure 12: The cytokine network. The figure depicts how each cell of the immune system secretes multiple cytokines which further induce other immune cells to express other type of cytokines (Zhang & An, 2009)

1.3.2 Pro-inflammatory cytokines

Proinflammatory cytokines are secreted mainly by activated macrophages, instigating an inflammatory reaction. IL - 1β, TNF-α, IL - 6, IL - 12 are the most important pro-inflammatory cytokines.

1.3.2.1 Interleukin - 1 (IL - 1)

Interleukin-1 consists of two major proteins, IL - 1α and IL - 1β which are important mediators of inflammatory responses. IL - 1 is secreted by monocytes and macrophages and by fibroblasts and endothelial cells as result of cell injury, infection or inflammation (Dinarello, 2018). Traumatic injury results in ischemia, hypoxia and necrosis. IL - 1α in precursor form is released from necrotic cells contributing to infiltration of neutrophils and initiation of sterile inflammation. It acts as an alarm signal in tissue injury and it is rarely secreted (Rider et al, 2011). IL - 1β is associated with pain, autoimmune conditions and with inflammation. IL - 1β promotes macrophages recruitment. Unlike IL - 1 α, IL - 1β is not expressed in homeostatic conditions. It is present in the inactive state in inflammasomes, where it can be activated by the cleavage of its precursor during inflammatory processes. Comparing IL - 1α with IL - 1β, the latter is dominant in later stages of inflammation and is involved in propagation of the inflammatory response. IL - 1 α released from dying cells initiate sterile inflammation inducing the recruitment of neutrophils, whereas IL - 1β promotes the recruitment and retention of macrophages (Rider et al., 2011).

1.3.2.2 Tumour necrosis factor-α (TNF - α)

TNF - α is a pleiotropic pro-inflammatory cytokine produced mainly by monocytes and macrophages. It can also be secreted by other non-immune cells such as smooth muscle cells, fibroblasts or neurons, microglia and astrocytes. TNF - α plays an important role in the inflammatory response to injury by recruiting immune cells such as macrophages and neutrophils at the site of injury. It acts as an acute-phase protein initiating a cascade of cytokines and growth factors (Liu & Tang, 2014; Woodcock & Morganti-Kossmann, 2013). High levels of TNF - α were detected in the first 4 hours after traumatic injury, after this time its values decreases rapidly. Animal model studies showed that there is a correlation

between high systemic concentration of TNF and the mortality due to haemorrhagic shock (DeLong & Born,2004). Anti-TNF - α therapies were successfully used as treatment for rheumatoid arthritis or inflammatory bowel disease. However, clinical trials involving approximatively 1000 patients with sepsis treated with TNF-neutralizing antibodies did not show any improvement in the 28-day survival rates, and more research is needed in this field. (Dinarello, 2000).

1.3.2.3 Interleukin- 6 (IL - 6)

IL - 6 is a small glycoprotein(21kDa) with four-α-helix-bundle structure, identified in mid-1980. IL - 6 is present in the blood of heathy individuals at as low as 1-5 pg/ml. Its levels can rise considerably during inflammatory events such as sepsis, when their concentration can be measured at levels of μg/ml. IL - 6 generation is activated by tissue damage or stress, such as ROS, also by the presence of lipopolysaccharides (LPS) or viral products. IL - 6 is also activated by pro-inflammatory cytokines TNF - α and IL - 1α which activate NF - kB binding site on the promoter region of IL - 6 gene, shown to have an important role in the inflammatory response to infection and tissue injury (Liberman & Baltimore, 1990).

Initially, IL - 6 was identified as B-cell differentiation factor inducing terminal B-cell differentiation and production of immunoglobulin G, (Figure 13). IL - 6 was also known as hepatocyte-stimulating factor for its capacity to induce acute-phase proteins production, such as C-reactive protein (CRP), β2-fibrinogen, amyloid or haptoglobin (Kishimoto, 2010). It has pro and anti-inflammatory proprieties, regulating the balance between regulatory and effector T-cells. IL - 6 orchestrates innate and adaptive immune response through regulation of the acute phase response, hemopoiesis and inflammation. IL - 6 is secreted by a variety of

cells, such as macrophages, dendritic and T-cells. However, B-cells, endothelial cells, fibroblasts, astrocytes and epithelial cells were shown to secrete IL - 6 as well.

IL - 6 is secreted by macrophages, monocytes, T-cells and endothelial cells, and released early after trauma. Its role is to initiate the hepatic acute-phase response, stimulating C-reactive protein (CRP) and other acute-phase proteins is necessary to promote an inflammatory response. There is evidence in scientific literature to suggest that the pattern of expression of IL - 6 in trauma correlates to severity of the injury Moreover, it was found that high levels in IL - 6 was common in non-survivors (Strecker et al., 2003; Okeny et al., 2015).

In the acute phase of inflammation, the IL - 6 pro-inflammatory effect was found to have an important role in initiating the systemic inflammatory response and was used as an early biomarker of severity of injury in trauma patients. The IL - 6 anti-inflammatory effect acts as down-regulator for TNF - α and IL - 1 secretion (Mihara et al, 2012; Rincon,2012; Stensballe et al., 2009). Increased levels of IL - 6 in the acute phase response or wound healing is considered protective in the short term, only when the response becomes chronically activated, then high concentrations of IL - 6 can become pathogenic to the host, being associated with fibrosis and chronic inflammation (Fontes et al., 2015).

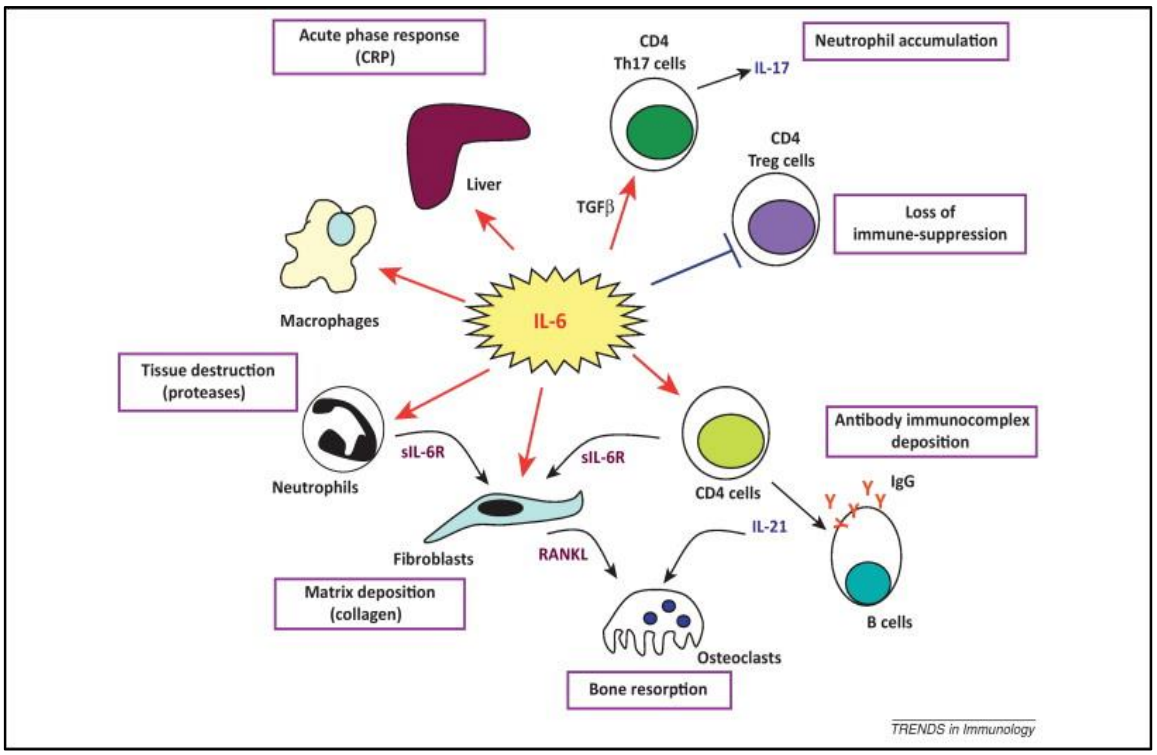


Figure 13: Biological activity of IL 6 and its effect on cells and organs (Rincon, 2012)

The presence of IL - 6 during antigen stimulation of CD4 T- cells, promotes a shift in Th1/Th2 balance towards Th2, acting like IL - 12 on naïve CD4 T-cell differentiation. Moreover, IL - 6 promotes autocrine production of IL - 4, and further, increases the Th2 differentiation through an auto-feedback loop (Dienz & Rincon, 2008). IL - 6 has proved to have an important role in the progression of auto-immune diseases, and its excess in serum levels is correlated to several chronic inflammatory diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis and multiple myeloma (Maeda et al, 2010). IL - 6 is part of the IL - 6 family which also includes IL - 11, leukaemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), cardiotrophin-like cytokine (CLC) and cardiotrophin-1 (CT-1).

IL - 6 acts upon the cells which express IL6 receptors (IL-6R), predominantly leukocytes and hepatocytes. IL-6R is an 80 kDa transmembrane protein with a short cytoplasmic domain, first identified in 1988.

IL - 6R exist as membrane-bound form and as a soluble form sIL - 6R. IL - 6 which bind to membrane-bound receptors is known as IL-6 classic signalling, whereas when binding via soluble IL - 6R, is known as IL - 6 trans signalling. IL - 6 binds to IL-6R and gp130, activating JAK/STAT pathway. STAT3 become phosphorylated forming homodimers which translocate to nucleus where regulate gene transcription (Heinrich et al., 1998). IL - 6R is mainly expressed on hepatocytes and many leukocytes, whereas sIL-6R is present in blood and urine, with concentration in serum around 25-35 ng/ml (Wolf et al, 2014). IL-6 binding to membrane-bound IL - 6R associates with the signal-transducing β-subunit glycoprotein gp130 (130kDa) which serves as signal transducer and formation of high affinity IL - 6 binding sites (Hibi et al., 1990).

IL-6 signals via the activation of Janus kinases (Jaks) and transcription factors of STAT family, known as the Jak/STAT signalling pathway (Fig 14). IL - 6 induces gp 130 homodimerization; gp-130-associated kinases Jak1, Jak2 and Tyk2 becomes activated and the cytoplasmic tail of gp 130 is phosphorylated. Phospho-tyrosine residues of gp 130 are docking sites for transcription factors STAT3 and STAT1, which become phosphorylated. They form dimers and translocate to the nucleus where they regulate the transcription of targeted genes, such as APP genes for C-reactive protein, lipopolysaccharide-binding protein, interferon regulatory factor or interstitial collagenase, and many other varieties of genes (Heinrich et al., 1998).

1.3.2.4 Interleukin - 12 (IL - 12)

IL - 12 is a cytokine, a 70kDa hetero dimer, composed of a light α-chain of 35 kDa (p35 subunit) and a heavy unit 40 kDa β-chain (p40 subunit). IL - 12 was first described in 1989 by Kobayashi et al. It was first known as natural killer cell stimulatory factor (NKSF) for its capacity to induce interferon-gamma (IFN - γ) production from T and NK cells (Oshimi & Hoshino, 1992). IL - 12 was also known to increase cytotoxic activity of resting peripheral blood NK cells as cytotoxic lymphocyte maturation factor (CLMF) for its proprieties as growth factor for activated T-cells (Gubler et al., 1991).

IL - 12 is an activator of cell-mediated immunity and promotes the differentiation of naïve CD4+ Th cells into Th1 cell, being an important link between innate and adaptive immunity. IL - 12 induces the proliferation of cytotoxic T lymphocytes, B cells and NK cells and is produced by activated dendritic cells and phagocytes (Shaashua et al., 2013). IL - 12 induces T cells and NK cells to produce several cytokines, the most important of them being IFN-γ (Trinchieri, 2003). In traumatic injury, a depressed IL - 12 production is correlated with a shift in differentiation of naïve CD4+ Th cells towards Th2 pattern (Keel & Trentz, 2004).

IL - 12 is mainly produced by antigen-presenting cells such as macrophages and dendritic cells and exert a proliferative activity on NK cells and T cells, increasing the production of interferon-gamma from these cells, as shown in figure 15. IL - 12 plays a critical role in differentiation of naïve $CD4^+$ T cells towards T helper1 cells (Th1). This polarisation towards Th1 cells, results in further IFN-γ secretion which stimulates bactericidal effect of phagocytic cells and promote cell-mediated immunity (D'Andrea et al., 1992; Stern et al., 1996). All this makes IL - 12 an important link between innate and adaptive immunity. IFN-γ in turn

regulates the production of IL - 12 in a positive feedback-loop (Zundler & Neurath, 2015; Watford et al., 2013).

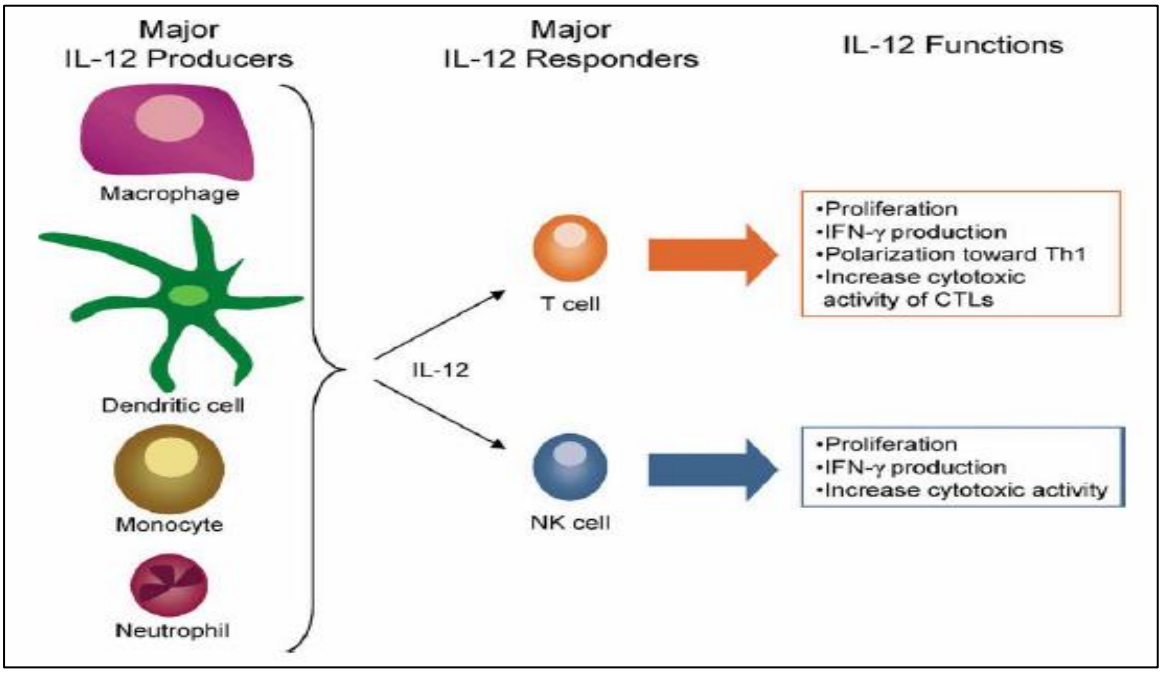


Figure 14: Cellular basis of IL - 12 secretion. The figure shows IL - 12 production by antigen-presenting cells and phagocytic cells, and its action on T cells and NK cells, inducing differentiation towards Th1 type cells, IFN-γ production and increased cytotoxic activity of T and NK cells (Watford et al., 2003).

Grohman et al., proved in 1998 that IL - 12 is the most potent initiator of the immune response by directly acting on professional antigen presenting cells such as dendritic cells which express high-affinity IL - 12R on their surface. Both IL - 12 subunits, α and β chains are encoded separately on human chromosomes 3 and 5. However, to obtain a biologically active IL - 12, the co-expression of both unit cDNAs are necessary (Gubler et al., 1991). IL - 12 may exist only as IL - 12 p40 which can be secreted in excess as free a monomer and can form homodimers $p40_2$, which was found to have role in directly recruiting macrophages; in the animal model IL - 12 p40 induces the expression of TNF - α (Ha et al., 1999; Jana et al., 2003).

IL - 12 receptor (IL-12R) is made up of two receptor subunits, IL - 12β1 and IL - 12β2. A high affinity IL - 12 binding site needs the expression of both β1 and β2 subunits.

IL - 12 p35 interacts with β2 subunit, whereas IL - 12 p40 interacts with β1 subunit. IL - 12 R subunits are expressed on T cells, NK-cells and DC cells. Upon high-affinity binding of IL - 12 to both subunits of IL - 12R, receptor-associate Jak2 is activated, which in turn phosphorylates IL - 12R Tyk2 situated in intracellular domain. This leads further to the phosphorylation of signal transducer and activation of transcription 4 (STAT4) which show to have an important role in IL - 12 signalling. STAT4 forms homodimers that translocate to the nucleus and binds to the IFN-γ promoter, inducing IFN-γ gene transcription (Ma et al., 2015; Watford et al., 2003). IFN-γ is the main activator for macrophages resulting in the synthesis of IL - 12 and IL - 12R and promotes Th1 development. INF-γ is known to suppress the expression of IL - 4 through interferon regulatory factors (IRF1 and IRF2) which acts as transcription repressors by binding to IL - 4 promoter, thus inhibiting Th2 responses (Elser et al., 2002).

1.3.3 Anti-inflammatory cytokines

The anti-inflammatory cytokines play a role of immunoregulators, modulating the inflammatory effect of pro-inflammatory cytokines and regulating the immune response toward homeostasis. The most important anti-inflammatory cytokines are IL - 10, IL - 4, IL - 1RA and IL - 1, to name a few.

1.3.3.1 Interleukin - 4 (IL - 4)

IL - 4 is a cytokine with molecular weight of 12-20 kDa. It was first known as B-cell stimulatory factor-1 for its ability to increase the expression of class-II major histocompatibility complex (MHC) on resting B cells and to stimulate the production of Ig G

and Ig E from B-cells (Paul & O'Hara, 1987). IL - 4 is mainly produced by Th2 lymphocytes, and by the mast cells (Figure.15), basophils and eosinophils. It has an important role in naïve $CD4^+$ T lymphocytes differentiation towards Th2 cells and their proliferation and has an inhibitory effect on Th1 differentiation through IFN-γ downregulation (Ul-Haq, Naz & Mesaik, 2016). IL - 4 has an autocrine effect on Th2 cells which produce more IL - 4. IL - 5, IL - 6 and IL - 10 are other types of cytokines secreted by the stimulated Th2 cells (Tato et al., 2006).

IL - 4 together with tumour necrosis factor (TNF) increases endothelial cell adhesiveness to T- cells and their migration at the site of inflammation, and can mediate chronic inflammation (Thornhill et al., 1990).

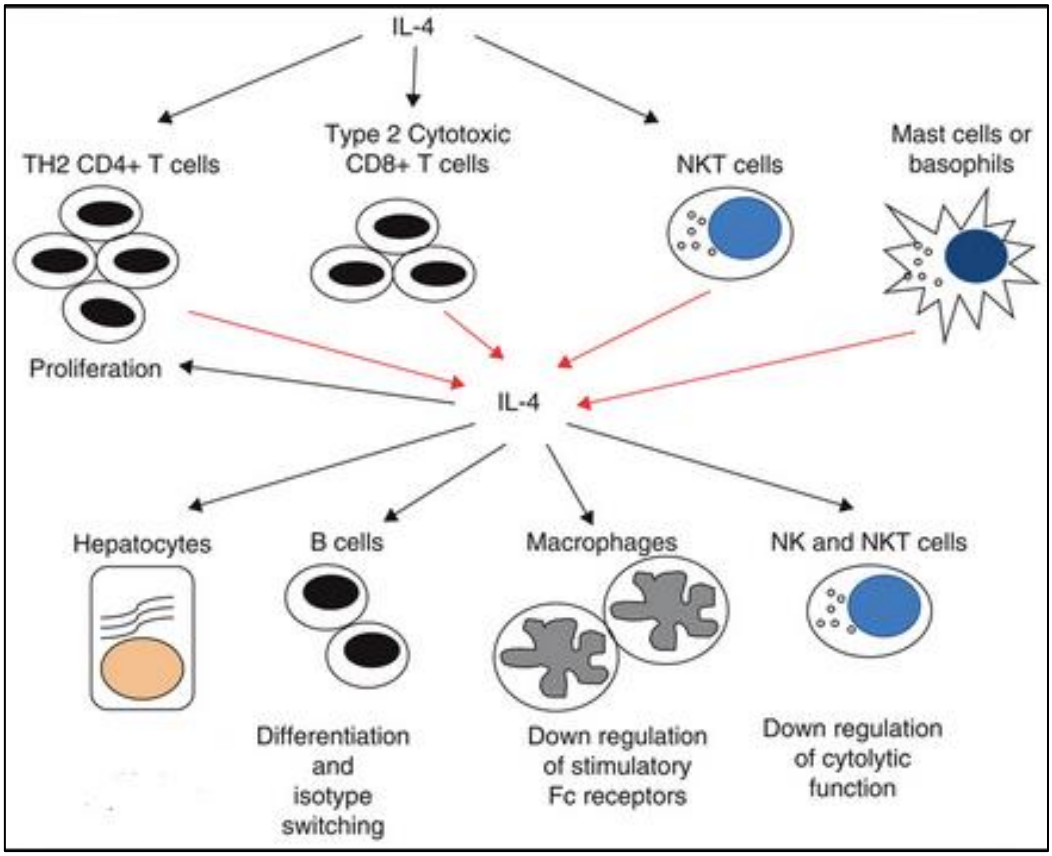


Figure 15: Biological activity of IL-4 (Njoku, 2010)

IL - 4 in association with IL - 13, both generated by Th2 lymphocytes, are involved in an alternative macrophage activation which is responsible for tissue repair and homeostasis

(Gordon,2003). IL - 4 is involved in several allergic and immune disorders, such as allergic asthma and atopic dermatitis. Increased IL - 4 and IL - 4R expression has been detected in breast, bladder and prostate cancer which may suggest that IL - 4 can increase tumour cells resistance to apoptosis (Ul-Haq, Naz, & Mesaik, 2016).

IL - 4 is a polyfunctional cytokine with receptors presenting on multiple types of cells. IL - 4 is produced mainly by Th2 cells and also by natural Killer cells (NK), basophils, mast cells and eosinophils (Paul, 2015). In recent years it was found that IL - 4 is also produced by T follicular cells (Tfh) as a major alternative source of IL - 4 (Sahoo et al., 2016). IL - 4 has an autocrine role, inducing naïve CD4+ Th cells to differentiate in Th2 cells (Noben-Trauth et al., 2002). Research has shown that an increased release of IL - 4 may be involved in the development of ARDS and SIRS (Li et al., 2002). Moreover, IL - 4 was found to be associated with the mortality in patients with SIRS, with higher levels in infectious SIRS. The high levels of the cytokine are reported to be associated with genetic variations in IL - 4 gene (Torre et al., 2000). Gu et al., (2011) showed that polymorphisms in the IL - 4 promoter are associated with the increase in expression of IL - 4, which might be associated with increase susceptibility to develop sepsis in trauma patients.

IL - 4 modulates macrophage activity by induction of inhibitory activity through increase in the expression of FcyRllb2, an inhibitory receptor on monocytes. These findings might help in taking a different approach in the treatment of chronic autoimmune diseases (Pricop et al., 2001). On the other hand, natural killer (NK) cells exposed to microenvironmental IL - 4 , have shown reduced cytolytic potential and generated dendritic cells which promote T-cell tolerance (Moretta et al., 2006).

IL- 4 receptors (IL-4R) consists of an IL - 4Rα chain which binds with high affinity to IL - 4, and IL - 4Rγ chains necessary for signalling pathway activation. The complex IL - 4Rα/IL - 4Rγ is expressed on many types of lymphocytes, such B-cells, T-cells and NK-cells, and on the endothelial cells. As figure 18 shows, upon ligand binding to IL - 4Rα with high affinity and the dimerization with gamma chain (γc), a signal transduction cascade initiates the activation of Jak1 and Jak3. It induces tyrosine phosphorylation on cytoplasmic tails of IL - 4Rα and γ-chain, followed by tyrosine phosphorylation of STAT6.

Phosphorylated STAT6 undergoes homodimerization, then translocate to the nucleus and stimulates gene expression (De Vries, Carballido & Aversa, 1999; Walford & Doherty, 2013). STAT 6 has an important role in T-cell survival and proliferation, Th2 differentiation and progression of allergic and immune disorders (Ul-Hak, Naz & Mesaik, 2016)

STAT6 induces expression of GATA3-Th2 specific transcription factor. GATA3 is known to downregulate IL - 12 expression which results in Th1 cytokine-INF-γ suppression, inhibiting Th1 responses, thereby, it intensifies Th2 immunity (Li-Weber & Krammer, 2003).

1.3.3.2 Interleukin - 10 (IL - 10)

Interleukin - 10 is a cytokine of approximatively 21 kDa with important immunoregulatory functions. Its primary role is to limit inflammatory responses to pathogens and to maintain immune homeostasis.

IL - 10 is a cytokine with anti-inflammatory properties. It is expressed by Th2, T-reg and Th-17 cells and by other cells of the immune system (dendritic cells, natural killer cells, macrophages and neutrophils), acting as a feedback regulator of diverse immune responses. IL - 10 inhibits the development of Th1 cells but also suppress Th2 cells and allergic responses (Saraiva, & O'Garra, 2010). IL - 10 is an important counter immune-regulator

during systemic inflammatory response syndrome, modulating exaggerated proinflammatory responses. IL - 10 is postulated to be the main driver for the compensatory anti-inflammatory response syndrome (Liu et al., 2017).

IL - 10 can be expressed by pathogen-activated dendritic cells, macrophages and neutrophils, and by T and B-cells. IL - 10 can also be expressed by other types of leukocytes such as eosinophils and mast cells. (Saraiva & O'Garra, 2010). Initially it was thought that IL - 10 to be produced only by only Th2 cells and was named as cytokine synthesis inhibitory factor (CSIF) for its property to inhibit Th1 cytokines production such as INF-γ, IL - 2 and GM-CSF (Fiorentino et al., 1989). Further studies showed that IL - 10 is also expressed by other cells specific to innate immunity (Siewe et al., 2006). Another sub-class of T lymphocyte known as T regulatory (Treg) cells mediate suppression of immune responses through IL-10 secreted by T regulatory1 (Tr1) and acting directly on effector cells (Arce-Sillas et al., 2016; Saraiva et al., 2010). IL - 10 can also be secreted by B-cell subpopulations able to suppress inflammation through paracrine mechanisms. In an autocrine manner IL - 10 regulates the differentiation of activated IL - 10-secreting B-cells in IgM and IgG secreting cells (Heine et al., 2014).

However, IL -10 can regulate either Th1 and Th2 responses by limiting T-cell activation and differentiation (Couper, Blount & Riley, 2008). IL - 10 inhibitory effects result in reduced secretion of pro-inflammatory cytokines (INF-γ, IL - 1, IL - 6, IL - 12, TNF, GM-CSF) and inhibition of production of chemokines (IL - 8 and IP-10) and prostaglandins (PG-E2) (Figure 16).

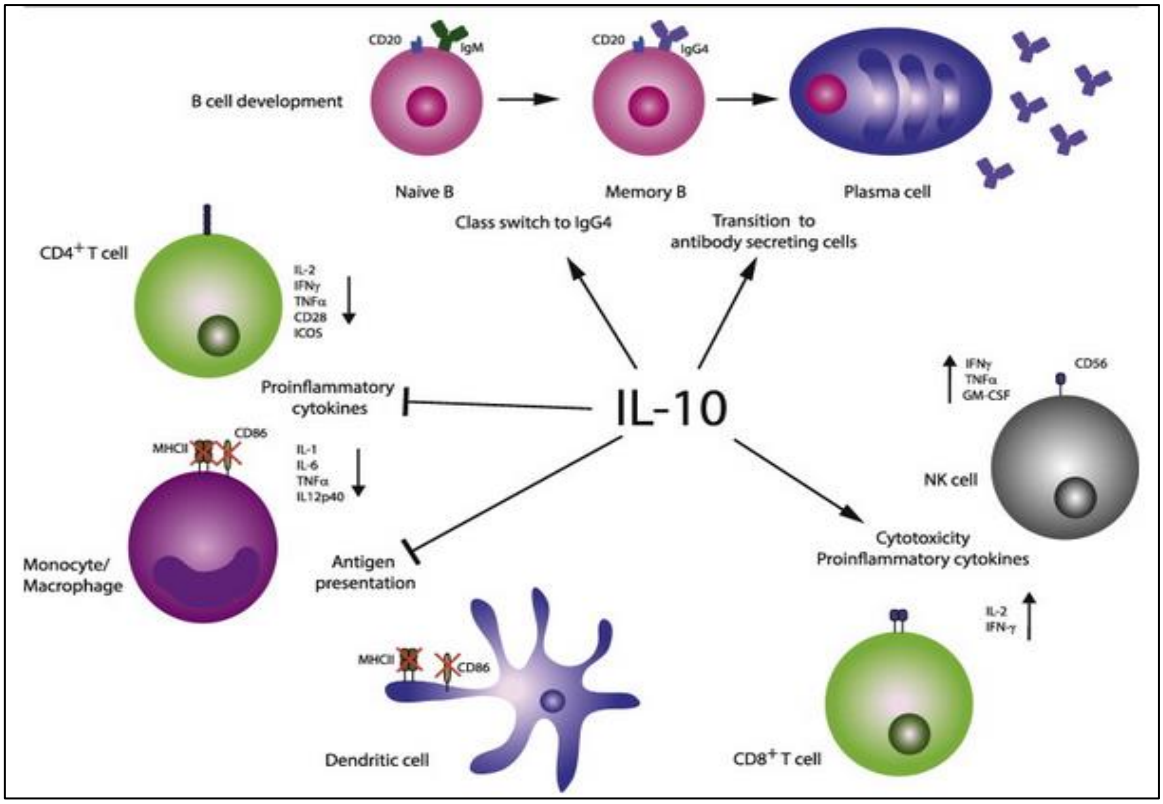


Figure 16: IL-10 function in maintaining homeostasis (Cho et al., 2015)

The inhibitory effect of IL - 10 exerted over monocytes results in downregulation of expression of MHC class II antigens on their surface, suppressing their capacity to function as APC for T-cells activation.

IL - 10 inhibits the maturation of DC and induces differentiation of immature DC into macrophages with reduced capacity of producing IL – 12. Therefore, IL - 10 can inhibit differentiation of naïve T-cells in Th1 cell type and favour the differentiation in Th2 cells (Moore et al., 2001) . IL - 10 has also an inhibitory effect through B cell class switching from IgE towards IgG4 which leads to T-cell anergy (Akdis et al., 1998). Furthermore, IL - 10 is shown to have an effect on the growth and differentiation of B-cells and mast cells (Cho et al., 2015). Although IL - 10 shows predominantly inhibitory immune activity, studies have revealed that IL - 10 in combination with IL - 2 can stimulate immune responses by inducing the proliferation and cytotoxic activity of $CD8^+$T lymphocytes (Santin et al., 2000), and

natural killer (NK) cells. This stimulation leads to increased production of IFN-γ, TNF-α and granulocyte-macrophage colony-stimulating factor (Carson et al., 1995).

IL - 10 exists in the form of a homodimer connected by two disulphide bridges, which makes it biologically active. IL - 10 receptor (IL - 10R) is a tetramer which comprises two IL - 10R-alpha and two IL - 10R-beta subunits.

Upon IL - 10 homodimer binding to IL - 10R-alpha subunit, the phosphorylation of Jak1-IL-10R-alpha takes place and is followed by Tyk2-IL-10Rbeta subunit phosphorylation. As figure 20 shows, their kinases will further phosphorylate tyrosine residues on the cytoplasmic domain of IL - 10R-alpha, which serve as docking site for STAT3. This leads to phosphorylation and homodimerization, followed by translocation to the nucleus, where it stimulates gene expression (Verma et al., 2016). IL - 10 is a potent suppressor for gene expression for pro-inflammatory cytokines such IFN-γ which activates STAT1; STAT3 activated by IL - 10 opposes STAT1, downregulating IFN-γ expression (Hu et al, 2007)

Macrophages and dendritic cells express high levels of IL - 10R on their surface, therefore IL - 10 regulates pro-inflammatory activity and their production, by regulation of gene expression. It targets proteins encoding for pro-inflammatory cytokines, chemokines through TLR signalling and their suppression (Murray, 2006).

Some of the pathogens have evolved to exploit IL - 10 pathway and to inhibit the expression of pro-inflammatory genes by activating STAT3, as IL - 10 does, resulting in macrophages inability to express IL - 12 and TNF necessary to suppress the parasite (Butcher et al., 2005).

1.4 METHODS FOR CYTOKINES DETECTION AND QUANTIFICATION

Cytokines are small secreted proteins with a vital role in intracellular communication. They are used as biomarkers for various diseases and in monitoring the progress of diseases or the response to a treatment. The cytokines are released into the intracellular space in minute quantities which makes them unsuitable for traditional protein detection technique such as gel electrophoresis or mass spectrometry, therefore immunoassays are the techniques of choice for cytokines detection (Kupcova Skalnikova et al., 2017).

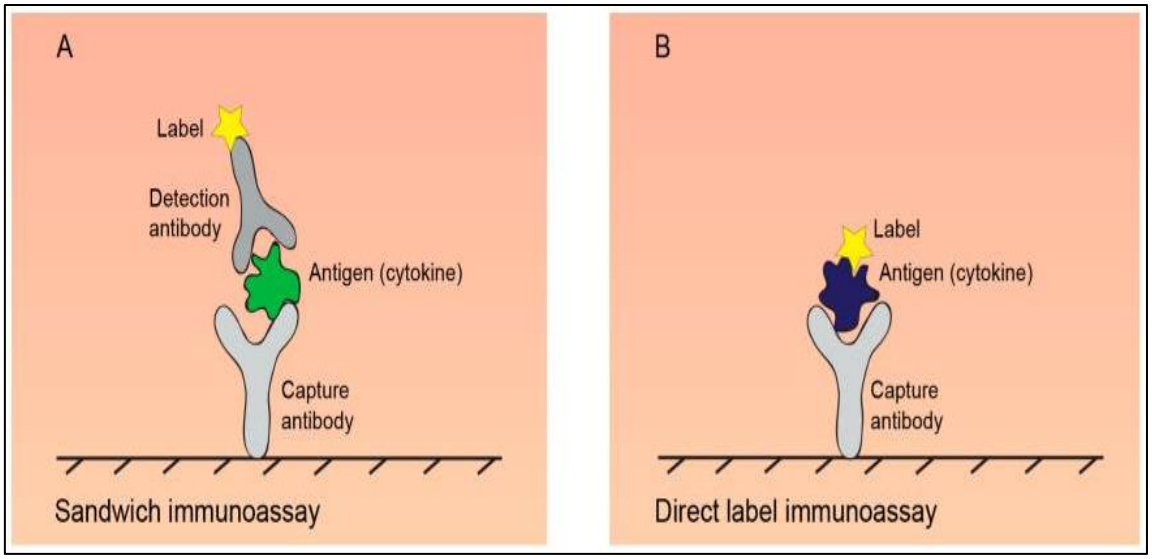


Figure 17: Schematic representing underlying principles of immunoassays. A) The cytokine binds to capture antibody then the cytokine is detected with the help of a detection antibody conjugated with a fluorescent tag. B) The antigen is directly enzyme-labelled and get immobilized on the capture antibody (Kupcova Skalnikova et al., 2017).

The most used techniques for cytokines detection are enzymes-linked immunosorbent assays (ELISA) (Figure 17), antibody arrays such as bead-based immunoassays or planar arrays. More recently new techniques based on microchip monochromatic strips were developed for rapid diagnoses to be used as point of care testing (POCT) (Man et al., 2014). Recently, scientists from University of Strathclyde have developed a biosensor device containing microelectrodes that can detect one of the most important markers of sepsis,

IL - 6. The device can be implanted and used on patients in intensive care for constant monitoring and deliver a bedside diagnosis in only two minutes instead of 72 hours as the current testing. The reduced cost of the device and the rapid diagnosis of sepsis can save many lives in the future (www.strath.ac.uk, 2019).

1.4.1 Enzyme-linked immunosorbent assay (ELISA)

The ELISA technique is a method developed in 1971 (Aydin, 2015) and is used in diagnostic or biomedical research to detect and quantify specific antigens. The technique uses antigen immobilized on the wall of a 96-well microtiter plate.

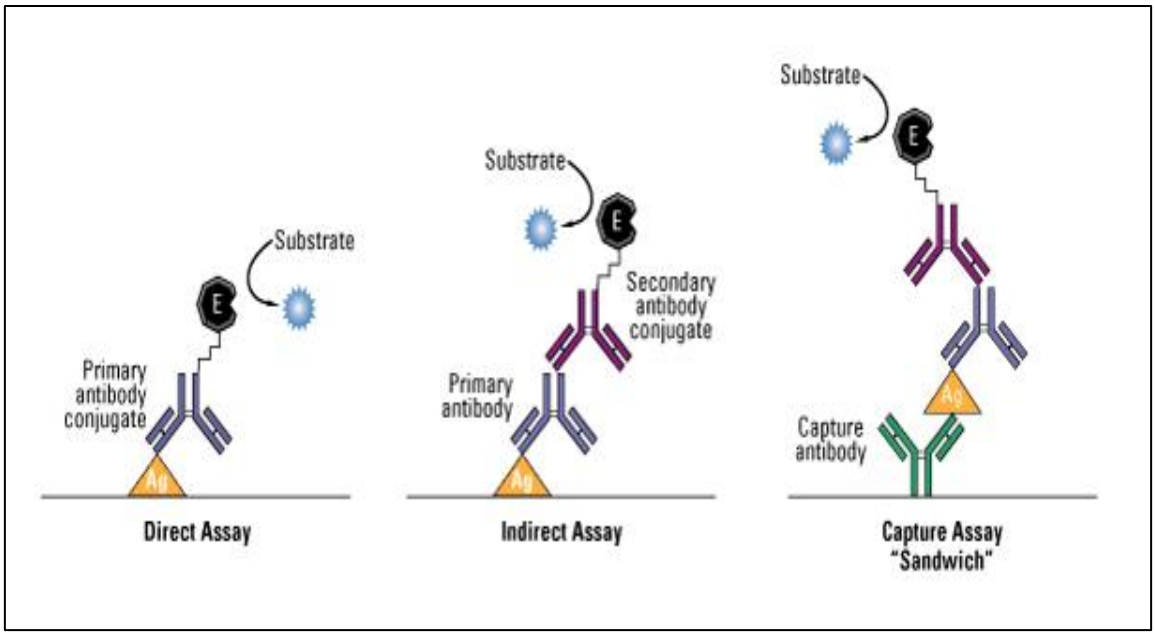


Figure 18: The principles of ELISA assays; direct, indirect and sandwich assay are shown (Image from Thermo Fisher Scientific)

The antibody specific to the antigen is added and binds to antigen, as shown in figure 18 for direct and indirect assays (Gan & Patel, 2013).

In the direct assay, the primary antibody conjugated to an enzyme binds to the antigen which is directly attached on the well surface. The indirect assay has the same principle as

the direct method, with the difference that a secondary antibody conjugated to an enzyme or fluorophore specific for the detection of the primary antibody is added and form an antigen-antibody complex. The most common enzymes used in ELISA assay is alkaline phosphatase, horseradish peroxidase or biotin.

Another type of ELISA assay known as capture or sandwich assay, has the wells coated in capture antibodies specific to the antigen of interest. The antigen binds to the antibody and is followed by washing steps. Further, antibodies tagged with enzymes specific to the antigen are added and will bind to them. Even though the methods show some differences, they all have in common the addition of a chromogenic substrate which will show a colour change or fluorescence in the presence of the antigens. The intensity of the colour or signal is directly proportional to the amount of antigen present. The signal is detected and measured with an ELISA reader (Aydin, 2015; Gan& Patel,2013).

The sandwich ELISA assay was found to be more sensitive than other types of ELISA methods. Indirect ELISA is more sensitive than direct method, this is achieved through the presence of multiple epitopes on the primary antibody which binds to the secondary antibody resulting in a better signal amplification. The large variety of secondary antibodies gives a great advantage to indirect method, but the direct method is quicker and eliminates cross-reactivity of a secondary antibody. Ready to use cytokine analysis kits are available from various companies. ELISA is a sensitive and specific method, it is easy to use, low-cost and rapidly produce results, but it needs a large volume of analyte and can be time consuming (Gan & Patel, 2013).

1.4.2 Antibody arrays

This method is similar to ELISA, but the capture antibodies are spotted on a nitrocellulose membrane or glass. The serum or other types of analyte is diluted and mixed with biotinylated antibodies and left to incubate. The cytokines of interest are immobilized on the membrane/glass slide by the capture antibody. The detection step is performed with the aid of streptavidin-horseradish peroxidase and chemiluminescent reagents. In the case of membrane, the detection process does not need specialized equipment for detection resembling to western blot, whereas for glass slides, an array scanner is needed for detection and quantification of proteins of interest (Kupcova Skalnikova et al., 2017).

1.4.3 Bead-Based immunoassays using flow cytometry

The assay uses a flow cytometer, an instrument that can detect fluorescent signals from individual cells which flow in the front of a narrow beam of light. Modern flowcytometers, though using the same principle, can detect other types of particles besides whole cells, such as nuclei, cytokines and hormones, to name a few (Givan, 2011). Curently, the flow cytometer measures optical and fluorescent characteristics of particles, such as light scattering and fluorescent emission from moving particles in the front of a laser beam. Physical characteristics measured are size, granularity, and fluorescence resulting from antibodies and dyes which are employed to identify and differentiate the particles of interest (Adan et al., 2017).

The main components of a flow cytometer are the fluidics and optic systems for signal detection and measurements. Through the fluidic system the analyte is transported to the optical system. The fluidic system is comprised of sheath fluid and the analyte in form of solution which is injected in the flow chamber at a high pressure. According to

hydrodynamic focusing principles, the particles will align in a single line while passing through the interrogation point, where the cell meet with the laser beam. (Figure 19). The optical system is made up of lasers and lenses with arole in focusing the laser beam on to the cell. The light scattered by cells divides in forward scatter (FSC) in a manner proportional to the size of the cell or particles, and side scatter (SSC), related to cell granularity or internal complexity, and also detects fluorescent-labelled antibodies or dyes (Givan, 2010).

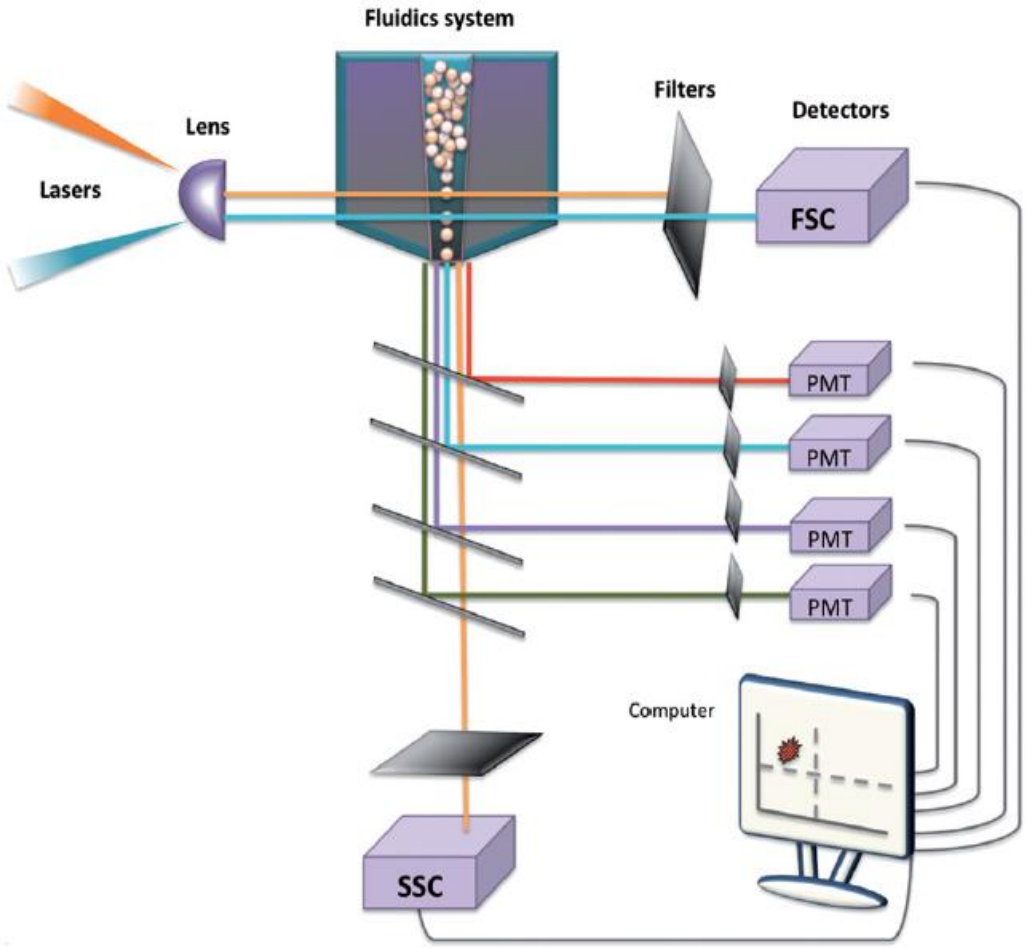


Figure 19: The principles of flow cytometry; the fluid flows in the front of a laser beam where optical and fluorescent characteristics of particles are measured. The light is deflected in contact with the cells resulting in forward scatter (FSC) and side scatter (SSC). The signals are then converted in voltages by photodetectors (PMT), then converted in digital data displayed on a computer (Adan et al., 2017)

The detection of the emitted fluorescent light is done with the aid of a series of dichroic mirrors and filters, which transmits and reflects the light of specific wavelengths onto photomultiplier tubes (PMT). PMTs are vacuum tubes containing a photocathode, electron focusing electrodes and dyodes for electron multiplication. Further, the voltage pulse created is amplified and digitized, then all the data collected is summarized in a distribution curve or a histogram (Cossarrizza et al., 2017).

Flow cytometric techiques can use surface and intracelluar markers to track the progression of cellular activity. It can also observe cellular events by using specific florophore-labeled antibodies to identify cell transition from one stage to another. Flow cytometry is also used for characterisation of intracellular epitopes, such as hormone receptors, cell cycle proteins, enzymes, DNA and mRNA. Flow cytometry has the advantage that it can measure simultaneously surface antigens and intracellulat antigens, analyze B cells vs T cells, or diseased cells against healthy cells, all in the same experiment(Krutzik et al., 2004).

Flow cytometric methods are also used to detect cytokine production, such as antigen-specific T-cells responses to proteins, viral lysate or peptide, and detect simultaneously CD4 and CD8 T-cells from the same sample used for stimulation (Maino & Maecker, 2004).

For decades, researchers in cell biology and immunology were interested in measurement of soluble mediators involved in immune regulation. ELISA methods are already used for measurements of soluble analyte analysis but has some limitations , such as labor-intensive at high cost, but it measures only one analyte at a time using an incresed volume of sample (Klein et al., 2012). As biomedical research became more complex, the necessity to improve the old methods increased, therefore, new methods such bead-based flow cytometric immunoassays were developed. Some techniques employ beads of different sizes or colours

as carriers for antigens and antibodies (McHugh, 1994). Others use beads coated with more than one antibody carrying different fluorochromes, each being able to detect specific antigens (Collins et al., 1998).

Bead-based flow cytometric immunoassays facilitate simultaneous measurement of multiple analytes in small volumes of biological fluids. With this methodology, one single sample can be analysed for inflammatory mediators and their receptors such as cytokines, chemokines and immunoglobulins, during a single test (Varro et al., 2007). The multiplex analysis is possible due to distinct high fluorescence intensity microspheres with different scatter characteristics, which differentiate them from other microspheres specific for other analyte. Each type of beads are conjugated with a specific high affinity antibody directed against a particular molecule from serum, cell culture or cell lysate.

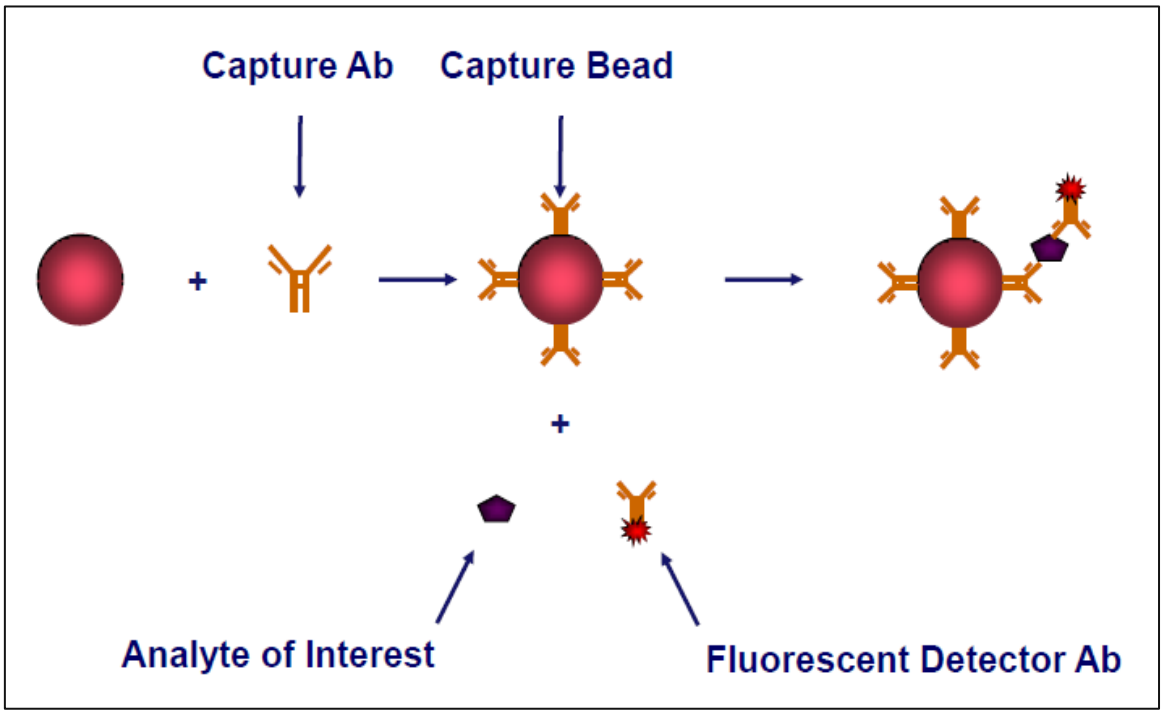


Figure 20: The principle underlying bead-based flow cytometric immune assays (BD Bioscience)

As figure 20 shows, detection antibodies are added to sandwich the complex of capture beads and the analyte, and mediate the detection of fluorescent signal, which is

proportional to the concentration of the analyte. The concentration for each analyte is then determined against standard curves using specific software provided by companies producing cytometric bead assays (Morgan et al., 2004; Varro et al., 2007).

The bead-based assay provides simplicity by eliminating some of washing steps without losing in specificity. The method is reliable and reproducible due to standardisation between assays provided by standard calibration beads. This ensures that tests performed in different places at different times can offer comparable results. Furthermore, due to the increased surface area of microspheres, the test requires a reduced volume of the sample, compared to ELISA methods. The method also offers simultaneous analysis of several analytes and time-efficiency but can be prohibitive for some institution or researchers due to high cost of the instruments (Young et al., 2008).

1.5 CLINICAL OUTCOME AS A RESPONSE TO TRAUMATIC INJURY

Tissue injury resulting from accidents or violence instigate immunologic and metabolic responses with a role in restoring the homeostasis. in the worst scenario, traumatic injury may result in metabolic, immunologic and physiological changes, correlated with the magnitude of the tissue injury. These changes lead to organ failure or coagulopathies and can trigger an overexuberant inflammatory process, and all together may lead to death (Keel & Trenz,2004).

Muir (2006), describes how trauma follows a trimodal distribution, comprised of an immediate death due to devastating trauma with over 30 % loss in total blood volume or due to brain injury. The next are the early deaths which occur between 2 and 24 hours from admission to hospital and associated with hypoxia due to ongoing haemorrhage, or due to cardiovascular or respiratory system deficiency. Seriously injured patients who survive these

two steps, risk to develop immunological dysfunctions leading to complications. The third category is defined by late deaths which happen days or weeks after the traumatic event and are consequent to cardiorespiratory failure, acid-base imbalance, systemic inflammatory response syndrome (SIRS), adult respiratory distress syndrome (ARDS) and multiple organ failure (MOF).

Soon after a massive traumatic injury, a vigorous pro-inflammatory response with increase in serum levels of cytokines takes place, with a role in mounting a response to the trauma, in the form of SIRS. The most important cytokines secreted in the beginning of the pro-inflammatory state are IL - 1, IL - 6, IL - 12, TNF-α and IFN-γ. They act as acute phase hormones or danger signals, with a role in secretion of C-reactive protein and procalcitonin. The more severe the injury, the higher the levels of these cytokines are, and correlate to injury severity scores (ISS) (Lenz et al., 2007).

A two-hit model was proposed in the late 1990s which states that the first traumatic event named the first hit, represents the priming event for a secondary event related to the traumatic injury. The secondary event results during the hospital stay as a response to reparatory surgical intervention, fluid replacement or nosocomial infections. It is considered that the priming event can drive the patient towards developing SIRS, and the second hit can lead to multiple organ failure and later to death (Morris et al., 2015).

The pro-inflammatory status is dampened down by anti-inflammatory mediators in a mechanism known as the counter inflammatory response. It runs parallel to the inflammation, opposing it in a balanced manner leading to healing and achieving homeostasis (Figure 21).

However, an unbalanced counter inflammatory response can lead to compensatory anti-inflammatory response syndrome (CARS) with the anti-inflammatory dominant state responsible for post-traumatic immunosuppression due to T-cells disfunction. This process leaves the patient vulnerable to opportunistic infections, sepsis, and in some cases can lead to multiple organ failure and death (Zedler & Faist, 2006; Morris et al., 2015).

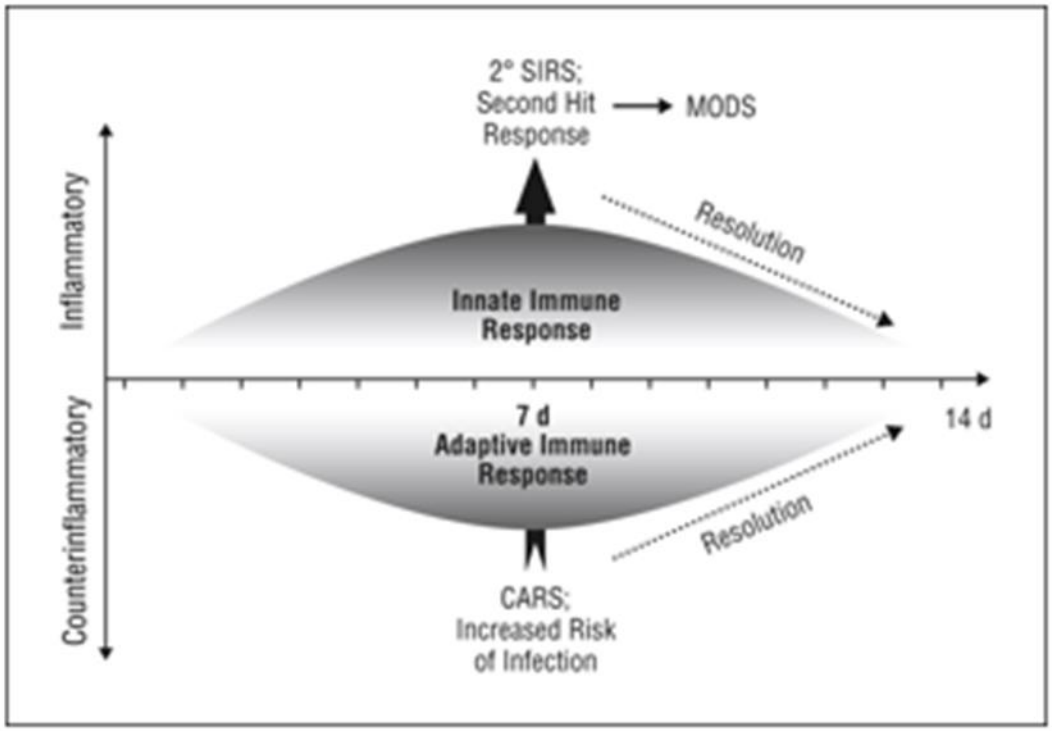


Figure 21: The model of injury for systemic inflammatory response syndrome (SIRS), compensatory anti-inflammatory response syndrome (CARS) (Choileain & Redmond, 2006)

Researches are showing that observing the inflammatory status provided by modulation of cytokine release before reparatory surgery, can help to avoid the development of SIRS, MOF, sepsis and also to reduce mortality in trauma patients (Harwood et al., 2005; Moghtadaei et al., 2016).

1.5.1 Systemic inflammatory response syndrome (SIRS)

SIRS is a result of a generalized aberrant inflammatory response in organs remote to the initial insult. SIRS can be associated to non-infectious(sterile) sources such as trauma, burns

or major surgery; when the process is associated to infection, SIRS is defined as sepsis (Bone et al., 1992). SIRS can be recognized following the observation on several clinical criteria, showed in table 1. When the patient presents two or more of the presented criteria, then there is a high possibility that the patient will develop SIRS (Martin &Badeaux, 2016).

Table 1: Parameters used as guide to determine the presence of systemic inflammatory response syndrome (Martin& Badeaux,2016).

SYSTEMIC INFLAMMATORY RESPONSE SYNDROM
• Temperature >38 ◦C or < 36◦C • Heart rate > 90 beats/min • Respiratory rate >20/min or $PaCO_2$ < 32 mmHg (4.3kPa) • White blood cell count >12,000/mm3 or <4000/m3 or >10% immature bands

The activation of the immune cells and secretion of cytokines have a role in achieving homeostasis, wound healing and tissue repair, being regulated by anti-inflammatory mediators. SIRS is driven by the strong systemic release of pro-inflammatory mediators from activated immune cells together with anti-inflammatory mediators. Its incapacity to restore the host's homeostasis leads to a dysregulated immune response and immune paralysis.

In the presence of disrupted cellular membranes or other danger signals released by injured cells, a series of mediators are released from immune cells in the area where the traumatic injury took place. Histamines, bradykinins and chemokines are released to act upon endothelium to facilitate the migration of neutrophils and monocytes towards the damaged site to restore tissue integrity. In severe trauma, a spill-over of inflammatory mediators in the peripheral blood stream increases the systemic inflammatory response. Furthermore, systemic activation of polymorphonuclear cells (PMC) leads to their sequestration in organs which were not affected by the initial insult (Lenz et al, 2007). One example is about primed

neutrophils sequestrated in tissues and organs such as liver or lung; they are responsible for liver failure, pulmonary failure and acute respiratory distress syndrome (ARDS) (Bhatia et al., 2005).

Polymorphonuclear cells have an important role in phagocytosis and killing of invading pathogens (Pechous, 2017), but at the same time they can damage the surrounding tissues through degranulation of elastase and metalloproteinases. Elastases degrade proteins in extracellular matrix and plasma proteins, whereas metalloproteinases are involved in destruction of structural proteins. In addition, the release of reactive oxygen species (ROS), hydrogen peroxide (H_2O_2), or other immune mediators, can lead to more damage added to the already damaged tissue or to death (Hu et al., 2017). ROS, H_2O_2 and hydroxyl radicals generated from infection and inflammation are the major cause of DNA damage, double strand DNA breaks, DNA-protein cross-links, leading to mutations and cell death (Ragu et al., 2007). Reactive nitrogen species (RNS) such as nitric oxide (NO) are also involved in tissue damage and vascular dysfunction (Keel& Trentz,2005).

There is a well-orchestrated interplay between multiple mediators as response to traumatic injury. During SIRS, the most important mediator is TNF-α, which is the most potent cytokine with role in coagulation, release of adhesion molecules and other molecules specific to early stages of traumatic injury. It also induces the secretion of other cytokines like IL - 6 and IL - 1β. TNF- α and IL - 1β were shown to be associated with the development of MODS, ARDS or sepsis. IL - 6 and the chemokine IL - 8 levels are found to be increased during the initial phase of traumatic injury. Moreover, increased levels of IL - 8 has been shown to be correlated with mortality from ARDS (Reikeras, 2010). Monocytes and macrophages are further involved in differentiation of T-helper lymphocytes. They produce IL - 12 which drives

the differentiation of Th cells in Th1 type cells which secrete IL - 2, IFN-γ and support the inflammation. Decrease in production of IL - 12 from macrophages leads to a shift towards Th2 lymphocytes which support the anti-inflammatory state (Keel & Trenz, 2005).

1.5.2 Compensatory anti-inflammatory response syndrome (CARS)

Parallel to the state of inflammation, many anti-inflammatory mediators are produced with a role to dampen down the inflammation. However, an overproduction of anti-inflammatory mediators may lead to a hypo-inflammatory phase. Macrophages and Th2 lymphocytes are responsible for the production of anti-inflammatory cytokines, such as IL - 4, IL - 10, IL - 13 and TGF-β. A contribution to anti-inflammation is provided by IL - 6 and also by cytokine receptor-antagonists, such as IL - 1Ra and TNFRI. IL - 10, however, is reported to be the most important anti-inflammatory agent in terms of suppressing the transcription of pro-inflammatory cytokines (Schroder et al., 2004). A cohort of patients which surviving the SIRS response are affected by a lengthy dangerous, state known as compensatory anti-inflammatory response syndrome (CARS). It is characterised by T-cells hypo-responsiveness and suppression of their proliferation, defects in antigen presentation and T-cell apoptosis. A hypothesis for these events was associated to IL-10 promoter polymorphism and its link to multiple organ dysfunction syndrome (MODS) (Gentile et al., 2012; Schroder et al., 2004).

The state of CARS leaves the patient in a profound immunosuppression allowing opportunistic infections to take over and resulting in septic complications. The immunosuppression process and ongoing inflammation consume energy derived from fat and protein catabolism, which results in loss of body mass, poor wound healing and recurrent infection. However, the immune system is very fine tuned and shows a very good

equilibrium between SIRS and CARS trying to avoid an overwhelming inflammation directed to its own self, and also to limit the access of infectious agents (Keel & Trentz, 2005).

1.6 CONSEQUENCES OF THE IMBALANCE BETWEEN SIRS AND CARS

Pro-inflammation (SIRS) and anti-inflammation (CARS) coexist from the beginning of the traumatic injury. The intensity and the balance of these two events can result in either a physiologic resolution or in organ dysfunction. If the traumatic event is followed by severe SIRS, the excessive pro-inflammation can cause early organ disfunction, while an early anti-inflammation inhibits pro-inflammatory mediators which may lead to immune-paralysis and susceptibility to nosocomial infections.

In a major injury the inflammatory response can become systemic and lead to serious consequences, including acute respiratory distress syndrome (ARDS) and multiple organ failure (MOF)

1.6.1 Acute respiratory distress syndrome (ARDS)

ARDS was first described in 1967 as an acute inflammatory lung injury, characterised by increased lung microvascular permeability with accumulation of protein-rich fluid, concluding in hypoxemic respiratory failure and poor lung compliance. It presents with dyspnoea and requires assisted ventilation. Statistics show that nearly 40% of patients with ARDS die, whereas survivors are left with lifelong consequences (Laffey, Misak & Kavanagh, 2017). Sepsis is the leading risk factor for ARDS and presents a higher rate of mortality, compared to only 7% trauma-associated ARDS with a mortality rate of 24% (XU & Song, 2017).

The American-European Consensus Conference (AECC) characterises ARDS through three diagnostic criteria:

- Presence of acute hypoxaemia: (PaO_2/FiO_2) >200 mmHg (26.7 kPa) and <300 mm Hg (40kPa)
- Bilateral infiltrates on chest radiography (CXR)
- Absence of raised pulmonary artery pressure < 18 mm Hg or no clinical evidence of left atrial hypertension

While the definition presents limitations from the point of view of reproducibility, lack of sensitivity and specificity, but nevertheless, those 3 criteria help to identify the onset of ARDS (Dushianthan et al., 2011).

The pathogenesis of ARDS shows a progressive, uncontrolled lung inflammation with accumulation of neutrophils, macrophages and erythrocytes in the alveolar space and hyaline membrane formation. ARDS is divided in three stages: exudative, proliferative and fibrotic. The first phase is characterised by increased lung microvascular permeability, leakage of proteinaceous fluid and leukocytes migration in the alveoli (Figure 26). The leukocytes release inflammatory mediators, such as ICAM-1, selectins and VEGF which disrupt the bonds of VE-cadherin (an adherens junction protein). It destabilizes the lung microvascular barrier function (Matthay et al., 2012), and are responsible for leukocytes accumulation and transmigration (Calfee et al., 2007). The second stage, 7-14 days after injury is characterised by proliferation of fibroblasts and Type II pneumocytes which attempt to replace necrotic alveolar Type I cells. The fibroblasts release extracellular matrix proteins and migrate into alveolar space, attaching to the denudated basement membrane. Some patients will progress to the third stage characterised by intra-alveolar fibrosis with deposition of alveolar collagen, which characterise the fibrotic stage (Fig 22).

Extended fibrosis reduces lung compliance with decreased tidal volume and CO_2 retention, poor gas exchange which contributes to hypoxia and ventilator dependence (Bellingan, 2002).

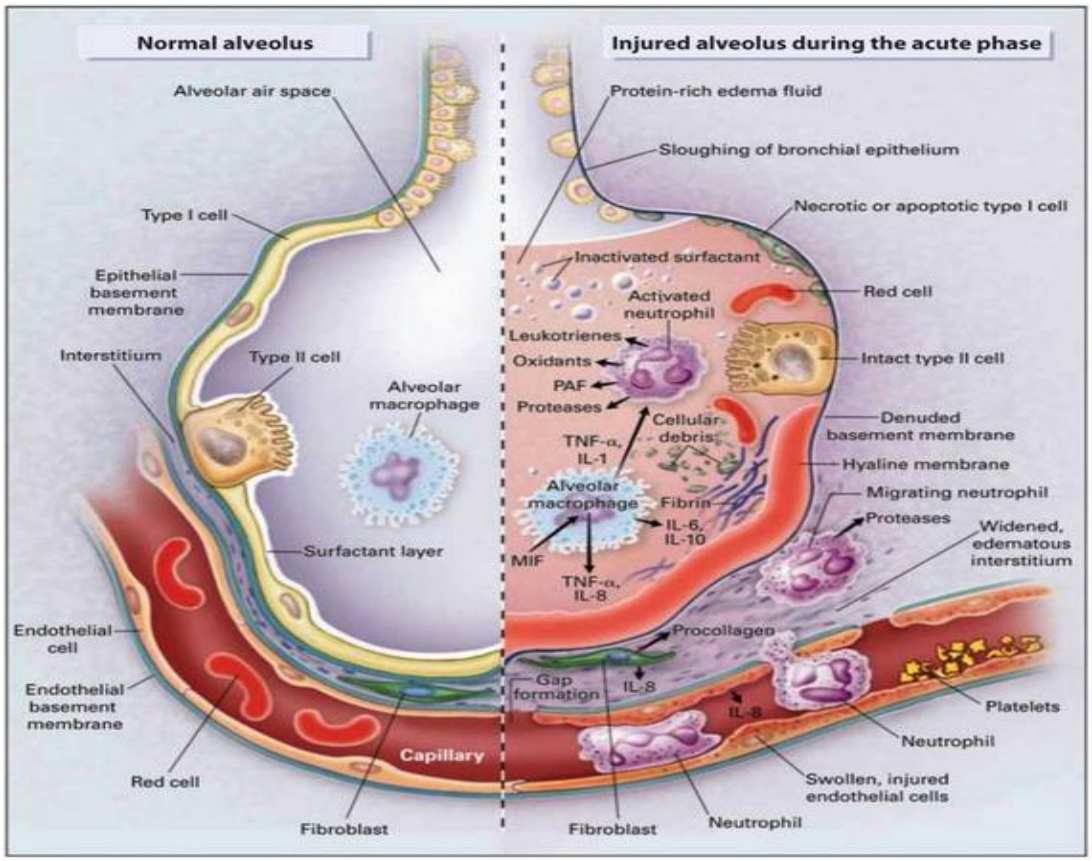


Figure 22: Acute phase alveolar injury (Calfee et al., 2007)

The inflammatory process is driven by TNF - α, IL - 1, IL - 6 and IL - 8. They promote neutrophil-endothelium adhesion and microvascular leakage. Neutrophil traps and histones, proteases and oxidants released by neutrophils contribute to endothelial and alveolar injury. TNF - α, IL - 1β and TGF-α have an important role in development of lung fibrosis. On the other hand, anti-inflammatory mediators IL - 4, IL - 10, IL - 1Ra, and low concentration of CO (carbon monoxide) have proven anti-inflammatory effect in ARDS (Bellingan, 2002). New therapies try to stabilize the lung and endothelial barriers targeting VE-cadherin bonds through administration of stabilizing ligands with role in cytoskeletal reorganization. VE-

cadherin is critical for the maintenance of endothelial barrier integrity in lung micro-vessels. Disruption of VE-cadherin bonds was shown to be associated with accumulation of leukocytes and platelets in micro-vessels proving the close relationship between barrier integrity and leukocyte transmigration. Restoring the barrier integrity using mesenchymal stem cells can help to reverse the major disfunctions associated with lung injury and ARDS (Matthay et al., 2012).

1.6.2 Multiple organ dysfunction syndrome (MODS) and multiple organ failure (MOF)

Multiple organ dysfunction syndrome is the result of a life-threatening injury, which develops in a potentially reversible dysfunction involving two or more organs, not involved in the initial injury (Marshall,2001). The organs taken into consideration are respiratory, cardiovascular, neurological, renal, hepatic, and haematologic systems. MODS is associated with irreversible organ damage and has a high rate of mortality resulting from hypovolemic shock, ischemia reperfusion injury, extensive injury, and an imbalanced immune system response (Barie et al., 2008). MODS is generally a consequence of sepsis but can also result from traumatic injuries and secondary to SIRS (Osterbur et al., 2014).

Due to improvements in trauma care, the incidence of MODS has decreased in the last decades but is still present in about 13-33 % of cases of traumatic incidents. Forty-four to 76 % of ICU patients with MODS may result in late deaths (Ostebur et al., 2014; van Wessem et Leenen, 2017). Researches show that age and highly traumatic injuries with prolonged hospital stay are factors that predispose to developing of MODS with increased of death (Barie et al., 2008; Ciesla, 2005).

The pathogenesis of MODS is still not well elucidated. Some described that the developing of MODS is due to excessive inflammation and to a dysregulated immune response or immune-

paralysis, others describe it as a result of the second-hit insult. MODS can be classified as a primary MODS when the organ dysfunction results directly from the primary insult, and secondary MODS when the organ dysfunction was resulted from systemic response developed in a distant organ different from the initial injury (Osterbur, 2014). However, the first minutes to hours show to be cardinal for the development of a normal or a dysregulated immune response and influence the development of MODS.

Table 2: Multiple organ dysfunction score (Marshal, 2001)

Organ system	0	1	2	3	4
Respiratory					
(PO_2/FIO_2 Ratio)	> 300	226–300	151–225	76–150	≤ 75
Renal					
(Serum Creatinine)	≤ 100	101–200	201–350	351–500	> 500
Hepatic					
(Serum Bilirubin)	≤ 20	21–60	61–120	121–240	> 240
Cardiovascular					
(R/P Ratio)	≤ 10.0	10.1–15.0	15.1–20.0	20.1–30.0	> 30.0
Hematologic					
(Platelet count)	> 120	81–120	51–80	21–50	≤ 20
Neurologic					
(Glasgow Coma Score)	15	13–14	10–12	7–9	≤ 6

(Abbreviations: PO2- partial oxygen pressure; FIO2 – fraction of inspired oxygen; Units : serum creatinine – µmole/l; serum bilirubin- µmole/l; platelet count –platelets/mL 10^{-3})

When organ dysfunction leads to failure of two or more organs it means that the MODS has developed into multiple organ failure (MOF). A classification of MOF distinguishes the early MOF characterised of multiorgan failure developed in the first 72 hours from injury, from the late MOF which develops three day away from the traumatic injury and represent approximatively 60% of total MOF (Llompart-Pou et al., 2014).

MOF is a consequence of SIRS, ischemia-reperfusion and haemorrhagic shock (Figure 23). The respiratory failure is present in 99% of all cases, showing that the lung plays a key role in

development of early MOF. The next organ to be affected is the heart, just few hours after respiratory failure, followed by liver and kidney dysfunction up to 5 days after the injury (Llompart-Pou et al., 2014). Eighty-seven per cent of single-organ failure is represented by lung dysfunctions, whereas in patients with 2 or more organ dysfunction 99% had lung dysfunction. These data suggest that lung dysfunction is a key component in development of post injury MOF, with the lung being a source of inflammatory cytokines and their systemic release (Suter et al., 1991; Ciesla et al., 2005).

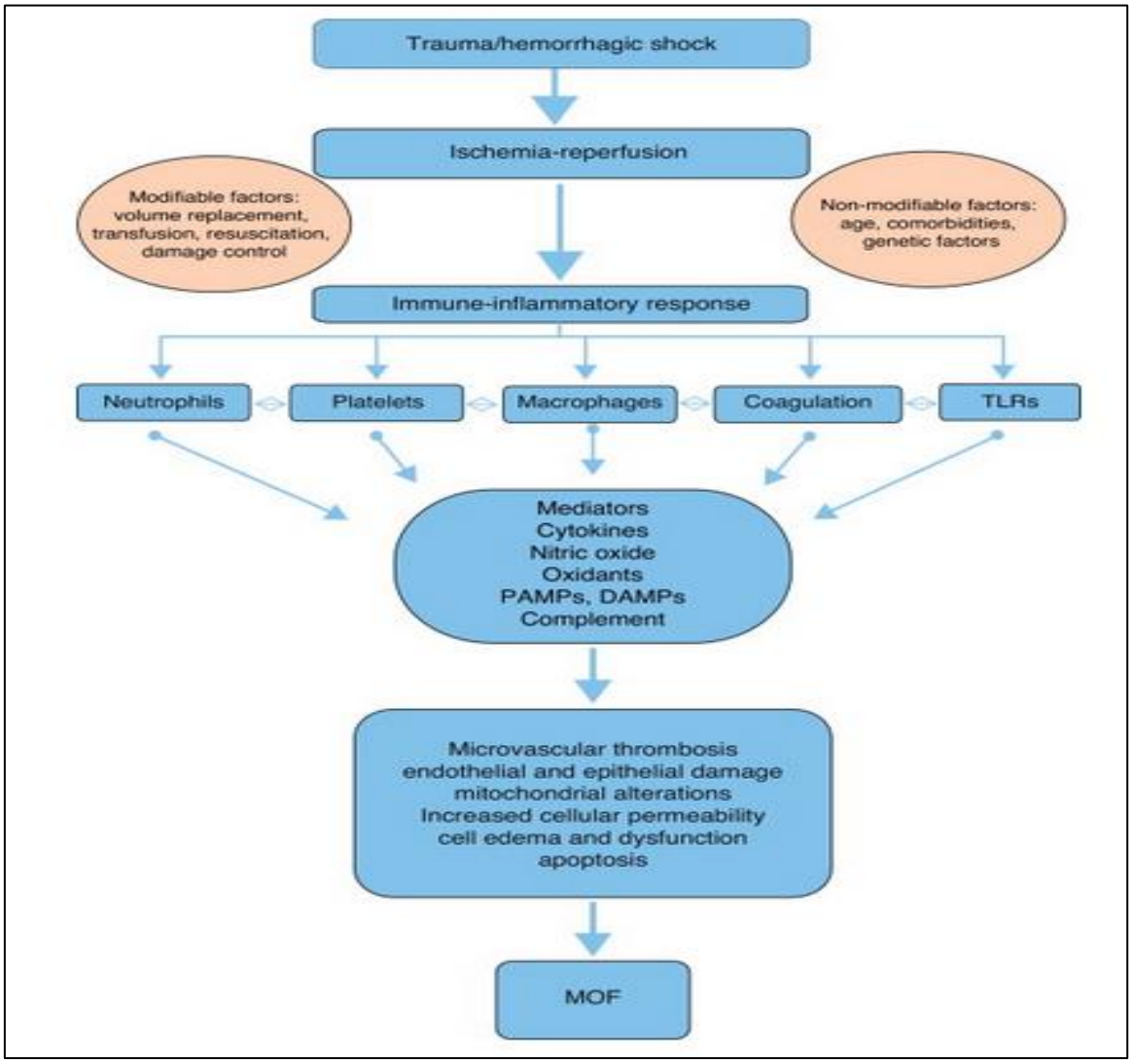


Figure 23: The pathophysiology of MOF following severe traumatic injury (Llompart-Pou et al., 2014)

A well-balanced immune system ensures the achievement of homeostasis after a traumatic event. Pro-inflammatory cytokines IL - 1β, IL - 6, IL - 8 and TNF-α are produced in excess after traumatic injury and together with acute phase proteins contribute to host defence and perpetuate local and systemic inflammatory response (Llompart-Pou et al., 2014). IL - 6 is a good indicator of trauma severity and outcome, whereas IL - 8 concentration is correlated to the development of ARDS. The complement system activation takes place at the site of injury with role in clearance of pathogens and act as a chemoattractant for phagocytes and other PMN cells towards the site of injury. Plasma protein complement C3 levels is also a hallmark for the outcome of traumatic injury (Charlie-Silva et al.,2019; Hecke et al., 1997).

IL - 8 has an important role in attracting the neutrophils at the site of damage, exerting their role in defence and phagocytosis of debris at the site of injury. Together with TNF-α and complement C5a contributes to the development of systemic inflammatory response syndrome and MOF. The ischemia-reperfusion characterised by temporary deprivation of blood supply and restoration of blood flow, plays an important part in development of MOF. The reperfusion phase is responsible for the formation of free radicals, such hydrogen peroxide (H_2O_2) and hydroxyl ions, leading to apoptosis and cell necrosis and predispose to multiple organ failure (Cryer, 2000).

As shown before, severe trauma causes systemic inflammatory response syndrome (SIRS) which can lead to multi organ failure (MOF) in absence of source of infection. Moreover, researches have shown that expression of innate immunity genes responsible for SIRS are expressed simultaneously with expression of genes responsible for counter-inflammatory response syndrome (CARS) (Xiao et al., 2011).

While in-hospital, mortality due to traumatic injury has decreased thanks to the introduction of the “Trauma system” (Leckey, 2018), and a new phenotype of late MOF developed. The patients belonging to this category remain critically ill in the ICU; they present with persistent inflammation, immunosuppression and catabolism syndrome (PICS) (Shepherd, Cole & Brohi, 2017; Vanzant et al., 2014). They exhibit persistent low-grade inflammation with IL - 6 and IL - 8 still elevated levels and increased white blood cell counts (WBC). Concomitant immunosuppression with elevated IL-10 and lymphopenia is associated with decreased HLA-DR on CD14+ monocytes. Genomic analysis shows that up-regulated pathways for IL - 10 and IL - 6 were associated with complicated recovery (Xiao et al., 2011). Patients in this category also present low albumin levels and lean muscle wasting with high levels of 3-methylhistidine to creatinine ratio, showing protein catabolism (Horiguchi et al., 2018). Patients affected with PICS fail to recover and are prone to infection and they will be further transferred to long-term acute care facilities (Vanzant et al., 2014).

1.6.2 Sepsis

The third international consensus definition for sepsis and septic shock revised the definition of sepsis as ‘a life-threatening organ dysfunction caused by dysregulated host response to infection ‘(Singer et al, 2016). Clinical findings in sepsis diagnosis are the presence of an infectious agent and a sequential organ failure assessment (SOFA) score ≥ 2 (Table 3). The patients with SOFA score ≥ 2 have an in-hospital rate of mortality > 10% compared with patients with SOFA score < 2 (Keeley, Hine & Nsutebu, 2016). Sepsis represents a major public health problem as its incidence is increasing due to aging population who present with multiple comorbidities or are under immunosuppressive therapies. Sepsis claims every year, approximately 5 million death worldwide (Brent, 2017).

Table 3: Parameters that defines sepsis (Brent, 2017)

Respiratory rate >22/ minute
Altered mentation status
Systolic blood pressure < 100mgHg
SOFA score ≥ 2

Systemic inflammatory response syndrome (SIRS), is a consequence of a spill-over of immune mediators from a localised inflammation into systemic circulation. The cause of inflammation can be due to an infectious agent such as bacteria , fungi or viruses, and can also be caused by non-infectious causes such as traumatic injuries, therefore, sepsis is an extreme response to inflammation.

There are many theories regarding sepsis, one theory supports the idea that there is an initial pro-inflammatory stage dominate by SIRS followed by an anti-inflammatory stage CARS. This leave the patient immunocompromised and predisposed to new infections leading to MOF (Hotchkiss &Karl, 2003). Newer studies find that there is a simultaneous activation of pro-inflammation and anti -inflammatory processes (Greenhalgh, 2017). Studies based on genetic analysis showed that traumatic injury resulted in SIRS might be caused by polymorphism in genes responsible for cytokines production (Zhang et al., 2015; Kingsley & Bhat, 2017) which can result in a persistent hyper-inflammatory state. However, other groups of researchers have found that immunosuppression is the cause of sepsis (Broomer et al., 2011; Hotchkiss et al., 2013). Moreover, trauma patients undergoing orthopaedic surgery are shown to develop post-operative septic complications at a rate of 1.6% higher when compared with non-trauma cohort (Lakomkin et al., 2017).

1.7 SCORING SYSTEMS IN TRAUMA

Trauma is the leading cause for mortality and morbidity in the developed world. In order to understand the magnitude of traumatic injury and the methods of treatment, scoring system are in place to assess the severity of injury and stratify patients in order to identify seriously injured patients and trigger a trauma team with an adequate treatment (Kingston & O'Flanagan, 2000). There are more types of trauma scoring systems available to assess traumatic injuries.

1.7.1 Injury severity score (ISS)

ISS is an anatomical scoring which observes an accurate description of the injury and is considered the "gold standard "score (Deng et al., 2016). ISS is calculated as a sum of the squares of the single highest abbreviated injury scale (AIS) score for each of the three most severely injured body regions, with one injury per body part taken in consideration (Deng et al., 2016; TARN).

Table 4: Abbreviated injury scale(Palmer & al., 2016)

AIS value	**Injury description**
0	No injury
1	Minor
2	Moderate
3	Serious
4	Severe
5	Critical
6	Un-survivable

The Abbreviated injury scale (Table 3) is an important tool for grading the severity of injury for trauma patients, with '0' for no injury to '6' for fatal injury (Kingston & O'Flanagan, 2000).

To calculate ISS the body is divided in 6 areas of the body: head and neck, face, thorax, abdomen and viscera, limbs and bony pelvis, and body surface. The sum of squares of three of the highest AIS scoring represent the ISS and can vary from 0 to 75 for un-survivable injuries (TARN.AC.UK). A cut off for ISS ≥ 16 was internationally accepted for severely traumatised patients which is associated with a mortality rate above 10% (Maduz et al., 2017; Palmer et al., 2016). Some of the information necessary to calculate ISS might be difficult to obtain in acute stages or only obtainable using CT scan. However, it is considered., as time-consuming scoring and incompatible with acute setting, resulting in data collection to be completed for only about 25% of cases (Kingston & O'Flanagan, 2000).

1.7.2 Glasgow coma scale (GCS)

GCS is part of a physiological scoring system which observes and measures vital signs to determine the level of physiological derangements caused by injury (Kingston & O'Flanagan, 2000). GCS was introduced in 1974 to assess the level of consciousness following head injury. It observes three aspects of responsiveness: eye-opening, motor, and verbal responsiveness.

The levels of response in all three components are scored from '1' for no response to '4' for normal values (eye-opening response), '5' (verbal response) and '6' (motor response). GCS score has cumulate values from '3' which means-no eye opening, no verbal response, no motor response, representing the worst score, and '15' which is the highest score for no head injury (Jain, Teasdale & Iverson, 2019) (Table 5). GCS is incorporated in clinical decision for management of the patients with brain injury, such as in a case of a patient with GCS equal to '8' or less which will require endotracheal intubation (Sternbach,2000).

Table 5: Glasgow coma scale (Jain, Teasdale & Iverson, 2019)

Feature	Scale Response	Score
Eye opening	Spontaneous To speech To pain None	4 3 2 1
Verbal response	Oriented Confused conversation Words(inappropriate) Sounds (Incomprehensible) None	5 4 3 2 1
Best motor response	Obey commands Localise pain Flexion-normal -abnormal Extend None	6 5 4 3 2 1

GCS has the advantage that it can rapidly and continually assess the patient's state and outcome and help to observe eventual improvements or deterioration. GCS was found to have good predictive values for hospital mortality (Gmec & Gasparovic, 2000; Asensio & Tunkey, 2015).

1.7.3 Sequential organ failure assessment (SOFA)

The sequential organ failure scoring system was introduced in 1994 to monitor the assessment of organ dysfunction or failure over time, and/or evaluate the patient's response to a treatment during the organ dysfunction (Llompart-Pou et al., 2014). SOFA score is not designated to predict an outcome, but to describe a sequence of complications in critically ill patients. SOFA calculates the sum values for the degree of dysfunction in six organs (Table

6.) The score ranges from '0' (normal) to '4' (most abnormal) for each organ. The higher the SOFA score, the highest the mortality rate was shown (Vincent et al., 1996). A SOFA score of 3 or greater for one organ systems is considered a failure of that organ.

Table 6: SOFA score (Llompart-Pou et al., 2014)

SOFA	0	1	2	3	4
Respiratory: PaO_2/FiO_2 (mmHg)	>400	≤ 400	≤300	≤200 with ventilatory support	≤100 with ventilatory support
Coagulation: Platelets $\times 10^3/\mu l$	>150	≤ 150	≤100	≤50	≤20
Liver: Bilirubin (mg/dl)	<1.2	1.2–1.9	2–5.9	6–11.9	>12
Cardiovascular: Hypotension (µg/kg/min)	No	MBP < 70	Dopamine ≤5 µg/kg/min or dobutamine (any dose)	Dopamine >5 µg/kg/min or adrenalin or noradrenalin ≤0.1 µg/kg/min	Dopamine >15 µg/kg/min or adrenalin or noradrenalin >0.1 µg/kg/min
CNS Glasgow	15	13–14	10–12	6–9	<6
Renal: Creatinine (mg/dl) or diuresis	<1.2	1.2–1.9	2–3.4	3.5–4.9 <500 ml/day	>5 <200 ml/day

Compared with other used scoring systems, SOFA score is considered to have the most balanced relation of sensitivity and specificity (Frohlich et al., 2016). The six systems taken in consideration for calculation of SOFA score are respiratory, coagulation, liver, cardiovascular, kidney and CNS.

While the SOFA score on admission could be used to stratify patients presenting in emergency departments, the late phase outcomes may not necessarily be predictive in major trauma patients with second phase complications. It is however a useful metric to monitor the onset of the second phase complications. The patients presenting with higher SOFA score need more urgent attention as organ dysfunction is associated with higher

mortality rates (Hu et al., 2017). This scoring system is easy to use and could provide valuable information which could be used as a proxy measure of poor clinical outcome.

Early identification of patients at risk of developing sepsis is crucial in order to receive focussed, early clinical interventions. A SOFA score higher than 3 describes organ failure (Antonelli et al., 1999; Fueglistaler et al., 2010) whereas SOFA ≥ 6 results in an increase in mortality rate of more than 25% (Lie et al., 2018; Shabir & Maqbool, 2017). Therefore, a cut-off of 3 and one of 6 for SOFA score were used for data analysis in the thesis.

SOFA score is considered to be a measure of multiple organ dysfunction resulting from SIRS and sepsis and is a reliable (albeit late) indicator of critically ill patients in an ICU setting. SOFA score of 0 means the patient does not present any organ failure (Raith et al., 2017), SOFA score represents cumulative organ dysfunction experienced by the patient with traumatic injuries. With the increase in SOFA score value, increases the number of organs failing to function during the hospital stay. Being able to predict from early stages the evolution of SOFA score can facilitate early diagnosis and support for failing organs and also to decrease the mortality rate (Ferreira et al, 2001). SOFA score ≥ 6 was found to be correlated with higher mortality (Lie et al., 2018; Keelley et al., 2016).whereas SOFA ≥ 3 is correlated to organ failure (Moreno et al., 1999; Keelley et al., 2016).

1.8 Aim of the study

The main aim of the study is to identify trends in IL - 4, IL - 6, IL - 10 and IL - 12 cytokines, with a view to identifying potential biomarkers for predicting poor clinical outcome in major trauma patients (n =80) admitted to Central Manchester Foundation Trust (CMFT) and Salford Royal Foundation Trust (SRFT). This will be accomplished through the following objectives:

- Recruitment and sampling of 80 patients admitted with major trauma to CMFT and SRFT.
- Analysis of serum samples obtained from Day 1, 3 and 5 post admission for cytokines IL - 4, IL - 6, IL - 10 and IL – 12 using optimised cytometric bead arrays and flowcytometry.
- Collation of associated clinical meta data from patients on Day 1, 3, 5 and 8 and the calculation of progressive SOFA scores as a proxy of multiple organ dysfunction and poor clinical outcome.
- Data analysis to establish cytokine trends on major trauma patients. Correlation of cytokine data to indices of poor clinical outcome (SOFA, creatinine, lactate, mortality etc.) to identify early (D1) cytokine profiles which could be used as predictors (potential biomarkers) of late, poor clinical outcomes.

CHAPTER 2- STUDY DESIGN, METHODS AND MATERIALS

2.1 STUDY DESIGN AND ETHICAL CONSIDERATIONS

The research project was designed to find correlations/associations between immune biomarkers and clinical outcome following traumatic injury in a large group of patients (n=80). The study was granted UKCRN-NIHR Portfolio status (BIT 19377), which supported research nurse funding for clinical activities in Central Manchester Foundation Trust (CMFT) and Salford Royal Foundation Trust (SRFT). The objective regarding the patient recruitment of the wider study is set to reach an overall number of 200 patients with traumatic injuries, accumulated from both hospitals.

For the Master by Research position, the data for a total 80 patients from both hospitals for IL-4, IL-6, IL-10 and IL-12 concentrations was analysed.

The request of ethical approval for the research study was accepted and fully approved by Local Ethics Research Committee Manchester, NHS/HSC Research and development offices- IRAS project ID 172620- and the Ethical Committee at the University of Salford, under ethics code ST1617-17.

2.1.1 Recruitment criteria for participation in the research study

The participants for the research study were recruited by a team of research nurses from Central Manchester Foundation Trust (CMFT) and Salford Royal Foundation Trust (SRFT)). An informed consent was obtained from the participants in conformity with the Mental Capacity Act (2007) (Appendix 1). The participants are allowed to withdraw from the study at any time if they wish to do so later and the samples are promptly destroyed from the biobanks. The suitability for participation in the research study is checked against a pre-set criterion as shown in table 7.

Table 7: Recruitment criteria

Recruitment criteria	
Age	> 16 and <90 Years
Gender	M/F
ISS	> 15
Immediate surgical intervention	
ICU admission	

ISS-injury severity score

The patients who meet the recruitment criteria will have 20 ml of venous blood collected within 24 hours of the injury. The protocol for this project provides that the collection of the same quantity of blood will be repeated for the day 3 (third day after traumatic injury) and day 5 (the fifth day after traumatic injury). Standard operating procedures and rigorous staff training were put in place to ensure homogeneity of the process and the integrity of the sample biobank resource.

Clinical data was collected for the days matching blood collection, and either in addition to an extra time point at day 8 following traumatic injury. The data collection sheet is shown in the Appendix 2. All blood samples and clinical data were collected ensuring that patient anonymity was respected at all times by using only a BIT identifier as per ethical stipulations. Moreover, all the data obtained from blood collection and analysis are stored under

In this study, IL - 4, IL - 6, IL - 10 and IL - 12 concentrations in the blood serum will be tested for D1 and D5 collection points and compared against SOFA score in the respective days. A SOFA score ≥ 3 for one organ system is considered as failure of this organ (Vincent et al., 1996; Frohlich et al., 2016). For this analysis, each serum sample had also D1/D5 ratio calculated for each cytokine concentration separately. The result obtained as increase or decrease in concentration are separated in two groups with a particular cut-off for each

cytokine to show the most visible changes. The two groups are then used to predict a clinical outcome correlated to SOFA score in D5.

These findings were taken in consideration for data analysis. Therefore, a patient with poor outcome is considered to have a SOFA score ≥ 3. C-reactive protein concentrations will be compared against IL - 4, IL - 6, IL - 10 and IL - 12 concentration, to find any correlation with the clinical outcome. . For our analysis, the patients were clustered in 2 groups at a cut-off of 100mg/l concentration of CRP in D5. The groups were further compared against the concentration of IL - 4, IL - 6, IL - 10 and IL - 12 in D1 to observe if they can be used as biomarkers of poor outcome.

The primary aim of this study was to investigate the hypothesis that immune imbalances (a hyperactive CARS response) result in the late phase (D5 post admission and beyond) morbidity and mortality. It is then highly likely that subcellular fingerprints of this immune imbalance can be detected much earlier than the presentation of gross clinical sign. Identification and detection of such early biomarkers (D1 post admission), will enable the crucial stratification of the cohort of patients destined for poor outcomes in the later phase of major trauma.

Early identification of patients at risk of developing sepsis is crucial in order to receive focussed, early clinical interventions. These analyses will help to determine the relationship between mentioned cytokines with the clinical outcome as predictive biomarkers. Elevated values for SOFA score are correlated to poor clinical outcome. A SOFA score higher than 3 describes organ failure (Antonelli et al., 1999; Fueglistaler et al., 2010) whereas SOFA ≥ 6 results in an increase in mortality rate of more than 25% (Lie et al., 2018; Shabir & Maqbool, 2017).

2.2 Sample handling

Patient blood samples are couriered to the University of Salford within 2-3 hours of collections. Serum is separated for cytokine detection and quantification. The serum is aliquoted and labelled appropriately and stored in the -80 annotated biobank until further experimentation. Peripheral blood mononuclear cells PBMCs are recovered for biobanking as described below and aliquoted for further use.

2.2.1 Serum separation

Major trauma patients are recruited following response to trauma calls by the research nurse teams at CMFT and SRFT. If the appropriate pre-specified inclusion /exclusion criteria are met, informed consent is obtained from the patient or next of kin, and the blood sample is collected and stored on ice. The research team at the University of Salford is informed and immediate transport of the sample is arranged. Once the blood sample has reached Cockcroft Building of the University of Salford, it transported to the laboratory for analysis.

The first step in processing the sample is serum separation from whole blood. For this process 10 mls of patient blood were transferred in two 15ml centrifuge tubes and centrifuged at 2000rpm (644xg) for 5 minutes. The serum was collected and 300 µl aliquots was transferred to each 8 labelled cryovials, then stored at -80°C in a blood components bank. The serum is used later for cytokines detection and quantification.

2.2.2 PBMCs collection

Peripheral blood mononuclear cells (PBMC) extraction is obtained by using Lymphoprep, which is a density gradient medium used for isolation of PBMCs. The isolation was obtained by carefully layering 5 ml of patient0-0 blood over 5ml Lymphoprep and centrifugation at 1800 rpm (522xg) for 20 minutes. After centrifugation the layer of PBMCs formed at the

interface between serum and Lymphoprep was harvested and transferred in another 15ml centrifuge tube. The PBMCs were washed in phosphate-buffered saline (PBS) and centrifuged at 1200 rpm for 10 minutes. At the bottom of the tube a pellet of PBMCs was formed which was re-suspended in 1ml cryopreservation media. One millilitre of cryopreservation media was prepared from 900 µl fetal bovine serum (FBS) and 100 µl of dimethyl-sulfoxide (DMSO). The re-suspended pellet is transferred into cryovials placed in the container "Mr. Frosty" containing isopropyl alcohol, and then transferred in the freezer at -80°C for further analysis.

2.3 CYTOMETRIC BEAD-ARRAY (CBA)

In order to detect and quantify the concentration for IL - 4, IL - 6, IL - 10 and IL - 12 from the blood serum, bead-based immunoassays were employed. CBA is a method of capturing an analyte with beads of known size and fluorescence, then detecting them using flow cytometry. The advantages of CBAs are that they are simple to deliver, they allow multiplex analysis of multiple proteins from a single sample. Moreover, a set of standards are provided to generate a standard curve for each analyte from where the concentration of the cytokines in unknown samples can be deducted.

CBA kits used for cytokines detection were acquired from BD Biosciences and contain the BD CBA Human Soluble Protein Flex Set and Human Soluble Protein Master Buffer Kit. A BD FACSVerse flow cytometer which provides dual-laser 488nm and 640nm lasers was used for cytokines detection. Each CBA Human Soluble Protein Flex Set provides CBA standards necessary to create the standard curve for each cytokine separately, and also contains a vial of capture beads and PE detection reagent. The capture beads and PE detection reagent are provided at 50X concentration and need dilution before use.

2.3.1 CBA Standards preparation

The BD CBA standards are provided in lyophilized form. For the start, the standards have to be reconstituted and serially diluted for use. The kit protocol was carried out according to manufacturer instruction.

2.3.2 The analysis of IL - 4, IL - 6, IL - 10 and IL - 12 concentration in major trauma samples using cytometric bead assay

For cytometric analysis, one tube with patient serum is retrieved from the blood components bank, stored at -80°C. The sample is allowed to defrost on the bench at room temperature. Each serum sample was analysed in triplicate. Fifty microliters of patient serum were added to each tube, followed by 50 µl of mixed capture beads each, and mixed gently. The tubes were left for incubation at room temperature for one hour.

Following one hour of incubation, 50 µl of PE detection reagent, prepared as shown for standards, was added to each tube and left to incubate for 2 hours, at room temperature and protected from direct light. Once the incubation ended, 1 ml of wash buffer was added to each tube and centrifuged for 5 minutes at 200xg. The supernatant was aspirated carefully to not disturb the pellet, then the pellet was resuspended in 300 µl of wash buffer, ready for flow cytometric analysis.

2.4 - DETECTION AND QUANTIFICATION OF CYTOKINES OF INTEREST USING FLOW CYTOMETRIC TECHNIQUES

FACS Verse flow cytometer (BD Bioscience, USA) with 488-nm and 640-nm lasers was used to detect and analyse blood samples collected from selected patients. FACSuite software is used to operate the machine, acquire samples and analyse the data. The sample flow through the cytometer and is interrogated by a laser for the existing fluorescent antibodies.

The fluorescence is captured and expressed in data as median PE fluorescence intensity (MFI) which is used for analysis.

2.5 Statistical analysis

The data is presented as mean, ± standard error of the mean for normal distribution and as median (inter quartile range) for non-normally distributed (Curran-Everett, 2008). Statistical analysis was performed to assess differences between the levels of cytokines from the blood collected in day 1 and from day 5, and also to observe relationship between the concentration of cytokines investigated in this thesis and clinical parameters such as SOFA score deduced from clinical meta data. Statistical analysis was performed using Minitab statistical software (Version 18, Minitab Inc. Pennsylvania State University, US). The deviation from Gaussian distribution was tested by Anderson-Darling test (Theodorsson, 1988). For normally distributed data, Two-sample T test were used, whereas for non-parametric data Mann-Whitney U-test was carried out. The statistical significance was determined at $p < 0.05$ level.

3. RESULTS

3.1 Optimization of the cytometric bead assay (CBA) for cytokine analysis

In this study, triplicate cytokine analyses (IL - 4, IL - 6, IL - 10 and IL - 12) were carried out in day 1 and day 5 (D5) post-admission serum samples of major trauma patients admitted to Central Manchester Foundation Trust and Salford Royal Foundation Trust. The data was compared with a range of clinical and biochemical parameters documented on day 1 (D1), day 5 (D5) and day 8 (D8) post-admission. The raw data from the study is detailed in Appendices 8, 9, 10 and 11.

3.1.1 Cytometric bead assay set-up and fluorescence compensation

The compensation was completed according to manufacturer's protocols, an assay to test manufacturer's standards of the relevant cytokines was created. Experiments for IL - 4, IL - 6, IL - 10 and IL - 12 were carried out to generate standard curves. The standard curve formula was used to convert median fluorescence intensity of PE in order to derive the corresponding concentrations for the respective cytokines. The standardised method was then applied to the clinical samples collected in the study to calculate serum cytokine levels and investigate the correlation between the previously mentioned cytokines and clinical outcome following traumatic injury

3.1.2 Optimization of IL - 4, IL - 6, IL - 10 and IL - 12 cytometric bead arrays

For IL - 4, IL - 6, IL - 10 and IL - 12 analyses, new experiments were created either in duplex (IL - 6 and IL – 10, IL – 4 and IL – 12) or multiplex (IL - 4, IL - 6, IL – 10, IL – 12) mode. The experiments contained new tube settings for standard samples and new designed plots, where the population of beads were correctly gated. The populations of capture beads were first interrogated using P1, being corrected with minimal voltage adjustment on FSC and SSC, as seen in first plot in Figures 24A and 25A. When standard samples were acquired, the beads are identified in the individually allocated space, as seen in the second panel (Figure 24B and 25B).

A) B) C)

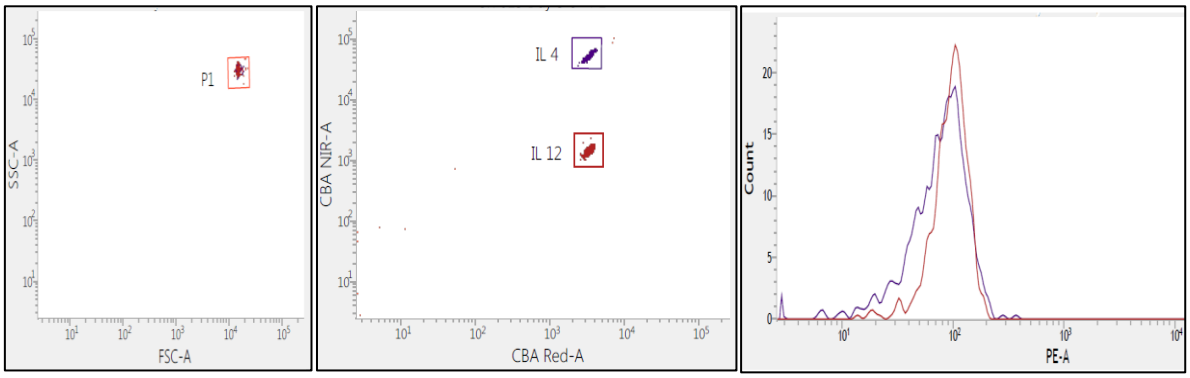


Figure 24. Duplex analysis for IL - 4 and IL - 12

Fig 24A. Gating of populations in P1, Fig 24B. relative positions in A5 for IL - 4 and E5 for IL - 12, Fig 24C. median fluorescence intensity of PE for IL - 4 and IL -12 standards.

Each bead population has an alphanumeric position, indicating the position relative to other beads. IL - 4 position can be found at position A5, IL - 6 at position A7, IL - 10 at B7 and IL - 12 at E5, as seen in the second panel of Figures 24B and252B. Panels 24C and 25C show median fluorescence intensity of PE for each bead population interrogated in the second panel.

A) B) C)

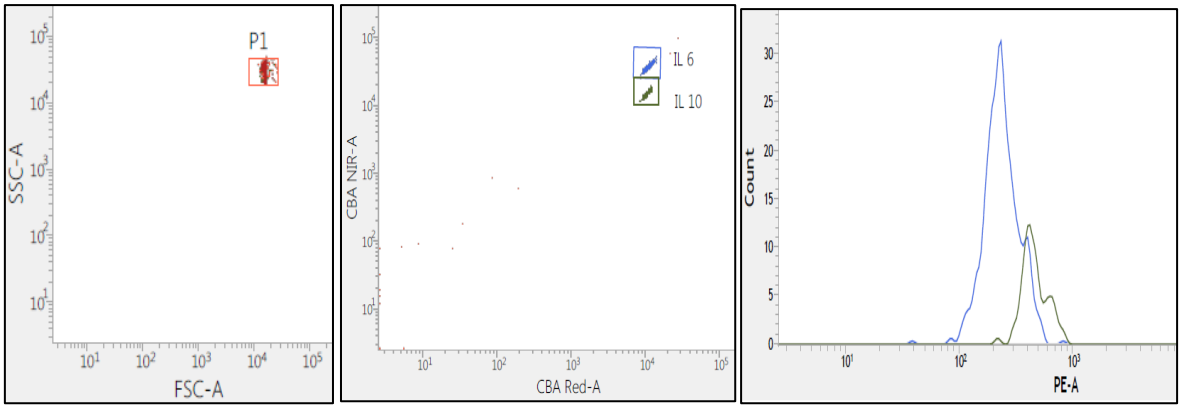


Figure 35: Duplex analysis for IL - 6 and IL – 10

Fig 25A. gating of populations in P1, Fig 25B. relative positions in A7 for IL - 6 and B7 for IL - 10, Fig 25C. median fluorescence intensity of PE for IL - 6 and IL - 10 standards.

Figures 26 and 27 represent the standard curves obtained for IL - 4 and IL - 12 standards respectively, while figures 35 and 36 show the standard curves obtained for IL - 6 and IL - 10 standards. The standard curves were derived through a range of pg/ml concentrations of the standards on the x axis /and plotted PE-median fluorescence intensity on y-axis to deduce cytometric measurements.

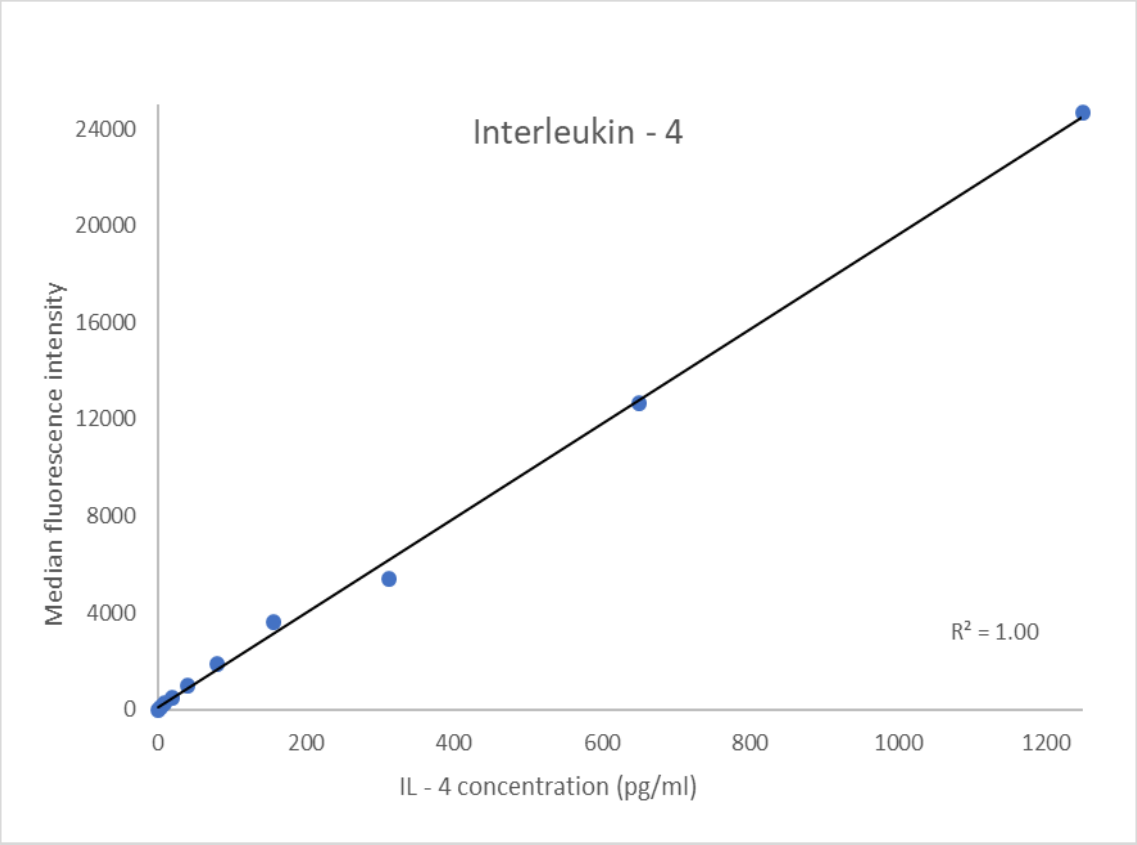


Figure 26: IL - 4 standard curve

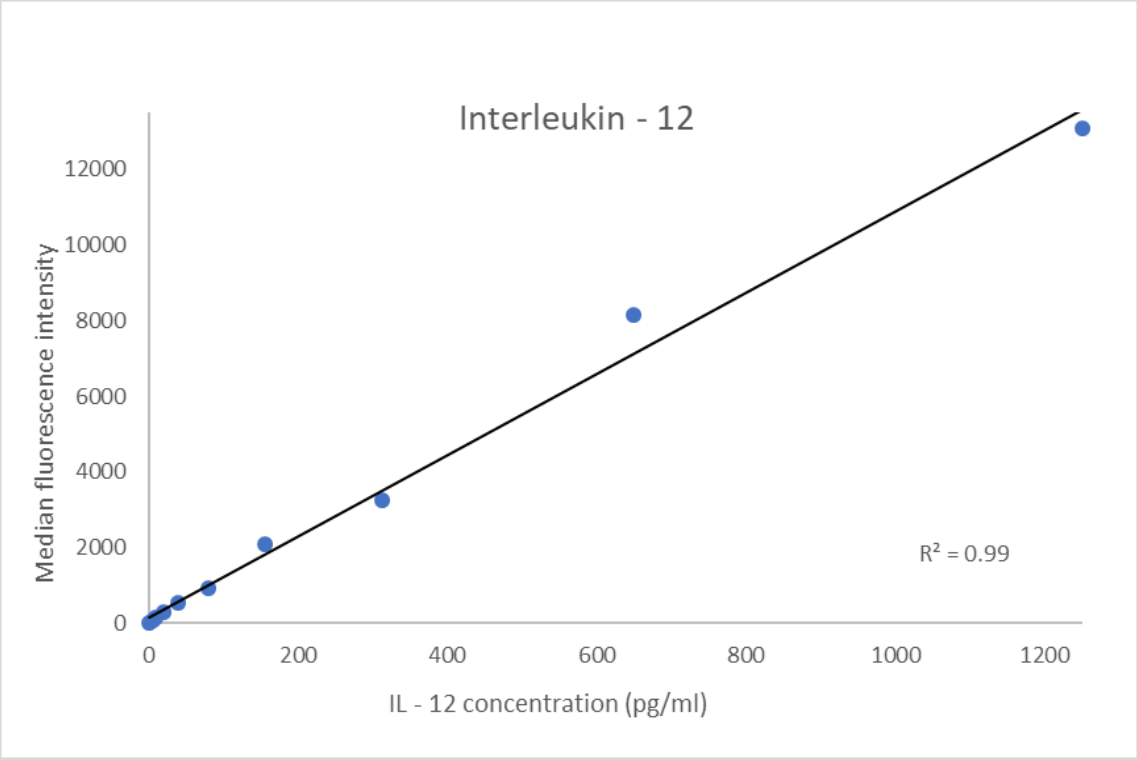


Figure 27: IL - 12 standard curve

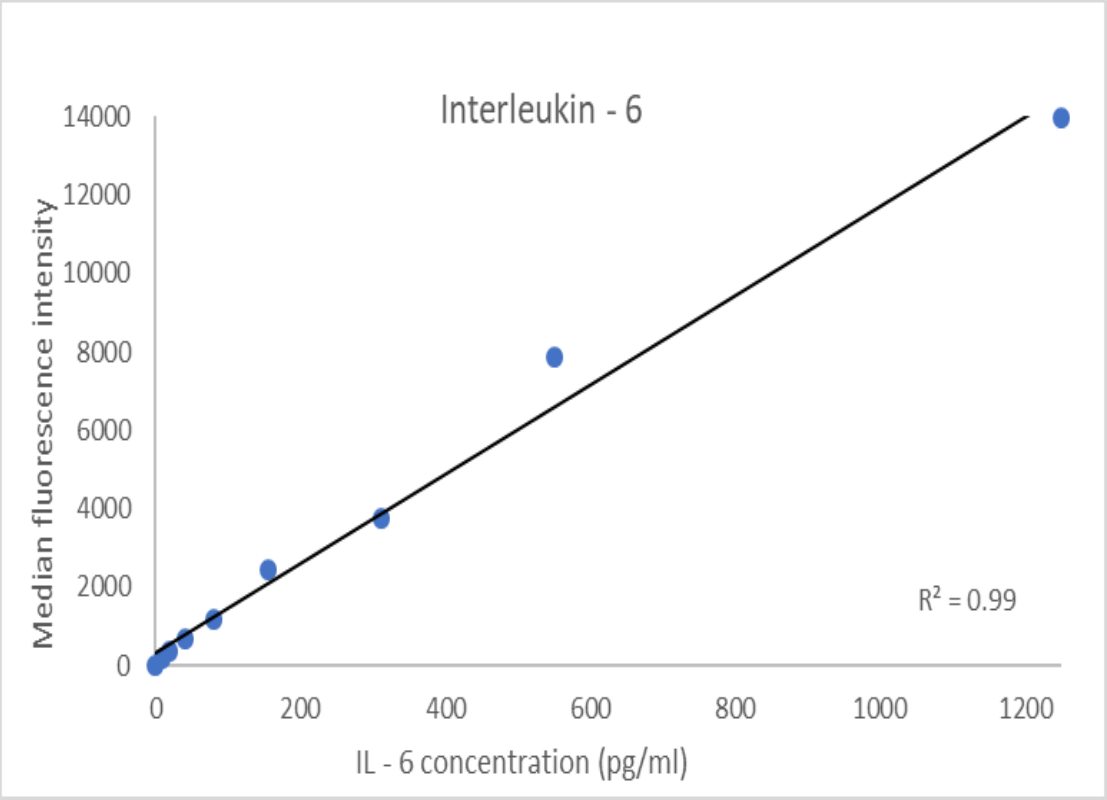


Figure 28: IL - 6 standard curve

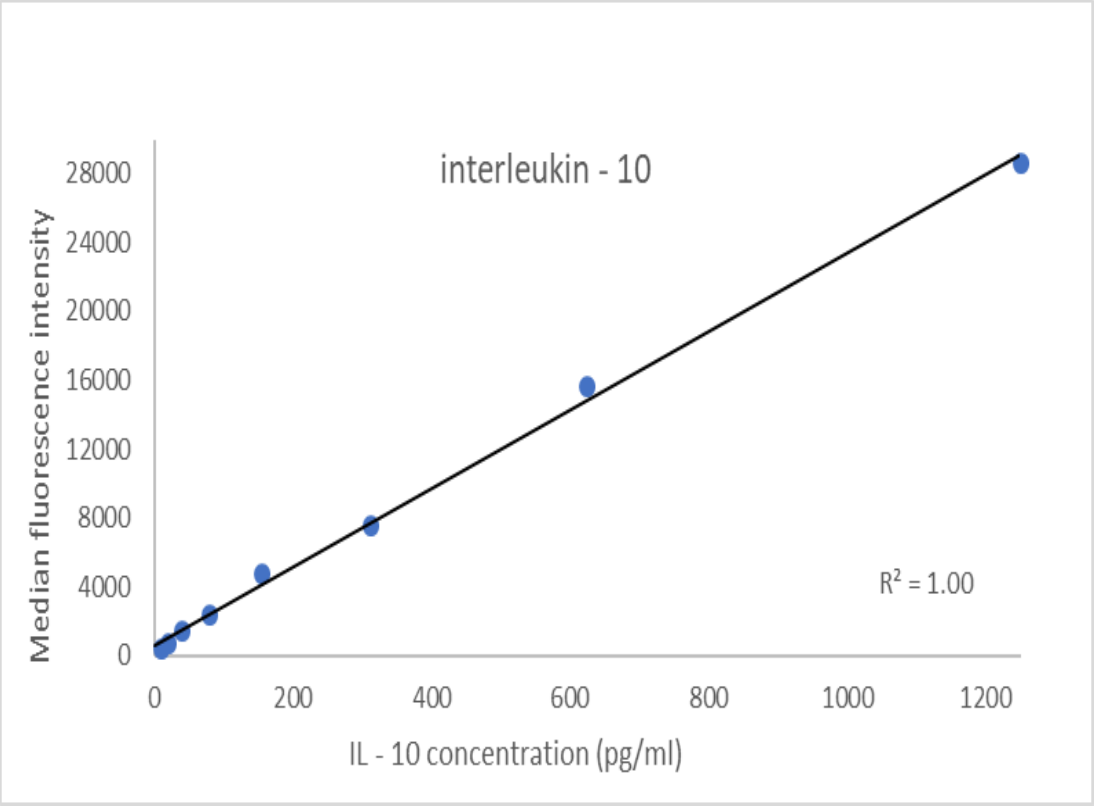


Figure 29: IL - 10 standard curve

The standard curves generated for the cytokines of interest (IL - 4, IL - 12, IL - 6 and IL – 10) were used for calculation of the concentration of respective cytokines in the serum of patients following traumatic injury.

Multiplex analysis for IL - 4, IL - 6, IL - 10 and IL - 12 was used for measurement of PE median fluorescence intensity necessary for cytokine quantification in the serum collected from patients after a traumatic injury. As per optimised protocol, the whole population of capture beads is first interrogated in P1, then recognised and individually gated in their allocated alphanumeric positions, as seen in the second panel in figure 30. The third panel in figure 30 shows the curve generated measuring median fluorescence intensity for PE. Median fluorescence intensity of PE values is further used to calculate the concentration of each cytokine individually, necessary for patient outcome analysis. The data verified that the sensitivity of the methodology was adequate to capture the cytokine levels in the patient serum.

3.1.3. Optimisation of the multiplex analysis of IL - 4, IL - 6, IL - 10 and IL - 12 concentrations in the serum of patients following traumatic injury

A) B) C)

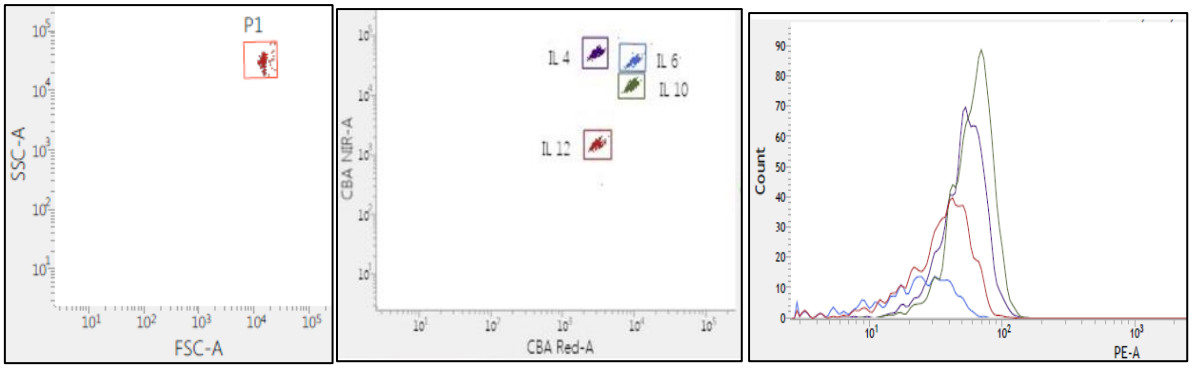


Figure 30: Multiplex analysis for IL-4, IL-6, IL-10 and IL-12

Fig 30A. gating of populations in P1, Fig 30B. relative positions in A7 for IL - 6 and B7 for IL - 10, A5 for IL - 4 and E5 for IL - 12. Fig 30C. median fluorescence intensity of PE for IL - 6 and IL - 10, IL - 4 and IL – 12. Each population has a distinct colour which matches the curve generated for PE MFI.

3.2 DATA ANALYSIS FOR CYTOKINE IL - 4, IL - 6, IL - 10 AND IL - 12 CONCENTRATIONS IN THE SERUM OF THE PATIENTS FOLLOWING TRAUMATIC INJURY

Comparison of D1 with D5 cytokine concentrations.

Averaged cytokine concentrations deduced from the patient cohort (n=80) was compared on Days 1 and 5 using the either the Mann-Whitney U-Test or a 2 Sample T-test as denoted in the figures below.

3.2.1 Interleukin - 4 concentration in serum sample from patients with taumatic injury

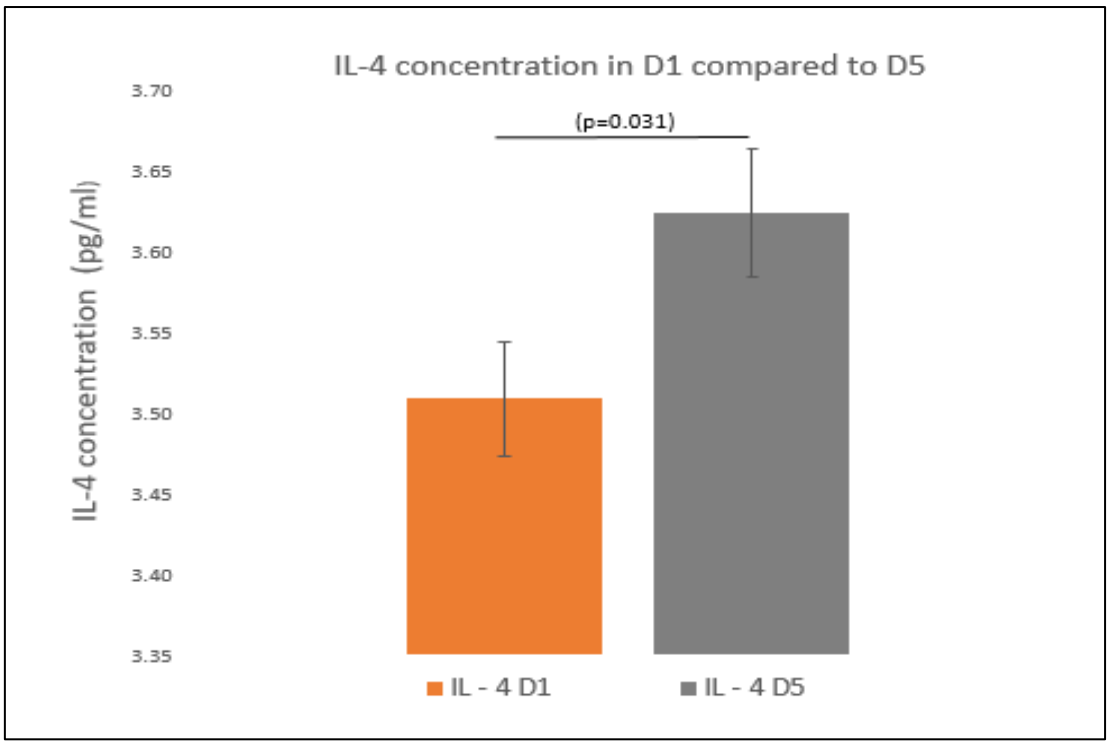


Figure 31: Mean IL - 4 concentrations (D1/D5 comparison) with standard error bars.

IL - 4 mean concentration in D1 was 3. 5083 pg/ml (range 2.9658 to 4.2648 pg/ml). IL - 4 values for D5 in the same cohort showed a mean concentration was 3.6233 pg/ml (range 2.4338 to 4.3709 pg/ml). The median value in D5 was 3. 6132. As shown in figure 31, a moderate but statistically significant increase in D5 compared to D1 was observed at p= 0.031 using a 2 Sample T-test.

3.2.2 Interleukin - 6 concentration in serum samples from patients with traumatic injury

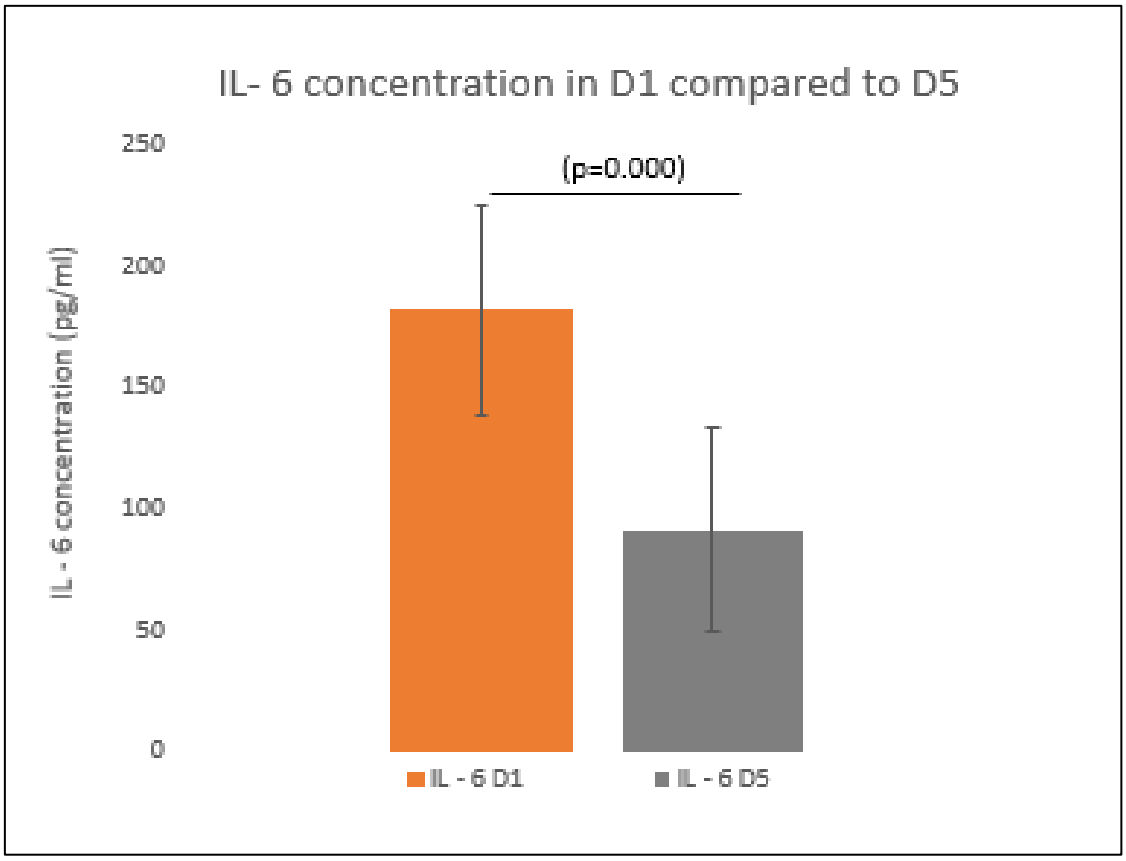


Figure 32: Averaged IL - 6 concentrations (D1/D5 comparison)

The mean IL - 6 value for D1 was 181.6190 pg/ml , whereas in D5 was equal to 91.2001 pg/ml. D1 values ranged from 0.8285 pg/ml to 3992.2299 pg/ml, whereas for D5, the values ranged from 0.2620 pg/ml to 4635.4485 pg/ml.

Statistical analysis shows a significant difference between D1 and D5 (p=0.000) with a significant decrease in IL - 6 concentration in D5 as shown in the figure 32. (P-value obtained with Mann-Whitney U-Test).

3.2.3. Interleukin - 10 concentration in serum samples from patients with traumatic injury

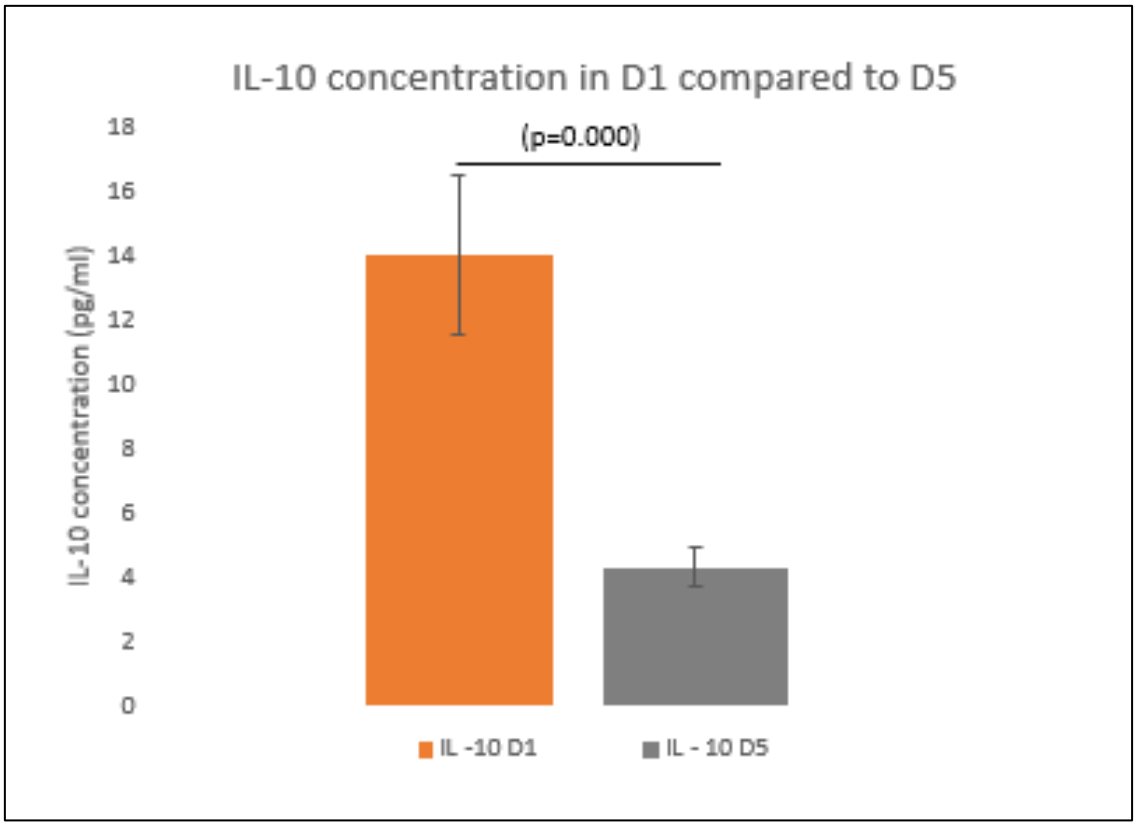


Figure 33: Mean IL - 10 concentrations (D1/D5 comparison)

As shown in figure 33, there is a significant decrease in the IL - 10 concentration from D1 to D5 at a value of p = 0.000. (P-value was obtained with Mann-Whitney U-Test). The mean value of IL - 10 in D1 was 14.0563 pg/ml with values ranging from 1.4881 pg/ml to 79.4539 pg/ml. IL - 10 mean concentration in D5 was 4.3129 pg/ml and the median value was 2.5558 pg/ml. D5 IL - 10 concentration values ranged from 0.3064 pg/ml to 57.5793 pg/ml.

3.2.4. Interleukin -12 concentration in serum samples from patients with traumatic injury

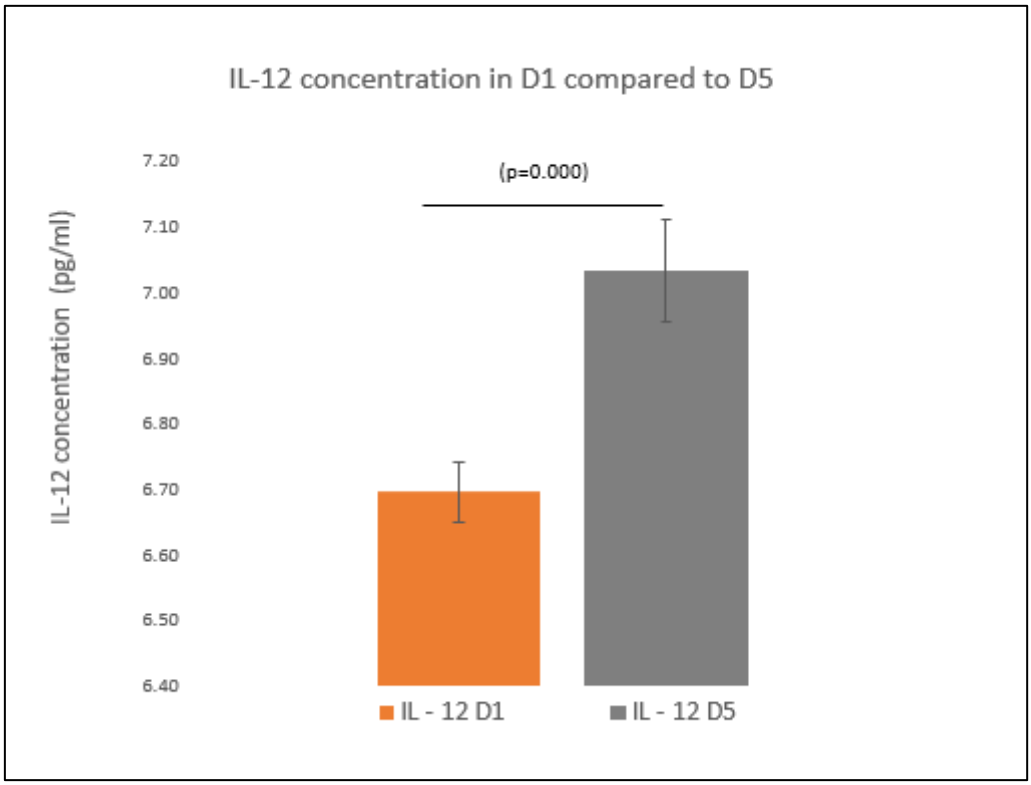


Figure 34: Mean IL – 12 concentrations (D1/D5 comparison)

From the figure 34 can be seen a significant increase in the concentration of IL - 12 in D5, at p= 0.000, (P-value was obtained with Mann-Whitney U-Test). The mean value obtained in D1 for IL - 12 concentration was 6.6956 pg/ ml, For D5 IL - 12 concentration, the mean concentration was 7.0324 pg/ml, the concentration values of D5 ranged from 6.1474 pg/ml to 10.9858 pg/ml.

3.3 Correlation between IL - 4, IL - 6, IL - 10 and IL - 12 concentration in D1 with SOFA scores in D5 and D8, as predictors of patient outcome

3.3.1 Cytokines concentration in D1 compared to D5 SOFA score (threshold of 3)

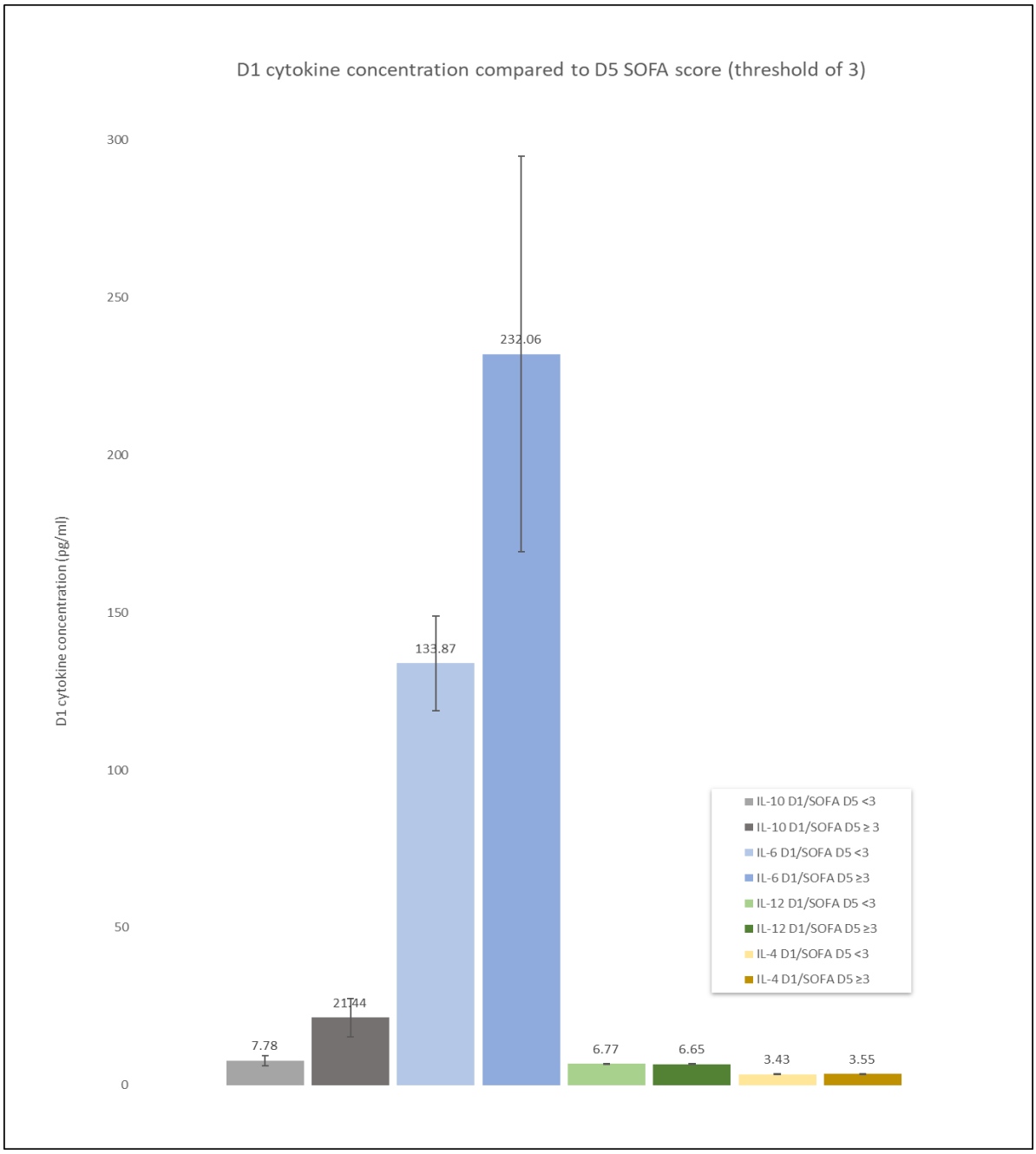
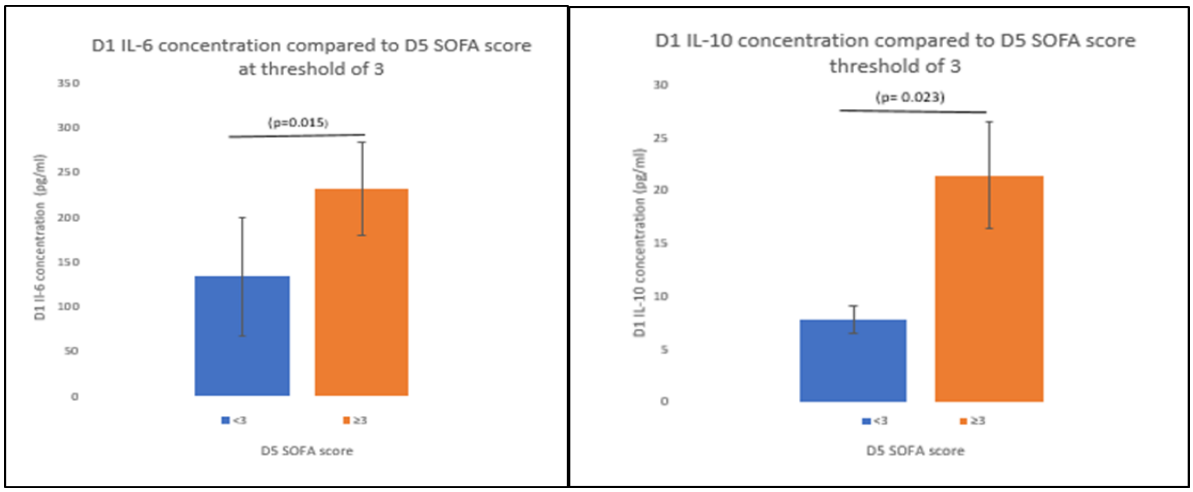


Figure 35: An overview of D1 IL - 4, IL - 6, IL - 10, and IL - 12 concentration associated with D5 SOFA score (threshold of 3)

Figure 35 present an overview for all 4 cytokine concentrations analysed in D1. SOFA score from D5 is employed to determine trends in early presence of cytokines in peripheral blood of trauma patient, to determine if these trends can be used as predictors for poor clinical outcome in later stages.

A) B)



C) D)

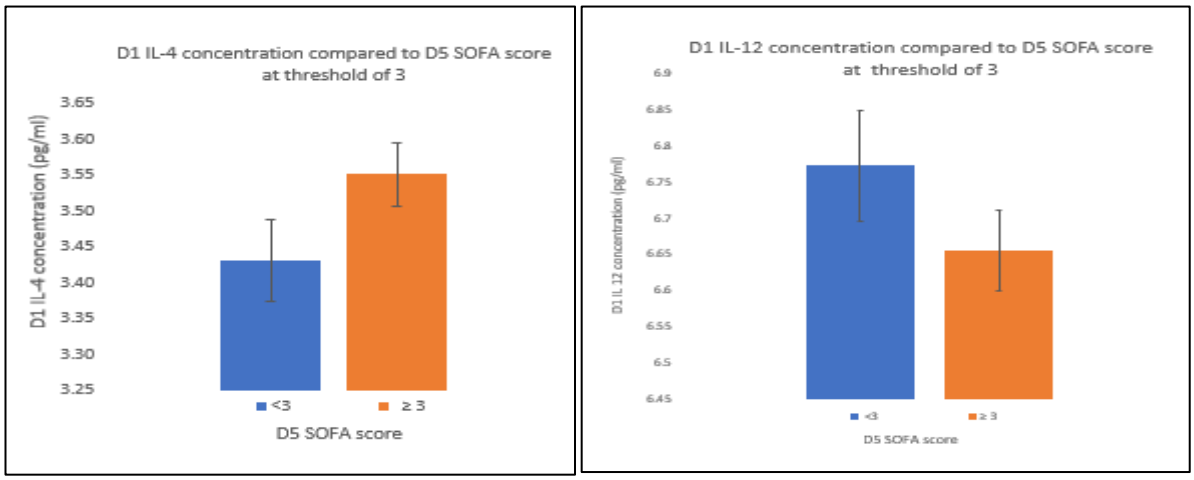


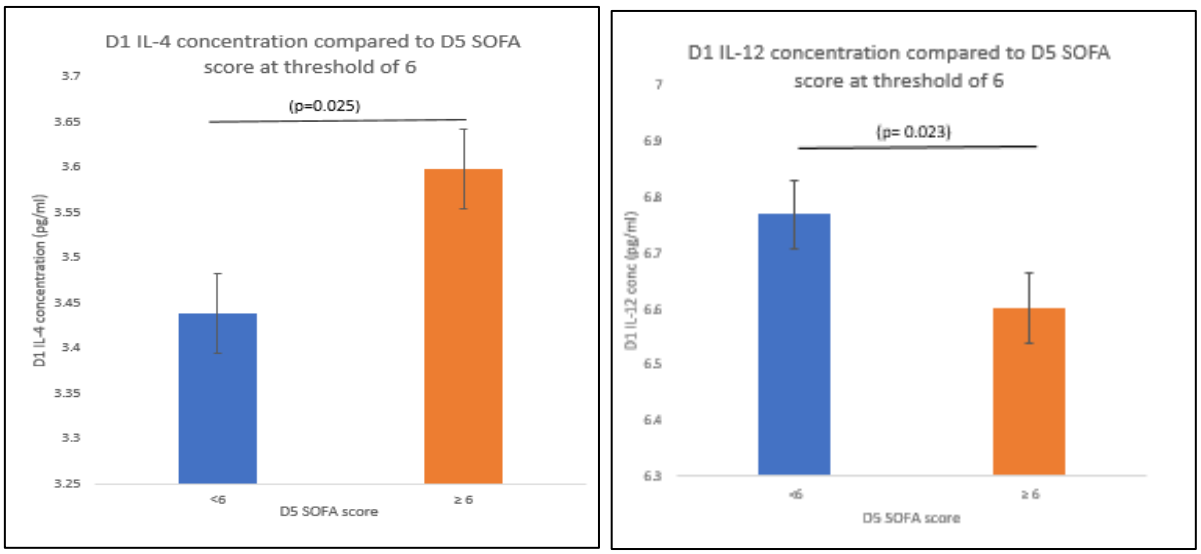
Figure 36: A comparison of cytokine concentration in D1 with D5 SOFA score (threshold of 3). (Mann-Whitney U-test was used to compare the data clustered in the groups of SOFA <3 and SOFA ≥ 3) Data is presented as mean values with standard error bars)

As seen in Figure 36, all 4 cytokines are represented in 4 different panels, each one representing one cytokine analysis. Figure 36A shows a statistically significant increase in IL - 6 concentration in D1 for patients which presented SOFA ≥ 3 in D5 (p=0.015), which suggest that higher value of IL - 6 in D1 might predict a poor clinical outcome. Figure 36B shows also

a statistically significant increase in IL - 10 concentration in D1 in the group of patients with SOFA ≥ 3 (p= 0.023). Figure 36C and 36D represent the data for IL - 4 and IL - 12, which show moderate differences between the two groups (SOFA < 3 and SOFA ≥ 3) but there are not statistically significant differences. (Mann-Whitney U-test was used to compare the two groups in each panel).

3.3.2 Cytokine concentration in D1 compared to D5 SOFA score (threshold of 6)

A) B)



C) D)

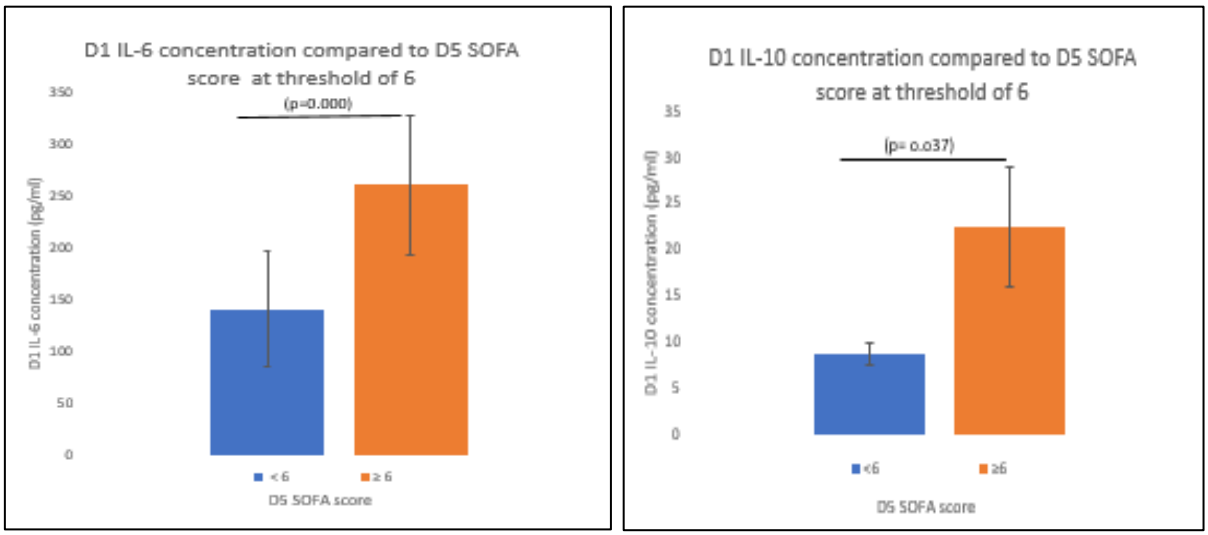
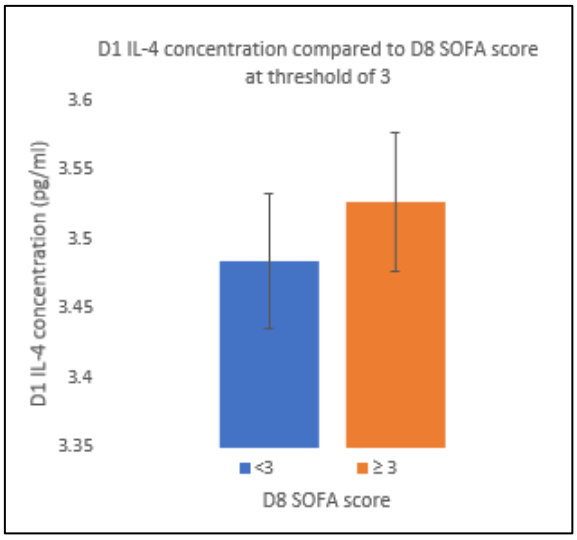


Figure 37: A comparison of D1 cytokine concentration correlated to D5 SOFA score (threshold of 6). (Mann-Whitney U-test was used to compare the data clustered in two groups of SOFA <3 and SOFA ≥ 3)

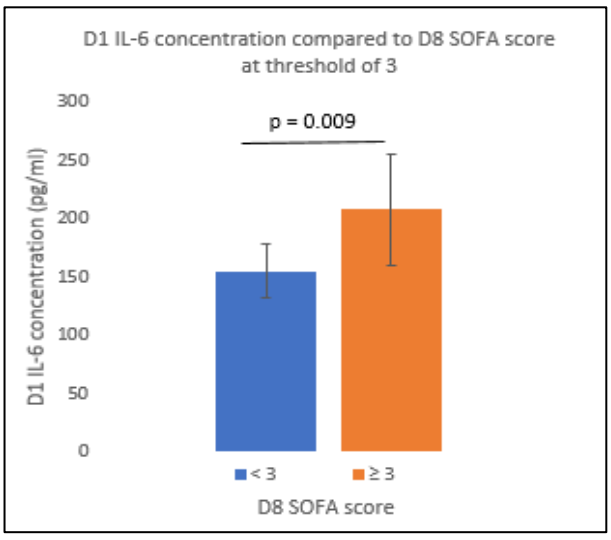
Figure 37 present all 4-cytokine concentration grouped separately at a threshold of 6 for SOFA score in D5. The threshold of 6 was chosen as it indicates that there is more than one organ starting to fail and increases the risk to develop sepsis with subsequent increased risk of death. These changes are reflected in the cytokine concentration changes and can be observed in each A), B), C) and D) panels in figure 37. Moreover, it can be observed that at the threshold of 6 for SOFA in D5, all our cytokines analysed show statistical deference between the groups sorted for SOFA score <6 compared with SOFA score ≥6. Figure 37A show a statistically significant increase in IL - 4 concentration when D5 SOFA ≥ 6 at p=0.025 (2 Sample T-test). IL - 12 is presented in figure 37B and shows a statistically significant decrease in IL - 12 concentration in the group of patients with D5 SOFA score ≥ 6 at p=0.025 (Mann-Whitney U-test). Figure 37C and 37D shows that IL - 6 and IL - 10 presents with statistically significant increase in their concentration in the blood serum collected form patients with SOFA ≥ 6 in the 5th day of their hospital stay (p= 0.000 for IL - 6 and respectively p= 0.037 for IL - 10; Mann-Whitney U-test).

3.3.3 Cytokine concentration in D1 compared to D8 SOFA score (threshold of 3)

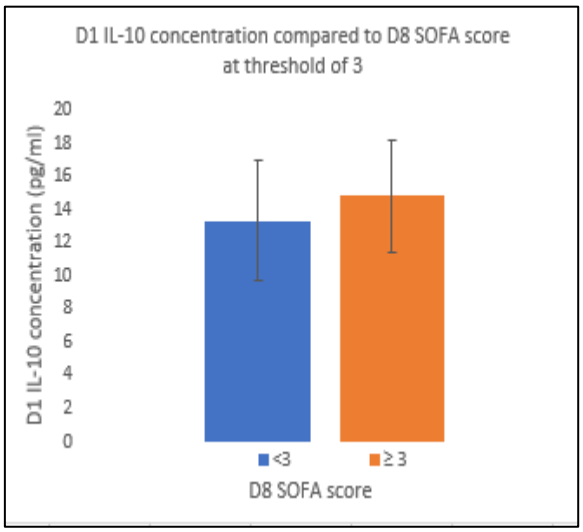
A)



B)



C)



D)

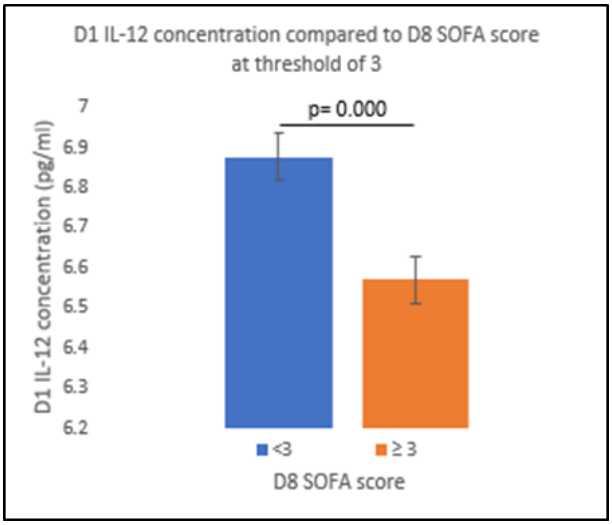
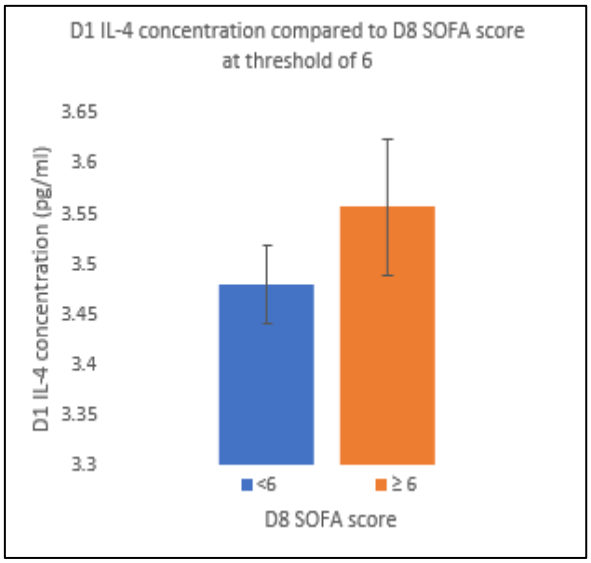


Figure 38: A comparison of D1 cytokines concentration with D8 SOFA score (threshold of 3). (Mann-Whitney U-test was used to compare the data clustered in two groups for SOFA <3 and SOFA ≥ 3)

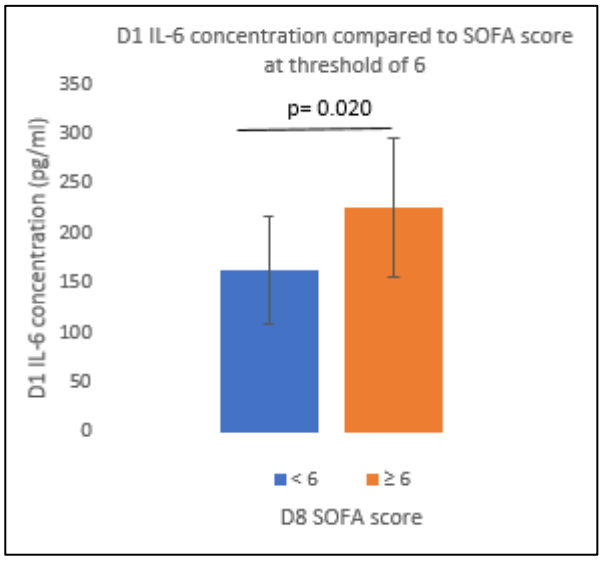
As seen in figure 38, the four cytokines are presented separately to test their ability as predictors of poor clinical outcome in D8 after a traumatic injury. From all 4 cytokines analysed, only IL - 6 and IL - 12 (38B and 38D) showed statistically significant differences between the concentrations clustered for D8 SOFA score at threshold of 3. The significant increase of IL - 6 concentration may predict a poor clinical outcome, as the higher concentration in D1 is associated to higher SOFA score 8 days after trauma (p=0.009, Mann-Whitney U-test). IL - 12 also shows statistically significant decrease in concentration in D1 and is correlated to higher values of SOFA score in D8 (p=0.000). No statistical significance was seen for IL - 4 and IL - 10 concentrations at threshold of 3 for SOFA score in D8.

3.3.4 Cytokine concentration in D1 compared to D8 SOFA score (threshold of 6)

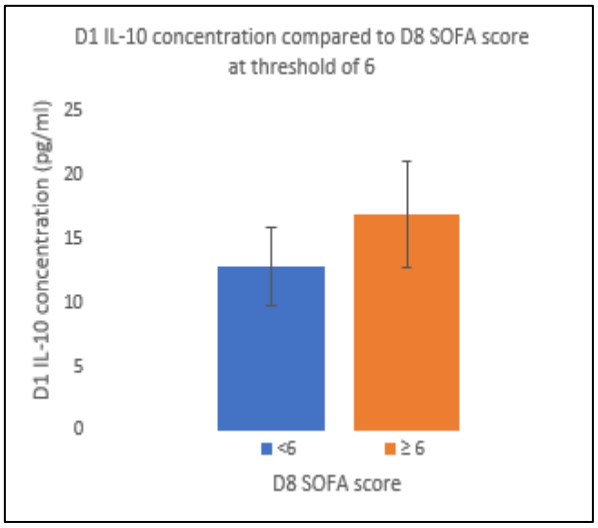
A)



B)



C)



D)

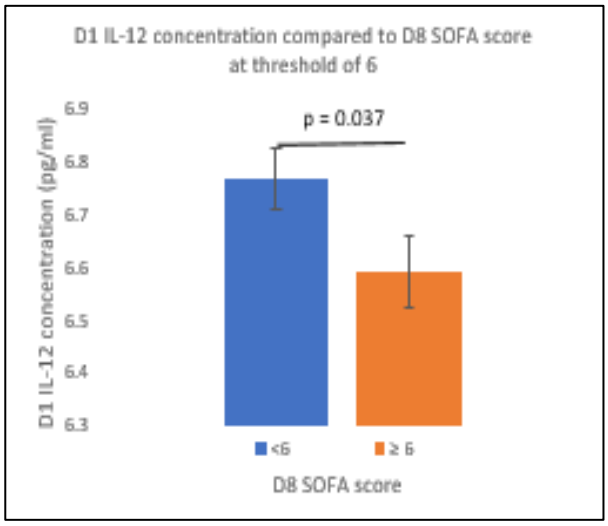


Figure 39: A comparison of D1 cytokine concentration with D8 SOFA score (threshold of 6). (Mann-Whitney U-test was used to compare the data clustered in two groups for SOFA <6 and SOFA ≥ 6)

From figure 39B can be observed that there is a statistically significant increase in IL - 6 concentration in D1 which correlates with high SOFA score in D8. Whereas IL - 12 concentration (figure 39D) shows a statistically significant decrease in its concentration (p=0.037), which correlate with poor clinical outcome in D8 defined by SOFA ≥ 6. IL - 4 and IL - 10 show differences between D8 SOFA <6 and D8 SOFA ≥ 6, but the differences are not statistical significant.

3.4 D1/D5 ratio for cytokine concentration and SOFA score in D5, as predictor of clinical outcome

3.4.1 IL - 4 D1/D5 concentration ratio and the association with D5 SOFA score

A) B)

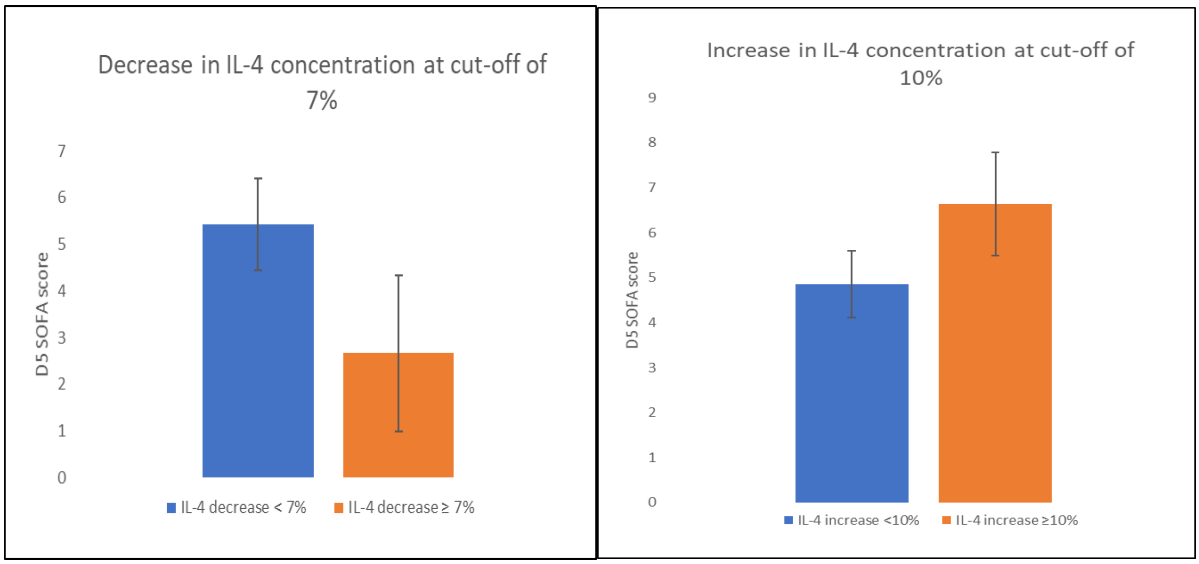


Figure 40: IL-4 D1/D5 concentration ratio reflected in D5 SOFA score (mean values with standard error bars)

The figure 40A shows that a decrease higher than of 7% in IL - 4 concentration from D1 to D5 results in a decrease in D5 SOFA score. Therefore, lower concentration of IL - 4 in D5 is correlated with a better outcome, whereas in figure 40B an increase in IL - 4 concentration over 5 days is corelated with higher SOFA score, therefore a poor clinical outcome.

3.4.2 IL - 6 D1/D5 concentration ratio and the association with D5 SOFA score

A) B)

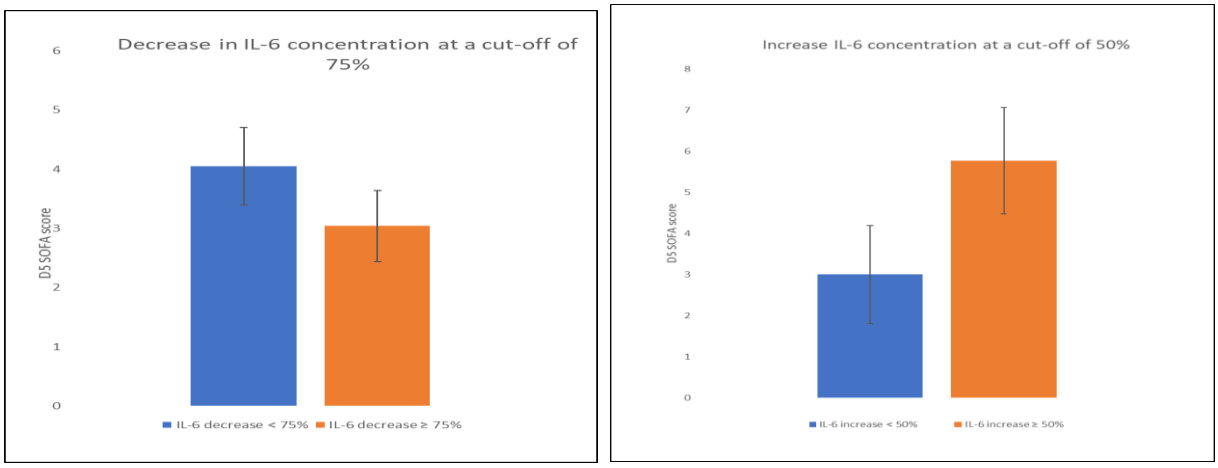


Figure 41: IL - 6 D1/D5 concentration ratio reflected in D5 SOFA score (mean values with standard error bars)

From the figure 41A we can observe that a decrease of 75% in IL - 6 concentration over 5 days of hospital stay, results in lower SOFA score, therefore a better clinical outcome. Whereas, an increase of 50% in IL - 6 concentration shows an increase in SOFA score with a poor clinical outcome, as shown in figure 41B.

3.4.3 IL - 10 D1/D5 ratio and the association with D5 SOFA score

A) B)

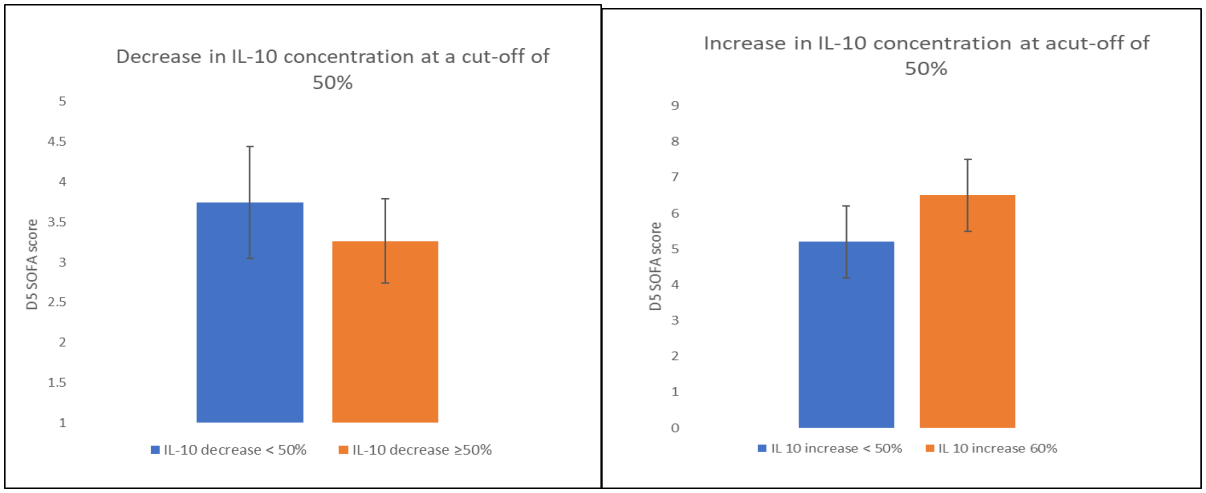


Figure 42: IL - 10 D1/D5 concentration ratio reflected in D5 SOFA score (mean values with standard error bars)

A decrease of 50% in IL - 10 concentration over 5 days of hospital stay results in a decrease in SOFA score, as shown in figure 42A. The figure 53B shows the opposite to figure 53A, providing that 50% increase in IL - 10 concentration results in an increase in SOFA score in D5. Therefore, an increase in IL – 10 concentration over 5 days results in poor clinical outcome.

3.4.4 IL - 12 D1/D5 concentration ratio and the association with D5 SOFA score

A) B)

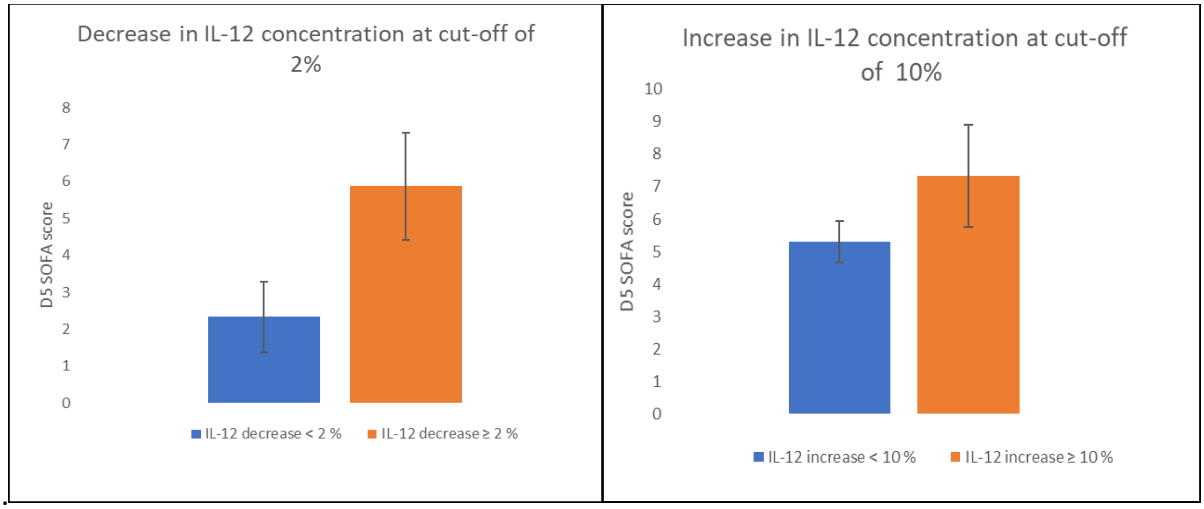


Figure 43: IL - 12 D1/D5 concentration ratio reflected in D5 SOFA score

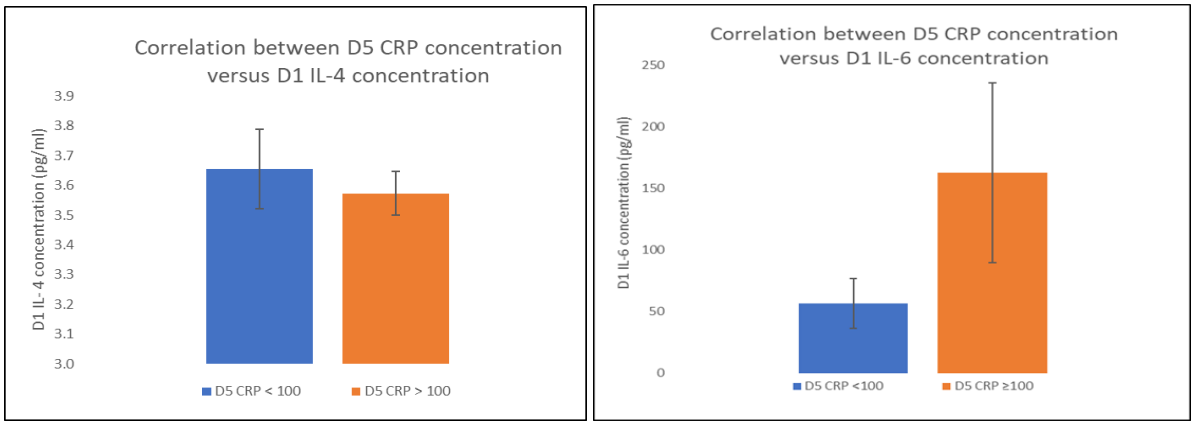
As figure 43A shows, a decrease in IL - 12 concentration higher than 2% over 5 days, results in an increase in SOFA score, whereas the increase in IL - 12 over 5 days up to 10 % and over 10 % results in higher SOFA scores.

All 4 cytokines analysed presented changes in concentration over 5 day of hospital stay and have resulted in changes in SOFA score. A decrease in IL - 4, IL - 6 and IL- 10 concentration show to be correlated with lower SOFA scores, therefore, they may predict a favourable clinical outcome. Whereas the increase in the mentioned cytokines is correlated with a poor outcome expressed as higher SOFA scores. On the other hand, D5 SOFA score show to be affected by too low levels of IL – 12 concentration and either by too high levels of IL - 12.

3.5. The association between IL – 4, IL - 6, IL - 10 and IL – 12 concentration and C - reactive protein levels in trauma patients.

As figure 44 show, there are different pictures of cytokines association to CRP. First, in figure 44A can be seen that there is no correlation between IL - 4 concentration in D1 and CRP concentration in D5 as both groups have nearly equal values of IL - 4 concentration in D1. A different picture is seen in figure 44B, showing that lower levels in CRP concentration in D5 are correlated with low levels of IL - 6 in D1.

A) B)



C) D)

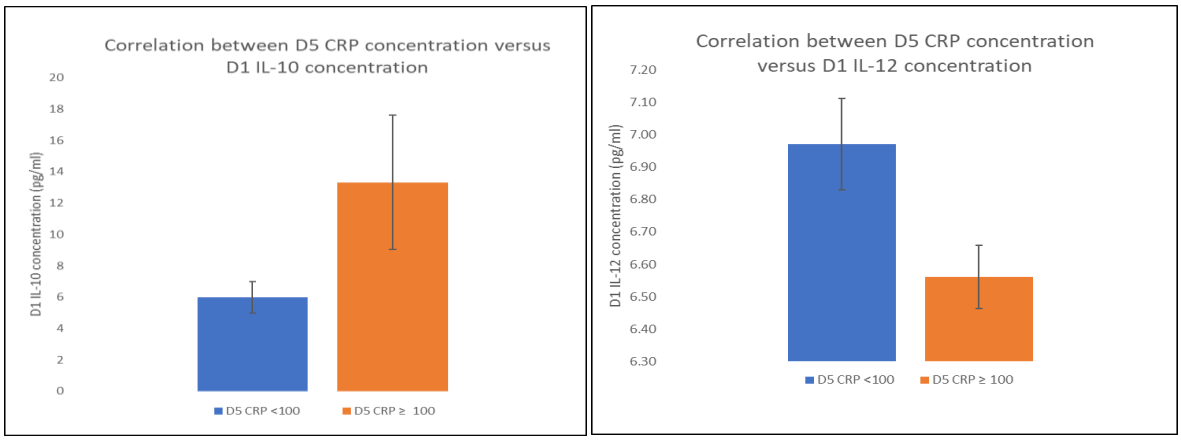


Figure 44: C-reactive protein concentration in D5 and the association with cytokine levels in D1

Figure 44C shows that high levels of IL - 10 concentration are associated with CRP levels higher than 100mg/l as the proxy measure for poor clinical outcome. The panel figure 44D, shows that higher levels of D5 CRP are associated with low levels of IL-12 concentrations on D1; similar results were described by Mold et al., in 2002. Overall, the results show that high levels of CRP in D5 are correlated to high levels of IL-10 and IL -6 and low levels of IL-12 in D1. The trends from this experiment did not show statistical significance, possibly due to the smaller cohort (n=30) of patients who had recorded CRP levels in their clinical metadata.

4. DISCUSSION

Traumatic injuries are among the top causes of death and disability worldwide, carrying a substantial economic burden. Approximatively 5.8 million people die each year as result of traumatic injuries. Globally, trauma affects mostly younger, male populations, being the leading cause of death in the under 45-year-old population. There are more fatalities due to traumatic injury than malaria, TB and HIV/AIDS summed together. The most common traumatic injuries acquired are through penetrating wounds, blunt or burn injury (Sakran et al., 2012), with traffic accidents accounting for 19 % and violence for 12 % of total injuries (Patton et al., 2009).

Major trauma continues to be a significant cause of mortality and morbidity in the UK. In England only, there are 20,000 major trauma cases each year resulting in 5400 deaths (TARN,2017). In contrast to global trends, the demographics of major trauma is recently reported to be changing quite significantly (TARN 2017). In UK, the median age of the patients presenting in hospitals with traumatic injuries has increased, showing that 42% of the trauma patients are over 65-years-old in contrast to the global patterns where young males predominate. The nature of the injuries has seen a shift as a result of this changing demographic. While the incidence of road traffic injuries continues as before, trauma resulting from falls of less than 2m in height increased to 47% of total major trauma injuries (N.H.S. England, 2018; Moran et al., 2018).

The implementation of the new 'Trauma care' pathway which focus on specialised major trauma centres has saved many lives which otherwise would have resulted in fatalities. The 19% percent increase reported in survival rates is attributed to this measure.

Traumatic injuries initiate a cascade of physiological adaptations, comprising cardiorespiratory and immune systems with the aims of achieving and maintaining

homeostasis. A local and systemic inflammatory response syndrome (SIRS) develops in order to control the haemorrhage and the function of vital organs. Ideally, a counter anti-inflammatory response syndrome (CARS) will replace the inflammatory state leading to an uneventful recovery (Keel & Trentz, 2004).

However, a major traumatic injury induces an intense immune-inflammatory response which depends on the initial trauma load and triggers the local and systemic release of pro-inflammatory mediators. A prolonged, intense pro-inflammatory state (overactivity of the SIRS response) leads to the development of early MODS, accounting for the early morbidity. On the other hand, an overwhelming hypo-inflammatory state from an overactivity of the CARS response may lead to immune-suppression that contribute to increased susceptibility to nosocomial infections and sepsis, which in turn result in late MODS and associated morbidity/mortality. Clinical evidence shows that another scenario has emerged comprising a persistent inflammation, immune suppression and catabolism syndrome (PICS) associated with poor clinical outcome (Gentile et al., 2012; Huber-Lang & Gebhard, 2016).

After a traumatic event the level of pro- and anti-inflammatory cytokines is normally altered. There are many studies focused on finding immune-modulators that can predict the evolution of the clinical outcome after a traumatic injury. The most investigated cytokines which show predictive qualities for development of SIRS and MOF in polytrauma patients are TNF-α, IL - 6, IL - 10 and IL - 8 (Valparaiso et al., 2014).

Investigating the cytokine concentrations over 5 days from the traumatic event and their correlation to clinical data will help to observe the trends in the cohort of trauma patients. Their quality as biomarkers of poor clinical outcome is investigated through comparison of the concentration of mentioned cytokines with SOFA score five days and eight days after the traumatic event. Serial measurements of serum levels were correlated to the severity of

organ dysfunction, time-course changes, and associated with another marker of inflammation and injury severity C-reactive protein, in order to evaluate their clinical utility.

Many of the published studies in major trauma have disadvantages with regards to small cohort sizes and single biomarker focus. While a significant body of literature pertaining to animal studies is available, there is increasing doubt cast on the translational relevance of such studies to humans. This study intends to analyse the predictive qualities of IL - 4, IL - 6, IL - 10 and IL - 12 in the development of complications after traumatic injuries, with the possibility of defining an early biomarker panel predicting poor clinical outcome in major trauma patient. The ability to stratify and identify such a cohort early in the post traumatic phase, will enable the focussed management of such patients with early, aggressive antibiotic treatments and thereby prevent the onset of complications. The inherent advantages of such a strategy in preventing antibiotic overuse are obvious. Furthermore, the ability to improve outcome in this cohort of patients will minimise the duration of hospital stays and have significant cost benefits for the NHS.

Interleukin-6

In this study, the serum IL - 6 concentration in D1 was compared with IL - 6 concentration in D5 to understand the general pattern of IL - 6 in the total cohort of patients. As expected, the mean concentration of IL - 6 in D1 was statistically significantly higher ($p<0.001$) than the concentration in D5, indicating a strong local inflammatory response early after injury. Studies on humans and animal model suggest that IL - 6 is a fast-acting cytokine and can be detected in high peaks in the first 24 hours after an traumatic event. It is associated to poor clinical outcome including mortality following traumatic injuries and its levels of concentration are correlated with the extent of tissue injury severity(Easton & Balogh, 2014).

To understand the association between IL-6 concentrations and the evolution of the clinical picture, and to investigate its predictive capacities for clinical outcome, the concentration of IL - 6 in D1 was tested against SOFA score calculated for D5 and for D8. As seen in the literature review, SOFA score ≥ 3 is correlated to single organ failure. This study shows that elevated levels of IL - 6 in D1 could predict poor clinical outcome in D5 ($p = 0.015$) and in D8 ($p = 0.009$), when SOFA clustering was based at a cut off of ≥ 3.

Secondly, it was investigated the relationship between the levels of IL - 6 as predictor of multiorgan failure and increased risk of death from sepsis expressed by SOFA score higher than 6. Indeed, it was found a relationship between higher levels of IL - 6 in D1 and SOFA score in D5, suggesting that the levels of IL - 6 in D1 may predict multiple organ failure in D5 ($p<0.001$); similar results showing an association between high levels of IL - 6 in D1 and high SOFA score in D8 were also found ($p= 0.020$). The concentration of IL - 6 ranging from 225,44 and 259.75 pg/ml may predict bad clinical outcome. Similar findings sowing an association between high levels of IL - 6 and increased SOFA score were also reported by Shimazui et al., (2017) and Takahashi et al., (2016). Moreover, Giannoudis et all., 2008, showed in their study that the cut off of 200 pg/ml can be used as diagnostic of SIRS state, and the concentration higher than 300 pg/ml of IL - 6 is associated with increased risk of MOF and death. As the results from this study are in concordance with the statements presented previously, it seems possible to say that IL - 6 may be a good biomarker for poor clinical outcome in traumatic injury.

The trends for IL - 6 were maintained (although not statistically significant) when the analyses were based on C- reactive protein levels as a proxy of late phase sepsis/MOF in the major trauma cohort. This might be due to IL - 6 stimulating properties over the production of CRP; concluding that too high levels of IL – 6 in D1 might sustain increased levels of CRP

over many days (Del Giudice & Gangestad, 2018). C-reactive protein is widely known to be a biomarker for inflammation and sepsis. CRP levels higher than 87 mg/l is associated with infection and with postoperative complication. Whereas levels of 100 mg/l and higher were associated with sepsis (Povoa et al., 2005). Higher values of CRP at D5 after surgical intervention is considered a marker of complications (Chapman et al., 2016).

In this study we intended to find correlations between the levels of IL - 6 in D1 and the levels of CRP in D5. While the trends corroborate our hypothesis, the lack of statistical significance is likely to be due to the reduced cohort size (n =30) where CRP levels were recorded.

Interleukin-10

With respect to the research question regarding the ability of IL - 10 to be used as an early biomarker of clinical outcome in patients with traumatic injuries, we first compared IL - 10 concentrations in D1 with D5 in the major trauma cohort. It can be noticed that the higher levels of IL - 10 in Day 1 post trauma showed a statistically significant decrease in D5 ($p=0.000$) with values of IL-10 in D1 of 14.06 pg/ml decreasing to 4.31 pg/ml in D5. This pattern is similar to the trend in IL - 6 concentration over the 5 days, showing that the pro-inflammatory cascade is dampened down by anti-inflammatory mediators, such as IL-10 in the attempt to restore the homeostasis for host survival. (Sapan et al., 2016).

The study went on to analyse the correlation between the levels of IL - 10 levels and SOFA score, in the attempt to observe if IL - 10 has predictive qualities or associations with poor clinical outcome. Similar to IL - 6 the patients were separated in to two groups on the basis of SOFA scores (cut off at 3 and 6) and were compared to observe the trends. High levels of IL - 10 concentration in D1 correlated significantly patients with high SOFA score in D5 at both cut off thresholds studied ($p = 0.023$ and respectively $p = 0.037$). These findings may

suggest that high levels of IL – 10 early in the post traumatic phase could indicate an adverse clinical outcome 5-8 days later and predict the cohort destines for poor clinical outcomes after a traumatic injury. There is evidence to suggest that abnormal (high) levels of IL - 10 concentrations might be due to IL - 10 gene polymorphisms present in some of the patients, making them prone to develop sepsis. The promoter of single nucleotide polymorphism (SNP) at position -1082 of IL - 10 gene has been found associated to a different production of IL - 10 and to susceptibility to sepsis and severe prognosis after septic shock (Palumbo et al., 2012). Moreover, Muehlstedt et al., (2002) reported that high IL - 10 was found in patients with lung injuries showing higher predisposition to nosocomial infections; therefore, the site of injury might be relevant when evaluating the immunologic status.

IL - 10 concentration in D1 was also tested against CRP in D5. Studies on humans and animal model have found that increased CRP concentration is related to adverse trauma progression (El- Ashker et al., 2014; Jiao et al., 2016). Once the patient improves and is successfully treated, the levels of CRP decrease accordingly. However, continually increased levels of CRP is related to a predisposition to complicated infections. In this study, the higher levels of IL - 10 concentration in D1 were correlated to CRP levels higher than 100 mg/l in D5. Similar correlations between levels of IL - 10 and CRP were presented by Jiao et al., (2016), showing that this correlation can be used predict progression and prognosis in trauma patients.

Interleukin-4

To observe the trends over 5 days of IL - 4 levels, the concentration in D1 was compared to IL - 4 levels in D5 (Figure 38). This comparison showed a moderate but statistically significant increase in IL - 4 values in D5 ($p=0.031$) for the full cohort. These results are in agreement with those obtained by Cohen et al., (2011) showing that low levels of IL - 4 at admission are

associated with the immediate reaction to acute injury when the levels of pro-inflammatory cytokines increase in attempt to fight the infection. The increase in IL - 4 concentration in later stages is correlated with the role of IL - 4 in suppression of pro-inflammatory mediators and restore the homeostasis. IL - 4 is secreted by Th2 helper cells and is depressed when the level of pro-inflammatory cytokines increase. Increase in IL - 4 in later stages of the immune response induces suppression of pro-inflammatory cytokines (Volpin et al., 2014). On the other hand, Torre et al., (2000) showed that higher levels of IL - 4 at admission were correlated with patients developing SIRS and were generally associated with younger aged patients , whereas lower levels of IL - 4 were associated with patients with higher age.

As with other cytokines analysed until now, the next step is to determine if IL - 4 can be used as biomarker and to predict the clinical outcome in patients following a traumatic injury. Similar to the other cytokines' analysis in this work, the trends in IL - 4 concentration were observed to enable stratification the patients in two groups regarding SOFA score at a threshold of 3. High levels of IL - 4 in D1 were found to be correlated to SOFA score ≥ 3 in D5, but the increase is not statistically significant. However, a statistically significant difference between the levels of IL - 4 in D1 at SOFA score ≥ 6 in D5 was observed ($p= 0.025$), showing that higher levels of IL - 4 in D1 are associated with higher SOFA scores in D5, indicating the Day 1 IL - 4 levels is a better discriminator of more ill patients. The work corroborates studies which have shown that high levels of IL - 4 correlate to MOFS, sepsis and with higher risk of mortality (DeLong & Born, 2004).

Further, we show that an increase of over 10 % in IL - 4 concentration over 5 days are associated to SOFA scores higher than 6. These results are in line with those of previous studies which shows that the levels of IL - 4 are higher in severe trauma than in moderate traumatic injury (Volpin et al., 2014).

Biomarkers are used for diagnostic and prognostic to determine the presence of disease or biological state. IL - 4 and IL - 6 were shown to be promising biomarkers for the detection of infection after reparatory joint intervention following traumatic events (Alvand et al., 2017). According to these findings, we can infer that highest levels of IL - 4 in D1 might be used as potential biomarker for predicting the development of SIRS in traumatic injury and a poor clinical outcome. However, in contrast with these results, other studies have found no correlation between IL - 4 and the severity of injury or the clinical outcome (Surbatovic et al., 2007). When IL - 4 levels were analysed in correlation with CRP levels, the study did not find any differences.

Interleukin-12

IL - 12 concentration in D1 was compared to IL - 12 concentration in D5 to assess differences between D1 and D5 IL - 12 concentration. The comparison showed a statistically significant increase in concentration of IL - 12 in D5 compared to D1($p<0.001$). In this study levels of IL - 10 were found to be higher in D1 and the levels of IL - 12 were lower. These findings are consistent with those in other studies such as Ertel et al., (1997), which showed in their research that low levels of IL - 12 might be correlated with higher values of anti-inflammatory mediators, such as IL - 10, IL - 4 and TGF-β. Furthermore, Wick et al., (2000) showed that normal or increased values of IL - 12 are correlated to survivors from traumatic events

The predictive abilities of the clinical outcome for IL - 12 were analysed in the next step. As for other cytokines, the patients were stratified in two groups according to SOFA score. The analysis showed that patients who showed lower values of IL - 12 in D1, presented higher SOFA score in D5 and D8. A decrease in IL - 12 concentration of more than 2 % over 5 days correlated with adverse outcomes. Similar findings were also reported by Koller et al., (1998)

showing that overall, after trauma the systemic IL - 12 show to be increased in non-septic patients and decreased in the patients who developed MOF.

Statistical analysis showed a significant association between lower values of IL - 12 with SOFA ≥ 6 in D5 ($p=0.023$). It was also found a statistically significant decrease in IL - 12 concentration in the groups at SOFA score ≥ 3 in D8 ($p<0.001$) and a decrease in concentration of IL - 12 in the group at SOFA ≥ 6 ($p=0.037$). In concordance with the present results, previous studies have demonstrated that lower levels of IL - 12 are correlated with multi-organ failure and high risk of dying from multiple organ failure (Wick et al., 2000). The data indicates that high IL - 12 levels in Day 1 are suggestive of a good clinical outcome or progression. Animal models have shown that in mice , survival has increased with IL – 12 administration, but IL -12 neutralization resulted in high mortality (DeLong et al., 2004).

Moreover, studies regarding the abilities of lymphocytes to produce IL - 12 after traumatic injury showed a decreased number of IL - 12-producing monocytes on D5 post injury which correlates with the development of MOF (Spolarics et al., 2003; Reikeras et al., 2014). These findings broadly supports the work of other studies regarding low levels of IL - 12 and their correlation with the development of ARDS, sepsis, predisposition to infection and generally adverse clinical outcome in trauma patients (Hensler et al., 1998; Spolarics et al., 2003; Bozza et al., 2007).

Further, the correlation of IL - 12 concentration with CPR is investigated. In this study the results show that low levels of IL - 12 are associated to higher levels of CRP ≥ 100mg/l, which in turn show a relationship with the development of sepsis. Therefore, low levels of IL-12 concentration are associated to high SOFA scores and with high levels of CPR, predicting the development of ARDS and sepsis. The trends from this experiment did not show statistical

significance, possibly due to the smaller cohort (n=30) of patients who had recorded CRP levels in their clinical metadata.

Overall, these results show that patients developing an adverse outcome present subnormal levels of IL - 12, whereas non-septic patients have increased levels of IL - 12. Following these analysis, high IL - 12 levels might be a good biomarker predicting good clinical outcome following traumatic injuries.

One limitation of this study was the delay in the retrieval of clinical meta data regarding the type of injury and length of stay, the type of organs affected by injury, further surgical intervention or about the blood loss during the incident. Although comparative data from Days 1-8 showed interesting trends, the lack of a cohort of controls similar to the normal heterogenous patient population from Greater Manchester was not available. Hence, the desirable a comparison with the normal levels of cytokines could not be carried out.

For the D1 samples, some variation would have been introduced as a result of clinical interventions including administering fluids or blood transfusion etc. The resulting haemodilution could introduce variations in the levels of cytokines, particularly for the cytokine that prevail at low concentrations in the serum.

Defining the profile of cytokines in trauma patients can help to observe and understand the different patterns in immune responses associated with the evolution of the clinical outcome. Multiplex analyses used in this study imply that having a wide panel of possible predictive biomarkers can help to detect the onset of sepsis and to stratify the patients in order to receive adequate health care. This will prevent the development of complications and ensures a better recovery from their injuries.

The current study has shown interesting overall trends for IL - 6, IL - 10, IL - 4 and IL - 12 cytokines in relation to their expression and association with clinical outcome in the later post traumatic phase. A parallel study conducted by Dr. Allarakia, (2018, data not published) on the same cohort of patients, showed that a high levels of T-reg cell expression (CD25+/CD127-) correlated with poor clinical outcome cohort resulting from immune-suppression. Treg cells are instrumental for driving immunosuppression through dampening of the T cell response. These findings together with low IL - 12 values, high IL - 10 and IL - 4 show a tendency towards immune-suppression in this cohort of patients presenting with higher SOFA scores in D5. Admittedly, the increased levels of IL - 6 for the same cohort shows a persistent pro-inflammatory status. These findings might suggest the presence of the newer phenotype described as 'persistent inflammatory-immunosuppressive and catabolic syndrome' (PICS).

The syndrome is characterised of high levels of IL - 6 and IL - 10, reduced effector T-cell function, elevated levels of C-reactive protein, length of hospital stay > 14 days, neutrophilia (Rosenthal & Moore, 2015). Another factor that defines PICS is that the phenomenon is more common to patients > 65 years of age. The data from the Trauma Audit research network (TARN) shows the shifting demographic in the UK whereby 42% of the major trauma patients are over 65 years old and suffering from multiple co-morbidities, with increased susceptibility of developing PICS. In support to this supposition is the data on length of hospital stay for the cohort of patients in this study, which shows that the average hospital stay is 41 days.

To fully define the presence of PICS syndrome for our trial, further research should include more cytokines profiles, such as IL - 8 and IFN-γ on the side of pro-inflammatory mediators,

IL - 13, TGF-β and IL - 17 as anti-inflammatory cytokines. This analysis could provide more definitive information regarding the presence of PICS. Unfortunately, the levels of albumin necessary to determine protein catabolism were not included in the data collected by the hospitals to support this hypothesis. It will be interesting to have a further check point at six months after initial injury and to collect further data to confirm or reject the hypothesis of presence of PICS syndrome.

Enormous investments in clinical research in the last decade has led to significant improvements in trauma and critical care services, resulting in better understanding of the underlying pathophysiological processes and higher survival rates for trauma patients (Dewar et al., 2009). However, the survival of severely injured trauma patients showed a rise in incidents of multiple organ failure. Despite the improvements in treatment strategies and a better understanding of the syndrome, multiple organ failure still continues to result in poor patient outcome, long ICU stays and high mortalities (Ciesla et al., 2005). An early biomarker-based warning system developed as a Point of Care diagnostic, could indeed make a difference.

At the time of conclusion of this study, the recruitment of patients for the clinical trial was still active, with the goal of achieving a complete cohort of 200 patients. The experiment described in this thesis was developed on 80 patients only, recruited from Central Manchester Foundation Trust (CMFT) and Salford Royal Foundation Trust (SRFT). Therefore, the data must be interpreted with caution and validated in the wider cohort of 200 patients. The findings presented are promising and could potentially have important implications in stratifying patients for better clinical management, improving clinical outcomes and minimising associated NHS costs.

However, there is abundant room for further progress in determining a wider panel of biomarkers and defining correlations between them.

CHAPTER 6: REFERENCES

1) Abul K. Abbas author. Andrew H. Lichtman author.; Shiv. Pillai author. Philadelphia, PA: Elsevier Saunders Eighth edition / Abul K. Abbas, MBBS, Andrew H. Lichtman, MD, PhD, Shiv Pillai, MBBS, PhD ; illustrations by David L. Baker, MA, Alexandra Baker, MS, CMI. 2015/

2) Adan, A., Alizada, G., Kiraz, Y., Baran, Y., & Nalbant, A. (2017). Flow cytometry: basic principles and applications. *Critical reviews in biotechnology*. 37(2), 163-176. doi: 10.3109/07388551.2015.1128876

3) Andersen, L. W., Mackenhauer, J., Roberts, J. C., Berg, K. M., Cocchi, M. N., & Donnino, M. W. (2013). Ethiology and therapeutic approach to elevated lactate levels. Mayo Clinic proceedings, 88(10), 1127–1140. doi: 10.1016/j.mayocp.2013.06.012

3) Andersson, U., & Tracey, K. J. (2003). HMGB1 in sepsis. *Scandinavian journal of infectious diseases.* 35(9), 577-584. Retrieved from: https://www.ncbi.nlm.nih.gov/pubmed/14620138

4) Akdis, C. A., Blesken, T., Akdis, M., Wüthrich, B., & Blaser, K. (1998). Role of interleukin 10 in specific immunotherapy. The Journal of clinical investigation, 102(1), 98–106. doi:10.1172/JCI2250

5) Amara, U., Rittirsch, D., Flierl, M., Bruckner, U., Klos, A., Gebhard, F… Huber-Lang, M. (2008). Interaction Between the Coagulation and Complement System. *Advances in Experimental Medicine and Biology*, 632, 71–79. Retrieved from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2713875/

6) Amara, U., Flierl, M. A., Rittirsch, D., Klos, A., Chen, H., Acker, B., … Huber-Lang, M. (2010). Molecular Intercommunication between the Complement and Coagulation Systems. *Journal of Immunology* (Baltimore, Md. : 1950), 185(9), 5628–5636. doi.org/10.4049/jimmunol.0903678

7) Antonelli, M., Moreno, R., Vincent, J. L., Sprung, C. L., Mendoça, A., Passariello, M., Riccioni, L.,& Osborn, J. (1999). Application of SOFA score to trauma patients. Sequential Organ Failure Assessment. *Intensive care medicine.*25(4), 389-94. Retrieved 06/05/2019 from: https://www.ncbi.nlm.nih.gov/pubmed/10342513

8) Arce-Sillas, A., Alvarez-Luquin, D., D. Tamaia-Dominguez, B…Adalid-Peralta, L. (2016). Regulatory T cells: Molecular actions on effector cells in immune regulation. *Journal of immunology research.* http://dx.doi.org/10.1155/2016/1720827

9) Asensio, A. J., & Trunkey, D. D. (2015). Current Therapy of Trauma and Surgical Critical Care. https: //books.google.co.uk/books?id=vkZ9CAAAQBAJ&pg=PA701&lpg=PA701&dq=trauma+first+hit&source=bl&ots

10) Aydin S. (2015). A short history, principles, and types of ELISA, and our laboratory experience with peptide/protein analyses using ELISA. *Peptides.* 72, 4-15. https://doi-org.salford.idm.oclc.org/10.1016/j.peptides.2015.04.012

11) Awosika, O., Kim, L., Mazhar, M., Rengifo-Pardo, M., & Ehrlich, A. (2018). Profile of dupilumab and its potential in the treatment of inadequately controlled moderate-to-severe atopic dermatitis. Clinical, cosmetic and investigational dermatology, 11, 41–49. doi:10.2147/CCID.S123329

12) Barie, S. P., Hydo, J. L., Shou, J., & Eachempati, R. S. (2008). Decreasing Magnitude of Multiple Organ Dysfunction Syndrome Despite Increasingly Severe Critical Surgical Illness: A 17-Year Longitudinal Study. *The Journal of Trauma Injury, Infection and Critical care.*65, 1227-1235. DOI: 10.1097/TA.0b013e31818c12dd

13) Beebe, M.A., Cua, J. D., & Malefyt, R de W. (2002). The role of interleukin-10 in autoimmune disease: systemic lupus erythematosus (SLE) and multiple sclerosis (MS). *Cytokine and growth factor reviews.* 13(4-5),4003-412. https://doi.org/10.1016/S1359-6101(02)00025-4

14) Bellingan, G. J. (2002). The pulmonary physician in critical care / The pathogenesis of ALI/ARDS. *Thorax*. 57, 540-546. http://dx.doi.org/10.1136/thorax.57.6.540

15) Berger, A. (2000). Th1 and Th2 responses: what are they? *BJM.* 2000, 321-424. doi: https://doi.org/10.1136/bmj.321.7258.424

16) Beltrame, M. H., Catarino, S. J., Goeldner, I., Boldt, A. B., & de Messias-Reason, I. J. (2015). The lectin pathway of complement and rheumatic heart disease. Frontiers in paediatrics, 2, 148. doi:10.3389/fped.2014.00148

17) Bertheloot, D., & Latz, E. (2017). HMGB1, IL-1α, IL-33 and S100 proteins: dual-function Alarmins. *Cellular & Molecular Immunology*. doi:10.1038/cmi

18) Bhardwaj, N., Khurana, S., Kumar, S., Sagar, S., Gupta, A., Mishra, B., … Mathur, P. (2018). CD14+ Monocytic Cytokines: Impact on Outcome in Severely Injured Patients. Indian Journal of Critical Care Medicine: Peer-Reviewed, Official Publication of Indian Society of Critical Care Medicine, 22(7), 528–532. http://doi.org/10.4103/ijccm.IJCCM_442_17

19) Bhatia, R. K., Pallister, I., Dent, C., Jones, S. A., & Topley, N. (2005). Enhanced neutrophil migratory activity following major blunt trauma. *Injury.* 36(8), 956-62. DOI: 10.1016/j.injury.2005.03.009

20) Bozza, A. F., Salluh, I. J., Japiassu, M. A., Soares, M., Assis, F. E., & Bozza, T. P. (2007). Cytokine profiles as markers of disease severity in sepsis: a multiplex analysis. *Critical care.* 11, R49. https://doi.org/10.1186/cc5783

21) Collin, M., & Bigley, V. (2018). Human dendritic cell subsets: an update. Immunology, 154(1), 3–20. doi:10.1111/imm.12888

22) Bone, C. R., Balk, A. R., Cerra, B. F., Dellinger, P. R., Fein, M. A., & Sibbald, J. W. (1992). Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM consensus conference committee. American College of chest physicians/ Society of critical care medicine. *Chest.* 101,1644-1655. DOI 10.1378/chest.101.6.1644

23) Boomer, J. S., To, K., Chang, K. C., Takasu, O., Osborne, D. F., Walton, A. H., … Hotchkiss, R. S. (2011). Immunosuppression in patients who die of sepsis and multiple organ failure. JAMA, 306(23), 2594–2605. doi:10.1001/jama.2011.1829

24) Brent, J. A. (2017). Sepsis. *Medicine.* 45(10), 649-653. https://doi-org.salford.idm.oclc.org/10.1016/j.mpmed.2017.07.010

25) Broere, F., van den Zee, R., & van Eden, W. (2011). Heat shock proteins are no DAMPs, rather 'DAMPERs'. *Nature Reviews Immunology.* 11, 565. doi:10.1038/nri2873-c1

26) Butcer, A. B., Kim, L., Panopoulus, D. A., & Denkers, Y. E. (2005). Cutting edge: IL-10-Independent STAT3 activation by *Toxoplama gondii* mediates suppression of IL-12 and TNF-α in host macrophages. 174(6), 3148-52. DOI: https://doi.org/10.4049/jimmunol.174.6.3148

27) Calfee, C. S., Eisner, M. D., Ware, L. B., Thompson, B. T., Parsons, P. E., Wheeler, A. P. (2007). Trauma-associated lung injury differs clinically and biologically from acute lung injury due to other clinical disorders. *Critical care medicine*, 35(10), 2243–2250.

28) Carbo, A., Hontecillas, R., Kronsteiner, B., Viladomiu, M., & Bassaganya-Riera, J. (2013). Systems Modelling of Molecular Mechanisms Controlling Cytokine-driven CD4+ T Cell Differentiation and Phenotype Plasticity. *PLOS/ Computational biology.* 9(4), e1003027. https://doi.org/10.1371/journal.pcbi.1003027

29) Carson, E.W., Lindemann, J. M., Baiocchi, R., Linett, M., Tan, C. J., & Caligiuri, A.M. (1995). The functional characterization of interleukin-10 receptor expression on human natural killer cells. *Blood.* 85(12), 3577-3585. Retrieved 13/04/19 from: https://www.ncbi.nlm.nih.gov/pubmed/7540068

30) Chapman, G., Holton, J., & Chapman, A. (2016). A threshold for concern? C-reactive protein levels following operatively managed neck of femur fractures can detect infectious complications with a simple formula. *Clinical biochemistry.* 49(3), 219-224. https://doi.org/10.1016/j.clinbiochem.2015.10.018

31) Charlie-Silva, I., Klein, A., Gomes, J., Prado, E., Moraes, A. C., Eto, S. F., … Belo, M. (2019). Acute-phase proteins during inflammatory reaction by bacterial infection: Fish-model. Scientific reports, 9(1), 4776. doi:10.1038/s41598-019-41312-z

32) Cho, J. M., Ellebrecht, T. C., & Payne, S. A. (2015). The dual nature of interleukin-10 in pemphigus vulgaris. *Cytokine.*73(2), 335-341. https://doi.org/10.1016/j.cyto.2014.11.002

33) Choi, G., Schultz, M.J., Levi, M., & van der Poll, T. (2006). The relationship between inflammation and the coagulation system. *Swiss Medical Weekly*, 136(9-10),139–144. DOI: 2006/09/smw-11059

34) Choileain, N. N., & Redmond, H. P. (2006). Cell response to surgery. *Arch Surg.* 141(11), 1132–1140. doi:10.1001/archsurg.141.11.1132

35) Chomarat, P., Dantin, C., Bennett, L., Banchereau, J., & Palucka. (2003). TNF Skews Monocyte Differentiation from Macrophages to Dendritic Cells. *The journal of immunology.* 171(5), 2262-2269. DOI: https://doi.org/10.4049/jimmunol.171.5.2262

36) Ciesla, J. D., Moore, E., Johnson, L. J., Burch, M. J., Cothren, C. C., & Sauaia, A. (2005). The role of the lung in postinjury multiple organ failure. *Surgery.* 138(4), 749-758. https://doi-org.salford.idm.oclc.org/10.1016/j.surg.2005.07.020

37) Ciesla, J. D., Moore, E.E., Johnson, L. J. (2005). A 12-Year Prospective Study of Postinjury Multiple Organ Failure. Has Anything Changed? *JAMA Surgery.*140(5), 432-440. doi:10.1001/archsurg.140.5.432

38) Cohen, M., Meir, T., Klein, E., Volpin, G., Assaf, M., & Pollack, S. (2011). Cytokine Levels as Potential Biomarkers for Predicting the Development of Posttraumatic Stress Symptoms in Casualties of Accidents. *The International Journal of Psychiatry in Medicine*, 42(2), 117–131. https://doi.org/10.2190/PM.42.2.b

39) Cohen, J. M., &Christie, S. A. (2016). New understandings of post injury coagulation and resuscitation. *International journal of surgery.* 33(B), 242-245. https://doi-org.salford.idm.oclc.org/10.1016/j.ijsu.2016.05.037

40) Collins, P. D., Luebering, J. B., & Shaut, M. D. (1999). T-lymphocyte functionality assessed by analysis of cytokine receptor expression, intracellular cytokine expression, and femtomolar detection of cytokines by quantitative flow cytometry. *Cytometry.* https://doi-org.salford.idm.oclc.org/10.1002/(SICI)1097-0320(19981001)33:2%3C249::AID-CYTO21%3E3.0.CO;2-T

41) Cossarizza, A., Chang, H. D., Radbruch, A., & Zimmermann, J. (2017). Guidelines for the use of flow cytometry and cell sorting in immunological studies. *European journal of immunology.* 47(10), 1584-1797. doi: 10.1002/eji.201646632.

42) Costantini, W. T., & Coimbra, R. (2017). Response to Major Injury. *Damage Control Management in the Polytrauma Patient.* DOI 10.1007/978-3-319-52429-0_4

43) Couper, N. K., Blount, G. D., & Riley, M.E. IL-10: The master regulator of immunity to infection. *The Journal of immunology.* 180(9), 5771-77. DOI: https://doi.org/10.4049/jimmunol.180.9.5771

44) Cray, C., Zaias, J., & Altman, N. H. (2009). Acute Phase Response in Animals: A Review. Comparative Medicine, 59(6), 517–526.

45) Cryer H.G. (2000). Ischemia and Reperfusion as a Cause of Multiple Organ Failure. In: Baue A.E., Faist E., Fry D.E. (eds) Multiple Organ Failure. Springer, New York. DOI https://doi-org.salford.idm.oclc.org/10.1007/978-1-4612-1222-5_12

46) Curran-Everett, D. (2008). Explorations in statistics: standard deviations and standard errors. *Advances in physiology education*. 32(3), 203-8. doi: 10.1152/advan.90123.2008.

47) D'Andrea, A., Rengaraju, M., Valiante, N. M., & Young, D. (1992). Production of natural killer cell stimulatory factor (interleukin 12) by peripheral blood mononuclear cells. *Journal of experimental medicine.* 176(5),1387. DOI: 10.1084/jem.176.5.1387

48) Del Giudice, M, & Gangestad, W. S. (2018). Rethinking IL-6 and CRP: Why they are more than inflammatory biomarkers, and why it matters. *Brain, Behaviour and Immunity.* 70,61-75. https://doi.org/10.1016/j.bbi.2018.02.013

49) Delneste, Y., Charbonnier, P., Herbault, N., Magistrelli, G., Caron, G., Bonnefoy, J. Y., & Jeannin, P. (2003). Interferon-gamma switches monocyte differentiation from dendritic cells to macrophages. *Blood.* 101(1, 143-150. DOI: 10.1182/blood-2002-04-1164

50) DeLong, W. G., & Born, C. G. (2004). Cytokines in patients with polytrauma. *Clinical Orthopaedics and related research/*. 422, 57-65. Doi: 10.1097/01.blo.0000130840.64528.1e

51) Deng, Q., Tang, B., Xue, C., Liu, Y., Liu, X., Lv, Y., & Zhang, L. (2016). Comparison of the Ability to Predict Mortality between the Injury Severity Score and the New Injury Severity Score: A Meta-Analysis. *International journal of environmental research and public health*, 13(8), 825. doi:10.3390/ijerph13080825

52) Dewar, D., Moore, F. A., Moore, E. E. & Balogh, Z. (2009). Postinjury multiple organ failure. *Injury*. 40(9), 912-8. doi: 10.1016/j.injury.2009.05.024

53) Dienz, O., & Rincon, M. (2008). The effects of IL-6 on CD4 T cell responses. *Clinical immunology (Orlando, Fla.)*, *130*(1), 27-33.

54) Dinarello, A. C. (2000). Proinflammatory Cytokines. Chest. 118(2), 503-508. https://doi.org/10.1378/chest.118.2.503

55) Dinarello C. A. (2018). Overview of the IL-1 family in innate inflammation and acquired immunity. Immunological reviews, 281(1), 8–27. doi:10.1111/imr.12621

56) DiPiro, J. T., Howdieshell, T. R., Goddard, J. K., Callaway, D. B., Hamilton, R. G., & Mansberger, A. R. Jr. (1995). Association of interleukin-4 plasma levels with traumatic injury and clinical course. *Archives of surgery.* 139(11), 1159-1162. Retrieved from: https://www.ncbi.nlm.nih.gov/pubmed/7487457

57) Dorweiler, B., Pruefer, D., Andrasi, T.B., Maksan, S. M., Schmiedt, W., Neufang, A., & Vahl, C. F. (2007). Ischemia-Reperfusion Injury: Pathophysiology and Clinical Implications. *European journal of trauma and emergency surgery.* 3396), 600-12. doi: 10.1007/s00068-007-7152-z.

58) Du Clos, W. T., & Mold, C. (2008). Complement and complement deficiencies. *Clinical immunology.* Third Ed. Principles and practice. https://doi-org.salford.idm.oclc.org/10.1016/B978-0-323-04404-2.10020-X

59) Dushianthan, A., Grocott, W. P. M. Postle, D. A., & Cusack, R. (2011). Acute respiratory distress syndrome and acute lung injury. *Postgraduate medical journal.* 87, 612-622. http://dx.doi.org/10.1136/pgmj.2011.118398.

60) Easton, R., & Balogh, J. Z. (2017). Peri-operative changes in serum immune markers after trauma: A systematic review. *Injury.* 45(6), 934-941. https://doi.org/10.1016/j.injury.2013.12.002

61) Eisinger, F., Patzelt, J., & Langer, H. F. (2018). The Platelet Response to Tissue Injury. Frontiers in medicine, 5, 317. doi:10.3389/fmed.2018.00317

62) Eom, S. H., Jin, S. J., Jeong, H. Y., Song, Y., Lim, Y. J., Kim, J. I … Kang, H. (2018). Kudzu Leaf Extract Suppresses the Production of Inducible Nitric Oxide Synthase, Cyclooxygenase-2, Tumor Necrosis Factor-Alpha, and Interleukin-6 via Inhibition of JNK, TBK1 and STAT1 in Inflammatory Macrophages. *International journal of molecular sciences*, *19*(5), 1536. doi:10.3390/ijms19051536

63) El-Ashker, M., Salama, M., Rizk, A., & El-Boshy, M. (2014). The use of inflammatory markers as a prognostic aid for traumatic reticuloperitonitis in water buffalo (Bubalus bubalis). *Veterinarni medicina.* 59(5), 239-246. Retrieved from: https://www.agriculturejournals.cz/publicFiles/126893.pdf

64) El-Benna, J., Hurtado-Nedelec, M., Marie, J-C., Gourgerot-Pocidalo, M-A., & My-Chang Dang, P. (2016). Priming the neutrophils respiratory burst: role in host defence and inflammation. *Immunological reviews*. 273(1), 180- 193. doi: 10.1111/imr.12447

65) Ertel, W., Keel, M., Neidhardt, R., Steckholzer, U., Kremer, J. P., Ungethuem, U., & Trentz, O. (199&). Inhibition of the Defence System Stimulating Interleukin-12 Interferon-γ Pathway During Critical Illness. *Blood journal.*89(5), 1612-1620. Retrieved from: http://www.bloodjournal.org/content/bloodjournal/89/5/1612.full.pdf

66) Feng, H., Zeng, Y., Graner, W. M., Likhacheva, A., & Katsanis, E. (2003). Exogenous stress proteins enhance the immunogenicity of apoptotic tumor cells and stimulate antitumor immunity. *Blood*. doi: https://doi.org/10.1182/blood-2002-05-1580

67) Ferreira, L. F., Bota, D. P., & Bross, A. (2001). Serial Evaluation of the SOFA Score to Predict Outcome in Critically Ill Patients. *JAMA.* 286(14), 1754-1758. doi:10.1001/jama.286.14.1754.

68) Fiorentino, D. F., Bond, M. W., & Mosmann, T. R. (1989). Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. The Journal of experimental medicine, 170(6), 2081-95

69) Fitschen- Oestem, S., Lippross, S., Klueter, T., Weuster, M., Varoga, D., & Neumbaer, C. (2017). A new multiple trauma model of the mouse. *BMS musculoskeletal disorders.* DOI 10.1186/s12891-017-1813-9

70) Fontes, J. A., Rose, N. R., & Čiháková, D. (2015). The varying faces of IL-6: From cardiac protection to cardiac failure. *Cytokine*, *74*(1), 62-8. doi: 10.1016/j.cyto.2014.12.024

71) Fox, S., I. (2008). Human Physiology. Chapter 15 The immune system. McGraw-Hill Education 11th ed.

72) Fröhlich, M., Wafaisade, A., Mansuri, A., Koenen, P., Probst, C., Maegele, M., … Sakka, S. G. (2016). Which score should be used for posttraumatic multiple organ failure? - Comparison of the MODS, Denver- and SOFA-Scores. Scandinavian journal of trauma, resuscitation and emergency medicine, 24(1), 130. doi:10.1186/s13049-016-0321-5

73) Fueglistaler, P., Amsler, F., Schüepp, M., Fueglistaler-Montali, I., Attenberger, C., Pargger, H., Jacob, A. L., & Gross, T. (2010). Prognostic value of Sequential Organ Failure Assessment and Simplified Acute Physiology II Score compared with trauma scores in the outcome of multiple-trauma patients. *American journal of surgery.* 200(2), 204-14. doi: 10.1016/j.amjsurg.2009.08.035.

74) Futosi, K., Fodor, S., & Mocsai, A. (2013). Neutrophil cell surface receptors and their intracellular signal transduction pathways. *International Immunopharmacology*. 17, 638-650. Doi: 10.1016/j.intimp.2013.06.034

75) Gan, D. S., & Patel, R. K. (20130. Enzyme immunoassay and enzyme- linked Immunosorbent assay. *Journal of investigative dermatology.* 133(9), 1-3. https://doi-org.salford.idm.oclc.org/10.1038/jid.2013.287

76) Gentile, L. F., Cuenca, A. G., Efron, P. A., Ang, D., Bihorac, A., McKinley, B. A., … Moore, F. A. (2012). Persistent inflammation and immunosuppression: a common syndrome and new horizon for surgical intensive care. The journal of trauma and acute care surgery, 72(6), 1491–1501. doi:10.1097/TA.0b013e318256e000

77) Gillani, S., Cao, J., Suzuki, T., & Hak, J. D. (2012). The effect of ischemia reperfusion injury on skeletal muscle. *Injury.* 43:6, 670-675. https://doi.org/10.1016/j.injury.2011.03.008

78) Givan, A. L. (2011). Flow cytometry: an introduction. *Methods in molecular biology.*699, 1-29. doi: 10.1007/978-1-61737-950-5_1.

79) Goettler, E. C., Giannoudis, V. P., & Rotondo, F. M. (2017). The concept of damage control. *Damage Control Management in the Polytrauma Patient.* H.-C. Pape et al. (eds.). DOI 10.1007/978-3-319-52429-0_2

80) Godfroy, I. J., Roostan, M., Moroz, S. Y., & Hubert Yin, H. (2012). Isolated Toll-like receptor transmembrane domains are capable of oligomerization. *PLoS ONE.* 7(11), e48875. DOI: 10.1371/journal.pone.0048875

81) Grant, A. M., & Aird, C., W. (2016). Molecular Evolution of the Vertebrate Blood Coagulation System. *Fastest Oncology & Haematology Insight Engine.* Retrieved from: https://oncohemakey.com/molecular-evolution-of-the-vertebrate-blood-coagulation-system

82) Greenhalgh DG. Sepsis in the burn patient: a different problem than sepsis in the general population. Burns Trauma. 2017 Aug 8; 5:23. doi: 10.1186/s41038-017-0089-5. PubMed PMID: 28795054; PubMed Central PMCID: PMC5547526.

83) Gotmaker, R., Peake, L. S., Forbes, A., & Bellomo, R. (2017). Mortality is Greater in Septic Patients with Hyperlactatemia Than with Refractory Hypotension. *Shock.* 48(3). https://doi-org.salford.idm.oclc.org/10.1097/SHK.0000000000000861

84) Grmec, S., & Gasparovic, V. (2000). Comparison of APACHE II, MEES and Glasgow Coma Scale in patients with nontraumatic coma for prediction of mortality. Acute Physiology and Chronic Health Evaluation. Mainz Emergency Evaluation System. *Critical care (London, England)*, *5*(1), 19–23. doi: 10.1186/cc973

85) Grohmann, u., Belladonna, l. M., Bianchi, R., & Puccetti, P. (1998). IL-12 acts directly on DC to promote nuclear localisation of NF-kB and primes DC for IL-12 production. *Immunity.* 9(3),315-323. https://doi.org/10.1016/S1074-7613(00)80614-7

86) Gruys, E., Toussaint, M. J., Niewold, T. A., & Koopmans, S. J. (2005). Acute phase reaction and acute phase proteins. Journal of Zhejiang University. Science. B, 6(11), 1045–1056. doi:10.1631/jzus. 2005.B1045

87) Gu. W., Zeng. L., Zhang, L.Y., Jiang, D. P., Du, D.Y., Hu, P., Wang, H. Y., Liu, Q., Huang, S. N., & Jiang, J. X. (2011). Association of interleukin 4 -589T/C polymorphism with T(H)1 and T(H)2 bias and sepsis in Chinese major trauma patients. *The journal of trauma.* 71(6), 1583-1587. doi: 10.1097/TA.0b013e3182115034.

88) Gauglitz, G. G., Finnerty, C. C., Herndon, D. N., Mlcak, R. P., & Jeschke, M. G. (2008). Are serum cytokines early predictors for the outcome of burn patients with inhalation injuries who do not survive? Critical care (London, England), 12(3), R81. doi:10.1186/cc6932

89) Gubler, U., Chua, O.A., Schoenhaut, D. S., & Familletti, C. P. (1991). Coexpression of two distinct genes is required to generate secreted bioactive cytotoxic lymphocyte maturation factor. *PNAS.*88(10), 4143-4147. https://doi.org/10.1073/pnas.88.10.4143

90) Gunst, M., Ghaemmaghami, V., Gruszecki, A., Urban, J., Frankel, H., & Shafi, S. (2010). Changing epidemiology of trauma deaths leads to a bimodal distribution. Proceedings (Baylor University. Medical Center), 23(4), 349–354.

91) Ha,j. s., Lee, H. C., Lee, B. S., & Sung, C. Y. (1999). A novel function of IL-12p40 as a chemotactic molecule for macrophages. *The Journal of immunology.*163(5), 2902-2908. Retrieved 17/02/2019 from: http://www.jimmunol.org/content/163/5/2902

92) Hamaguchi, S., Hirose, T., Akeda, Y., Matsumoto, N., Irisawa, T., Seki, M…Tomoko, K. (2013). Identification of neutrophil extracellular traps in the blood of patients with systemic inflammatory response syndrome. *The Journal of international medical research.* 41(1), 162-8. doi: 10.1177/0300060513475958

93) Harboe, M., & Mollnes, T. E. (2008). The alternative complement pathway revisited. Journal of cellular and molecular medicine, 12(4), 1074–1084. doi:10.1111/j.1582-4934.2008. 00350.x

94) Hayakawa, M. (2017). Pathophysiology of trauma-induced coagulopathy: disseminated intravascular coagulation with the fibrinolytic phenotype. *Journal of intensive care.* 5,14 doi: 10.1186/s40560-016-0200-1

95) Hamidzadeh, K., Christensen, S. M., Dalby, E., Chandrasekaran, P., & Mosser, D. M. (2017). Macrophages and the Recovery from Acute and Chronic Inflammation. Annual Review of Physiology, 79, 567–592. http://doi.org/10.1146/annurev-physiol-022516-034348

96) Hallstrom, T., & Riesbeck, K. (2010). *Haemophilus influenzae* and the complement system. *Trends in microbiology.* DOI: http://dx.doi.org/10.1016/j.tim.2010.03.007

97) Harwood, P. J., Giannoudis, P. V., van Griensven, M., Krettek, C.,& Pape, H.C. (2005). Alterations in the systemic inflammatory response after early total care and damage control procedures for femoral shaft fracture in severely injured patients. *The journal of trauma.* 58(3), 446-54. DOI: 10.1097/01.TA.0000153942.28015.77

98) Hazeldine, J., Hampson, P., & Lord, M., J. (2014). The impact of trauma on neutrophil function. *Injury.* http://dx.doi.org/10.1016/j.injury.2014.06.021

99) Hamidzadeh, K., Christensen, S. M., Dalby, E., Chandrasekaran, P., & Mosser, D. M. (2017). Macrophages and the Recovery from Acute and Chronic Inflammation. Annual Review of Physiology, 79, 567–592. http://doi.org/10.1146/annurev-physiol-022516-034348

100) Hawrylowicz, C. M., & O'Garra, A. (2005). Potential role of interleukin-10-secreting regulatory T cells in allergy and asthma. *Nature reviews. Immunology.* 5(4), 271-83. DOI:10.1038/nri1589

101) Heinrich, P. C., Behrmann, I., Müller-Newen, G., Schaper, F., & Graeve, L. (1998). Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway. *The Biochemical journal*, *334 (Pt 2)*(Pt 2), 297-314.

102) Hecke, F., Schmidt, U., Kola, A., Bautsch, W., Klos, A., & Kohl, J. (1997). Circulating complement proteins in multiple trauma patients-Correlation with injury severity, development of sepsis, and outcome. *Critical care medicine.* 25(12), 2015-2024. Retrieved 07/04/2019 from: https://www.ncbi.nlm.nih.gov/pubmed/9403752

103) Hengst, M. J. (2003). The Role of C-Reactive Protein in the Evaluation and Management of Infants with Suspected Sepsis*. Advances in neonatal care*. 3(1). Retrieved 25/08/2018 from: https://www.medscape.com/viewarticle/450937_3

104) Hensler, T., Heidecke, C-D., Hecker, H., Heeg, K., & Holzmann, B. (1998). Increased Susceptibility to Postoperative Sepsis in Patients with Impaired Monocyte IL-12 Production. *The journal of immunology.* 161(5),2655-2659.Retrieved from: http://www.jimmunol.org/content/161/5/2655/tab-article-info

105) Hibi, M., Murakami, M., Saito, M., Hirano, T., Taga, T., & Kishimoto. T. (1990). Molecular cloning and expression of an IL-6 signal transducer, gp 130. *Cell.* 63 (6), 1149-1157. doi.org/10.1016/0092-8674(90)90411-7

106) Heine, G., Drozdenko, G., Grun, R. J., Chang, H-D., Radbruch, A., & Worm, M. (2014). Autocrine IL-10 promotes human B-cell differentiation into IgM or IgG secreting Plasmablasts. *European Journal of Immunology.* 44(6). https://doi-org.salford.idm.oclc.org/10.1002/eji.201343822

107) Heinrich, P. C., Behrmann, I., Haan, S., Hermanns, H. M., Müller-Newen, G., & Schaper, F. (2003). Principles of interleukin (IL)-6-type cytokine signalling and its regulation. *The Biochemical journal*, *374*(Pt 1), 1-20.

108) Hietbrink, F., Koenderman, l., Rijkers, G. T., & Leenen, L. P. H. (2006). Trauma: the role of the innate immune system. *World Journal of Emergency Surgery.* 1:15. https://doi.org/10.1186/1749-7922-1-15

109) Hirsinger, S., Simmen, H-P., Werner, L. M. C., Wanner, A. Guido., & Rittirsch, D. (2012). Danger Signals Act the Immune Response after Trauma. *Hindawi Publishing Corporation Mediators of Inflammation.* doi:10.1155/2012/315941

110) Horiguchi, H., Loftus, T. J., Hawkins, R. B., Raymond, S. L., Stortz, J. A., Hollen, M. K., … Sepsis and Critical Illness Research Center Investigators (2018). Innate Immunity in the Persistent Inflammation, Immunosuppression, and Catabolism Syndrome and Its Implications for Therapy. Frontiers in immunology, 9, 595. doi:10.3389/fimmu.2018.00595

111) Hotchkiss, S. R., & Katl, E. I. (2003). The Pathophysiology and Treatment of Sepsis. *New England journal of medicine.* 348, 138-150. DOI: 10.1056/NEJMra021333

112) Hotchkiss RS, Monneret G, Payen D. Sepsis-induced immunosuppression: from cellular dysfunctions to immunotherapy. Nat Rev Immunol. 2013 Dec;13(12):862-74. doi: 10.1038/nri3552. Epub 2013 Nov 15. PubMed PMID: 24232462; PubMed Central PMCID: PMC4077177.

113) Hyun, Y. M., Lefort, C. T., & Kim, M. (2009). Leukocyte integrins and their ligand interactions. *Immunologic research.* 45(2-3), 195-208. doi: 10.1007/s12026-009-8101-1

114) Hu, X., Hen, J., Wang, L., & Ivashkiv, B. L. (2006). Crosstalk among Jak-STAT, Tool-like receptors, and ITAM-dependant pathways in macrophage activation. *Journal of leukocyte biology.* 82(2). https://doi-org.salford.idm.oclc.org/10.1189/jlb.1206763

115) Hu, H., Du, X., He, Y., & Yu, C. (2017). Application of SOFA score as a predict tool for mortality of delayed admission to ICU on patients with acute respiratory failure. *The American journal of emergency medicine.*35(6),914-915.https://www-sciencedirect-com.salford.idm.oclc.org/science/journal/07356757

116) Huber-Lang, M., Sarma, J. V., Zetoune, S. F., & Ward, A.P. (2006). Generation of C5a in the absence of C3: a new complement activation pathway. *Nature medicine.* doi:10.1038/nm1419

117) Huber-Lang, M., & Gebhard, F. (2016). Inflammatory Changes and Coagulopathy in Multiply Injured Patients. *The Poly-Traumatized Patient with Fractures: A Multi-Disciplinary Approach*. H.-C. Pape et al. (eds.). DOI: 10.1007/978-3-662-47212-5_4

118) Hu, Y. P., Peng, Y. B., Zhang, Y. F., Wang, Y., Yu, W. R., Yao, M., & Fu, X. J. (2017). Reactive Oxygen Species Mediated Prostaglandin E2 Contributes to Acute Response of Epithelial Injury. *Oxidative medicine and cellular longevity*, 2017, 4123854. doi:10.1155/2017/4123854

119) Hur, J., Yang, H. T., Chun, W., Kim, J. H., Shin, S. H., Kang, H. J., & Kim, H. S. (2015). Inflammatory cytokines and their prognostic ability in cases of major burn injury. Annals of laboratory medicine, 35(1), 105–110. doi:10.3343/alm.2015.35.1.105

120) Hwang, F. P., Portefield, N., Pannell, D., Davis, A. T., & Elster, A. E. (2011). Trauma is danger. *Journal of translational medicine.* 9:92. https://doi.org/10.1186/1479-5876-9-92

121) Huber-Lang, M & Gebhard, F. (2016). Inflammatory Changes and Coagulopathy in Multiply Injured Patients. *The Poly-Traumatized Patient with Fractures: A Multi-Disciplinary Approach*. DOI 10.1007/978-3-662-47212-5_4

122) Ingersoll, A. M., Platt, M. A., Potteaux, S. & Randolph,J. G. (2011). Monocyte trafficking in acute and chronic inflammation. *Trends in immunology.*32910), 470-477. doi:10.1016/j.it.2011.05.001.

123) Ito T. (2014). PAMPs and DAMPs as triggers for DIC. Journal of intensive care, 2(1), 67. doi:10.1186/s40560-014-0065-0

124) Jain, S., Gautam, V., & Naseem, S. (2011). Acute-phase proteins: As diagnostic tool. Journal of Pharmacy and Bioallied Sciences, 3(1), 118–127. http://doi.org/10.4103/0975-7406.76489

125) Jain, s., Teasdale, M. G., & Iverson, M. L. (2019). Glasgow coma scale. *StatPearl Publishing.* Retrieved 07/04/2019 from: https://www.ncbi.nlm.nih.gov/books/NBK513298/

126) Jana, M., Dasgupta, S., Saha, R. N., Liu, X., & Pahan, K. (2003). Induction of tumor necrosis factor-alpha (TNF-alpha) by interleukin-12 p40 monomer and homodimer in microglia and macrophages. *Journal of neurochemistry*, *86*(2), 519-28. doi: 10.1046/j.1471-4159.2003. 01864.x

127) Janeway, C., A., Jr., Travers, P., Walport, M. (2001). The complement system and innate immunity. *Immunobiology: The Immune System in Health and Disease* 5th ed. New York: Garland Science. Retrieved from: https://www.ncbi.nlm.nih.gov/books/NBK27100/

127) Jiao, J., Wang, J. W., Xiao, F., & Huang, Y. C. (2016). The association between the levels of CRP, IL-10, PLA2, Fbg and prognosis in traumatic fracture of lower limb. Experimental and therapeutic medicine, 12(5), 3209–3212. doi:10.3892/etm.2016.3746

128) Jones, M. A. (2017). Interleukin-6 and Interleukin-10 concentrations as predictors of patient outcome following major traumatic injury. Retrieved from: http://usir.salford.ac.uk.salford.idm.oclc.org/id/eprint/43970/

129) Kovtun, A., Messerer, D. A. C., Scharffetter-Kochanek, K., Huber-Lang, M., & Ignatius, A. (2018). Neutrophils in Tissue Trauma of the Skin, Bone, and Lung: Two Sides of the Same Coin. Journal of Immunology Research, 2018, 8173983. http://doi.org/10.1155/2018/8173983

130) Kawai, T & Akira, S. (2011). Toll-like Receptors and Their Crosstalk with Other Innate Receptors in Infection and Immunity. *Immunity.*34:5, 637-650. https://doi.org/10.1016/j.immuni.2011.05.006

131) Kawasaki, T.& Kawai, T. (2014). Toll-like receptor signalling pathways. *Frontiers in immunology*. 5:461. doi: 10.3389/fimmu.2014.00461

132) Keel, M., & Trentz, O. (2005). Pathophysiology of polytrauma. *Injury.*36(6), 691-709. DOI: 10.1016/j.injury.2004.12.037

133) Keelley, A., Hine, P., & Nsutebu, E. (2016). The recognition and management of sepsis and septic shock: a guide for non-intensivists. *Postgraduate Medical Journal.* 93,626-634. http://dx.doi.org/10.1136/postgradmedj-2016-134519

134) Kemmer, A. (2006). Crush Injury and Other Acute Traumatic Ischemia. *Handbook in hyperbaric medicine.* Mathieu D. (eds) Springer, Dordrecht. DOI https://doi-org.salford.idm.oclc.org/10.1007/1-4020-4448-8_17

135) Kemp, F. S., & Lockey, F. R. (2002). Anaphylaxis: A review of causes and mechanisms. *Journal of allergy and clinical immunology.* 110(3), 341-348. https://doi.org/10.1067/mai.2002.126811

136) Kingsley, S. M. K., & Bhat, B.V. (2017). Role of microRNAs in sepsis. *Inflammation research.* 66(7), 553-569. doi: 10.1007/s00011-017-1031-9

137) Kirchhoff, C., Biberthaler, P., Mutschler, W. E., Faist, E., Jochum, M., & Zedler, S. (2009). Early down-regulation of the pro-inflammatory potential of monocytes is correlated to organ dysfunction in patients after severe multiple injury: a cohort study. Critical Care, 13(3), R88. http://doi.org/10.1186/cc7914

137) Kingston, R., & O'Flanagan, J. S. (2000). Scoring systems in trauma. *Irish Journal of Medical Science.* 169(3), 168-172. https://doi-org.salford.idm.oclc.org/10.1007/BF03167688

138) Kimura, F., Shimizu, H., Yoshidome, H., Ohtsuka, M., & Miyazaki, M. (2010). Immunosuppression following surgical and traumatic injury. *Surgery today.* 40(9), 793-808. doi: 10.1007/s00595-010-4323-z.

139) Klein, L. D., Martinez, E. J., Hickey, H. M., Hassouna, F., Zaman, K., & Steinhoff, M. (2012). Development and characterisation of a multiplex bead-based immunoassay to quantify pneumococcal capsular polysaccharide-specific antibodies. *Clinical and vaccine immunology.* https://doi.org/10.1128/CVI.05535-11

140) Kobayashi, m., Fitz, L., Hewick, M. R., & Trincheri, G. (1989). Identification and purification of natural killer cells stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. *Journal of Experimental Medicine.* 170(3), 827. DOI: 10.1084/jem.170.3.827

150) Kooler, M., David, A., Hahn, M. P., & Muhr, G. (1998). Liberation of interleukin 12 (IL12) after trauma and polytrauma*. Langenbecks Arch Chir Suppl Kongressbd.* 115(Suppl), 453-6. Retrieved from: https://www.ncbi.nlm.nih.gov/pubmed/14518295

151) Koller, M., David, A., Hahn, M.P., & Muhr, G. (1998). Liberation of interleukin 12 (IL12) after trauma and polytrauma. *Deutsche Gesellschaft fur chirurgie.* 115(suppl l), 453-6. Retrieved from: https://www.ncbi.nlm.nih.gov/pubmed/14518295

152) Kono, H., & Rock, L. K. (2008) . How dying cells alert the immune system to danger. *Nature reviews Immunology.* 8:(4), 279-289. doi:10.1038/nri2215

153) Komai, K., Shichita, T., Ito, M., Kanamori, M., Chikuma, S., & Yoshimura, A. (2016). Role of scavenger receptors as damage-associated molecular pattern receptors in Toll-like receptor activation. *International immunology.* 29(2), 59-70. doi:10.1093/intimm/dxx010

154) Kumar, H., Kawai, T., & Akira, S. (2009). Toll-like receptors and innate immunity. *Biochemical and biophysical research communications.*388(4), 621-625. https://doi.org/10.1016/j.bbrc.2009.08.062

155) Kunai, V., Abbas, K. A., Aster, C. J., & Stanley, R. (2015). Robbins and Cotran pathologic basis of disease. 9th ed. Philadelphia, PA: Elsevier

156) Kupcova Skalnikova, H., Cizkova, J., Cervenka, J., & Vodicka, P. (2017). Advances in Proteomic Techniques for Cytokine Analysis: Focus on Melanoma Research. International journal of molecular sciences, 18(12), 2697. doi:10.3390/ijms18122697

157) Lachmann, J. P. (2018). Looking back on the alternative complement pathway. *Immunobiology.* 223(8-9), 519-523. https://doi.org/10.1016/j.imbio.2018.02.001

158) Laffey, J. G., Misak, C., & Kavanagh, B. P. (2017). Acute respiratory distress syndrome. *British medical journal.* 359. doi: http://dx.doi.org.salford.idm.oclc.org/10.1136/bmj.j5055

159) Lakomkin, N., Sathiyakumar, V., Wick, B., Shen, M. S., Jahangir, A. A., Mir, H., … Sethi, M. K. (2016). Incidence and predictive risk factors of postoperative sepsis in orthopaedic trauma patients. *Journal of orthopaedics and traumatology: official journal of the Italian Society of Orthopaedics and Traumatology*, *18*(2), 151–158. doi:10.1007/s10195-016-0437-4

160) Lavery, R.F., Livingston, D. H., Tortella, B. J., Sambol, J.T., Slomovitz, B. M., & Siegel, J. H. (2000). The utility of venous lactate to triage injured patients in the trauma center. *Journal of the American college of surgeons.* 190(6),656-64. https://doi.org/10.1016/S1072-7515(00)00271-4

161) Lecky, F., Bouamra, O., & Woodford, M. (2017). Changing Epidemiology of Polytrauma. *Damage Control Management in the Polytrauma Patient.* 27-32. Doi: 10.1007/978-3-319-52429-0_3. Retrieved from: https://link-springer-com.salford.idm.oclc.org/chapter/10.1007/978-3-319-52429-0_3

162) Leclerc, E., Fritz, G., Vetter, W. S., & Heizmann, W. C. (200). Binding of S100 proteins to RAGE: An update. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research.* 1793(6), 993-1007. https://doi.org/10.1016/j.bbamcr.2008.11.016

163) Lelubre, C., Anselin, S., Zouaoui Boudjeltia, K., Biston, P., & Piagnerelli, M. (2013). Interpretation of C-reactive protein concentrations in critically ill patients. BioMed research international, 2013, 124021. doi:10.1155/2013/124021

164) Lendrum, A. R., & Lockey, J. D. (2012). Trauma system development. *Anaestesia.*68(1). https://doi-org.salford.idm.oclc.org/10.1111/anae.12049

165) Lenz, A., Franklin, A. G., & Cheadle, G. W. (2007). Systemic inflammation after trauma. *Injury.* 38, 1336-1345. doi: 10.1016/j.injury.2007.10.003

166) Lee, D-G., Lee, K-S., Shim, J-J., Yoon, S-M., & Bae, H. G. (2005). Prognostic Value of the C-reactive Protein Levels in the Head Injury. Korean journal of noerotraumatology .1(1), 57-60. Retrieved 08/05/2019 from: https://www.kjnt.org/Synapse/Data/PDFData/1203JKNTS/jknts-1-57.pdf

167) Lee, H. K., & Iwasaki, A. (2007). Innate control of adaptive immunity: dendritic cells and beyond. *Seminars in immunology.* 19(1), 48- 55. DOI: 10.1016/j.smim.2006.12.001

168) Li, Q., Qian, G., Zhang, Q., Gong, J., Tang, Z., & Gao, Z. (2002). Changes of plasma interleukin-4, interleukin-10 and interleukin-13 in patients with acute respiratory distress syndrome. *Zhonghua Jie He He Hu Xi Za Zhi.* 25(11), 661-664. Retrieved from: https://www.ncbi.nlm.nih.gov/pubmed/12490119

169) Libermann, T. A., & Baltimore, D. (1990). Activation of interleukin-6 gene expression through the NF-kappa B transcription factor. *Molecular and cellular biology*, *10*(5), 2327-34. DOI:10.1128/MCB.10.5.2327

170) Lie, K. C., Lau, C-Y., Chau, N. V. V., West, T. E., & Limmathurotsakul, D. (2018). Utility of SOFA score, management and outcomes of sepsis in Southeast Asia: a multinational multicenter prospective observational study. *Journal of intensive care.* 6,9. https://doi.org/10.1186/s40560-018-0279-7

171) Lipinska-Gediga, M. (2017). Neutrophils, NETs, NETosis- old or new factors in sepsis and septic shock? *Anaesthesiology Intensive Therapy.* 49(3), 235-240. doi: 10.5603/AIT.2017.0041.

172) Liu, C., & Tang, J. (2014). Expression levels of tumor necrosis factor-α and the corresponding receptors are correlated with trauma severity. *Oncology letters*, *8*(6), 2747-2751.

173) Liu, C. H., Kuo, S. W., Ko, W. J., Tsai, P. R., Wu, S. W., Lai, C. H., … Jou, T. S. (2017). Early measurement of IL-10 predicts the outcomes of patients with acute respiratory distress syndrome receiving extracorporeal membrane oxygenation. Scientific reports, 7(1), 1021. doi:10.1038/s41598-017-01225-1

174) Liu, G., Zhang, L., & Zhao, Y. (2009). Modulation of immune responses through direct activation of Toll-like receptors to T cells. *Clinical and experimental immunology.* doi:10.1111/j.1365-2249.2010. 04091.x

175) Lobo, S. Lobo, F., Peres Bota, D., & Lopez-Ferreira, F. (2003). C-reactive protein levels correlate with mortality and organ failure in critically ill patients. *Chest*. 123(6), 2043-9. https://doi.org/10.1378/chest.123.6.2043

176) Lockey, J. D. (2018). Improved Trauma Outcomes after the Introduction of a Trauma System in England. *The Lancet.* 1(2), 3-4. Doi: http//doi.org/10.1016/j.eclinm.2018.08.002

177) Lonza.com retrieved 15/12/2018 from: https://www.lonza.com/products-services/bioresearch/primary-cells/hematopoietic-cells/hematopoietic-knowledge-center/macrophages.aspx

178) Lord, J. M., Midwinter, M. J., Chen, Y.-F., Belli, A., Brohi, K., Kovacs, E. J., … Lilford, R. J. (2014). The systemic immune response to trauma: an overview of pathophysiology and treatment. *Lancet (London, England)*, 384(9952), 1455–1465. http://doi.org/10.1016/S0140-6736(14)60687-5

179) Lopmpart-Pou, A. J., Talayero, M., Homar, J., & Royo, C. (2014). Multiorgan failure in the serious trauma patient. Medicina intensiva. 38(7), 455-462. https://doi-org.salford.idm.oclc.org/10.1016/j.medine.2014.05.002

180) Luckheeram. R., Zhou, R., Verma, D. A., & Xia, B. (2012). CD4+T Cells: Differentiation and Functions. *Clinical and Developmental Immunology*. 2012, Article ID 925135. https://doi.org/10.1155/2012/925135

181) Luyendyk, P. J., Schoenecker, G. J., &Flick, J. M. (2018). The multifaceted role of fibrinogen in tissue injury and inflammation. Blood. doi.org/10.1182/blood-2018-07-818211

182) Maduz, R., Kugelmeier, P., Meili, S., Döring, R., Meier, C., & Wahl, P. (2017). Major influence of interobserver reliability on polytrauma identification with the Injury Severity Score (ISS): Time for a centralised coding in trauma registries? *Injury.* 48(4), 885-889. doi: 10.1016/j.injury.2017.02.015

183) Maeda, K., Mehta, H., Drevets, A., D. & Coggeshall, K. M. (2010). IL-6 increases B-cell Ig G production in a feed-forward proinflammatory mechanism to skew haematopoiesis and elevate myeloid production. B*lood.* 115, 4699-4706. doi: https://doi.org/10.1182/blood-2009-07-230631

184) Man, Y., Lv, X., Iqbal, J., & Peng, G… Deng, Y. (2015). Microchip based and immunochromatographic strip assay for the visual detection of interleukin-6 and of tumour necrosis factor α using gold nanoparticles as labels. *Microchimica acta*. 182, 3-4. DOI: 10.1007/s00604-014-1362-y

185) Mann, G. K., & Lorand, L. (1993). Introduction: Blood coagulation. *Methods in enzymology.* 222,1010. https://doi.org/10.1016/0076-6879(93)22003-X

186) Martin, B. J., & Badeaux, E. J. (2016). Making sense of biomarkers in sepsis and systemic inflammatory response syndrome for intensive care unit patients. *Critical care nursing.* http://dx.doi.org/10.1016/j.cnc.2016.09.004

187) Martini W. Z. (2016). Coagulation complications following trauma. *Military Medical Research*, 3, 35. doi:10.1186/s40779-016-0105-2

188) Marshall, J. C. (2001). The multiple organ dysfunction syndrome. Surgical Treatment: Evidence-Based and Problem-Oriented. Munich: Zuckschwerdt; : Holzheimer RG, Mannick JA, ed. Retrieved 07/04/2019 from: https://www.ncbi.nlm.nih.gov/books/NBK6868

189) Martin, J. S., (2016). Cell death and inflammation: the case for IL-1 family cytokines as the canonical DAMPs of the immune system. *The FEBS Journal.* doi:10.1111/febs.13775

190) Matthay, A. M., Ware, B. L., & Zimmerman, A. G. (2012). The acute respiratory distress syndrome. *J. Clin. Invest*. 122(8), 2731-2740. https://doi.org/10.1172/JCI60331.

191) Matzinger, P. (1994). Tolerance, Danger and the Extended Family. *Annual review of Immunology.* 12:991-1045. DOI: 10.1146/annurev.iy.12.040194.005015

192) McCullough, K. C., Ruggli, N., & Summerfield, A. (2009). Dendritic cells-at the front-line of pathogen attack. *Veterinary immunology and Immunopathology.* 128(1-3): 7-15. doi: 10.1016/j.vetimm.2008.10.290

193) McIllroy, D.J., Jarnicki,A.G., Au, G. G., Lott, N., Smith, D. W., Hansbro, P. M., & Balogh, Z.J. (2014). Mitochondrial DNA neutrophil extracellular traps are formed after trauma and subsequent surgery. *Journal of Critical care.*29(6). doi: 10.1016/j.jcrc.2014.07.013.

194) McInnes, B.I. (2017). Cytokines. *Kelley and Firestein's Textbook of Rheumatology*. 1, 396-407. https://doi.org/10.1016/B978-0-323-31696-5.00026-7

195) McHugh, M. T. (1994). Flow microsphere immunoassay for the quantitative and simultaneous detection of multiple soluble analytes. *Methods in cell biology. 42, 575-595.* https://doi-org.salford.idm.oclc.org/10.1016/S0091-679X(08)61096-1

196) Medzhitov, R., & JanewayJr, C. (2000). Innate immunity. *The New England journal of medicine.* 338-344. DOI: 10.1056/NEJM200008033430506

197) Mellman, I. (2013). Dendritic Cells: Master Regulators of the Immune Response. *Cancer immunology researche.*1(3), 145-149. Retrieved 28/08/2018 from: http://cancerimmunolres.aacrjournals.org/content/1/3/145.long. DOI: 10.1158/2326-6066.CIR-13-0102

198) Mihara, M., Hashizume, M., Yoshida, H., Suzuki, M., & Shiina, M. (2012). IL-6/IL-6 receptor system and its role in physiological conditions. *Clinical Science.* 122(4), 143-159. DOI: 10.1042/CS20110340

199) Miralda, I., Uriarte, S. M., & McLeish, K. R. (2017). Multiple Phenotypic Changes Define Neutrophil Priming. *Frontiers in Cellular and Infection Microbiology*, *7*, 217. http://doi.org/10.3389/fcimb.2017.00217

200) Moghtadaei, M., Otoukesh, B., Pazoki-Toroudi, H., Boddouhi, B., & Yeganeh, A. (2016). Evaluation of inflammatory response in patients undergoing surgical treatment for early and delayed femoral fractures. *Archives of medical science: AMS*, *15*(1), 141–145. doi:10.5114/aoms.2016.63013

201) Mold, C., Rodriguez, W. Rodic-Polic, B., & Du Clos, T, W. (2002). C-Reactive Protein Mediates Protection from Lipopolysaccharide Through Interactions with FcγR. *The journal of Immunology.* 169(12), 7019-7025. DOI: https://doi.org/10.4049/jimmunol.169.12.7019

202) Mold, C., West, S., & Du Clos, T. (2011). Role of C-reactive protein in regulatory monocyte induction and activation in trauma patients (110.18). *The journal of Immunology.*186(1 Supplement),110.18. Retrieved 08/05/2019 from: http://www.jimmunol.org/content/186/1_Supplement/110.18

203) Moreno, R., Vincent, J-L., & Matos, R. G. (1999). The use of maximum SOFA score to quantify organ dysfunction/failure in intensive care. Results of a prospective, multicentre study. *Intensive care medicine.* 25(7), 686-696. DOI: 10.1007/s001340050931

204) Moreno, S. E., Alves-Filho, J. C., Alfaya, T. M., da Silva, J. S., Ferreira, S. H., & Liew, F. Y. (2006). IL-12, but not IL-18, is critical to neutrophil activation and resistance to polymicrobial sepsis induced by cecal ligation and puncture. *Journal of immunology.* 177(5), 3218-24. DOI: 10.4049/jimmunol.177.5.3218

205) Moretta, L., Ferlazzo, G., Bottino, C., Vitale, M., Pende, D., Mingari, M. C., & Moretta, A. (2006). Effector and regulatory events during natural killer-dendritic cell interactions. *Immunological reviews.* 214, 219-228. DOI:10.1111/j.1600-065X.2006. 00450.x

206) Morgan, E., Varro, r., Sepulveda, H., Ember, A. E…Gaur, A. (2004). Cytometric bead array: a multiplexed assay platform with applications of biology in various areas of biology. *Clinical immunology*. 10:3, 252-266. https://doi-org.salford.idm.oclc.org/10.1016/j.clim.2003.11.017

207) Morgan, T., E. (2017). Regulation of Drug-Metabolizing Enzymes and Drug Metabolism by Inflammatory Responses. *Academic Press.* 2(21-58). https://doi.org/10.1016/B978-0-12-802949-7.00002-X

208) Morris, M. F. C., Tahir, M., Arshid, S., Castro, s. M., & Fontes, W. (2015). Reconciling the IPC and two-hit models: Dissecting the underlying cellular and molecular mechanisms of two seemingly opposing frameworks. *Journal of immunology research.* http://dx.doi.org/10.1155/2015/697193

209) Moore, W. K., Malefyt, deW. R., Coffman, L. R., & O'Garra, A. (2001). Interleukin -10 and Interleukin-10 receptor. *Annual reviews of Immunology.* 19, 683-765. Retrieved 23/02/2019 from: https://search-proquest-com.salford.idm.oclc.org/docview/201638169/ECA0AEA21ADE4619PQ/4?accountid=8058

210) Moran, G. C., Lecky, F., Bouamra, O., Lawrence, t., Edwards, A., & Woodford, M. (2018). Changing the System - Major Trauma Patients and Their Outcomes in the NHS (England) 2008–17. *The Lancet.* 2, 13-21. DOI: https://doi.org/10.1016/j.eclinm.2018.07.001

211) Moussion, C., Ortega, N., & Girard, J-P. (2008). The IL-1-Like Cytokine IL-33 Is Constitutively Expressed in the Nucleus of Endothelial Cells and Epithelial Cells In viva: A novel alarmin? *PloS ONE* 3(10): e3331. doi: 10.1371/journal.pone.0003331

212) Muir, W. (2006). Trauma: physiology, pathophysiology, and clinical implications. *Journal of veterinary emergency and critical care.* 16(4), 253-263. doi:10.1111/j.1476-4431.2006. 00185.x

213) Muller, A. W. (2013). Getting Leukocytes to the Site of Inflammation. *Vet pathology.* 50(1), 7-22. doi:10.1177/0300985812469883.

214) Murray, P. J. (2006). Understanding and exploiting the endogenous interleukin-10/STAT3-mediated anti-inflammatory response. *Current opinion in pharmacology.* 6(4), 379-86. DOI: 10.1016/j.coph.2006.01.010

215) Murphy, K., & Weaver, C. (2017). Innate immunity: The first lines of defence. *Janeway's Immunology.* 9th ed. Garland Science. Taylors & Francis group, USA.

216) National Trauma Data Bank (2016) , Annual review, Retrieved on 12th October 2017 from: https://www.facs.org/~/media/files/quality%20programs/trauma/ntdb/ntdb%20annual%20report202016.ashx

217) Neumaier, m., & Scherer, M.A. (200). C-reactive protein levels for early detection of postoperative infection after fracture surgery in 787 patients. *Acta orthopaedica.* 79(3), 428-32. doi: 10.1080/17453670710015355.

218) National Health Services England. (2018). More than 1,600 extra trauma victims alive today says major new study. Retrieved 09/05/2019 from: https://www.england.nhs.uk/2018/08/more-than-1600-extra-trauma-victims-alive-today-says-major-new-study/

219) Nicola, N. A. (1994). Cytokine pleiotropy and redundancy: a view from the receptor. *Stem cells.* 12 Supl 193-12), 12-4. Retrieved 13/09/2018 from: https://www.ncbi.nlm.nih.gov/pubmed/7696967

220) Njoku D. B. (2010). Suppressive and pro-inflammatory roles for IL-4 in the pathogenesis of experimental drug-induced liver injury: a review. *Expert opinion on drug metabolism & toxicology*, *6*(5), 519–531. doi:10.1517/17425251003601979

221) Noben- Trauth, N., Hu-Li, J., & Paul, W. E. (2002). IL-4 secreted from individual naive $CD4^+$ T cells acts in an autocrine manner to induce Th2 differentiation. *European journal of immunology.* 32, 1428-1433.https://doi-org.salford.idm.oclc.org/10.1002/1521-4141(200205)32:5<1428: AID-IMMU1428>3.0.CO;2-0

223) Nourshargh, S. Alon, R. (2014). Leukocyte Migration into Inflamed Tissues. *Immunity.* 41(5), 694-707. https://doi.org/10.1016/j.immuni.2014.10.008

224) O'Garra, A. & Arai, N. (2000). The molecular basis of T helper 1 and T helper 2 cell differentiation. *Trends in cell biology.* 10(12), 542- 550. DOI: https://doi.org/10.1016/S0962-8924(00)01856-0

225) Okello, M., Makobore, P., Wangoda, R., Upoki, A., & Galukande, M. (2014). Serum lactate as a predictor of early outcomes among trauma patients in Uganda. International journal of emergency medicine, 7, 20. doi:10.1186/s12245-014-0020-9

226) Okeny, P. K., Ongom, P., & Kituuka, O. (2015). Serum interleukin-6 level as an early marker of injury severity in trauma patients in an urban low-income setting: a cross-sectional study. BMC emergency medicine, 15, 22. doi:10.1186/s12873-015-0048-z

227) Osterbur, K., Mann, F. A., Kuroki, K., & DeClue, A. (2014). Multiple organ dysfunction syndrome in humans and animals. *Journal of veterinary internal medicine,* 28(4),1141–1151. doi:10.1111/jvim.12364

228) Osugi, Y., Hara, J., Tagawa, S., Takai, K., & Tawa, A. (1997). Cytokine Production Regulating Th1 and Th2 Cytokines in Hemophagocytic Lymphohistiocytosis. *Blood.* 89, 4100-4103. Retrieved 07/09/2018 from: http://www.bloodjournal.org/content/89/11/4100?sso-checked=true

229) Ozaki, k., & Leonard, J. W. (2002). Cytokine and Cytokine Receptor Pleiotropy and Redundancy. The *Journal of Biological Chemistry.* 277, 29355-29358. doi: 10.1074/jbc. R200003200

230) Oshimi, K., & Hosino, S. (1992). Function and molecular structure of IL-12. *Nihon Rinsho. Japanese journal of clinical medicine.* 50(8), 1840-4. Retrieved on 17/02/2019, from: https://www.ncbi.nlm.nih.gov/pubmed/1359171

231) Palmer, S. C., Gabbe, J. B., & Cameron, A. P. (2016). Defining major trauma using the 2008 Abbreviated Injury Scale. *Injury.* 47(1), 109-115. https://doi-org.salford.idm.oclc.org/10.1016/j.injury.2015.07.003

232) Palta, S., Saroa, R., & Palta, A. (2014). Overview of the coagulation system. *Indian Journal of* Anaesthesia. 58(5), 515-523. Retrieved from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4260295

233) Palumbo, A. A., Forte, I. C., PIleri, D., Vaccarino, V., Conte, F., D'Amelio, L…Lio, D. (2012). Analysis of IL-6, IL-10 and IL-17 genetic polymorphisms as risk factors for sepsis development in burned patients. *Burns*.38(2),208-213. https://doi.org/10.1016/j.burns.2011.07.022

234) Patton, G. C., Coffey, C., Sawyer, S. M., Viner, R. M., Haller, D. M., Bose, K., Vos, T., Ferguson, J., & Mathers, C. D.(2009). Global patterns of mortality in young people: a systematic analysis of population health data. Lancet. 374(9693), 881-92. doi: 10.1016/S0140-6736(09)60741-8.

235) Paul, E. W. (2015). History of interleukin-4. *Cytokine.* 75(1), 3-7. https://doi.org/10.1016/j.cyto.2015.01.038

236) Pechous R. D. (2017). With Friends Like These: The Complex Role of Neutrophils in the Progression of Severe Pneumonia. Frontiers in cellular and infection microbiology, 7, 160. doi:10.3389/fcimb.2017.00160

237) Reikeras, O., Borgen, P., Reseland, J. E., & Lyngstadaas, S., P. (2014). Changes in serum cytokines in response to musculoskeletal surgical trauma. *BMC Research notes.*7, 128. Retrieved from: https://link-springer-com.salford.idm.oclc.org/article/10.1186/1756-0500-7-128

238) Povoa, P., Coelho, L., Almeida, E., Fernandes, A., Mealha, R., Moreira, R., & Sabino, H. (2005). C-reactive protein as a marker of infection in critically ill patients. *Clinical microbiology and infection.* 11(2), 101-108. https://doi.org/10.1111/j.1469-0691.2004.01044.x

239) Pricop, L., Redecha, P., Tieillaurd, J-L., Frey. J…Salmon, E. J. (2001). Differential Modulation of Stimulatory and Inhibitory FcγR Receptors on Human Monocytes by Th1 and Th2 Cytokines. The journal of Immunology. 166(1), 531-537. DOI: https://doi.org/10.4049/jimmunol.166.1.531

240) Proebstl, D., Voisin, M.-B., Woodfin, A., Whiteford, J., D'Acquisto, F., Jones, G. E., … Nourshargh, S. (2012). Pericytes support neutrophil subendothelial cell crawling and breaching of venular walls in vivo. The Journal of Experimental Medicine, 209(6), 1219–1234. http://doi.org/10.1084/jem.20111622

241) Ragu, S., Faye, G., Iraqui, I., Masurel-Heneman, A., Kolodner, R. D., & Huang, M. E. (2007). Oxygen metabolism and reactive oxygen species cause chromosomal rearrangements and cell death. Proceedings of the National Academy of Sciences of the United States of America, 104(23), 9747–9752. doi:10.1073/pnas.0703192104

242) Raith, E. P., Udy, A. A., Bayley, M. (2017). Prognostic Accuracy of the SOFA Score, SIRS Criteria, and qSOFA Score for In-Hospital Mortality Among Adults with Suspected Infection Admitted to the Intensive Care Unit. *JAMA*. 317(3), 290-300. doi:10.1001/jama.2016.20328

243) Ratter, J. M., Rooijackers, H., Hooiveld, G. J., Hijmans, A., de Galan, B. E., Tack, C. J., & Stienstra, R. (2018). In vitro and in vivo Effects of Lactate on Metabolism and Cytokine Production of Human Primary PBMCs and Monocytes. Frontiers in immunology, 9, 2564. doi:10.3389/fimmu.2018.02564

244) Rieder, P., Carmi, Y., Guttman, O., & Apte, N. R. (2011). IL-1α and IL-1β Recruit Different Myeloid Cells and Promote Different Stages of Sterile Inflammation. *The Journal of Immunology*. 187 (9) 4835-4843. DOI: https://doi.org/10.4049/jimmunol.1102048

245) Reikeras, O. (2010). Immune depression in musculoskeletal trauma. *Inflammation research*. 59 (6), 409-414. https://doi-org.salford.idm.oclc.org/10.1007/s00011-010-0167-7

246) Rincon, M. (2012). Interleukin-6: from an inflammatory marker to a target for inflammatory diseases. *Trends in immunology*. 33(11), 571 -577. DOI:10.1016/j.it.2012.07.003. Retrieved from: https://search-proquest-com.salford.idm.oclc.org/docview/1506966904?accountid=8058

247) Rosenthal, M. D., & Moore, F. A. (2015). Persistent inflammatory, immunosuppressed, catabolic syndrome (PICS): A new phenotype of multiple organ failure. Journal of advanced nutritional and human metabolism, 1(1), e784. doi:10.14800/janhm.784

248) Rotstein, O. D. (2003). Modelling the two-hit hypothesis for evaluating strategies to prevent organ injury after shock/resuscitation. The journal of trauma. 54(5 Suppl.): S203-206. DOI: 10.1097/01.TA.0000064512.62949.92

249) Russel, M., & Suarez, C. (2015). Hematologic Disorders and Oncologic Emergencies: Overview of Coagulation and Fibrinolysis. Critical care medicine. Retrieved 14th November 2017 from: https://clinicalgate.com/hematologic-disorders-and-oncologic-emergencies Vivo: A Novel 'Alarmin'? PLoS ONE 3(10): e3331. doi: 10.1371/journal.pone.0003331

250) Rider, P., Voronov, E., Dinarello, C.A., Apte, R. N., & Cohen, I. (2017). Alarmins: Feel the Stress. Journal of Immunology.194(4), 1395-1402. doi: 10.4049/jimmunol.1601342.

251) Saadia, R & Schein, M. (1999). Multiple organ failure. How valid is the "two hit" model? Journal of accident and emergency medicine. 16(3), 163-166. Retrieved from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1343323/

252) Sahoo, A., Wali, S., & Nurieva, R. (2016). T helper 2 and T follicular helper cells: Regulation and function of interleukin-4. Cytokine & growth factor reviews, 30, 29–37. doi: 10.1016/j.cytogfr.2016.03.011

253) Sakran, V. J., Greer, E. S., Werlin, E., & McCunn, M. (2012). Care of the injured worldwide: trauma still the neglected disease of modern society. Scandinavian journal of trauma, resuscitation and emergency medicine. 20, 64. https://doi.org/10.1186/1757-7241-20-64

254) Santin, D. A., Hemonat, L. P., Ravaggi, A., Bellone, S., & Cannon, J. M. (2000). Interleukin-10 Increases Th1 Cytokine Production and Cytotoxic Potential in Human Papillomavirus-Specific CD8+ Cytotoxic T Lymphocytes. Journal of virology. DOI: 10.1128/JVI.74.10.4729-4737.2000

255) Sapan, H. B., Paturusi, I., Jusuf, I., Patellongi, I., Massi, M. N., Pusponegoro, A. D., … Hatta, M. (2016). Pattern of cytokine (IL-6 and IL-10) level as inflammation and anti-inflammation mediator of multiple organ dysfunction syndrome (MODS) in polytrauma. International journal of burns and trauma, 6(2), 37–43. Retrieved 13 May 2019 from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4913232/

256) Saraiva, M., & O'Garra, A. (2010). The regulation of IL-10 production by immune cells. Nature reviews. Immunology. 10(3), 170-81. DOI:10.1038/nri2711

157) Schenkel, A. (2007). Leukocytomics: A Systems Biology Approach to Leukocyte Population and Molecular Dynamics. Retrieved on 23/10/2017 from: http://www.cvmbs.colostate.edu/mip/leukocytomics

258) Scenten, D., & Medzhitov, R. (2011). The control of adaptive immune responses by the innate immune system. Advances in immunology. 109, 87-124. doi: 10.1016/B978-0-12-387664-5.00003-0.

259) Schnoor, M., Alcaide, P., Voisin, M. B., & van Buul, J. D. (2015). Crossing the Vascular Wall: Common and Unique Mechanisms Exploited by Different Leukocyte Subsets during Extravasation. Mediators of inflammation, 2015, 946509. doi:10.1155/2015/946509

260) Schröder, O., Laun, R. A., Held, B., Ekkernkamp, A., & Schulte, K-M. (2004). Association of interleukin-10 promoter polymorphism with the incidence of multiple organ dysfunction following major trauma: results of a prospective pilot study. Shock.21(4), 306-310. Retrieved 2/04/2019 from: https://journals.lww.com/shockjournal/Fulltext/2004/04000/ASSOCIATION_OF_INTERLEUKIN_10_PROMOTER.3.aspx

261) Sherwin, C., Broadbent, R., Young, S., Worth, J., McCaffrey. F., Medlicott, N. J., & Reith, D. (2008). Utility of interleukin-12 and interleukin-10 in comparison with other cytokines and acute-phase reactants in the diagnosis of neonatal sepsis. *American journal of perinatology*. 25(10), 629-36. doi: 10.1055/s-0028-1090585.

262) Shimazui, T., Matsumura, Y., Nakada, T. A., & Oda, S. (2017). Serum levels of interleukin-6 may predict organ dysfunction earlier than SOFA score. Acute medicine & surgery, 4(3), 255–261. doi:10.1002/ams2.263

263) Schröder, H., Grösche, M., Adler, M., Spengler, M., & Neimeyer, C. M. (2017). Immuno-PCR with digital readout. *Biochemical and Biophysical Research Communications.*488(2). 311-315. https://doi.org/10.1016/j.bbrc.2017.04.162

264) Schrödl W, Büchler R, Wendler S, Reinhold P, Muckova P, Reindl J, Rhode H. (2016) . Acute phase proteins as promising biomarkers: Perspectives and limitations for human and veterinary medicine. Proteomic. Clinical applications. 10(11), 1077-1092. doi: 10.1002/prca.201600028.

265) Seneviratne, N., A., Sivagurunathan, B., Monaco, C. (2012). Toll-like receptors and macrophage activation in atherosclerosis. Clinica chimica acta. 413:(1-2),3-14. https://doi.org/10.1016/j.cca.2011.08.021

266) Sears, B. W., Stover, M. D., & Callaci, J. (2009). Pathoanatomy and clinical correlates of the immunoinflammatory response following orthopaedic trauma. The Journal of the American Academy of Orthopaedic Surgeons, 17(4), 255–265.

267) Semerao, F., Ammollo, T. C., & Esmon, T. C. (2011). Extracellular histones promote thrombin generation through platelet-dependent mechanisms: involvement of platelet TLR2 and TLR4. Blood. 118(7), 1952-1961. doi: 10.1182/blood-2011-03-343061.

268) Shaashua, L., Rosenne, E., Neeman, E., Sorski, L., Sominsky, L., Matzner, P., … Ben-Eliyahu, S. (2014). Plasma IL-12 levels are suppressed in vivo by stress and surgery through endogenous release of glucocorticoids and prostaglandins but not catecholamines or opioids. Psychoneuroendocrinology, 42, 11–23. doi: 10.1016/j.psyneuen.2013.12.001

269) Shabir, A., & Maqbool, M. (2017). Accuracy of SOFA Score in Predicting Outcome in Medical Patients with Various Diagnosis in Intensive Care Unit in a Tertiary Care Hospital in Northern India. International journal of contemporary medical research. 4(1), 168-172. Retrieved 06/05/2019 from: https://www.ijcmr.com/uploads/7/7/4/6/77464738/ijcmr_1210_feb_6.pdf

270) Shafi, S., Renfro, L. A., Barnes, S., Rayan, N., Gentilello, L. M., Fleming, N., & Ballard, D.(2012). Chronic consequences of acute injuries: worse survival after discharge. *The journal of trauma and acute care surgery*. 73(3), 699-703. DOI: 10.1097/TA.0b013e318253b5db

271) Shepherd, J. M., Cole, E., & Brohi, K. (2017). Contemporary Patterns of Multiple Organ Dysfunction in Trauma. Shock (Augusta, Ga.), 47(4), 429–435. doi:10.1097/SHK.0000000000000779

272) Siewe, L., Bollati-Fogolin, M., Wickenhauser, C., Krieg, T., Müller, W., & Roers, A. (2006). Interleukin-10 derived from macrophages and/or neutrophils regulates the inflammatory response to LPS but not the response to CpG DNA. *European journal of Immunology*. 36(12), 3248-55. DOI: 10.1002/eji.200636012

273) Singer, M., Deutschman, C. S., Seymour, C. W., Shankar-Hari, M…Angus, C. D. et al. The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). *JAMA.* 315(8),801–810. doi:10.1001/jama.2016.0287

274) Sobrino, J., & Shafi, S. (2013). Timing and causes of death after injuries. Proceedings (Baylor University. Medical Center), 26(2), 120–123.

275) Song, G. Y., Chung, C. S., Chaudry, I. H., & Ayala, A. (2000). IL-4-induced activation of the Stat6 pathway contributes to the suppression of cell-mediated immunity and death in sepsis. *Surgery.* 128("), 133-8. DOI: 10.1067/msy.2000.107282

276) Spangers, S., deVries, T., & Everts, V. (2016). Monocytes heterogeneity: Consequences for monocytes-derived immune cells. *Journal of immunology research*. Retrieved 27/08/2017 from:https://www.hindawi.com/journals/jir/2016/1475435/.

277) Spolarics, Z., Siddiqi, M., Siegel, J. H., Garcia, Z. C., Stein, D. S., Denny, T., & Deitch, E. A. (2003). Depressed interleukin-12-producing activity by monocytes correlates with adverse clinical course and a shift toward Th2-type lymphocyte pattern in severely injured male trauma patients*. Critical care medicine*. 31(6),1722-9. DOI: 10.1097/01.ccm.0000063579. 43470.aa

278) Strecker, W., Gebhard, F., Perl, M., Rager, J., Buttenshon, K Kinzl, L., & Beck, A. (2003). Biochemical characterization of individual injury pattern and injury severity. *Injury*. 34(12), 879-887. https://doi.org/10.1016/S0020-1383(03)00022-6

279) Subatovic, M., Filipovic, N., Radakovic, S., Stankovic, N., & Slavcovic, Z. (2007). Immune cytokine response in combat casualties: blast or explosive trauma with or without secondary sepsis. *Military medicine.* 172(2), 190-195. DOI: 10.7205/milmed.172.2.190

280) Suter, M. P., Suter, s., Giardin, E., Roux-Lombard, P., Grau, E.G., & Dayer, J-M. (1991). High Bronchoalveolar Levels of Tumor Necrosis Factor and Its Inhibitors, Interleukin-1, Interferon, and Elastase, in Patients with Adult Respiratory Distress Syndrome after Trauma, Shock, or Sepsis. *American review of respiratory disease.* 145(5), https://doi.org/10.1164/ajrccm/145.5.1016

281) Stensballe, J., Christiansen, M., Tonnesen, E., Espersen, K., Lippert, F. K., & Rasmussen, L.S. (2009). The early IL-6 and IL-10 response in trauma is correlated with injury severity and mortality. *Acta Anaesthesiologica Scandinavica.* 53(4).https://doi-org.salford.idm.oclc.org/10.1111/j.1399-6576.2008.01801.xft

282) Stern, A. S., Magram, J., & Persky, D. H. (1996). Interleukin-12 an integral cytokine in the immune response. *Life sciences.* 58(8), 639-54. Retrieved 17/02/2019 from: https://www.ncbi.nlm.nih.gov/pubmed/8594314

283) Sternbach, L. G. (2000). The Glasgow coma scale. *The Journal of emergency medicine.*19(1), 67-71. DOI: https://doi.org/10.1016/S0736-4679(00)00182-7

284) Surbatovic, M., Popovic, N., Vojvodic, D., Milosevic, I…Radakovic, S. (2015). Cytokine profile in severe gram-positive and gram-negative abdominal sepsis. *Scientific reports.* 5, 11355. Retrieved from: https://www-nature-com.salford.idm.oclc.org/articles/srep11355

285) Takahashi, W., Nakada, T. A., Yazaki, M., & Oda, s. (2016). Interleukin-6 Levels Act as a Diagnostic Marker for Infection and a Prognostic Marker in Patients with Organ Dysfunction in Intensive Care Units. *Shock*. doi: 10.1097/SHK.0000000000000616.

286) Tarmey, T. N., & Kirkman, E. (2016). Coagulopathy and Inflammation: An Overview of Blast Effects. *Blast Injury Science and Engineering*. 229-237. https://doi-org.salford.idm.oclc.org/10.1007/978-3-319-21867-0_19

287) Tato, C. M., Laurence, A., & O'Shea, J. J. (2006). Helper T cell differentiation enters a new era: le roi est mort; vive le roi! *The Journal of experimental medicine*, *203*(4), 809–812. doi:10.1084/jem.20060522

288) The Trauma audit & Research Network. (2017). Major trauma in older people – 2017 Report. Retrieved on 12th October 2017 from: https://www.tarn.ac.uk/Content.aspx?c=3793

289) The Trauma audit & Research Network. (2019). Performance Comparison: Trauma Care. Trauma Care in England and Wales. Retrieved 13 April 2019 from: https://www.tarn.ac.uk/Content.aspx?ca=15

290) The Royal college of surgeons of England (2009). Regional Trauma Systems: Interim guidance for commissioners. *Publisher: RCSENG - Professional Standards and Regulation.* Retrieved from: https://www.rcseng.ac.uk/library-and-publications/rcs-publications/docs/regional-trauma-systems-interim-guidance-for-commissioners/,

291) Theodorsson, E. (1988). BASIC computer program to summarize data using nonparametric and parametric statistics including Anderson-Darling test for normality. *Computer Methods and Programs in Biomedicine.* 26(2), 207-213. https://doi-org.salford.idm.oclc.org/10.1016/0169-2607(88)90046-6

292) Thermo Fisher Scientific. Overviews of ELISA. Retrieved 26/02/2019 from: https://www.thermofisher.com/uk/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/overview-elisa.html

293) Torre, D., Tambini, R., Aristodemo, S., Gavazzeni, G., Goglio, A., Cantamessa, C., … Biondi, G. (2000). Anti-inflammatory response of IL-4, IL-10 and TGF-beta in patients with systemic inflammatory response syndrome. Mediators of inflammation, 9(3-4), 193–195. doi:10.1080/09629350020002912

294) Trinchieri, G. (2003). Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nature reviews. Immunology.* 3(2), 133-46. DOI:10.1038/nri1001

295) Tsao, C-M., Ho, S-T., & Wu, C_C. (2015). Coagulation abnormalities in sepsis. *Acta anaesthesiologica Taiwanica.* 53(1), 16-22. https://doi.org/10.1016/j.aat.2014.11.002

296) University of Florida Health, (2018). Traumatic injury. Retrieved 21/11/2018 from: https://ufhealth.org/traumatic-injury

297) Valparaiso, P. A., Vicente, A.D., Bograd, A. B., Elster, A. E., & Davis, A. T. (2014). Modelling acute traumatic injury. *Journal of surgical research.*194, 220-232. doi.org/10.1016/j.jss.2014.10.025

298) Van Wessem, P. J. K., & Leenen, P.H.L. (2017). Reduction in mortality rates of postinjury multiple organ dysfunction syndrome: A shifting paradigm? *Shock.* DOI: 10.1097/SHK.0000000000000938

299) Vanzant, E. L., Lopez, C. M., Ozrazgat-Baslanti, T., Ungaro, R., Davis, R., Cuenca, A. G., … Efron, P. A. (2014). Persistent inflammation, immunosuppression, and catabolism syndrome after severe blunt trauma. The journal of trauma and acute care surgery, 76(1), 21–30. doi: 10.1097/TA.0b013e3182ab1ab5

300) Verma, R., Balakrishnan, L., Sharma, K., Khan, A. A…. Shankar, S. (2016). A network of Interleukin-10 signalling pathway. *Journal of cell commun signal. 10, 61-67.* DOI 10.1007/s12079-015-0302-x

301) Vincent, J.L., Moreno, R., Takala, J., Willats, S., & Thijs, G. L. (1996). The SOFA (Sepsis-related Organ Failure Assessment) score to describe organ dysfunction/failure. *Intensive Care Med* (1996) 22: 707. https://doi-org.salford.idm.oclc.org/10.1007/BF01709751

302) Volpin, G., Cohen, M., Assaf, M., Meir, T., Katz, R., & Pollack, S. (2014). Cytokine levels (IL-4, IL-6, IL-8 and TGFβ) as potential biomarkers of systemic inflammatory response in trauma patients. International orthopaedics, 38(6), 1303–1309. doi:10.1007/s00264-013-2261-2

303) Watford, T. W., Moriguchi, M., Morinobu, A., & O'Shea, J. J. (2003). The biology of Il-12: coordinating innate and adaptive immune responses. *Cytokine & Growth factor reviews.*14, 361-368. doi:10.1016/S1359-6101(03)00043-1

304) Wang, S., Voisin, M.-B., Larbi, K. Y., Dangerfield, J., Scheiermann, C., Tran, M., … Nourshargh, S. (2006). Venular basement membranes contain specific matrix protein low expression regions that act as exit points for emigrating neutrophils. The Journal of Experimental Medicine, 203(6), 1519–1532. http://doi.org/10.1084/jem.20051210

305) Werman, A., Werman-Venkert, R., White, R., Lee, J.-K., Werman, B., Krelin, Y., … Apte, R. N. (2004). The precursor form of IL-1α is an intracrine proinflammatory activator of transcription. Proceedings of the National Academy of Sciences of the United States of America, 101(8), 2434–2439. http://doi.org/10.1073/pnas.0308705101

306) Wick, M., Kolliq, E., Walz, M., Muhr, G., & Koller, M. (2000). Does liberation of interleukin-12 correlate with the clinical course of polytraumatized patients? *Der chirurg.* 71(9), 1126-1131. Retrieved from: https://www.ncbi.nlm.nih.gov/pubmed/11043131

307) Wolf, J., Rose-John, S., & Garbers, C. (2014). Interleukin-6 and its receptors: A highly regulated and dynamic system. *Cytokine.*70(1), 11-20. https://doi.org/10.1016/j.cyto.2014.05.024

308) Woodcock, T., & Morganti-Kossmann, M. C. (2013). The role of markers of inflammation in traumatic brain injury. *Frontiers in neurology*, *4*, 18. doi:10.3389/fneur.2013.00018

309) World health organisation. (2018). Road traffic injuries. Retrieved from: https://www.who.int/news-room/fact-sheets/detail/road-traffic-injuries

310) Varro R., Chen R., Sepulveda H., Apgar J. (2007) Bead-Based Multianalyte Flow Immunoassays. In: Albitar M. (eds) Monoclonal Antibodies. Methods in Molecular Biology, vol 378. Humana Press. DOI https://doi-org.salford.idm.oclc.org/10.1007/978-1-59745-323-3_9

311) Vincent, J. L., de Mendonca, A., Cantraine, F., Moreno, R., Takala, J., Suter, P. M…Blecher, S. (1998). Use of the SOFA score to assess the incidence of organ dysfunction/failure in intensive care units: results of a multicentre, prospective study. Working group on ‘‘sepsis-related problems’’ of the European Society of Intensive Care Medicine. *Critical CareMedicine.*26(11),1793Y1800. PMID:9824069

312) Voll, R. E., Urbonaviciute, V., Herrmann, M., & Kalden, R. J. (2008). The Mosaic of Autoimmunity. *Israel medical association journal.* 10(1), 26-28. Retrieved from: https://www.ima.org.il/MedicineIMAJ/viewarticle.aspx?year=2008&month=01&page=26

313) Voisin, M.B. & Nourshargh, S. (2013). Neutrophil transmigration: emergence of an adhesive cascade within venular walls*. Journal of innate immunity*. 5(4), 336-347. doi: 10.1159/000346659

314) Young, S-H., Antonini, M. J., Roberts, R. J., Erdely, d. a., ZIedler-Erdely, C. P. (2008). Performance evaluation of cytometric bead assays for the measurement of lung cytokines in two rodent models. *Journal of immunological methods*. 331:1-2, 59-68. https://doi-org.salford.idm.oclc.org/10.1016/j.jim.2007.11.004

315) Xiao, W., Mindrinos, M. N., Seok, J., Cuschieri, J., Cuenca, A. G., Gao, H. (2011). Inflammation and Host Response to Injury Large-Scale Collaborative Research Program. A genomic storm in critically injured humans. *The Journal of experimental medicine*, 208(13), 2581–2590. doi:10.1084/jem.20111354

316) Xu, W., & Song, Y. (2017). Biomarkers for patients with trauma associated acute respiratory distress syndrome. Military Medical Research, 4, 25. doi:10.1186/s40779-017-0134-5

317) Zedle, S., & Faist, E. (2006). The impact of endogenous triggers on trauma- associated inflammation. *Current opinion in critical care.* 12(6), 595-601. doi: 10.1097/MCC.0b013e3280106806

318) Zhang, J.-M., & An, J. (2007). Cytokines, Inflammation and Pain. International Anesthesiology Clinics, 45(2), 27–37. http://doi.org/10.1097/AIA.0b013e318034194e

319) Zhang, Q., Itagaki, K., & Hauser, C. J. (2010). Mitochondrial DNA is released by shock and activates neutrophils via p38 map kinase. *Shock.* 34(1): 55-59. doi: 10.1097/SHK.0b013e3181cd8c08.

320) Zhang, Y., Zhang, Y., Gu, W., & Sun, B. (2014). Th1/Th2 Cell Differentiation and Molecular Signals. In: Sun B. (eds) T Helper Cell Differentiation and Their Function. *Advances in Experimental Medicine and Biology*, vol 841. Springer, Dordrecht. DOI: 10.1007/978-94-017-9487-9_9

321) Zhang, a., Gu, w., Zeng, L., Zhang, L., Du, D…Jiang, J. (2015). Genetic Variants of microRNA Sequences and Susceptibility to Sepsis in Patients with Major Blunt Trauma. *Annals of surgery.* 261(1), 198-196. doi: 10.1097/SLA.0000000000000687

322) Zhi, H., Xie, J., & Skare, J. T. (2018). The Classical Complement Pathway Is Required to Control Borrelia burgdorferi Levels During Experimental Infection*. Frontiers in immunology*, 9, 959. doi:10.3389/fimmu.2018.00959

323) Zinkernagel, R. M. (2015). Interactions between cells of the immune system. Medical biology. retrieved 04/09/2018 from: http://almerja.com/reading.php?idm=23133

324) Zundler, S., & Neurath, F. M. (2015). Interleukin -12: Functional activities and implications for diseases. *Cytokine & Growth facto reviews.* 26(5), 559-568. https://doi.org/10.1016/j.cytogfr.2015.07.003

325) University of Strathclyde. (2019). New rapid test for sepsis could save thousands of lives. Retrieved 28/02/2019 from: https://www.strath.ac.uk/whystrathclyde/news/rapidtestforsepsisdevelopedbystrathclyderesearcherscouldsavethousandsoflives/

CHAPTER 7: 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 – Clinical data collection sheet.

Day			Date		
HR		BP	__/__	Temp	__C◦
Hb		WCC		PLT	
eGFR		Creatinine		Billirubin	
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			
Antibiotic treatment	Y/N	Source of sepsis		__empirical/unknown	

Appendix 3 – Day 1 - clinical data for the patients recruited on Central Manchester Foundation Trust.

0	Day 1	Sys BP	Dia BP	MAP	Temp	Hb	WCC	PLT	eGFR	Creat	Bili	PT	Intubated	NIV / CPA	FiO2	P/F	Lactate	Norad	CRP
BIT001	115	110	60	76.66667	37.8	134	31.6	356	42	132	34	15.6	N	N	0.21	N	1.2	0	19
BIT002	125	122	84	96.66667	38	122	18.6	142	50	134	28	16.6	N	N	0.28	38.2	3.9	0	63
BIT003	94	70	45	53.33333	36	123	11.5	180	55	116	6	15.4	N	N	0.3	N	N	0	2
BIT004	129	101	61	74.33333	36.5	112	16.2	213	76	85	7	14.4	N	N	0.21	63.1	0.9	0	102
BIT005	82	135	85	101.6667	37.9	110	8.4	199	90	70	7	15.9	N	N	0.28	N	0.4	0	32
BIT006	84	111	71	84.33333	36.2	79	9.8	472	81	74	3	15.4	N	N	0.21	N	N	0	15
BIT007	110	126	66	86	37.5	140	19	298	90	76	12	14.7	N	N	0.35	48.3	0.9	0	60
BIT008	110	85	60	68.33333	37.7	99	5.9	152	65	69	13	20.8	Y	N	0.21	58	2	0	23
BIT009	90	102	55	70.66667	37.9	108	22	232	81	101	15	16.3	N	N	0.28	N	N	0	6
BIT010	116	99	35	56.33333	37.8	130	9.9	286	80	68	7	13.7	N	N	0.3	21.6	1.3	0	100
BIT011	90	103	71	81.66667	36.4	114	15.4	318	27	158	8	19.7	N	N	0.4	N	N	0	117
BIT014	112	102	56	71.33333	36.2	78	16.6	129	78	88		16.3	Y	N	0.4	29.2	3.4	0	8
BIT016	117	72	48	56	36.3	115	26.6	150	50	126	9	15.7	N	N	0.35	34.7	3.5	0	5
BIT017	127	94	63	73.33333	36.2	120	10.9	222	72	83	6	13.7	Y	N	0.6	71.9	1.7	0	6
BIT018	109	175	90	118.3333	35.4	146	16.8	260	90	49	6	11.3	N	N	1	N	0.7	0	89
BIT021	146	90	70	76.66667	35.2	109	33.5	235	50	146	10	14.2	Y	N	0.3	50	3.4	0	1
BIT022	97	117	75	89	35.6	128	27.1	408	90	79	5	10.8	N	N	0.28	N	N	0	3
BIT023	98	88	65	72.66667	35.7	112	22.1	156	83	91	18	12.3	N	N	0.32	43.6	1.1	0	17
BIT024	115	74	40	51.33333	38.2	83	16.4	155	90	56	8	11.2	Y	N	0.6	62.3	3.8	0	170
BIT025	122	85	45	58.33333	33.9	97	16.1	130	31	241	20	11.9	N	Y	0.35	9.34	6.3	0.07	345
BIT027	129	163	65	97.66667	38	100	28.8	203		111	61	10.7	Y	N	1	27.6	1.4	0	30
BIT028	130	89	50	63	35	87	16	191	43	166	4	11.2	N	N	0.4	N	N	0	38
BIT029	94	159	84	109	35.5	119	6.8	132	90	74	28	11.2	N	N	1	N	N	0	226
BIT034	75	88	55	66	37.8	129	9.7	235	26	229	10	11.4	N	N	0.6	24.2	1.4	0	N
BIT035	89	168	93	118	36.2	139	14	295	67	83	15	11.2	N	N	0.85	N	N	0	43
BIT040	110	109	88	95	36.1	131	33.2	226	80	99	11	11.4	N	N	0.6	61.6	1.5	0	10
BIT042	99	135	67	89.66667	38.4	118	19.5	213	N	93	5	11.9	N	N	0.21	N	N	0	1
BIT043	109	79	30	46.33333	37.7	117	19	194	81	101	9	12	N	N	0.35	33.4	3.3	0	
BIT044	126	135	81	99	35.9	136	14	237	59	128	5	11.4	N	N	0.21	N	2.2	0	1
BIT046	92	80	50	60	35.5	73	29.6	78	59	81	16	13.4	N	N	0.85	31.4	1.8	0	6
BIT047	160	77	56	63	35	147	28.7	248	67	114	12	12.4	Y	N	0.7	41.9	4.8	1.01	75
BIT048				0		145	27.1	287	81	91	12	12.1	y	n					71
BIT049	123	177	77		35.1	121	16.4	129	89	102	27	12	n	n	0.32	39.2	7.7	0	1
BIT050	97	123	59		35/6	149	8.7	359	64	116	10	11.5	y	n	0.28	0	2	0	20
BIT052	112	151	76		36.5	94	13.3	161	66	88	12	13	y	n	0.35	40.3	4.1	0	2
BIT053	85	103	52	69	34.6	117	8.1	154	62	77	7	11.5	n	n	0.85	30.6	1.7	0	32
BIT055	88	165	75	105	38.2	105	8.9	146	82	81	18	12.3	n	n	0.28	49.8	1.6	0	38
BIT060	130	70	40	50	35.7	80	13.9	101	45	146	25	12.7	Y	N	0.95	27.7	5.4	0.19	
BIT061	116	180	105	130	38.1	128	14.4	220	89	63	10.9	11	y	n	0.8	19.9	2	0	18
BIT064	95	148	93	111.3333	35.6	134	19.4	154	90	69	6	10.9	n	n	0.85	n	2.8	0	1
BIT065	100	106	61	76	35.6	123	29.1	338	90	47	19	11.2	N	N	0.24	44	0.7	0	41
BIT066	ZZ?	162	85	110.6667	35	138	13.2	178	81	83	8	11.9	n	n	0.28	n	n	0	3
BIT069	112	95	55	68.33333	35.5	104	19.7	257	79	104	15	11.9	y	n	0.85			0	4
BIT070	110	178	117	137.3333	35.8	143	26.9	339	89	88	11	11.8	y	n				0	n
BIT072	53	138	72	94	35.6	130	6.8	242	n	76	20	12.6	n	n	0.21	n	n	0	1
BIT073	53	138	72	94	35.6	129	16	210	56	87	32	11.7	n	n	0.7	9.79	2	0	116
BIT074	90	130	90	103.3333	36.2	110	11.3	238	90	65	13	11.4	n	n	0.21			0	29
BIT076	112	87	52	63.66667	35.7	105	17.3	381	46	93	<3	11.1	y	n	0.3	30.2	2.8	0.11	20
BIT030	71	70	48	55.33333	36.2	113	6	146	90	67	5	10	y	n	0.85	n	0.8	0	4
BIT078	88	102	51	68	35.7	118	16.4	174	83	92	11	11.2	y	y	0.85	32.4	2.6	0.11	9
BIT079	144	219	136	163.6667	38.3	155	16	240	70	102	25	12.2	y	n	0.35	n	7.6	0	272
BIT084	78	108	59	75.33333	36.1	80	11	168	70	102	11	12.5	y	n	0.5	n/k	n/k	n	40
BIT087				0		138	17.7	199	90	82	9	11.7							
BIT088				0		135	38.5	409	76	111	30	10.9							
BIT091				0		87	9.1	235	30	152	10								8
BIT092				0		136	143	274	90	89	12								61
BIT093				0		84	21.2	259	38	165	18								
BIT094		60	25	36.66667	36.2	123	4.2	307	40	137		11.8							
BIT095	103	147	22	63.66667	36.6	119	28	200	28	230	7	11.6	N				0.7		
BIT096				0		146	20.7	141	90	74	13	10.6							
BIT098	107	100	66	77.33333	36.7	152	11	301				10.8	N				2.9		
BIT099	95	130	90	103.3333		144	5	187	90	50			N						
BIT100				0		98	3.9	289	90	86		11.4	N	N			10.3		
BIT102	82	110	60	76.66667	37	165	12.8	233	46	136	11	11.2	Y	N	40%		0.8		
BIT103	121	84	53	63.33333	33.1	85	3.2	142	61	120	3	10.4	Y	N	50	49	15		3
BIT104	90	100	50	66.66667	37.4	89	11.6	195	90	43	6	10.3	N	N	21%		0.7		15
BIT105	118	93	52	65.66667	37	102	11.6	171	90	99			N	N	60		2		5
BIT107	99	77	44	55	38	113	4.8	134	65	101	30	11	Y	N	70	11.25	12.6	0.1	7
BIT109	100	94	54	67.33333	35.8	107	6.6	57	90	50	16	12.8	N	N	21		2		
BIT111	75	130	62	84.66667	36.9	83	6.8	216	80	66	10		Y	N	30				111
BIT120	98	158	98	118	37.5	126	12.7	199	>90	69	15	10.7	N	N	35%	n/a	2.4	n/a	15
BIT121	54	109	75	86.33333	36.8	104	11.2	135	90	64	NA	10	N	N	50%	NA	0.7	NA	32
BIT123	135	61	44	49.66667	37	113	22.9	137	>90	86	16	10.9	Y	N	70%	17.9	1.9	3	7
BIT124	109	177	76	109.6667	35.5	110	13.3	211	69	70	7	9.8	N	N	35%	31.4	1.1	N	37
BIT125	59	94	37	56	35.5	98	11.1	446	83	79	11	11	n	N	60	16	2.4	N	N
BIT126	113	168	83	111.3333	38.1	125	23	147	74	68	16	10.8	N	N	60	n/a	2.7	n	80
BIT127	91	118	69	85.33333	36.8	101	10.4	197	77	90	N	11	N	N	40	NA	NA	n	28
BIT128	108	140	90	106.6667	35.4	121	22.1	198	61	108	6	n/a	N	Y	60	27	3.3	n	39
BIT129	59	93	88	89.66667	37.2	119	9.3	200	>90	58	15	12	N	N	32	34	2.9	0	37

	CRP	CVVH/HD	Sedated	GCS	Antibiotic	Septic source	MAP SOFA	PLT SOFA	CREAT SOFA	BILI SOFA	RESP SOFA	GCS SOFA	SOFA DAY 1
BIT001	19	N	N	15	Y		0	0	1	2	0	0	3
BIT002	63	N	N	15	Y		0	1	1	1	2	0	5
BIT003	2	N	N	15	Y		1	0	1	0	0	0	2
BIT004	102	N	N	15	N		0	0	0	0	0	0	0
BIT005	32	N	N	15	N		0	0	0	0	0	0	0
BIT006	15	N	N	15			0	0	0	0	0	0	0
BIT007	60	N	N	15	Y		0	0	0	0	1	0	1
BIT008	23	N	Y	N	Y	empirical	1	0	0	0	0	0	1
BIT009	6	N	N	14	Y		0	0	0	0	0	1	1
BIT010	100	N	N	15	N		1	0	0	0	3	0	4
BIT011	117	N	N	15	N		0	0	1	0	0	0	1
BIT014	8	N	Y	N	Y	empirical	0	1	0	0	2	0	3
BIT016	5	N	N	15	Y		1	1	1	0	2	0	5
BIT017	6	N	Y	N	Y	empirical	0	0	0	0	0	0	0
BIT018	89	N	N	15	Y	empirical	0	0	0	0	0	0	0
BIT021	1	N	N	15	Y	empirical	0	0	1	0	1	0	2
BIT022	3	N	N	15	Y	empirical	0	0	0	0	0	0	0
BIT023	17	N	N	14	Y	empirical	0	0	0	0	1	1	2
BIT024	170	N	Y	N	Y		1	0	0	0	0	0	1
BIT025	345	N	N	15	Y	empirical	3	1	2	1	4	0	11
BIT027	30	N	Y	N	Y	empirical	0	0	1	2	2	0	5
BIT028	38	N	N	15	N		1	0	1	0	0	0	2
BIT029	226	N	N	15	Y	empirical	0	1	0	1	0	0	2
BIT034	N	N	N	15	N		1	0	2	0	3	0	6
BIT035	43	N	N	15	N		0	0	0	0	0	0	0
BIT040	10	N	N	15	Y	empirical	0	0	0	0	0	0	0
BIT042	1	N	N	15	Y	empirical	0	0	0	0	0	0	0
BIT043		N	N	15	N		1	0	0	0	2	0	3
BIT044	1	N	N	15	Y	empirical	0	0	1	0	0	0	1
BIT046	6	N	N	15	Y	empirical	1	2	0	0	2	0	5
BIT047	75	N	Y	N	Y	empirical	4	0	1	0	1	0	6
BIT048	71	n	y		y	e	1	0	0	0	4	4	9
BIT049	1	n	n	y15	y	empirical	1	1	0	1	2	0	5
BIT050	20	n	y		y	empirical	1	0	1	0	4	4	10
BIT052	2	n	y		y	empirical	1	0	0	0	1	4	6
BIT053	32	n	n	15	y	empirical	1	0	0	0	2	0	3
BIT055	38	n	n	15	y	empirical	0	1	0	0	1	0	2
BIT060		N	Y	N	Y	empirical	4	1	1	1	2	0	9
BIT061	18	n	y		y	empirical	0	0	0	0	3	4	7
BIT064	1	n	n	15	y	empirical	0	0	0	0	0	0	0
BIT065	41	N	N	13	N		0	0	0	0	1	1	2
BIT066	3	n	n	15	y	empirical	0	0	0	0	0	0	0
BIT069	4	n	y		y	empirical	1	0	0	0	4	4	9
BIT070	n	n	y		y	empirical	0	0	0	0	4	4	8
BIT072	1	n	n	14	y	eye	0	0	0	1	0	1	2
BIT073	116		n	15	y	empirical		0	0	1	4	0	5
BIT074	29	n	n	15	y	empirical	0	0	0	0	4	0	4
BIT076	20	n	y	3	y	empirical	4	0	0	4	2	4	14
BIT030	4	n	y		y	e	1	1	0	0	0	4	6
BIT078	9	n	y	3	y	emp	4	0	0	0	2	4	10
BIT079	272	n	y	15	y	emp	0	0	0	1	0	0	1
BIT084	40	n	y	15	y	emp	4	0	0	0	0	0	4
BIT087					N		1	0	0	0	4	4	9
BIT088					Y	empirical	1	0	1	1	4	4	11
BIT091	8				Y	empirical	1	0	1	0	4	4	10
BIT092	61	N					1	0	0	0	4	4	9
BIT093						empirical	1	0	1	0	4	4	10
BIT094		N		15	Y	unknown	1	0	1	0	4	0	6
BIT095				15	Y	empirical	1	0	2	0	4	0	7
BIT096							1	1	0	0	4	4	10
BIT098				15			0	0	0	0	4	0	4
BIT099				15			0	0	0	0	4	0	4
BIT100					Y	Empirical	1	0	0	0	4	4	9
BIT102		N		10			0	0	1	0	4	2	7
BIT103	3	N				Empirical	1	1	1	0	1	4	8
BIT104	15	N		15		Empirical	1	0	0	0	4	0	5
BIT105	5	N					1	0	0	0	4	4	DISCHARGED
BIT107	7	N		3		Empirical	3	1	0	1	4	4	13
BIT109		N				Empirical	1	2	0	0	4	4	11
BIT111	111	N		15			0	0	0	0	4	0	4
BIT120	15	N	N	15	Y	empirical	0	0	0	0	na	0	0
BIT121	32	N	N	3	Y	Emiprical	0	1	0	NA	0	3	4
BIT123	7	n	Y	15	n		3	1	0	0	3	0	7
BIT124	37	N	N	15	Y	Emiprical	0	0	0	0	2	0	2
BIT125	N	N	N	15/15	N	n/a	1	0	0	0	2	0	3
BIT126	80	N	N	15/15	N	n/a	0	0	0	0	0	0	0
BIT127	28	N	N	15/15	N	n/a	0	0	0	0	0	0	0
BIT128	39	N	N	15/15	N	n/a	0	0	0	0	2	0	2
BIT129	37	N	N	15/15	Y	empirical	0	0	0	0	2	0	2

Appendix 4 - Day 5 - clinical data for the patients recruited on Central Manchester Foundation Trust.

	HR	Sys BP	Dia BP	MAP	Temp	Hb	WCC	PLT	eGFR	Creat	Bili	PT	Intubated	NIV / CPA	FiO2	P/F	Lactate	Norad	CRP
BIT001	94	111	78	89	37.2	102	10.1	356	90	84	N	N	N	N	0.21	N	N	0	18
BIT002	107	131	79	96.33333	36.6	91	7.5	191	90	65	18	16.9	N	N	0.28	45.2	0.8	0	244
BIT003	110	85	65	71.66667	36.6	83	6.3	211	86	78	13	14	N	N	0.21	N	N	0	113
BIT004	117	92	76	81.33333	37.3	102	12	221	90	45	17	15	N	N	0.21	53.6	0.5	0	329
BIT005	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	0	N
BIT006	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	0	N
BIT007	85	115	85	95	36.5	122	7.7	217	90	61	10	13.1	N	N	0.21	N	N	0	55
BIT008	108	102	62	75.33333	35.7	85	20.3	199	90	48	22	12.5	Y	N	0.28		1.3	0	210
BIT009	80	115	47	69.66667	37.1	83	7.6	233	90	91	15	14.7	N	N	0.21	N	N	0	95
BIT010	124	126	94	104.6667	36.2	114	6.7	270	90	38	7	13.6	N	N	0.4	N	N	0	181
BIT011	98	115	60	78.33333	36.9	N	N	N	N	N	N	N	N	N	0.32	N	N	0	
BIT014	103	130	64	86	35.9	91	10.6	357	90	78	N	N	N	N	N	N	N	0	194
BIT016	100	113	70	84.33333	36.4	83	10	125	58	111	11	14.1	N	N	0.4	29.8	1.4	0	174
BIT017	112	140	65	90	37.7	82	6.2	85	90	60	8	13.6	N	N	N	N	N	0	124
BIT018	113	176	96	122.6667	37.6	111	8.5	228	90	53	13	10.7	Y	N	0.4	25.8	1.2	0	92
BIT021	98	133	78	96.33333	36	109	9.7	339	90	70	9	10.4	N	N	0.21	N	N	0	26
BIT022	54	130	75	93.33333	36.1	N	N	N	N	N	N	N	N	N	N	N	N	0	
BIT023	80	111	58	75.66667	37.1	N	N	N	N	N	N	N	N	N	0.21	N	N	0	
BIT024	95	105	50	68.33333	36.7	82	9	198	90	52	6	9.9	Y	N	0.3	45.4	0.6	0	
BIT025	75	96	46	62.66667	34.7	92	14.9	166	83	79	16	11.1	N	N	0.6	22.1	1.4	0	
BIT027	66	116	80	92	36.4	118	11.9	350	90	75	N	N	N	N	0.21	N	N	0	33
BIT028	60	132	65	87.33333	37.7	96	9	346	90	70	11		N	N	0.21	N	N	0	105
BIT029	86	107	69	81.66667	35.4	N	N	N	N	N	N	N	N	N	0.21	N	N	0	N
BIT034	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	0	N
BIT035	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	0	N
BIT040	117	134	82	99.33333	38.5	103	12.2	409	90	86	21	N	N	N	0.24	N	N	0	309
BIT042	106	127	73	91	37.4	101	8.6	239	N	64	8	11.3	N	N	0.21	N	N	0	150
BIT043	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	0	N
BIT044	N	N	N	#VALUE!	N	N	N	N	N	N	N	N	N	N	N	N	N	Norad	N
BIT046	110	86	37	53.33333	35.8	80	48.7	84	83	60	24	13.4	N	N	0.5	23.5	0.9	0	181
BIT047	112	139	65	89.66667	38.2	86	7.1	179	90	76	22	11.4	N	N	0.28	43.9	0.8	0	227
BIT048				0		74	12.7	245	19	321	12	11	y	n					350
BIT049	97	147	81		37.2	123	8.2	248	90	71	8	n	n	n	0.21	n	n	0	3
BIT050	98	115	74	87.66667	36.1	119	7.2	339	90	69	11	n	n	n	0.28	n	n	0	267
BIT052	98	132	87	102	35.9	n	n	n	n	n	n	n	n	n	0.21	n	n	0	n
BIT053	105	155	72		37.5	102	6.2	207	90	48	n	11	n	n	0.32	n	n	0	123
BIT055	90	98	63	74.66667	37.5	n	n	n	n	n	n	n	n	n	0.21	n	n	0	n
BIT060	125	105	50	68.33333	37.7	77	6.5	165	75	94	15	10.8	Y	N	0.6	11.3	1.2	0	
BIT061	97	140	55	83.33333	35.8	82	8.8	213	90	38	14	11.1	n	n	0.35	27.4	0.9	0	152
BIT064	DISCHARGED			0															
BIT065	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	0	N
BIT066	87	145	72		35.7	129	9.9	261	90	62	13	n	n	n	0.21	n	n	0	53
BIT069	110	149	88	108.3333	37.1	125	8.4	242	90	67	7	11.1	n	n	0.28	63.7	1.1	0	n
BIT070	56	145	63	90.33333	36	123	10.6	349	78	98	n	n	n	n	0.21	n	n	0	n
BIT072	104	136	71	92.66667	35.7	n	n	n	n	n	n	n	n	n	n	n	n	0	n
BIT073	83	131	83		36.9	127	5.6	254	76	67	7	n	n	n	0.28	n	n	0	22
BIT074	89	137	77	97	37.8	n	n	n	n	n	n	n	n	n	0.21	n	n	0	n
BIT076	121	112	70	84	37.8	89	7.8	282	>90	48	4	n/d	n	n	0.24	n	n	n	n
BIT030	74	91	52	65	35.9	n	n	n	n	n	n	n	n	n	0.21	n	n	0	n
BIT078	105	141	70	93.66667	37.9	101	6.2	217	>90	80	n	n	n	n	0.21	n	n	n	127
BIT079	n/k	n/k	n/k	#VALUE!	n/k	96	7.5	239	86	85	9	10.7	n	n	n/k	n	n/k	n	151
BIT084	n/k	n/k	n/k	#VALUE!	n/k	78	20.7	404	>90	57	9	n/d	n	n	n/k	n/k	n/k	0	243
BIT087	DISCHARGED			0															
BIT088				0		85	15.5	293	90	86	19	10.5							
BIT091				0		79	10	262	17	128	7								
BIT092				0		100	11.6	233	90	71	7								
BIT093				0		94	12.6	313	88	79	26								103
BIT094				0		108	4.8	45	90	77									
BIT095	DISCHARGED			0															
BIT096				0															
BIT098	97	140	86	104	36.9	124	16.9	247	90	59	15	11.1	N	N					
BIT099				0		132	8.8	182	90	53	31		N	N					178
BIT100	105	88	64	72	37.3	84	1.6	155	84	98	8		N	N					
BIT102	91	100	70	80	36.6	126	11.1	185	67	98	15	10.9	Y	N	40%		0.8		251
BIT103	109	115	63	80.33333	36.8	79	12.4	158	90	65	13	10.3	N	N	35		0.9		350
BIT104	80	102	56	71.33333	39.2	62	8.6	302	90	49	8	11	N	N	21				110
BIT105				0															
BIT107	113	94	50	64.66667	38	75	9.1	125	64	102	21	11	Y	N	60		1	0.4	372
BIT109	101	140	79	99.33333	36.4	73	3.4	88	90	35	31		N	Y	21%		1.5		
BIT111				0		79	6.4	207	90	47	10		N	N					
BIT120	60	142	82	102	36.2	127	5.1	266	>90	65	12	10.5	N	N	35%	n/a	n/a	n/a	n/a
BIT121	55	112	43	66	36.3	104	NA	185	90	60	N	9.4	N	N	21%	N	NA	N	NA
BIT123	111	141	84	103	36.2	101	9.1	292	>90	54	16	NA	N	N	28%	N	NA	NA	110
BIT124	87	171	70	104	35.7	122	7.2	190	90	47	10	9.9	N	N	35%	N	NA	N	107
BIT125	66	91	34	53	36.8	84	10.9	256	87	76	14	10	N	N	21	27	1.7	N	N
BIT126				0				n											
BIT127	78	103	64	77	37.1	95	5.3	217	90	79	N	NA	N	N	28	N	NA	0	186
BIT128	136	137	72	93.66667	37.9	71	31.1	391	41	152	56	10	Y	N	80	16	1		289
BIT129	64	116	68	84	37.2	123	7.6	217	90	58	N	NA	N	N	21%	N	N		NA

	CVVH/HD	Sedated	GCS	Antibiotic	Septic sou	Steroids	CAM +ve	MAP SOFA	PLT SOFA	CREAT SOFA	BILI SOFA	RESP SOFA	GCS SOFA	SOFA DAY 5
BIT001	N	N	15	Y		N	N	0	0	0	0	0	0	0
BIT002	N	N	15	Y		N	N	0	0	0	0	1	0	1
BIT003	N	N	15	Y		N	N	0	0	0	0	0	0	0
BIT004	N	N	15	N		N	N	0	0	0	0	0	0	0
BIT005	N	N	N	N	N	N	N	0	0	0	0	0	0	0
BIT006	N	N	N	N	N	N	N	0	0	0	0	0	0	0
BIT007	N	N	15	N		N	N	0	0	0	0	0	0	0
BIT008	N	Y	N	Y	Abdomen	Y	N	0	0	0	1	4	0	5
BIT009	N	N	15	Y		N	N	1	0	0	0	0	0	1
BIT010	N	N	15	Y	Chest	N	N	0	0	0	0	0	0	0
BIT011	N	N	15	N		N	N	0	0	0	0	0	0	0
BIT014	N	N	15	N		N	N	0	0	0	0	0	0	0
BIT016	N	N	15	Y		N	N	0	1	1	0	2	0	4
BIT017	N	N	15	N		N	N	0	2	0	0	0	0	2
BIT018	N	Y	N	Y	empirical	N	N	0	0	0	0	3	0	3
BIT021	N	N	15	N		N	N	0	0	0	0	0	0	0
BIT022	N	N	15	N		N	N	0	0	0	0	0	0	0
BIT023	N	N	15	N		N	N	0	0	0	0	0	0	0
BIT024	N	Y	N	Y		N	Y	1	0	0	0	1	0	2
BIT025	N	N	15	N		N	N	1	0	0	0	3	0	4
BIT027	N	N	15	Y	POST OP	N	N	0	0	0	0	0	0	0
BIT028	N	N	15	N		N	N	0	0	0	0	0	0	0
BIT029	N	N	15	N		N	N	0	0	0	0	0	0	0
BIT034	N	N	N	N	N	N	N	0	0	0	0	0	0	0
BIT035	N	N	N	N	N	N	N	0	0	0	0	0	0	0
BIT040	N	N	15	Y	empirical	N	N	0	0	0	1	0	0	1
BIT042	N	N	15	N		N	N	0	0	0	0	0	0	0
BIT043	N	N	N	N	N	N	N	0	0	0	0	0	0	0
BIT044	N	N	N	N	N	N	N	N	N	N	N	N	N	N
BIT046	N	N	13	N	empirical	N	N	1	2	0	1	3	1	8
BIT047	N	N	15	N		N	N	0	0	0	1	1	0	2
BIT048	n	y		y	empirical	n	n	1	0	3	0	4	4	12
BIT049	n	n	15	n		n	n	1	0	0	0	0	0	1
BIT050	n	n	15	n		n	n	0	0	0	0	0	0	0
BIT052	n	n	15	n		n	n	0	0	0	0	0	0	0
BIT053	n	n	15	n		n	n	1	0	0	0	0	0	1
BIT055	n	n	15	n		n	n	0	0	0	0	0	0	0
BIT060	N	Y	N	Y	empirical	Y	Y	1	0	0	0	4	0	5
BIT061	n	n	15	y	empirical	n	n	0	0	0	0	2	0	2
BIT064								1	4	0	0	4	4	13
BIT065	N	N	N	N	N	N	N	0	0	0	0	0	0	0
BIT066	n	n	n	n		n	n	1	0	0	0	0	0	1
BIT069	n	n	15	n		n	n	0	0	0	0	0	0	0
BIT070	n	n	15	y	empirical	n	n	0	0	0	0	0	0	0
BIT072	n	n	15	y	eye	y	n	0	0	0	0	0	0	0
BIT073	n	y	15	y	empirical	y	n	1	0	0	0	0	0	1
BIT074	n	n	15	y	empirical	n	n	0	0	0	0	0	0	0
BIT076	n	n	15	y	emp	n	n	4	0	0	0	0	0	4
BIT030	n	n	15	n		n	n	1	0	0	0	0	0	1
BIT078	n	n	15	y	emp	n	n	4	0	0	0	0	0	4
BIT079	y	n	15	y	emp	n	n	4	0	0	0	0	0	4
BIT084	n	n	15	y	abdo	n	n	#VALUE!	0	0	0	0	0	0
BIT087								1	4	0	0	4	4	13
BIT088								1	0	0	0	4	4	9
BIT091								1	0	1	0	4	4	10
BIT092								1	0	0	0	4	4	9
BIT093				Y	spleen			1	0	0	1	4	4	10
BIT094	N		15	Y	CHEST			1	3	0	0	4	0	8
BIT095								1	4	0	0	4	4	13
BIT096								1	4	0	0	4	4	13
BIT098				Y	empirical			0	0	0	0	4	4	8
BIT099	N	N	15					0	1	0	1		0	1
BIT100								0		0	0			
BIT102	N	N		Y	empirical	N		0	0	0	0	1		
BIT103	N	N						0	4	0	0	2		
BIT104	N	N	15					1	4	0	0	2	0	7
BIT105														
BIT107	N	Y	3					4	2	0	1	0	4	11
BIT109	N	N	15					0	3	0	1	2	0	6
BIT111	N	N	15					0	0	0	0		0	
BIT120	N	N	15	N	n/a	N	N	4	0	0	0	0	0	4
BIT121	N	N	15	Y	EMPIRICA	N	N	0	0	0	0	0	0	0
BIT123	N	N	15	N	N	N	N	0	0	0	0	0	0	0
BIT124	N	N	15	Y	Wound	N	N	0	0	0	0	0	0	0
BIT125	N	N	15	N	N	N	N	1	0	0	0	2	0	3
BIT126								1	0	0		4		SR 067
BIT127	N	N	15	N	N	N	N	0	0	0	0	0	0	0
BIT128	N	Y	3	Y	empirical	N	N	0	0	1	2	3	4	10
BIT129	N	N	15	Y	Empirical	N	N	0	0	0	0	0	0	0

Appendix 5- Day 8 - clinical data for the patients recruited on Central Manchester Foundation Trust

HR	Sys BP	Dia BP	MAP	Temp	Hb	WCC	PLT	eGFR	Creat	Bili	PT	Intubated	NIV / CPAP	FiO2	P/F	Lactate	Norad	CRP
80	117	78	91	36.9	108	27.5	460	90	86	N	N	N	N	0.21	N	N	0	88
111	134	95	108	36.6	91	12.5	184	90	64	29	16.9	N	N	0.35	N	N	0	219
89	93	53	66.33333333	37.1	N	N	N	N	N	N	N	N	N	0.21	N	N	0	N
92	114	64	80.66666667	36.6	N	N	N	N	N	N	N	N	N	0.21	N	N	0	N
N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	0	N
N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	0	N
75	134	65	88	36.3	N	N	N	N	N	N	N	N	N	0.21	N	N	0	N
113	121	68	85.66666667	36	94	29.5	241	90	36	23	13.8	Y	N	0.4	N	1.3	0	52
80	119	63	81.66666667	36.9	96	10.5	337	90	79	19	15.2	N	N	0.21	N	N	0	
125	134	100	111.3333333	37.3	121	9.6	546	90	44	6	14.2	N	N	0.35	N	N	0	45
91	121	68	85.66666667	36.9	73	15.8	123	59	79		15.1	N	N	0.21	N	N	0	74
100	112	72	85.33333333	36.2	101	15.2	481	88	90	N	N	N	N	N	N	N	0	158
93	97	66	76.33333333	35.9	105	11.9	233	64	101	16	14.4	N	N	0.28	43.3	1.3	0	134
N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	0	N
105	151	94	113	35.7	94	6.4	193	90	41	9	11.4	N	N	0.28	N	N	0	206
N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	0	N
N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	0	N
90	105	57	73	37.8	N	N	N	N	N	N	N	N	N	21	N	N	0	
99	110	65	80	37.3	107	N	N	N	N	N	N	N	N	0.21	N	0.6	0	
87	83	45	57.66666667	35.3	80	14.2	329	75	86	9	10.7	N	N	8	19.2	1.1	0	1
N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	0	N
N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	0	N
N	104	68	80	36	N	N	N	N	N	N	N	N	N	0.21	N	N	0	N
N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	0	N
N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	0	N
112	149	73	98.33333333	39	100	17.3	708	90	82	26	N	N	N	0.28	N	N	0	329
			0															
N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	0	N
N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	Norad	N
154	86	55	65.33333333	35.9	77	44	96	90	54	96	15	N	N	0.45	20.7	1.6	0	73
120	150	62	91.33333333	38.8	72	8.7	302	90	68	40	11.5	N	N	0.28	52.1	0.8	0	N
			0		84	20.4	270	12	470	18	10.9	y	n					563
76	126	63	84	36.2	n	n	n	n	n	n	n	n	n	0.21	n	n	0	n
86	101	57	71.66666667	36.3	125	7.1	351	90	64	13	n	n	n	0.21	n	n	0	166
104	139	74	95.66666667	36.2	n	n	n	n	n	n	n	n	n	0.21	n	n	0	n
95	160	95	116.6666667	37	101	3.6	100	n	n	n	n	n	n	0.32	n	n	0	n
71	80	51	60.66666667	37.4	n	n	n	n	n	n	n	n	n	0.21	n	n	0	n
118	106	46	66	37.3	92	14.6	317	90	80	17	10.6	Y	Y	0.65	12.7	1.3	0.07	
99	140	77	98	35.5	n	n	n	n	n	n	n	n	n	0.21	n	n	0	n
			0															
N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	0	N
61	140	65	90	35.6	n	n	n	n	n	n	n	n	n	0.21	n	n	0	n
56	120	69		36.5	n	n	n	n	n	n	n	n	n	0.21	n	n	0	n
81	132	73		35.5	n	n	n	n	n	n	n	n	n	0.21	n	n	0	n
DISCHARGED			0															
n			0	n	126	5.9	271	68	74	n	11.4	n	n	0.21	n	n	0	15
100	133	81	98.33333333	37.5	115	11.3	470	90	72	10	n	n	n	0.21	n	n	0	70
111	132	83	99.33333333	37.1	93	10	404	>90	46	n	11.3	n	n	n	n	n	n	302
88	127	58	81	36.2	n	n	n	n	n	n	n	n	n	0.21	n	n	0	n
99	146	79	101.3333333	37.3	n	n	n	n	n	n	n	N	n	0.21	n	n	n	n
n/k			0	n/k	87	11.1	198	36	179	7	12.2	n	n	n/k	n	n/k	n	170
n/k			0	n/k	74	18	624	>90	54	9	n/d	n	n	n/k	n/k	n/k	0	295
			0															
			0		101	15.6	644	90	78	19	10.5							
			0		66	12.7	386	17	179	5								69
			0		90	8.9	281	90	65	6								
			0		94	12.6	413	78	88	25	11							88
DISCHARGED			0															
			0															
			0															
93	123	77	92.33333333	36.4	122	11.1	389	90	63	15	10.9		N					
99	104	78	86.66666667	36.5	113	7	219	90	43			N	N					225
105	88	64	72	37.2	103	18.4	475	81	101	13		N	N					
75	104	53	70	36	133	9.1	274	82	83	11		N	N	4075				
109	115	61	79	36.7	80	15	293	90	58	9		N				0.5		284
80	102	56	71.33333333	37	119	10.3	611	90	52	12	11	N		0.21				82
			0															
121	87	47	60.33333333	38.1	77	18.7	553	75	89	11	11	y	N	0.4	34.75	0.7		309
109	136	70	92	36	87	1.4	111	90	35	23		N	N	0.4				67
			0															
80	126	72	90	35.9	n/a	n/a	n/a	n/a	n/a	n/a	n/a	N	N	0.21	n/a	n/a	n/a	n/a
	DISCHARGED HOME.		#VALUE!															
109	126	74	91.33333333	35	103	9.5	323	>90	54	15	10.4	N	N	0.21	N	NA	NA	108
95	140	68	92	35.8	NO BLOODS DONE							N	N	0.21	NA	NA	N	N
62	112	53	72.66666667	37.9	90	12.6	454	79	83	NA	11.3	N	N	21	NA	NA	N	45
SR 067			0															
94	102	62	75.33333333	36.6	103	5.4	348	90	79			N	N	21	NA	NA	N	NA
136	66	51	56	38.2	82	40	272	17	322	81	12	Y	N	100	11	3.9	1.54	130
63	111	57	75	37.8	NO BLOODS DONE							N	N	0.21	NA	NA	N	NA

CVVH/HD	Sedated	GCS	Antibiotic	Septic sou	Steroids	CAM +ve	Tranexam	Transfusion	ISS	MAP SOFA	PLT SOFA	CREAT SOFA	BILI SOFA	RESP SOFA	GCS SOFA	SOFA DAY 8
N	N	15	N	N	N	N	N	N	14	0	0	0	0	0	0	0
N	N	15	Y	N	N	N	N	N	29	0	0	0	1	0	0	1
N	N	15	N	N	N	N	N	Y	25	1	0	0	0	0	0	1
N	N	15	N	N	N	N	Y	Y	25	0	0	0	0	0	0	0
N	N	N	N	N	N	N	N	N	33	0	0	0	0	0	0	0
N	N	N	N	N	N	N	N	N	N	0	0	0	0	0	0	0
N	N	15	N	N	N	N	N	N	20	0	0	0	0	0	0	0
N	Y	N	Y	Abdomen	Y	N	N	Y	8	0	0	0	1	0	0	1
N	N	15	N	N	N	N	N	N	29	0	0	0	0	0	0	0
N	N	15	Y	Chest	N	N	N	N	26	0	0	0	0	0	0	0
N	N	15	N	N	N	N	N	Y	25	0	1	0	0	0	0	1
N	N	15	N	N	N	N	Y	Y	16	0	0	0	0	0	0	0
N	N	15	N	N	N	N	N	N	29	0	0	0	0	1	0	1
N	N	N	N	N	N	N	N	N	4	0	0	0	0	0	0	0
N	N	15	Y	empirical	N	N	N	N	41	0	0	0	0	0	0	0
N	N	N	N	N	N	N	N	N	16	0	0	0	0	0	0	0
N	N	N	N	N	N	N	N	N	25	0	0	0	0	0	0	0
N	N	15	N	N	N	N	Y	N	9	0	0	0	0	0	0	0
N	N	14	Y	N	N	N	N	Y	29	0	0	0	0	0	1	1
N	N	15	Y	Chest	N	N	N	N		1	0	0	0	3	0	4
N	N	N	N	N	N	N	N	N	30	0	0	0	0	0	0	0
N	N	N	N	N	N	N	Y	Y	16	0	0	0	0	0	0	0
N	N	15	N	N	N	N	N	N	20	0	0	0	0	0	0	0
N	N	N	N	N	N	N	N	N	N	0	0	0	0	0	0	0
N	N	N	N	N	N	N	N	N	17	0	0	0	0	0	0	0
N	N	15	Y	empirical	N	N	N	N	25	0	0	0	1	0	0	1
							Y	N	14	1	4	0	0	4	4	13
N	N	N	N	N	N	N	N	N	19	0	0	0	0	0	0	0
N	N	N	N	N	N	N	Y	N	14	4	0	0	0	0	0	4
N	N	14	Y	empirical	N	N	Y	Y	36	1	2	0	2	3	1	9
N	N	15	N		Y	N	Y	Y	10	0	0	0	2	1	0	3
y	y		y	empirical	y	n	y	y	Not coded	1	0	4	0	4	4	13
n	n	15	n		n	n	y	n	35	0	0	0	0	0	0	0
n	n	15	n		n	n	y	n	9	0	0	0	0	0	0	0
n	n	15	n		n	n	y	y	36	0	0	0	0	0	0	0
n	n	15	n		n	n	y	y	25	0	1	0	0	0	0	1
n	n	15	n		n	n	y	y	N	1	0	0	0	0	0	1
N	Y	12	N		Y	Y	Y	Y	n	3	0	0	0	4	2	9
n	n	15	n		n	n	y	y	n	0	0	0	0	0	0	0
							y	n	9	1	4	0	0	4	4	13
N	N	N	N	N	N	N	N	N	N	0	0	0	0	0	0	0
n	n	15	n		n	n	n	n	n	0	0	0	0	0	0	0
n	n	15	n		n	n	y	y	Noyt yet	1	0	0	0	0	0	1
n	n	15	y	empirical	n	n	y	n	Not yet	1	0	0	0	0	0	1
DISCHARGED			y	eye	y	n	n	n	Rejected	1	4	0	0	4	4	13
n	n	15	y	empirical	y	n	y	n	Not yet	1	0	0	0	0	0	1
n	n	15	y	empirical	n	n	n	n	Not yet	0	0	0	0	0	0	0
n	n	15	n		n	n	y	y	Not yet	4	0	0	0	0	0	4
n	n	15	n		n	n	y	y		0	0	0	0	0	0	0
n	n	15	n		n	n	y	y		4	0	0	0	0	0	4
y	n	15	n	n	n	n	n	n		4	0	2	0	0	0	6
n	n	15	y	abdo	n	n	y	y		1	0	0	0	0	0	1
										1	4	0	0	4	4	13
										1	0	0	0	4	4	9
										1	0	2	0	4	4	11
										1	0	0	0	4	4	9
										1	0	0	1	4	4	10
DISCHARGED										1	4	0	0	4	4	13
										1	4	0	0	4	4	13
										1	4	0	0	4	4	13
			Y	empirical	N					0	0	0	0	4	4	8
N	N	15								0	0	0	0	4	0	4
			Y					Y		0	0	0	0	4	4	8
							Y	Y								
N	N				N		Y	Y								
N	N	15														
N	Y	3	Y	empirical			Y	Y								
N	N	15	Y	chest			Y									
N	N	15	N	n/a	N	N	N	N		4	0					
									17		4					
N	N	15	N	N	N	N	N	N	17	0	0	0	0	0	0	0
N	N	15	Y	WOUND	N	N	N	Y	32	0	4	NA	NA	0	0	4
N	N	15	N	N	N	N	N	N	not coded	4	0	0	NA	0	0	4
SR 067									20	1	0				4	5
N	N	15	N	N	N	N	y	y	Not coded	0	0	0	0	0	0	0
Y	Y	3	Y	empirical	Y	N	N	N		4	0	3		4	4	15
N	N	15	Y	eMPIRICA	N	N	Y	Y	9	4	NO BLOODS DONE			0	0	4

Appendix 6 - Day 1 – clinical data for the patients recruited on Salford Royal Foundation Trust

	Day 1																	
	HR	Sys BP	Dia BP	Noradrenaline	MAP	MAP SOFA	PLT	PLT SOFA	Creat(µmol/l)	Creat SOFA	Bili (µmol/l)	Bili SOFA	P/F	P/F (mm Hg)	RESP SOFA	GCS	GCS SOFA	SOFA DAY 1
BIT SR001	115	110	60	0	76.66666667	0	356	0	132	1	34	2	38.2	290.32	2	15	0	5
BIT SR002	84	149	59	5	167	4	161	0	47	0	7	0		0	4	3	4	12
BIT SR003	126	138	80	30	206	4	186	0	127	1	12	0	0.22	1.672	4	N	0	9
BIT SR007	110	158	60	0	172	0	205	0	58	0	13	0	11.32	86.032	4	11	2	6
BIT SR008	132	119	80	0	93	0	169	0	53	0	11	0	n	N	0	13	1	1
BIT SR009	108	209	139	0	162	0	307	0	84	0	13	0	n	N	0	6	3	3
BITSR010	101	147	78	0	101	0	250	0	79	0	10	0	n	N	4	3	4	8
BITSR011	84	125	50	5	75	4	193	0	84	0	3	0	n	N	4	N	0	8
BITSR012	97	180	101	0	127	0	124	1	75	0	14	0	N	N	4	3	4	9
BITSR013	95	105	74	0	84	0	235	0	59	0	6	0		N	4	3	4	8
BIT SR014	58	158	61		93	0	246	0	54	0	8	0	375.05	2850.38	0	N	0	0
BITSR015	70	120	80	0	93.3	0	175	0	69	0	7	0	N	N	0	15	0	0
BIT SR018	92	104	97	0	99.3	0	169	0	76	0	7	0	n	N	0	15	0	0
BIT SR019	101	126	59	12	81.3	4	251	0	85	0	9	0	N	N	4	3	4	12
BIT SR020	76	132	66	5.5	88	4	237	0	75	0	22	1	N	N	4	3	4	13
BIT SR021	69	110	60	6	76.7	4	87	2	49	0	23	1	N	N	0	14	1	8
BIT SR022	101	121	65	0	83.7	0	121	1	56	0	15	0	N	N	4	N	0	5
BIT SR023	53	110	52	6	71.3	4	165	0	42	0	5	0	N	N	4	3	4	12
BIT SR024	103	110	50	0	70	0	246	0	57	0	7	0	32.9	250.04	4	3	4	8
BIT SR025	50	111	41	0	64.3	1	308	0	53	0	12	0	21.3	161.88	3	N	0	4
BIT SR026	101	102	71	0	81.3	0	348	0	130	1	8	0	172	1307.2	0	3	4	5
BIT SR027	86	130	68	0	88.7	0	78	2	67	0	28	1	N	N	4	6	3	10
BIT SR028	58	91	49	0	63	1	242	0	79	0	21	1	N	N	0	14	1	3
BIT SR029	100	114	70	0	84.67	0	310	0	51	0	9	0	N	N	0	14	1	1
BIT SR030	117	113	64	2	93.67	4	276	0	120	1	11	0	N	N	4	3	4	13
BIT SR031	80	123	53	6	76.33	4	162	0	47	0	5	0	N	N	4	3	4	12
BIT SR032	95	115	60	0	78.33	0	208	0	62	0	21	1	N	N	0	15	0	1
BIT SR033	90	136	62	0	86.67	0	185	0	102	0	12	0	N	N	0	15	0	0
BIT SR034	104	121	73	4	89	4	109	1	148	1	15	0	N	N	4	3	4	14
BIT SR035	60	97	48	0	64.33	1	198	0	68	0	13	0	N	N	0	14	1	2
BIT SR036	60	136	72	7	93.33	4	202	0	61	0	5	0	N	N	4	3	4	12
BIT SR037	54	118	95	0	102.67	0	212	0	66	0	12	0	N	N	4	3	4	8
BIT SR039	63	114	58	0	76.67	0	82	2	97	0	16	0	N	N	0	15	0	2
BIT SR041	126	110	80	0	90	0	207	0	82	0	21	1	N	N	4	N	0	5
BIT SR044	106	60	98	2	75.33	4	116	1	97	0	28	1	0.5	3.8	4	15	0	10
BIT SR045	66	98	50	0.1	65	3	193	0	192	2	10	0	N	N	4	3	4	13
BIT SR046	102	161	69		99.7	0	248	0	71	0	12	0	2	15.2	4	15	0	4
BIT SR047	95	150	72	N	94	4	214	0	81	0	19	0	0.23	1.748	4	15	0	8
BIT SR051	85	123	55	0.03	78	3	270	0	67	0	14	0	0.56	4.256	4	ND	0	7
BIT SR052	72	123	58	N	75	4	200	0	80	0	14	0	0.4	3.04	4	3	4	12
BIT SR053	100	128	99	N	108.67	4	274	0	95	0	7	0	N	N	0	15	0	4
BIT SR054	79	138	50	N	68	4	327	0	57	0	5	0	0.26	1.976	4	14	1	9
BIT SR055	94	130	70	N	90	4	146	1	51	0	7	0	0.36	2.736	4	8	3	12
BIT SR056	99	143	82	N	102	4	328	0	102	0	7	0	ND	N	0	15	0	4
BIT SR057	58	165	88	N	115	4	177	0	45	0	11	0	0.51	3.876	4	15	0	8
BIT SR058	94	135	82	N	99.67	4	236	0	77	0	13	0	ND	N	0	14	1	5
BIT SR060	100	130	67	N	88	4	307	0	86	0	17	0	ND	N	0	15	0	4
BIT SR061	73	120	39	0.26	64	4	211	0	57	0	12	0	ND	N	4	3	4	12
BIT SR062	106	102	56	N	72	4	174	0	62	0	29	1	ND	N	4	6	3	12
BIT SR063	70	105	58	N	73.67	4	156	0	56	0	6	0	0.22	1.672	4	7	3	11
BIT SR064	55	120	60	0.03	80	3	196	0	68	0	16	0	0.25	1.9	4	15	0	7
BIT SR066	86	122	58	0.33	78	4	129	1	70	0	12	0	0.33	2.508	4	3	4	13
BIT SR067	MRI					1		4		0		0		N	0		4	9
BIT SR069	46	130	58	10	76	4	171	0	89	0	7	0	0.42	3.192	4	3	4	12
BIT SR070	76	126	74		91.33	0	213	0	57	0	14	0	0.51	3.876	4	13	1	5
BIT SR071	44	135	67		89.67	0	160	0	61	0	19	0	0.22	1.672	4	3	4	8
BIT SR073	66	166	77	0.18	107	4	185	0	67	0	12	0	0.68	5.168	4	14	1	9
BIT SR074	95	116	60		78.67	0	205	0	56	0	4	0	ND	N	0	13	1	1
BIT SR076	72	178	60	0.133	94	4	235	0	66	0	24	1	0.57	4.332	4	3	4	13
BIT SR077	72	129	76		93	0	233	0	75	0	4	0	0.58	4.408	4	5	4	8
BIT SR078	85	135	57		83	0	269	0	63	0	6	0	0.31	2.356	4	15	0	4
BIT SR079	94	140	70	0	93.33333333	0	171	0	101	0	8	0	0.34	2.584	4	15	0	4
BIT SR080	72	150	90	0	110	0	220	0	72	0	23	1	0.4	3.04	4	13	1	6
BIT SR081	55	168	58	0.098	94.66666667	3	229	0	62	0	10	0	0.405	3.078	4	3	4	11
BIT SR082	100	110	52	0.288	71.33333333	4	267	0	101	0	18	0	0.446	3.3896	4	6	3	11
BIT SR083	114	107	54	0.062	71.66666667	3	267	0	101	0	18	0	0.432	3.2832	4	3	4	11
BIT SR084	88	125	68	0	87	0	259	0	68	0	16	0	0.39	2.964	4	15	0	4
BIT SR085	90	182	94	0	123.3333333	0	327	0	78	0	4	0	0.23	1.748	4	14	1	5
BIT SR086	84	105	55	No Weight	71.66666667	4	188	0	67	0	30	1	0.62	4.712	4	15	0	9

	Temp	Hb	WCC	eGFR	PT	Intubated	NIV / CPAP	FiO2	Lactate	CRP	CVVH/HD	Sedated	Antibiotics	Septic source
BIT SR001	37.8	134	31.6	42	15.6	N	N	0.21	1.2	19	N	N	Y	empirical/unknown
BITSR002	38.7	108	10.9	N	11.7	Y	N	98%	3.6	N	N	Y	N	N
BITSR003	39.1	136	28.5	62	12.1	Y	N	80%	2.3	N	N	Y	N	N
BITSR007	36.7	132	7	>90	12.8	N	N	28%	0.9	N	N	N	N	N
BITSR008	35	104	8.8	>90	11.9	N	N	21%	1	N	N	N	N	N
BITSR009	37.1	97	14.4	60	9.9	N	N	100%	4.3	N	N	N	N	N
BITSR010	37.5	134	18.3	>90	12.4	Y	N	25%	7.1	N	N	Y	N	N
BITSR011	38.2	127.9	15.4	>90	10.7	Y	N	25%	2.9	N	N	Y	Y	N
BITSR012	37	134	13.7	85	12.4	Y	N	21%	1	N	N	Y	N	N
BITSR013	36.1	85	8.5	85	11.2	Y	N	40%	1.8	N	N	Y	N	N
BITSR014	37.7	145	15.6	>90	14.1	Y	N	21%	2.1	N	N	Y	Y	N
BITSR015	36.8	115	11.4	73	11.7	N	N	30%	N	4.5	N	N	N	N
BITSR018	36.8	110	7	>90	13.3	N	N	0%	2	N	N	N	Y	5
BITSR019	37.6	89	15	87	13.1	Y	N	21%	1.6	N	N	Y	Y	6
BITSR020	36.9	134	17.1	N	13.9	Y	N	21%	2.2	N	N	Y	N	N
BITSR021	36.6	104	9.5	N	13.6	N	N	21%	2	N	N	N	Y	6
BITSR022	38	104	10	>90	13.5	Y	N	21%	1.8	N	N	Y	Y	6
BITSR023	36.7	130	15.2	>90	11.5	Y	N	30%	1.5	N	N	Y	Y	1
BITSR024	36.7	135	10.2	>90	13.1	Y	N	40%	1.7	N		Y	Y	6
BITSR025	33.1	95	6.7	>90	16.6	Y	N	40%	2.7	N	N	Y	N	N
BITSR026	36.4	155	30.8	N	12.4	Y	N	21%	1.7	N	N	Y	Y	
BITSR027	37.9	103	9.1	>90	13.7	Y	N	21%	3.1	N	N	N	N	N
BITSR028	37.8	134	16.6	89	11.6	N	N	21%	1.2	N	N	N	Y	6
BITSR029	37	127	16.5	>90	11.9	N	N	21%	3.9	N	N	N	Y	6
BITSR030	36.7	124.1	14.6	53	11.7	Y	N	30%	6.6	N	N	Y	N	N
BITSR031	37.9	125.8	9.6	N	12.7	Y	N	21%	3.9	N	N	Y	N	N
BITSR032	36.4	118	6.1	>90	11.4	N	N	21%	0.8	N	N	N	Y	6
BITSR033	37.5	106	11.4	73	11.6	N	N	21%	1.7	N	N	N	Y	6
BITSR034	38.7	94	16.8	32	12.6	Y	N	30%	4.8	N	N	Y	Y	6
BITSR035	38.6	135	16.3	>90	11.6		Y	40%	1.3	N	N	N	Y	6
BITSR036	36.6	132.9	8.4	>90	12.8	Y	N	40%	2.6	N	N	Y	Y	6
BITSR037	37	116	9.2	89	12.2	Y	N	25%	0.25	N	N	Y	Y	1
BITSR039	37.4	117	11.5	64	14.2	N	N	40%	2.6	N	N	N	N	N
BITSR041	39.2	130.1	15.9	81	15.4	Y	N	35%	1.5	N	N	Y	Y	6
BITSR044	36.5	85	10	N	11.6	N	N	21%	3	58	N	N	Y	6
BITSR045	37	121	10.4	24	14.4	Y	N	40%	1.4	N	N	Y	Y	1
BITSR046	38.2	157	12.9	>90	11.1	N	N	50%	3.4	N	N	N	N	N
BITSR047	37.5	134	15.2	87	11.6	N	N	40%	1.6	N	N	N	N	N
BITSR051	37.2	121	11.6	79	12	Y	N	21%	2.4	N	N	Y	N	N
BITSR052	38	118	8.9	>90	13.4	Y	N	21%	0.9	ND	N	Y	Y	6
BITSR053	36.8	160	16.6	73	12	N	N	21%	1.9	ND	N	Y	Y	6
BITSR054	37.7	113	10	ND	11.5	Y	N	21%	4.6	ND	N	Y	N	N
BITSR055	36.4	135	14.3	>90	14.1	Y	N	30%	1.1	ND	N	Y	N	N
BITSR056	37.2	145	12.9	ND	12.4	N	N	21%	ND	ND	N	N	Y	6
BITSR057	36.3	150	12.7	ND	11.1	N	N	21%	0.9	ND	N	N	Y	6
BITSR058	37.1	128	14.6	>90	ND	N	N	25%	1.1	ND	N	N	Y	6
BITSR060	36	128	12.7	76	11.4	N	N	21%	1.5	ND	N	N	Y	1
BITSR061	36.9	104	17.8	89	ND	Y	N	28%	5	ND	N	Y	N	N
BITSR062	36	96	18.8	>90	10.8	Y	N	30%	0.4	ND	N	Y	Y	6
BITSR063	37.2	124	14.8	>90	12.2	Y	N	25%	2.77	ND	N	Y	N	N
BITSR064	38	146	8.1	>90	11.4	N	N	21%	1.1	ND	N	N	N	N
BITSR066	36.7	82	19	>90	12.8	Y	N	35%	3.8	ND	N	Y	Y	6
BITSR067														
BITSR069	37.8	134	13.1	78	171	Y	N	0.3	4.07	ND	N	Y	Y	6
BITSR070	37	121	9.4	>90	12.7	N	N	0.21	1	ND	N	N	N	N
BITSR071	35.5	134	19.4	>90	12.4	Y	N	0.5	1.2	ND	N	Y	Y	N
BITSR073	36.6	104	14.4	>90	11.5	N	N	0.28	1.5	ND	N	N	N	N
BITSR074	35.9	113	7.7	>90	10.8	N	N	0.21	1.5	ND	N	N	Y	6
BITSR076	36.4	139	18.2	>90	13	Y	Y	0.21	0.9	ND	N	Y	Y	6
BITSR077	36.9	137	8.6	>90	10.2	Y	N	0.25	1.32	ND	N	N	N	N
BITSR078	37.8	101	6.5	82	12.4	N	N	0.35	0.8	ND	N	N	Y	1
BITSR079	36.9	99	10.9	46	10.9	N	N	0.21	1.7	N	N	N	Y	6
BITSR080	36.3	138	20.4	90	13.4	N	N	0.25	N	N	N	N	N	N
BITSR081	37	138	16	>90	12	Y	N	0.3	N	32	N	Y	N	N
BITSR082	36.8	136	20	N	12.3	Y	N	0.5	4.1	N	N	Y	Y	6
BITSR083	38.3	136	20	N	12.3	Y	N	0.25	2.8	N	N	Y	N	N
BITSR084	37.8	149	13.7	>90	12.4	N	N	0.24	0.83	N	N	N	N	N
BITSR085	38.6	139	11.6	61	30	N	N	0.3	1.9	36	N	N	N	N
BITSR086	35.9	121	20.7	N	17	N	N	0.21	3.9	N	N	N	Y	6

Appendix 7 - Day 5 – clinical data for the patients recruited on Salford Royal Foundation Trust.

	Day 5																	
	HR	Sys BP	Dia BP	Norad	MAP	MAP SOFA	PLT	PLT SOFA	Creat	Creat SOFA	Bili	Bili SOFA	P/F	P/F	RESP SOFA	GCS	GCS SOFFA	SOFA DAY 5
BITSR001	94	111	78	0	89	0	356	0	84	0	N	0	40	304	1	15	0	1
BITSR002	100	169	81	N		4	195	0	57	0	9	0	N	N	4	5	4	12
BITSR003	134	172	82	9	112	4	176	0	85	0	10	0	0.34	2.584	4	N	0	8
BITSR007	67	130	72	0	187	0	309	0	60	0	15	0	N	N	0	14	1	1
BITSR008	74	112	50	0	70	0	207	0	207	2	N	0	N	N	0	15	0	2
BITSR009	123	162	52	0	88	0	188	0	110	1	3	0	N	N	4	N	0	5
BITSR010	96	150	70	10	96	4	194	0	59	0	5	0	N	N	4	N	0	8
BITSR011	85	125	61	0	82	0	174	0	46	0	10	0	N	N	0	15	0	0
BITSR012	122	137	58	N	84	4	192	0	76	0	6	0	N	N	0	13	1	5
BITSR013	RIP																	RIP
BITSR014	83	150	50	7	83.3	4	328	0	51	0	7	0	295.03	2242.228	0	N	0	4
BITSR015	68	120	80	0	93.3	0	163	0	58	0	9	0	N	N	0	15	0	0
BITSR018	71	124	64	0	84	0	N	0	N	0	N	0	N	N	0	15	0	0
BITSR019	90	171	64	0	99.7	0	225	0	55	0	10	0	N	N	4	3	4	8
BITSR020	76	152	59	4	90	4	228	0	48	0	6	0	N	N	4	N	0	8
BITSR021	67	111	43	0	65.7	1	104	1	35	0	19	0	N	N	0	14	1	3
BITSR022	90	188	80	0	116	0	N	0	N	0	N	0	N	N	0	14	1	1
BITSR023	86	119	60	9	83	4	224	0	44	0	15	0	59	448.4	0	6	3	7
BITSR024	59	140	50	0	80	0	229	0	72	0	17	0	N	N	0	10	2	2
BITSR025	72	172	66	0	101.3	0	207	0	42	0	10	0	N	N	0	9	3	3
BITSR026	66	160	90	0	113.33	0	N	0	N	0	N	0	N	N	0	15	0	0
BITSR027	88	142	70	0	94	0	222	0	55	0	6	0	N	N	4	5	4	8
BITSR028	65	117	63	0	80.33	0	208	0	43	0	14	0	N	N	0	13	1	1
BITSR029	72	112	84	0	93.33	0	142	1	50	0	6	0	N	N	0	15	0	1
BITSR030	93	148	50	0	82.67	0	282	0	58	0	13	0	35.1	266.76	2	3	4	6
BITSR031	61	150	80	0	103.33	0	N	0	N	0	N	0	N	N	0	14	1	1
BITSR032	82	126	73	0.5	90.67	4	211	0	55	0	8	0	N	N	0	15	0	4
BITSR033	88	142	69	0	93.33	0	261	0	74	0	13	0	N	N	0	15	0	0
BITSR034	75	140	70	0	93.33	0	151	0	205	2	35	2	N	N	4	10	2	10
BITSR035	74	112	62	0	78.67	0	N	0	N	0	N	0	N	N	0	15	0	0
BITSR036	62	162	54	5.5	90	4	175	0	43	0	7	0	N	N	4	3	4	12
BITSR037	82	138	62	1	86	4	200	0	61	0	6	0	N	N	4	3	4	12
BITSR039	64	118	72	0	87.33	0	175	0	40	0	33	2	N	N	0	15	0	2
BITSR041	68	193	66	12	108.33	4	397	0	60	0	8	0	344	2614.4	0	11	2	6
BITSR044	97	172	56	0	94.67	0	173	0	44	0	19	0	N	N	0	15	0	0
BITSR045	78	120	76	0	91	0	430	0	67	0	12	0	N	N	0	15	0	0
BITSR046	103	135	82	N	100	4	N	0	N	0	N	0	N	N	0	15	0	4
BITSR047	81	110	51	0.05	70	3	298	0	74	0	11	0	N	N	4	3	4	11
BITSR051	68	122	78	ND	92.67	4	ND	0	ND	4	ND	4	ND	N	0	15	0	12
BITSR052	75	120	78	N	92	4	288	0	50	0	11	0	ND	N	0	15	0	4
BITSR053	81	122	76	N	91.33	4	274	0	64	0	12	0	ND	N	0	15	0	4
BITSR054	74	124	44	0.06	74	3	232	0	30	0	7	0	0.58	4.408	4	3	4	11
BITSR055	85	111	54	ND	73	4	176	0	140	1	8	0	ND	N	0	14	1	6
BITSR056	98	120	70	ND	86.67	4	225	0	119	1	9	0	ND	N	0	15	0	5
BITSR057	84	132	82	N	50	4	ND	0	ND	4	ND	4	ND	N	0	15	0	12
BITSR058	100	146	84	N	104.67	4	316	0	48	0	ND	4	ND	N	0	15	0	8
BITSR060	67	104	58	N	73.33	4	ND	0	85	0	4	0	ND	N	0	15	0	4
BITSR061	82	132	45	0.07	80	3	237	0	44	0	9	0	0.17	1.292	4	3	4	11
BITSR062	70	120	70	ND	86.67	4	385	0	41	0	10	0	ND	N	0	14	1	5
BITSR063						1		4		0		0			4		4	13
BITSR064						1		4		0		0			4		4	13
BITSR066	136	138	70	ND	94	4	247	0	39	0	12	0	0.65		4	3	4	12
BITSR067	88	171	69	ND	103	4	188	0	51	0	15	0	ND		4	15	0	8
BITSR069	62	122	70	ND	87	4	ND	0	ND	4	ND	4	ND		4	15	0	16
BITSR070	52	120	50	ND	73.33	4	ND	0	ND	4	ND	4	ND		4	14	1	17
BITSR071	75	136	64	ND	88	4	ND	0	49	0	16	0	ND		4	15	0	8
BITSR073	76	113	46	ND	54	4	235	0	55	0	15	0	ND		4	14	1	9
BITSR074	95	130	90	ND	103	4	ND	0	ND	4	ND	4	ND		4	14	1	17
BITSR076	68	132	80	ND	98	4	ND	0	ND	4	ND	4	ND		4	14	1	17
BITSR077	DISCHARGED					1		4		0		0			4		4	13
BITSR078	120	115	64	0.233	81	4	311	0	58	0	6	0	0.3514		4	3	4	12
BITSR079	102	131	43	0	72.33	0	320	0	45	0	15	0		n	0	15	0	0
BITSR080	85	149	70	0	96.33	0	344	0	58	0	20	1		n	0	14	1	2
BITSR081	98	185	70	0.368	108.33	4	200	0	60	0	10	0	0.34	2.584	4	3	4	12
BITSR082	95	145	75	0	98.33	0	547	0	46	0	25	1	0.42	3.192	4	10	2	7
BITSR083	95	121	60	0.021	80.33	3	247	0	45	0	12	0	0.44	3.344	4	3	4	11
BITSR084	75	135	55	0	81.67	0	263	0	53	0	16	0		n	0	15	0	0
BITSR085	98	130	62	0	84.67	0	305	0	93	0	15	0		n	0	15	0	0
BITSR086	N	N	N	0	n	0	N	0	N	0	N	0		n	0	N	N	0

	Temp	Hb	WCC	eGFR	PT	Intubated	NIV / CPAP	FiO2	Lactate	CRP	CVVH/HD	Sedated	Antibiotics	Septic source	Steroids	CAM+ve
BITSR001	37.2	102	10.1	90	N	N	N	0.21	N	18	N	N	Y	empirical/unknown	N	N
BITSR002	37.7	11.5	10.5	>90	N	Y	N	30%	0.9	N	N	N	N	N	N	N
BITSR003	39.4	78	12.5	>90	13.2	Y	N	35%	1	N	N	N	N	N	N	N
BITSR007	36.7	142	6.3	>90	N	N	N	N	N	N	N	N	N	N	N	N
BITSR008	37.9	74	8.3	>90	N	N	N	N	N	N	N	N	Y	1	N	N
BITSR009	38.2	69	2.6	44	10.7	Y	N	45%	2.3	N	N	Y	N	N	Y	N
BITSR010	38.4	121	10.9	>90	16.8	Y	N	40%	1.3	N	N	Y	Y	1	N	N
BITSR011	37	118	7.1	N	12	N	N	21%	N	N	N	N	N	N	N	N
BITSR012	37.6	94	14.9	74	N	N	N	21%	N	N	N	N	Y	1	N	N
BITSR013																
BITSR014	37.7	137	13.6	>90	13.4	Y	N	30%	1.1	N	N	Y	N	N	N	N
BITSR015	36.6	134	6.6	>90	11.7	N	N	21%	N	N	N	N	N	N	N	N
BITSR018	37	N	N	N	N	N	N	21%	N	N	N	N	N	N	N	N
BITSR019	37.5	84	11.5	N	13.6	Y	N	35%	0.7	N	N	Y	N	N	N	N
BITSR020	27.5	108	9.1	N	13.7	Y	N	21%	1.2	N	N	Y	Y	6	N	N
BITSR021	37.2	70	8.7	N	11.7	N	N	21%	N	N	N	N	Y	6	N	N
BITSR022	37.9	N	N	N	N	N	N	21%	N	N	N	N	Y	6	Y	N
BITSR023	37.4	119	9.4	N	13.9	Y	N	21%	1.1	N	N	Y	Y	1	N	N
BITSR024	36.8	85.1	10.1	>90	14.8	N	N	30%	0.7	N	N	N	N	N	N	N
BITSR025	36.4	91	7.4	N	11.9	N	N	22%	1.4	N	N	N	N	N	N	N
BITSR026	36.2	N	N	N	N	N	N	21%	N	N	N	N	Y	6	N	N
BITSR027	38.4	63.7	10.3	>90	12.8	Y	N	21%	2.2	N	N	N	Y	6	N	N
BITSR028	37.6	92	90	N	11	N	N	30%	0.9	N	N	N	N	N	N	N
BITSR029	37	104	7.8	>90	N	N	N	21%	N	50	N	N	Y	6	Y	N
BITSR030	38.6	81.3	12.5	>90	13.2	Y	N	30%	1.6	N	N	Y	Y	1	N	N
BITSR031	36.8	N	N	N	N	N	N	21%	N	N	N	N	N	N	N	N
BITSR032	37.2	116	6.3	>90	12.3	N	N	21%	N	N	N	N	Y	6	N	N
BITSR033	37.4	90	7.5	>90	N	N	N	21%	N	N	N	N	Y	1	N	N
BITSR034	37.2	97	20.1	33	11.6	Y	N	35%	1.2	N	N	Y	N	N	N	N
BITSR035	37.1	N	N	N	N	N	N	21%	N	N	N	N	N	N	N	N
BITSR036	37.7	108.3	14.1	N	14.4	Y	N	21%	0.8	N	N	Y	Y	6	N	N
BITSR037	37.8	89	7.5	>90	13.6	Y	N	35%	1	N	N	Y	Y	1	N	N
BITSR039	37	106	8.6	>90	N	N	N	21%	N	122	N	N	Y	1	N	N
BITSR041	38.7	86	19.5	>90	12.3	Y	N	45%	2.1	N	N	N	Y	6	N	N
BITSR044	37.7	77	10.3	N	10.8	N	N	21%	1.2	N	N	N	Y	6	Y	N
BITSR045	36.9		12.1	81	14.4	N	N	21%	N	13	N	N	Y	1	N	N
BITSR046	37	N	N	N	N	N	N	21%	N	N	N	N	N	N	N	N
BITSR047	37.6	77	10.8	>90	12.4	Y	N	55%	1.4	N	N	Y	Y	1	N	N
BITSR051	37	ND	ND	ND	ND	N	N	21	N	ND	N	N	Y	4	N	N
BITSR052	37.1	109	11.4	>90	ND	N	N	21	N	ND	N	N	Y	6	Y	N
BITSR053	36.6	94	6.8	>90	12.3	N	N	21%	N	ND	N	N	N	N	Y	N
BITSR054	35.6	77	9	ND	11	Y	N	21%	2	ND	N	N	N	N	N	N
BITSR055	36.7	116	6	ND	ND	N	N	24%	N	222	N	N	Y	1	N	N
BITSR056	37.4	101	8.3	53	ND	N	N	21%	N	281	N	N	Y	1	N	N
BITSR057	36.3	ND	ND	ND	ND	N	N	21%	N	ND	N	N	N	N	Y	N
BITSR058	36.5	113	8.1	ND	ND	N	N	21%	N	ND	N	N	N	N	N	N
BITSR060	37.2	ND	ND	77	ND	N	N	21%	ND	ND	N	N	N	N	N	N
BITSR061	38	89.2	19.9	ND	15.3	Y	N	25%	1.2	ND	N	Y	N	N	N	N
BITSR062	37	89	5.4	ND	ND	N	N	21%	ND	100	N	N	Y	6	N	N
BITSR063																
BITSR064																
BITSR066	39.4	74	11.5	ND	14.5	Y	N	21%	1.4	ND	N	Y	Y	6	N	N
BITSR067	37.5	82	8.4	>90	10.2	N	N	30%	ND	ND	N	N	Y	6	Y	N
BITSR069	36.6	ND	ND	ND	ND	ND	ND	0.21	ND	ND	N	N	N	N	N	N
BITSR070	36.1	ND	ND	ND	ND	N	N	0.21	ND	ND	N	N	N	N	N	N
BITSR071	36.5	ND	ND	ND	ND	N	N	0.21	ND	ND	N	N	Y	6	N	N
BITSR073	36.5	90	8.6	>90	ND	N	N	0.21	ND	ND	N	N	N	N	Y	N
BITSR074	36.8	ND	ND	ND	ND	N	N	0.21	ND	ND	N	N	Y	6	N	N
BITSR076	36.8	ND	ND	ND	ND	N	N	0.21	ND	ND	N	N	N	N	N	N
BITSR077																
BITSR078	37.9	87	13.8	90	14	Y	Y	0.35	1.5	ND	N	Y	N	N	N	N
BITSR079	36.5	91	10	N	87	N	N	0.24	N	60	N	N	Y	1	N	N
BITSR080	37.3	129	11.3	90	N	N	N	0.21	N	135	N	N	N	N	N	N
BITSR081	38.9	98	16.5	>90	13.5	Y	N	0.45	N	309	N	Y	Y	1	N	N
BITSR082	38.2	88	25	N	13.7	N	N	0.24	1.1	N	N	N	Y	1+6	N	Y
BITSR083	38.6	94	10.4	N	12.4	Y	N	0.42	0.9	N	N	Y	Y	1	N	N
BITSR084	37.1	131	9.8	>90	N	N	N	0.28	N	N	N	N	Y	6	N	N
BITSR085	38.3	122	15.2	50	N	N	N	0.21	N	125	N	N	N	N	Y	N
BITSR086	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N

Appendix 8 - Day 8 – clinical data for the patients recruited on Salford Royal Foundation Trust

	Day 8																	
	HR	Sys BP	Dia BP	Norad	MAP	MAP SOFA	PLT	PLT SOFA	Creat	Creat SOFA	Bili	Bili SOFA	P/F	P/F	RESP SOFA	GCS	GCS SOFA	SOFA D8
BIT SR001	80	117	78	0	91	0	460	0	86	0	N	0	10	76	4	15	0	4
BIT SR002	RIP	RIP	RIP	RIP	RIP	RIP	RIP	RIP	RIP	RIP	RIP	RIP	RIP	RIP	RIP	RIP	RIP	RIP
BIT SR003	95	120	60	N	160	4	453	0	63	0	9	0	0.5	3.8	4	4	4	12
BIT SR007	76	142	82	0	211	0	387	0	52	0	N	0	N	N	0	15	0	0
BIT SR008	70	122	54	0	73	0	325	0	49	0	N	0	N	N	0	15	0	0
BIT SR009	RIP	RIP	RIP	RIP	RIP	RIP	RIP	RIP	RIP	RIP	RIP	RIP	RIP	RIP	RIP	RIP	RIP	RIP
BITSR010	97	125	74	14.5	91	4	212	0	59	0	5	0	N	N	4	N	0	8
BITSR011	80	118	80	N	92	4	218	0	49	0	N	0	N	N	0	15	0	4
BITSR012	72	130	72	0	91	0	401	0	98	0	N	0	N	N	0	13	1	1
BITSR013	RIP					1		4		0		0			4		4	13
BIT SR014	88	137	63	7	87.7	4	N	0	40	0	7	0	N		4	13	1	9
BITSR015	59	105	70	0	81.67	0	N	0	N	0	N	0	N	N	0	15	0	0
BIT SR018	68	118	70	0	86	0	N	0	N	0	N	0	N	N	0	15	0	0
BIT SR019	85	130	52	0	78	0	422	0	54	0	7	0	N	N	4	3	4	8
BIT SR020	98	118	63	6	81.3	4	406	0	47	0	26	1	N	N	4	3	4	13
BIT SR021	72	104	70	0	81.3	0	211	0	27	0	18	0	N	N	0	14	1	1
BIT SR022	99	140	70	0	93.3	0	N	N	N	0	N	0	N	N	0	14	1	1
BIT SR023	69	115	64	9	81	4	214	0	39	0	14	0	N	N	4		4	12
BIT SR024	66	160	62	0	94.7	0	390	0	51	0	16	0	N	N	0	13	1	1
BIT SR025	83	160	80	0	106.7	0	242	0	45	0	7	0	N	N	0	14	1	1
BIT SR026	105	115	60	0	78.33	0	211	0	56	0	N	0	N	N	0	15	0	0
BIT SR027	77	147	78	0	101	0	426	0	43	0	11	0	N	N	4	6	3	7
BIT SR028	82	130	80	0	96.67	0	276	0	47	0	N	0	N	N	0	13	1	1
BIT SR029	88	118	72	0	87.33	0	N	0	40	0	12	0	N	N	0	15	0	0
BIT SR030	74	118	44	0	68.6	1	347	0	48	0	9	0	N	N	4	12	2	7
BIT SR031	66	130	80	0	96.67	0	N	0	50	0	N	0	N	N	0	14	1	1
BIT SR032	74	128	68	0	88	0	N	0	N	0	N	0	N	N	0	15	0	0
BIT SR033	100	143	73	0	96.33	0	11.7	4	71	0	11	0	N	N	0	15	0	4
BIT SR034	79	148	79	0	102	0	377	0	295	2	21	1	N	N	4	3	4	11
BIT SR035	78	122	84	0	96.67	0	N	0	N	0	N	0	N	N	0	15	0	0
BIT SR036	52	144	77	0	99.33	0	200	0	43	0	5	0	N	N	4	10	2	6
BIT SR037	RIP	RIP	RIP	RIP	RIP	RIP	RIP	0	RIP		RIP		RIP	RIP		RIP		RIP
BIT SR039	60	104	66	0	78.67	0	426	0	39	0	23	1	N	N	0	15	0	1
BIT SR041	70	172	67	8.5	102	4	519	0	43	0	8	0	N	N	0	14	1	5
BIT SR044	91	158	74	0	102	0	283	0	46	0	24	1	N	N	0	15	0	1
BIT SR045	N	N	N	N	N	N	N	N	N	N	N	N	N	N	0	N	0	N
BIT SR046	N	N	N	N	N	N	N	N	N	N	N	N		N	0	N	0	0
BIT SR047	68	130	78	N	78	4	362	0	74		12	0	0.35	2.66	4	7	3	11
BIT SR051	72	140	78	N	98.67	4	356	0	59	0	N	0	ND	N	0	15	0	4
BIT SR052	DISCHARGED					1		4		0		0		N	0		4	9
BIT SR053	80	116	68	N	84	4	N	0	N	0	N	0	ND	N	0	15	0	4
BIT SR054	69	155	58	0.34	97.67	4	301	0	30	0	7	0	ND	N	4	3	4	12
BIT SR055	88	120	68	ND	85.33	4	ND	0	N	0	N	0	ND	NN	0	14	1	5
BIT SR056	94	140	78	N	98.67	4	ND	0	N	0	N	0	ND	N	0	15	0	4
BIT SR057	83	135	85	N	101.67	4	ND	0	N	0	N	0	ND	N	0	15	0	4
BIT SR058	88	138	66	ND	90	4	ND	0	N	0	N	0	ND	N	0	15	0	4
BIT SR060	DISCHARGED							4		0		0		N	0		4	8
BIT SR061						1		4		0		0		N	0		4	9
BIT SR062						1		4		0		0		N	0		4	9
BIT SR063						1		4		0		0		N	0		4	9
BIT SR064						1		4		0		0		N	0		4	9
BIT SR066	110	151	75	1.3	85	4	ND	0	37	0	13	0	0.552	4.1952	4	3	4	12
BIT SR067	86	144	83	ND	105	4	ND	0	N	0	N	0	ND	N	0	15	0	4
BIT SR069	68	130	80	N	96.67	4	ND	0	N	0	N	0	ND	N	0	15	0	4
BIT SR070	49	110	64	N	79	4	ND	0	N	0	N	0	ND	N	0	14	1	5
BIT SR071	64	108	64	N	79	4	ND	0	N	0	N	0	ND	N	0	15	0	4
BIT SR073	67	133	61	N	84.33	4	ND	0	N	0	N	0	ND	N	0	15	0	4
BIT SR074	DISCHARGED							4		0		0		N	0		4	8
BIT SR076	80	125	83	N	97	4	ND	0	N	0	N	0	ND	N	0	14	1	5
BIT SR077						1		4		0		0		N	0		4	9
BIT SR078	88	157	65	0.186	96	4	708	0	58	0	3	0	0.45	3.42	4	5	4	12
BIT SR079	87	139	58	0	85	0	424	0	50	0	11	0	N	N	0	15	0	0
BIT SR080	77	136	88	0	104	0	N	0	N	0	N	0	N	N	0	15	0	0
BIT SR081	75	168	65	0	99.33333	0	401	0	44	0	5	0	0.38	2.888	4	4	4	8
BIT SR082	103	133	67	0	89	0	763	0	54	0	17	0	N	N	0	12	2	2
BIT SR083	95	150	66	0.082	94	3	384	0	48	0	28	1	0.19	1.444	4	3	4	12
BIT SR084	80	115	65	0	81.66667	0	N	0	N	0	N	0	N	N	0	15	0	0
BIT SR085	103	148	65	0	92.66667	0	N	0	N	0	N	0	N	N	0	15	0	0
BIT SR086	N	N	N	N	N	N	N	0	N	0	N	0	N	N	N	N	0	N

	Temp	Hb	WCC	eGFR	PT	Intubated	NIV / CPAP	FiO2	Lactate	CRP	CVVH/HD	Sedated	Antibiotics	Septic source	Steroids	CAM+ve	Tranexamic acid	Transfusion	ISS
BIT SR001	36.9	108	27.5	90	N	N	N	0.21	1	88	N	N	N	empirical/unknown	N	N	N	N	16
BIT SR002	RIP	RIP	RIP	RIP	RIP	RIP	RIP	RIP	RIP	RIP	RIP	RIP	RIP	RIP	RIP	RIP	RIP	RIP	RIP
BIT SR003	38.5	86	13.6	>90	13.8	Y	N	30%	1.1	N	N	Y	Y	1	N	N	N	N	
BIT SR007	36.6	126	7.1	>90	N	N	N	N	N	N	N	N	N	N	N	N	N	N	
BIT SR008	37.3	94	11.9	N	N	N	N	21	N	24	N	N	Y	1	N	N	N	N	
BIT SR009	RIP	RIP	RIP	RIP	RIP	RIP	RIP	RIP	RIP	RIP	RIP	RIP	RIP	RIP	RIP	RIP	RIP	RIP	RIP
BITSR010	36.4	110	16	>90	16	Y	N	30	1.5	N	N	Y	Y	1	N	N	N	N	
BITSR011	36.8	121	15	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	
BITSR012	37.3	101	13.3	63	N	N	N	N	N	264	N	N	Y	1	N	N	N	N	RIP
BITSR013																			RIP
BIT SR014	37.3	N	N	N	N	N	N	21	N	N	N	N	Y	1	N	N	N	N	
BITSR015	36.6	N	N	N	N	N	N	21	N	N	N	N	N	N	N	N	N	N	
BIT SR018	36.6	N	N	N	N	N	N	21	N	N	N	N	N	N	N	N	N	N	
BIT SR019	37.5	88	12.6	N	13.8	Y	N	30	0.9	N	N	Y	N	N	N	N	N	N	
BIT SR020	37.7	102.4	12.3	N	14.9	Y	N	21	0.7	N	N	Y	Y	6	N	N	N	N	
BIT SR021	36.7	87	14.4	N	N	N	N	21	N	N	N	N	N	N	N	N	N	N	
BIT SR022	36.9	N	N	N	N	N	N	21	N	N	N	N	Y	6	N	N	Y	Y	
BIT SR023	38.1	106	13.3	N	13.8	Y	N	21	1.6	N	N	Y	Y	1	N	N	N	N	
BIT SR024	36.7	88	11.8	>90	14.6	N	N	21	N	N	N	N	Y	6	N	N	N	N	
BIT SR025	36.3	100	7.6	N	N	N	N	21	N	N	N	N	N	N	N	N	N	Y	
BIT SR026	36.6	119	17.8	>90	N	N	N	21	1.4	198	N	N	N	N	N	N	N	N	
BIT SR027	37.5	107.1	15	N	13.2	Y	Y	21	2	N	N	N	N	N	N	N	N	Y	
BIT SR028	36.8	99	10.2	N	N	N	N	21	N	N	N	N	N	N	N	N	N	N	
BIT SR029	37.1	N	N	N	N	N	N	21	N	61	N	N	Y	6	Y	N	N	N	
BIT SR030	37.1	84	12.2	N	13.7	Y	N	21	1.4	22	N	Y	Y	1	N	N	N	N	
BIT SR031	37	N	N	>90	N	N	N	21	N	N	N	N	N	N	N	N	N	N	
BIT SR032	38.2	N	N	N	N	N	N	21	N	N	N	N	Y	6	N	N	N	N	
BIT SR033	37.2	90	6.5	>90	11.7		N	21	N	N	N	N	Y	1	N	N	N	N	
BIT SR034	37.1	78.3	22.8	22	10.9	Y	N	30	1.1	N	Y	Y	Y	6	N	N	Y	Y	
BIT SR035	37	N	N	N	N	N	N	21	N	N	N	N	N	N	N	N	N	N	
BIT SR036	38.4	95	7.8	N	13.7	Y	N	21	0.8	N	N	Y	N	N	N	N	N	N	
BIT SR037	RIP	RIP	RIP	RIP	RIP	RIP	RIP	RIP	RIP	RIP	RIP	RIP	RIP	RIP	RIP	RIP	N	N	
BIT SR039	37.1	117	7.8	N	N	N	N	21	N	61	N	N	Y	1	N	N	N	N	
BIT SR041	38	82	12.1	N	13.3	N	Y	35	N	N	N	N	Y	6	N	N	N	N	
BIT SR044	37.9	97	11.4	N	11.1	N	N	21	N	N	N	N	Y	6	N	N	Y	N	
BIT SR045	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	
BIT SR046	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	16
BIT SR047	39.1	77.5	12.4	>90	13.2	Y	Y	35%	1	N	N	Y	Y	1	N	N	Y	Y	22
BIT SR051	37.6	121	10	>90	ND	N	N	21	ND	46	N	N	Y	4	N	N	N	N	9
BIT SR052																	Y	N	34
BIT SR053	37	ND	ND	ND	ND	N	ND	21	N	N	N	N	Y	6	Y	N	N	N	13
BIT SR054	37.3	77	9	ND	12.4	Y	ND	21	1.2	N	N	Y	N	N	N	N	N	N	20
BIT SR055	36.5	ND	ND	ND	ND	N	ND	21	N	N	N	N	Y	1	N	N	N	N	4
BIT SR056	36.8	ND	ND	ND	ND	N	ND	21	N	N	N	N	N	N	N	N	N	N	4
BIT SR057	37	ND	ND	ND	ND	N	ND	21	N	N	N	N	N	N	N	N	N	N	30
BIT SR058	36.8	ND	ND	ND	ND	N	ND	21	N	N	N	N	N	N	N	N	Y	N	26
BIT SR060																	N	N	16
BIT SR061																	IN CRASH3 STUDY -50% CHAN	N	16
BIT SR062																	N	N	13
BIT SR063																	N	N	29
BIT SR064																	N	N	16
BIT SR066	37.4	73	14.5	ND	13.4	Y	N	25	1.6	ND	N	Y	Y	6	N	N	Y	Y	22
BIT SR067	36.7	ND	ND	ND	ND	N		N	N	N	N	N	Y	6	N	N	N	N	9
BIT SR069	36.6	ND	ND	ND	ND	N	N	21	N	N	N	N	N	N	N	N	N	N	10
BIT SR070	36.2	ND	ND	ND	ND	N	N	21	N	N	N	N	N	N	N	N	N	Y	24
BIT SR071	37.2	ND	ND	ND	ND	N	N	21	N	N	N	N	Y	6	N	N	Y	Y	22
BIT SR073	36.2		ND	ND	ND	N	N	21	N	ND	N	N	N	N	Y	N	Y	N	25
BIT SR074																	Y	Y	16
BIT SR076	36.8		ND	ND	ND	N	N	21	ND	ND	N	N	N	N	N	N	N	N	25
BIT SR077																	N	N	13
BIT SR078	37.6		10	90	12.5	Y	Y	30	1.2	ND	N	Y	Y	1	N	N	N	N	9
BIT SR079	36.8	89	9.4	90	N	N	N	0.21	0	41	N	N	Y	6	N	N	N	N	18
BIT SR080	36.7	N	N	N	N	N	N	0.21	0	N	N	N	N	N	N	N	Y	Y	27
BIT SR081	38.1	92	20.5	N	12.4	Y	N	0.4	1.2	N	N	N	Y	1+6	N	N	N	N	17
BIT SR082	38	89	22.2	>90	N	N	N	0.21	N	N	N	N	Y	1	N	Y	Y	Y	38
BIT SR083	38.3	90	12.1	N	13.3	Y	N	0.75	0.8	N	N	Y	Y	1	N	N	N	N	18
BIT SR084	36.9	N	N	N	N	N	N	0.25	N	N	N	N	N	N	Y	N	Y	N	34
BIT SR085	37	N	N	50	N	N	N	0.21	N	N	N	N	Y	1	Y	N	N	N	9
BIT SR086	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	Y	Y	9

Appendix 9 - Interleukin-4 concentration, SOFA scores and C-RP concentration.

Patient BIT	D1 IL-4 concentration	D5 IL-4 concentration	SOFA D1	SOFA D5	SOFA D8	IL4 % D1-D5	C-RP D1	C-RP D5
MRI 001	3.6875	3.7214	3	0	0	0.92	19	18
MRI 009	4.0050	3.7070	1	1	0	-7.44	6	110
MRI 010	3.6442	3.6637	4	0	0	0.53	100	181
MRI 011	3.7741	3.8513	1	0	1	2.05	117	
MRI 014	3.6875	3.7936	3	0	0	2.88	8	194
MRI 016	3.5720	3.9090	5	4	1	9.43	5	174
MRI 017	3.7164	3.5482	0	2	0	-4.52	6	124
MRI 021	3.8174	3.8802	2	0	0	1.64	1	26
MRI 023	3.8607	4.3709	2	0	0	13.22	17	
MRI 024	3.5432	3.4905	1	2	1	-1.49	170	
MRI 070	2.9947	2.9709	8	0	1	-0.80		
MRI 074	3.4854	3.6637	4	0	0	5.11	29	
MRI 078	3.8318	4.1544	10	4	4	8.42	9	127
MRI 079	3.5287	3.4183	1	4	6	-3.13	272	151
MRI 084	3.7452	4.0245	4	0	0	7.46	40	243
MRI 085	3.4133	3.6781	3	3	3	7.76		
MRI 088	3.7885	4.2410	3	3	0	11.94		
MRI 091	3.7452	3.8080	10	10	11	1.68	8	
MRI 092	3.9617	3.7647	9	9	9	-4.97	61	
MRI 093	3.7164	3.9235	10	10	8	5.57		103
MRI 098	3.6586	3.8657	4	8	8	5.66		
MRI 099	3.3555	3.7936	4	6	4	13.05		178
MRI 100	3.5287	3.6925	9	9	8	4.64		
MRI 102	4.0050	4.1544	7	8	8	3.73		251
MRI 103	3.9329	4.1111	8	8	8	4.53	3	350
MRI 104	3.8751	3.7214	5	4	4	-3.97	15	110
MRI 107	4.0483	3.9956	13	14	7	-1.30	7	372
MRI 120	3.0524	3.2163	0	4		5.37	15	
MRI 123	3.2401	3.2307	7	0	0	-0.29	7	110
MRI 124	3.2256	3.0142	2	0	4	-6.56	37	107
MRI 125	3.0669	3.4905	3	3	4	13.81		
MRI 127	2.9947	3.2884	0	0	0	9.81	28	168
MRI 128	3.0669	3.1441	2	0	15	2.52	39	289
MRI 129	3.0813	3.1585	2	0	4	2.51	37	
SR 007	3.6298	3.7070	6	1	0	2.13		
SR 009	3.1535	3.1297	3	5		-0.76		
SR 011	2.9803	3.2163	8	0	8	7.92		
SR 012	3.1390	3.5626	9	5		13.49		
SR 014	3.2689	3.2451	0	4	9	-0.73		
SR 015	3.5287	3.5915	0	0	0	1.78	4.5	
SR 019	3.8607	4.3709	12	8	8	13.22		
SR 020	3.5432	3.5482	13	8	13	0.14		
SR 021	3.6009	3.4616	8	3	1	-3.87		
SR 022	3.3411	2.4338	5	1	1	-27.15		
SR 023	3.8607	4.3709	12	7	12	13.22		
SR 024	3.1390	2.9998	8	2	1	-4.44		
SR 025	3.8607	4.0822	4	3	1	5.74		
SR 028	2.9658	3.7358	3	1	1	25.96		
SR 029	3.5576	3.5193	1	1	0	-1.08		50
SR 030	3.0813	3.5771	13	6	7	16.09		
SR 032	3.5432	3.6348	1	4	0	2.59		
SR 033	3.4133	3.4327	0	0	4	0.57		
SR 034	3.2834	3.4265	14	10	11	4.36		
SR 036	3.3122	3.4327	12	12	6	3.64		
SR 039	3.3411	3.6059	2	2	1	7.93		122
SR 041	3.4421	3.4183	5	6	5	-0.69		
SR 044	3.3700	3.5049	10	0	1	4.00	58	
SR 045	3.2112	3.4327	13	0		6.90		13
SR 046	3.3122	3.2740	4	4	0	-1.15		
SR 047	3.4710	3.6781	8	11	0	5.97		
SR 051	3.3555	3.5193	7	12	4	4.88		
SR 052	3.5720	3.6059	12	4	3	0.95		
SR 054	3.5576	3.3750	9	11	12	-5.13		
SR 055	3.8318	3.8369	12	6	5	0.13		222
SR 057	3.6875	3.5482	8	12	4	-3.78		
SR 058	3.5287	3.6204	5	8	4	2.60		
SR 061	3.6442	3.4905	12	11	9	-4.22		
SR 062	3.1102	3.4905	12	5	9	12.23		100
SR 063	3.0524	3.3750	11	13	9	10.57		
SR 064	3.0524	3.3606	7	13	9	10.10		
SR 066	3.0236	3.2451	13	12	12	7.33		
SR 067	3.1390	3.2018	9	8	4	2.00		
SR 074	3.6875	3.8080	1	17	4	3.27		
SR 078	3.9040	3.8946	4	12	12	-0.24		
SR 082	3.6875	3.9379	11	7	2	6.79		
SR 083	3.4999	3.8513	11	11	12	10.04		
SR 087	4.2648	4.2410	12	8	12	-0.56		
SR 088	3.7885	4.0101	12	12	10	5.85		
SR 089	4.0195	3.7070	12	6	6	-7.77		
SR 094	3.3988	3.8946	12	12	12	14.59		

Appendix 10 - Interleukin-6 concentration, SOFA scores and C-RP concentration.

Patient BIT	D1 IL-6 concentration	D5 IL-6 concentration	SOFA D1	SOFA D5	SOFA D8	IL6% D1-D5	C-RP D1	C-RP D5
MRI 001	11.3283	3.0025	3	0	0	-73.50	19	18
MRI 003	4.7401	3.7344	2	0	1	-21.22	6	110
MRI 004	2.7799	4.9771	0	0	0	79.04	100	181
MRI 007	25.3024	4.4768	1	0	0	-82.31	117	
MRI 010	60.3894	14.8551	4	0	0	-75.40	8	194
MRI 011	32.3502	11.3450	1	0	1	-64.93	5	174
MRI 014	255.4891	27.0436	3	0	0	-89.41	6	124
MRI 021	55.0690	2.4316	2	0	0	-95.58	1	26
MRI 022	25.9795	2.4178	0	0	0	-90.69	17	
MRI 023	62.0062	5.7620	2	0	0	-90.71	170	
MRI 070	18.2546	5.1401	0	0	1	-71.84		
MRI 072	0.8285	0.2620	2	0	5	-68.38	29	
MRI 073	55.5665	0.9944	5	1	1	-98.21	9	127
MRI 078	65.4753	18.2659	10	4	4	-72.10	272	151
MRI 084	22.6878	107.7671	4	0	0	375.00	40	243
MRI 088	520.3382	181.8963	11	9	0	-65.04		
MRI 091	50.2787	85.7805	10	10	11	70.61		
MRI 092	543.5319	16.8894	9	9	9	-96.89	8	
MRI 093	79.9175	31.7842	10	10	10	-60.23	61	
MRI 098	829.8959	72.9766	4	8	8	-91.21		103
MRI 099	44.1354	221.7738	4	6	4	402.49		
MRI 100	77.1584	255.4003	9	8	8	231.01		178
MRI 102	57.7800	50.6236	7	8		-12.39		
MRI 103	665.2555	255.0339	8	8		-61.66		251
MRI 104	43.5534	60.1727	5	4	4	38.16	3	350
MRI 107	2173.1447	240.7857	13	14	7	-88.92	15	110
MRI 120	93.4543	25.1235	0	0		-73.12	7	372
MRI 123	37.6688	19.6053	8	0	0	-47.95	15	
MRI 124	258.1378	26.6324	2	0	4	-89.68	7	110
MRI 125	364.4924	100.5891	4	6		-72.40	37	107
MRI 127	59.2889	15.0140	0	0	0	-74.68		
MRI 128	143.8293	201.6626	2	10	15	40.21	28	168
MRI 129	143.8293	201.6626	2	4	4	40.21	39	289
SR 008	110.8641	13.8048	6	1	0	-87.55	37	
SR 009	146.0823	4635.4485	3	5		3073.18		
SR 010	28.0939	34.0638	8	0	8	21.25		
SR 011	53.9082	2.0999	8	0	4	-96.10		
SR 019	70.8642	12.9232	8	4	8	-81.76		
SR 023	119.1915	28.0120	8	7	12	-76.50		
SR 025	179.5252	170.0839	4	3	1	-5.26	4.5	
SR 028	119.5795	19.6916	3	1	1	-83.53		
SR 029	9.1750	12.8585	1	1	0	40.15		
SR 030	122.4895	15.3374	13	8	7	-87.48		
SR 032	17.3420	5.9176	1	4	0	-65.88		
SR 033	47.6490	19.7778	0	0	4	-58.49		
SR 034	70.2175	126.9514	14	10	11	80.80		
SR 036	62.9749	17.7947	12	12	6	-71.74		
SR 039	183.5991	28.4431	2	2	1	-84.51		
SR 041	504.9045	447.0282	5	10	5	-11.46		50
SR 044	32.8404	29.5208	10	0	1	-10.11		
SR 045	284.3924	6.9954	13	0		-97.54		
SR 046	128.2232	31.0728	4	0	0	-75.77		
SR 047	88.4750	33.2715	8	11	11	-62.39		
SR 051	59.0734	10.2934	11	0	4	-82.58		
SR 052	97.0540	6.8014	12	0	3	-92.99		122
SR 054	84.2717	29.3484	9	11	12	-65.17		
SR 055	82.8059	21.9549	12	2	5	-73.49	58	
SR 057	17.0834	13.1387	8	1	4	-23.09		13
SR 058	18.5922	18.7000	9	0	4	0.58		
SR 061	256.2841	93.1525	12	11	9	-63.65		
SR 062	122.9852	8.3318	7	1	9	-93.23		
SR 063	123.0715	10.7676	7	9	9	-91.25		
SR 064	32.7542	8.1163	7	5	9	-75.22		
SR 066	149.4769	46.3125	13	8	12	-69.02		222
SR 067	62.0911	31.4177	1	0	4	-49.40		
SR 069	225.1796	13.6776	12	12	4	-93.93		
SR 070	47.2179	9.8838	5	13	5	-79.07		
SR 071	83.1508	33.1422	8	4	4	-60.14		100
SR 074	35.1684	6.3703	1	1	8	-81.89		
SR 078	27.3222	229.2967	4	12	12	739.23		
SR 079	67.8680	11.9963	4	0	0	-82.32		
SR 082	111.5393	26.2660	11	7	2	-76.45		
SR 083	82.0083	36.6988	11	11	12	-55.25		
SR 084	38.6819	107.1851	4	0	0	177.09		
SR 087	103.1542	99.4251	12	8	12	-3.62		
SR 088	105.4176	15.4451	12	12	10	-85.35		
SR 089	283.5871	17.2989	12	6	6	-93.90		
SR 092	6.2410	2.6628	12	0	0	-57.33		
SR 093	192.6093	26.8695	4	0	0	-86.05		
SR 094	19.7347	128.1801	12	12	12	549.52		

Appendix 11 - Interleukin-10 concentration, SOFA scores and C-RP concentration.

Patient BIT	D1 IL-10 concentration	D5 IL-10 concentration	SOFA D1	SOFA D5	SOFA D8	IL-10 % D1-D5	C-RP D1	C-RP D5
MRI 001	5.2654	2.6244	3	0	0	-50.16	19	18
MRI 003	2.4980	2.4130	2	0	1	-3.40	6	110
MRI 004	2.8831	2.8713	0	0	0	-0.41	100	181
MRI 007	5.8730	0.6899	1	0	0	-88.25	117	
MRI 010	6.1736	0.3064	4	0	0	-95.04	8	194
MRI 011	2.1515	1.0631	1	0	1	-50.59	5	174
MRI 014	19.3904	1.9753	3	0	0	-89.81	6	124
MRI 021	15.2025	1.7680	2	0	0	-88.37	1	26
MRI 022	1.4881	0.3375	0	0	0	-77.32	17	
MRI 023	5.5827	1.7680	2	0	0	-68.33	170	
MRI 070	2.4003	1.9028	0	0	1	-20.73		
MRI 072	3.0119	1.9857	2	0	5	-34.07	29	
MRI 073	3.8827	2.4936	5	1	1	-35.78	9	127
MRI 078	3.1920	2.7283	10	4	4	-14.53	272	151
MRI 084	5.0921	4.3795	4	0	0	-13.99	40	243
MRI 088	188.2993	5.9516	11	9	0	-96.84		
MRI 091	8.6999	7.8516	10	10	11	-9.75		
MRI 092	24.7824	4.7301	9	9	9	-80.91	8	
MRI 093	10.1923	5.7593	10	10	10	-43.49	61	
MRI 098	18.0757	4.1420	4	8	8	-77.09		103
MRI 099	2.6039	5.9968	4	8	4	130.30		
MRI 100	4.5718	11.7761	9	8	8	157.58		178
MRI 102	4.3230	3.4182	7	8	8	-20.93		
MRI 103	112.8630	14.2417	8	8		-87.38		251
MRI 104	4.0516	3.2938	5	4	4	-18.70	3	350
MRI 107	79.4539	8.2362	13	14	7	-89.63	15	110
MRI 120	2.7057	1.4277	0	0		-47.23	7	372
MRI 123	2.1741	1.5408	8	0	0	-29.13	15	
MRI 124	3.6105	1.6991	2	0	4	-52.94	7	110
MRI 125	10.0005	6.3022	4	6	4	-36.98	37	107
MRI 127	3.4521	1.9140	0	0	0	-44.56		
MRI 128	13.5970	20.4621	2	10	15	50.49	28	168
MRI 129	13.5970	20.4621	2	4	4	50.49	39	289
SR 008	7.6819	1.9028	6	1		-75.23	37	
SR 009	4.5565	57.5793	3	5		1163.67		
SR 010	5.9766	2.8979	8	0		-51.51		
SR 011	3.4473	2.5040	8	0		-27.36		
SR 019	5.7593	2.8075	8	4		-51.25		
SR 023	12.4774	3.4861	8	7		-72.06		
SR 025	13.7101	6.3927	4	3		-53.37	4.5	
SR 028	48.1597	2.9319	3	1		-93.91		
SR 029	6.5623	2.1968	1	1		-66.52		
SR 030	12.5339	3.1242	13	8		-75.07		
SR 032	1.6200	1.6426	1	4		1.40		
SR 033	2.2081	1.8575	0	0		-15.88		
SR 034	35.4701	5.9403	14	10		-83.25		
SR 036	2.3890	2.1854	12	12		-8.52		
SR 039	12.8393	1.1110	2	2	1	-91.35		
SR 041	20.8579	12.3529	5	10	5	-40.78		50
SR 044	7.9874	3.8254	10	0	1	-52.11		
SR 045	5.8724	1.6878	13	0		-71.26		
SR 046	6.3248	2.5587	4	0	0	-59.55		
SR 047	8.6094	5.1260	8	11	0	-40.46		
SR 051	1.8575	1.4503	11	0	4	-21.92		
SR 052	6.1891	1.8688	12	0	3	-69.81		122
SR 054	5.4766	2.0045	9	11	12	-63.40		
SR 055	8.2023	2.5926	12	2	5	-68.39	58	
SR 057	2.2759	1.6426	8	1	4	-27.83		13
SR 058	1.6765	1.7896	9	0	4	6.75		
SR 061	3.5200	9.3898	12	11	9	166.76		
SR 062	5.0016	1.4390	7	1	9	-71.23		
SR 063	14.7959	1.7670	7	9	9	-88.06		
SR 064	1.6991	1.1449	7	5	9	-32.62		
SR 066	9.8987	3.1807	13	8	12	-67.87		222
SR 067	2.0497	3.3164	1	0	4	61.80		
SR 069	3.1015	1.7557	12	12	4	-43.39		
SR 070	3.2599	1.6878	5	13	5	-48.23		
SR 071	6.0760	5.5105	8	4	4	-9.31		100
SR 074	4.8998	2.6265	1	1	8	-46.40		
SR 078	2.6152	3.6896	4	12	12	41.08		
SR 079	11.0297	2.8075	4	0	0	-74.55		
SR 082	78.4021	4.1760	11	7	2	-94.67		
SR 083	6.2344	2.4116	11	11	12	-61.32		
SR 084	5.4766	3.4295	4	0	0	-37.38		
SR 087	2.8527	2.8075	12	8	12	-1.58		
SR 088	6.5850	2.5134	12	12	10	-61.83		
SR 089	26.4789	3.9611	12	6	6	-85.04		
SR 092	1.6313	1.1789	12	0	0	-27.73		
SR 093	9.5368	2.7170	4	0	0	-71.51		
SR 094	1.8122	3.1242	12	12	12	72.40		

Appendix 12 - Interleukin-12 concentration, SOFA scores and C-RP concentration.

Patient BIT	D1 IL-12 concentration	D5 IL-12 concentration	SOFA D1	SOFA D5	SOFA D8	IL12 % D1-D5	C-RP D1	C-RP D5
MRI 001	6.6621	6.7136	3	0	0	0.77	19	18
MRI 009	6.6106	7.0224	1	1	0	6.23	6	110
MRI 010	6.8680	7.0481	4	0	0	2.62	100	181
MRI 011	6.7136	6.9709	1	0	1	3.83	117	
MRI 014	6.5849	7.0739	3	0	0	7.43	8	194
MRI 016	6.6878	7.2798	5	4	1	8.85	5	174
MRI 017	6.7908	6.8423	0	2	0	0.76	6	124
MRI 024	7.0996	6.9709	2	0	0	-1.81	1	26
MRI 021	6.8680	7.1511	2	0	0	4.12	17	
MRI 023	6.8423	7.3312	1	2	1	7.15	170	
MRI 070	6.7651	6.7393	8	0	1	-0.38		
MRI 074	7.0481	6.9195	4	0	0	-1.83	29	
MRI 078	6.8423	7.3055	10	4	4	6.77	9	127
MRI 079	7.2026	7.2283	1	4	6	0.36	272	151
MRI 084	6.7136	7.0996	4	0	0	5.75	40	243
MRI 085	7.0996	7.2026	3	3	3	1.45		
MRI 088	6.7651	7.1511	3	3	0	5.71		
MRI 091	6.6878	6.7651	10	10	11	1.15	8	
MRI 092	6.8423	7.3312	9	9	9	7.15	61	
MRI 093	6.0702	6.6878	10	10	8	10.18		103
MRI 098	6.2246	7.0996	4	8	8	14.06		
MRI 099	6.3018	8.1033	4	6	4	28.59		178
MRI 100	6.5334	6.6621	9	9	8	1.97		
MRI 102	7.0481	7.3827	7	8	8	4.75		251
MRI 103	6.7393	6.9452	8	8	8	3.06	3	350
MRI 104	6.9195	7.0996	5	4	4	2.60	15	110
MRI 107	6.8165	7.1768	13	14	7	5.29	7	372
MRI 120	6.5334	6.2246	0	4		-4.73	15	
MRI 123	6.1731	6.4305	7	0	0	4.17	7	110
MRI 124	6.2503	6.3533	2	0	4	1.65	37	107
MRI 125	5.9672	6.2761	3	3	4	5.18		
MRI 127	5.6841	6.4820	0	0	0	14.04	28	168
MRI 128	6.3533	6.8165	2	0	15	7.29	39	289
MRI 129	5.9415	6.1474	2	0	4	3.47	37	
SR 007	7.3827	7.2540	6	1	0	-1.74		
SR 009	7.2026	6.9709	3	5		-3.22		
SR 011	6.5592	6.7908	8	0	8	3.53		
SR 012	6.9967	7.1511	9	5		2.21		
SR 014	6.5077	6.7651	0	4	9	3.95		
SR 015	7.1254	6.9967	0	0	0	-1.81	4.5	
SR 019	6.8680	7.1254	12	8	8	3.75		
SR 020	7.1254	7.0224	13	8	13	-1.44		
SR 021	7.2798	7.2283	8	3	1	-0.71		
SR 022	6.9452	6.8165	5	1	1	-1.85		
SR 023	6.8423	7.3312	12	7	12	7.15		
SR 024	6.8165	6.6106	8	2	1	-3.02		
SR 025	6.7393	7.0224	4	3	1	4.20		
SR 028	7.3055	7.4342	3	1	1	1.76		
SR 029	7.3055	10.2652	1	1	0	40.51		50
SR 030	7.2798	7.6401	13	6	7	4.95		
SR 032	6.9195	7.3570	1	4	0	6.32		
SR 033	6.8423	7.1511	0	0	4	4.51		
SR 034	6.8937	7.3312	14	10	11	6.35		
SR 036	6.9452	7.1511	12	12	6	2.96		
SR 039	7.1061	7.5629	2	2	1	6.43		122
SR 041	7.5114	6.9709	5	6	5	-7.20		
SR 044	7.0996	7.3827	10	0	1	3.99	58	
SR 045	7.1768	6.9967	13	0		-2.51		13
SR 046	7.0996	7.1511	4	4	0	0.72		
SR 047	6.3533	7.2283	8	11	0	13.77		
SR 051	6.4562	6.4562	7	12	4	0.00		
SR 052	6.5592	6.6621	12	4	3	1.57		
SR 054	6.5077	6.7136	9	11	12	3.16		
SR 055	6.4047	6.3018	12	6	5	-1.61		222
SR 057	6.4562	6.3018	8	12	4	-2.39		
SR 058	6.3275	6.3533	5	8	4	0.41		
SR 061	6.5592	6.4047	12	11	9	-2.35		
SR 062	5.6584	7.1511	12	5	9	26.38		100
SR 063	6.0702	6.8680	11	13	9	13.14		
SR 064	5.9415	6.2761	7	13	9	5.63		
SR 066	5.9930	6.1731	13	12	12	3.01		
SR 067	5.9158	6.2761	9	8	4	6.09		
SR 074	6.6621	7.0224	1	17	4	5.41		
SR 078	6.6621	6.7136	4	12	12	0.77		
SR 082	6.9709	6.7651	11	7	2	-2.95		
SR 083	6.4305	6.8937	11	11	12	7.20		
SR 087	7.1254	7.5629	12	8	12	6.14		
SR 088	6.4820	6.8423	12	12	10	5.56		
SR 089	6.6364	7.1254	12	6	6	7.37		
SR 094	6.3533	10.9858	12	12	12	72.91		