The Department of Bioengineering

Evaluation of adsorbent haemoperfusion in interventional therapy of sepsis

By

Euan Ross Coutts

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Declaration

This thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination which has led to the award of a degree.

Signed:

Date:

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Abstract

Objectives: Sepsis and its sequelae, is one of the major clinical challenges facing intensive care units across the globe, and the greatest cause of intensive care mortality. The aim of the project is to evaluate the suitability of a newly developed adsorbent for use as an interventional therapy in the treatment of sepsis. In order to achieve this, a rat model comparable to the human clinical condition and its symptoms was developed. This rat model was further developed and used to evaluate the effect of deployment of the adsorbent in a miniaturised extracorporeal haemoperfusion circuit.

Materials & Methods: In order to achieve this, a rat model (adult male Sprague Dawley 480g-590g) comparable to the acute phase physiological symptoms of a systemic sepsis condition in humans was developed. Sepsis was induced by intraperitoneal injection of lipopolysacharide (LPS). Key physiological characteristics, heart rate, body temperature, and mean arterial blood pressure, were measured dynamically and recorded over a six hour time course. Cytokine profiles were also measured by time interval blood sampling and subsequent ELISAs to characterise progression. In the final phases of experimentation, a miniaturised extracorporeal haemoperfusion circuit was developed to evaluate the impact of deployment in the progression of the condition.

Results: Clear trends are evident. Falling blood pressure in the sepsis group over time when compared to controls. In addition, the sepsis group exhibited a very

dynamic temperature response, progressing from hyperthermia to hypothermia over 6 hours. This would appear to be in-line with documented literature and clinical sepsis observations.

There was a difference in cytokine upregulation between the control and sepsis groups. Controls exhibited an upregulation in cytokines associated with the surgical procedure, however this was dramatically lower than compared to the LPS-induced sepsis group.

With confidence in the sepsis model, an *in vivo* experimental extracorporeal haemoperfusion circuit involving an adsorbent device was developed to investigate its impact on the sepsis condition in further groups. This resulted in an attenuation of cytokine upregulation, and impact on the physiological markers of sepsis. Cytokine regulation, particularly of TNFα, was dramatically mitigated in the treatment group. A more stable and mitigated falling blood pressure was observed in the treatment group compared to a dramatic response in the sepsis group and a similar observation was also made in regard to body temperature. It is concluded that the deployment of a novel adsorbent is a valid area of further investigation in the treatment of the progression of the sepsis condition based upon the outcome of iterative phases of experimentation using an in-vivo rodent model.

Conclusions: The deviation in physiological response and cytokine levels between the sepsis and control groups is in line with documented theory and clinical observations. Progression of the condition in control, sepsis and attenuated progression in experimental treatment models are discussed. The key physiological

parameters and inflammatory mediators of the condition were monitored and evaluated to assess the efficacy of this therapy. It is concluded that the deployment of the adsorbent was associated with attenuation of the cytokine response in the LPS-induced sepsis animals and modification of the physiological response. This work confirms that the model is a suitable analogue for clinical sepsis and that deployment of a novel adsorbent has a demonstrable mitigating impact upon the progression of the condition representing a valid area for further development of interventional therapy in the ICU setting.

The following are the main achievements of this work;

- A stable and repeatable *in-vivo* animal model capable of running a 6 hour time course to model the acute stage of sepsis and a 6 hour control model was developed for use in the Bioengineering facility at the University of Strathclyde.

- This model provided a means of dynamically and iteratively monitoring key physiological parameters and inflammatory mediators of sepsis respectively.

- A series of experiments was carried out using these models; clear trends are evident in results which appear to be in-line with documented literature and clinical sepsis observations.

- A device was developed for suspending an adsorbent within a miniaturised extracorporeal circuit allowing circulating whole blood to contact the

adsorbent presenting a means to assess its impact on the parameters being monitored.

- The animal models were further developed to provide a stable and repeatable *in-vivo* extracorporeal haemoperfusion model capable of running a 6 hour time course and, utilising the test device, suspending the adsorbent within the circuit; this provided an in-vivo control, sepsis and treatment model.

- This is significant as any novel therapy capitalising upon a potential window of intervention must be applicable on a systemic scale due to the nature of the sepsis condition.
- A further series of experiments was carried out using these models; clear trends are evident in results which demonstrate a mitigating effect on the progression of the condition in a treatment group compared to an LPS-induced sepsis group and a control group.

- It is a concluding recommendation that the potential therapy be developed further in order to assess its potential impact upon the progression of the condition and potential applications in the ICU setting.

List of Abbreviations

ACCP	American College of Chest Physicians
ALI	Acute Lung Injury
ARDS	Acute Respiratory Distress Syndrome
ARF	Acute Renal Failure
CARS	Compensatory Anti-inflammatory Response Syndrome
CASP	Colon Ascendens Stent Peritonitis
CHD	Continuous Haemodialysis
CHF	Continuous Haemofiltration
CHFD	Continuous High-Flux Dialysis
CLP	Cecal Ligation and Puncture
CO	Cardiac Output
CRRT	Continuous Renal Replacement Therapy
DAMP	Damage Associated Molecular Pattern
DIC	Disseminated Intravascular Coagulation
ECMO	Extracorporeal Membrane Oxygenation
FDA	Federal Drug Administration
HVHF	High-Volume Haemofiltration
ICU	Intensive Care Unit
IL-6	Interleukin 6
IL-1β	Interleukin - 1β
IL-8	Interleukin – 8
IP	Intraperitoneal
LPS	Lipopolysaccharide

LTA	Lipoteichoic Acid
MODS	Multiple Organ Dysfunction Syndrome
MOF	Multiple Organ Failure
NHS	National Health Service
PAMP	Pathogen Associated Molecular Pattern
PBS	Phosphate Buffered Saline
PE	Plasma Exchange
PMF	Polymyxin Fibre
PMN	Polymorphonuclear
PRR	Pattern Recognition Receptor
PVC	Polyvinylchloride
RBC	Red Blood Cell
SCCM	Society of Critical Care Medicine
SEM	Scanning Electron Microscope
SIRS	Systemic Inflammatory Response Syndrome
SSC	Surviving Sepsis Campaign
SVR	Systemic Vascular Resistance
TNF-α	Tumour Necrosis Factor-α
TFPI	Tissue Factor Inhibiting Pathway
TLR	Toll-Like Receptor
VAD	Ventricular Assist Device

Thesis Outline

In chapter 1, an introduction to the topic of sepsis and its associated sequelae including Systematic Inflammatory Response Syndrome (SIRS) is addressed. The evolving understanding and definitions of the condition and its variations are discussed then the condition is further explored in terms of clinical and financial burden.

In chapter 2, the pathophysiology of sepsis is explored both in terms of the underlying mechanisms of the condition and the current treatment in the clinical setting before concluding with an exploration of current developmental therapeutic options.

Chapter 3 concisely defines the aims and objectives of this research and hypothesis statements.

In chapter 4, the development of control and sepsis rodent models and the development of a miniaturised haemoperfusion circuit involving a suspended adsorbent for use with these models will be discussed. Three phases of experiments are carried out utilising this model which are discussed in this chapter:

1. Establishment of control model.
2. Experimental investigation and data collection of control model and induced sepsis model.

3. Experimental investigation and data collection of adsorbent based haemoperfusion circuit as a treatment modality in the progression of induced sepsis.

Phase 1 was an initial pilot study to ensure validity of the model and check the ability to appropriately monitor key physiological and inflammatory characteristics of the condition. Phase 2 was a larger study to collect data and evaluate the clinical relevance of the acute phase of the sepsis model in contrast to a control model. Phase 3 focuses on the development of a method for evaluating the efficacy of the adsorbent as a means of therapy. A device for suspending the adsorbent in circulating whole blood and a rodent extracorporeal circuit model were developed. The results and discussion are presented for phases 1, 2 and 3 However while phases 1 and 2 were successful in achieving their objectives, phase 3 was not. The reasons for this are discussed and recommendations made.

Chapter 5 discusses the refinement of the experimental design and treatment modality following the unsuccessful phase 3. The experimental protocol was refined and a new 4^{th} phase of experiments was conducted incorporating a control, sepsis and treatment group. The results are presented and discussed for this 4^{th} phase.

Chapter 6 provides conclusions from the body of work conducted, limitations involved in the work and makes recommendations for future research based upon the developed models, device and protocols.

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1.0 Introduction

Sepsis and Systemic Inflammatory Response Syndrome (SIRS) currently represent a major healthcare concern. As a condition, sepsis is commonplace, expensive and often fatal. In Europe, it affects 3 people in every 1,000 and kills at least 135,000 each year, approximately the same number of deaths caused by lung cancer in the EU, costing approximately €7.6 billion (Hunter, 2006). In the USA it is responsible for as many fatalities as acute myocardial infarction, developing among 900,000 people each year in the United States, a third of whom die when the condition is complicated by acute organ dysfunction (Angus et al., 2001). The demand for a therapeutic intervention in the treatment of sepsis is profound.

It has been argued that as modern medicine advances, new techniques could be increasing the risk of patients developing the condition. This has been attributed to the widespread use of indwelling catheters, chest drains, invasive monitoring equipment, surgery involving increased endotracheal intubation and use of prolonged mechanical ventilation (Yoshihiko Kanno et al., 2003). In addition, improvements in medical care have given longer life spans to the elderly and to patients with metabolic, neoplastic, or immunodeficiency disorders—but these groups remain at increased risk (Roger C. Bone, 1991).

Despite these statistics, the management and therapy of sepsis is a major clinical challenge largely due to the fact that the condition is so complex. Currently, management still revolves around support of organ function and prevention of

complications until recovery occurs (Singer, 1998). The development and progression of sepsis is multifactorial, affecting the cardiovascular, immunological and endocrine systems of the body (Buras et al., 2005), and the underlying pathophysiology is still the subject of much research and debate, along with its resulting clinical symptoms and effects (Rittirsch et al., 2009, Riedemann et al., 2003). Sepsis typically arises as a complication in patients with severe trauma, burns, blood loss or patients having recently undergone major surgery, most notably surgeries involving extracorporeal circulation (Angus et al., 2001). As a result, sepsis is arguably the most common condition encountered in the intensive care unit, arising as a complication in approximately 30 to 40% of intensive care unit (ICU) admissions (Jean-Louis Vincent, 2006).

The reason for the complexity and difficulties in defining the condition and its underlying pathology is because sepsis is not a disease in the traditional sense of the word, but rather a syndrome describing a systemic response, in which invading pathogens release endotoxins into the bloodstream, which, in turn, activate various immunological and coagulatory responses whose function can grow out of control (Hunter, 2006). Contemporary thinking and research in this regard are covered in greater detail throughout this thesis.

The use of an adsorbent, developed at the University of Strathclyde, has been successful in mediating circulating levels of key mediators of the condition, particular cytokines, from whole blood, principally in human *in-vitro* experimentation and *in-vivo* large animal studies in the context of extracorporeal bypass. It is the aim of this project to evaluate the application of this adsorbent as a

device, which can provide an interventional therapy in the treatment of sepsis in the intensive care unit (ICU) by eliminating the mediating cytokines from blood in an extracorporeal haemoperfusion circuit, thus mitigating the progression of the potentially fatal condition.

1.1 Definition of Sepsis & Sequelae

"Interest in restoring balance in body elements has enamored physicians since ancient times. Claudius Galen, physician to Roman emperors, first popularized Hippocrates' principles of the body's four humors—blood, phlegm, black bile, and yellow bile—and emphasized the need to balance the humors for health. Modern researchers continue to seek this balance." (Fortenberry and Paden, 2006)

1.1.1 A Historical Perspective

In its most basic terms, sepsis describes the systemic response to infection (Bone et al., 1992). Bacteraemia, Blood Poisoning, Septicaemia, Sepsis and Toxic Shock are all terminologies which have been used at one point or another to describe the condition of sepsis, anecdotally and professionally. The contemporary understanding and definition of the condition is most significantly influenced by the definitions of Cornelius Celsus (45 BC – 25AD), who recorded the condition of inflammation as presenting with heat (Color), pain (Dolor), redness (rubor) and swelling (tumor). This was subsequently supplemented with loss of function by Claudius Galen (129 AD – approximately 200 AD). This inflammatory response arises as a part of the immune response system, designed to heal some form of insult, see Figure 1 below.

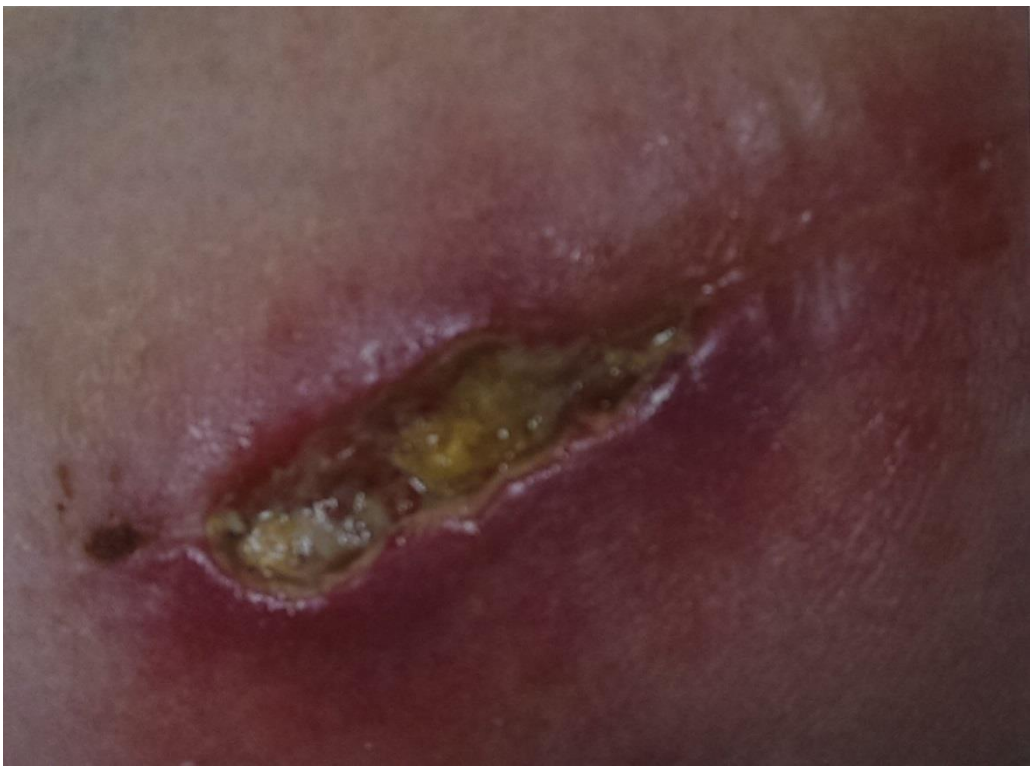

Figure 1: Typical inflammatory response to some insult to the body, demonstrating redness (rubor), swelling (tumor), pain (dolor) and heat (calor) (Daniels, 2009). Figure reproduced with permission of John Wiley and Sons licence number 3846451470627.

Contemporary understanding of the condition has advanced significantly from these broad criteria. However historically, the condition can be traced back even earlier than the works of Celsus in records as far back as 4000 years ago in Egypt (Majno, 1991, Botero and Pérez, 2012) . Without the aid of microscopy, the Egyptians were only able to postulate as to the nature of the phenomenon but they did have the concept of some malicious humour originating from the intestine (Majno, 1991), demonstrated in the original hieroglyphs shown in Figure 2, which was viewed as a source of autointoxication. It was even believed that *"it could find its way into the vessels, settle anywhere in the body, or even "rise to the heart" and kill"* (Majno, 1991). This ancient idea arguably prompted the search for the antithesis of such a

humour, investigating possible methods of preventing wounds from festering and becoming rotten and in doing so developing wound salves, some of which incorporated honey and appear to have had some degree of success (Broughton et al., 2006). A papyrus from 1600 BC, and believed to be a reproduction of an even earlier papyrus from 3000 BC, found in Luxor, Egypt (widely considered to be the oldest known manuscript on surgery) records cases of traumatic lesions resulting from wounds, factures and dislocations. A surprising awareness of secondary complications is evident; references are made to fever as a secondary phenomenon in the wound and in some cases modifies the treatment and prognosis undertaken (Botero and Pérez, 2012). The presence of pus, flesh turning black, convulsions and odour emanating from the wound are all also described as secondary developments with negative connotations. Without a full understanding of the mechanisms involved, this manuscript, and the ancient physicians responsible for its production, identified tentative indicators of infection and inflammatory response as it is now known.

Figure 2: The ancient Egyptian Concept of WHDW, a dangerous principle contained in the terminal portion of the intestine. The fifth hieroglyph (not pronounced) simply means "something disgusting" (Majno, 1991). Figure reproduced with permission of Oxford University Press licence number 3830830350434.

WHDW, although believed to originate in the gut, was perceived to be able to move through blood vessels and intoxicate the entire body (Majno, 1991). Ancient Egyptians accordingly made use of substances which were not subject to the petrification process in an attempt to heal wounds and prevent the identified secondary complications. Honey and grease were among the substances used to this effect (Broughton et al., 2006).

Later in the ancient Greek civilisation (approximately 800 BC to 600 AD), the concept was also upheld. It is thought that these principles entered Greek medicine by physicians who were trained in Egypt, although it can be disputed as there is no way to ascertain if this was an informed development of the Egyptian concept or an original one derived in response to observation of similar phenomenon. Sepsis as a term is first mentioned in Homer's poems (debated to be 850 BC or the early 12th century BC) , where sepsis is derived from the verb sepo (σηπω) meaning "I rot" (Geroulanos and Douka, 2006).

The Greek era marked some of the most significant events in the evolution of medicine including the Hippocratic texts (400 BC) (which also make note of sepsis (σ̃ηψιζ) and "pepsis", which have been conceptualised as a form of yin and yang, as demonstrated in Figure 3 (Majno, 1991) Pepsis was positive, representing processes of maturation and fermentation. On the other hand sepsis was negative, representing putrefaction, characterized by bad smell (Marshall et al., 2005). There is evidence that both Egyptian and Greek physicians practised wound treatment to support haemostasis and attempted to prevent pus formation; washing wounds with beer, hot water and honey, subsequently covering them with grease and herbs; even surgical

drainage of pus (Majno, 1975). Such methods were retrospectively investigated and found that some of these mixtures had *in-vitro* antibacterial action (Broughton et al., 2006, Majno, 1975). It can be postulated that these were used because of their sweet aromas and lack of putrefaction in line with the views held at the time promoting such characteristics as antithesis of WHDW in Egypt and of sepsis in Greece. Lamentably such practices were to fall to out of favour in lieu of another school of Greek thought; humourism.

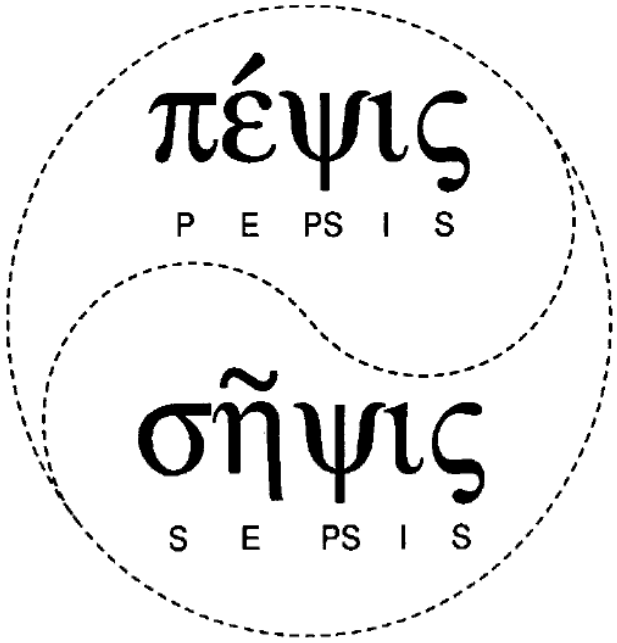

Figure 3: The Greek concepts of pepsis and sepsis referred to two manners of biological breakdown. They were, in a broad sense, complementary and interrelated, recalling the Chinese concept of yin and yang (Majno, 1991). Figure reproduced with permission of Oxford University Press licence number 3830830350434.

The Greeks noticed that particular diseases presenting with fever could affect people who were related or living in close quarters. They postulated that some kind of intoxication was the initiating cause. This would alter humours and could affect large

groups of people at once in a particular location. Due to similarities in the stench of those taken ill and part of the environment, the stench of marshes, a relationship was believed to exist. The thinking at the time was that rotten substances and putrefaction, sepsis, gave rise to odious exhalations, dubbed "miasmas" or "miasmata" (Majno, 1991). For years to follow, feverish diseases were associated with bad environmental odours that poisoned the body. Indeed, the Romans noted the connection of the presence of mosquitos in marshes to the appearance of malaria; even the work malaria is derived from the Roman expression "bad air"; mal'aria (Botero and Pérez, 2012). This was also compounded by contagion, a concept known to the Greeks but brought into language from the Latin 'contigere' meaning 'to touch'.

The Hippocratic texts suggested that the body consists of four fundamental humours; blood, phlegm, yellow bile, and black bile, which were associated with the four elements; air, water, fire, and earth. Good health required equilibrium of these humours as well as harmony with the environment, a state referred to as eucrasia. Any disequilibrium of these humours was the cause of disease, a state referred to as dyscracia (Botero and Pérez, 2012). This theory dominated medical thinking for centuries. Under this theory, suppuration of pus could be considered either beneficial or harmful. Within the Greek theories of sepsis and pepsis, sepsis was associated with rot and putrefaction whereas pepsis was associated with maturation and fermentation (Botero and Pérez, 2012). The formation of pus could be viewed within either classification. Pus of an interpreted benign nature was associated with pepsis and favourable whereas pus of an excessive, dark and odious nature was associated with sepsis and a poor prognosis, however a total absence of pus was also associated

with poor prognosis. This notion lead to the practice, although the specifics are unknown, of maintaining a perceptibly beneficial amount of suppuration of wounds, leading to the invention, by a Greek barber and physician in 280 BC, of the "pyúlkos" or pus extractor, the predicator of the present day syringe (Majno, 1975). Venesection was practiced on those exhibiting feverish-septic symptoms to extract blood from a wound, samples of which would be observed and if considered dark, it was concluded that there was an increase in black bile, an observation now known to be due to decreased oxygenation. Records from this time, including the Hippocratic texts, discuss a range of methods to remedy wounds, ranging from dressing with substances and salves as discussed and site drainage to more dramatic interventions such as cauterisation, importantly methods would be employed as deemed appropriate by the physician based upon observation. However moving forward to the Roman and Medieval ages, particular practices fell out of favour in lieu of proliferation of medical dogma.

The medical theories and practices of the Roman Empire (approximately 27 BC – 610 AD) were heavily influenced by the Greeks. It was during this time that, as previously mentioned, Cornelius Celsus (45 BC – 25AD) first characterised the four cardinal signs of inflammation "Notae vera inflammationis sunt quattuor; rubor et tumor cum calore et dolore" identifying redness, swelling, heat and pain (Shiga et al., 2014, Forrest, 1982). He further described a variety of different wounds and gave detailed descriptions of their varied treatment, classifying topical preparations according to their effects on the wound and also described the surgical instruments in use at the time and their use (Forrest, 1982). Celsus arguably provided the first documented advocation of haemostasis:

"When a man has been wounded who can be saved, there are in the first place two things to be kept in mind: that he should not die from hemorrhage or inflammation" (Majno, 1975)

However, his text "De Medicina" was lost until 1443 (Botero and Pérez, 2012). Despite the Greek influence on Roman medicine, which influenced the likes of Celsus, certain methods were propagated more than others. The most significant medical figure of the time became Claudius Galen of Pergamon (AD 129–199) physician to the Emperor Marcus Aurelius who would establish a medical dogma what would become unshakable for 15 centuries. He appreciated the core concepts of anatomy and physiology as well as the importance of scientific experimentation. However, he was limited to only what could be observed with the naked eye, and explained a number of phenomena by philosophical and teleological arguments (Forrest, 1982). Most significantly for the history of sepsis, Galen was a proponent of suppuration, instigating the concept of "Pus bonum et laudabile", which advocated that pus was useful and laudable, stating that wounds were cured on second intention and that pus formation was fundamental for their healing (Botero and Pérez, 2012, Thurston, 2000). This concept stood fast as medical dogma for the next fifteen centuries. The concept instigated the widespread use of cauterisation, rotten materials and caustic substances to promote pus generation in a misguided attempt to promote healing. Despite the well documented Roman awareness of the important of health and wellbeing, prompting athletic events, constructing bathhouses and prompting athletic events, they had no solid concept of infection by contact or of infection in general. Subsequently, no meaningful attempts to challenge the Galen approach or to develop infection preventative measures were developed (Thurston, 2000). The

Greek theories of conservative moderation in wound treatment in response to observed characteristics were lamentably superseded by this approach.

However there was some thinking, even during these times, by authors which offered theories which were of a more ontological leaning. Aristotle (384 BC - 322 BC) considered that new animals were born from putrification and that sepsis produced small creatures in putrid mud such as swamps, a point further noted later by Marcus Terentius Varro (116 - 27 BC), who wrote of *"a multitude of small animals develop which can not be perceived by the eye, but the penetrate the body through the mouth and the nose, and cause terrible diseases"* (Majno, 1991). These theories still fell within the Humoral system and it would be many centuries before the concept of microorganisms and germ theory would be conceived.

Throughout the following Medieval age (approximately 400 – 1400 AD), it can be imagined that sepsis was a major cause of death quietly fatally concluding the work of the medieval physician and their favoured methods of cauterisation and "Pus bonum et laudabile". During this time, the problems were further compounded by increasingly poor sanitary conditions, typified by insufficient water supplies, poor, if not absent drainage and overcrowding (Thurston, 2000).It was to be some time, bold thinking, the advent of firearms and an inconvenience of circumstance which would eventually alter this practice.

It was the not until the 13th century that some authors were bold enough to contradict these practices in earnest. Wound cleaning and attempting to keep wounds dry began to be promoted as opposed to prompting pus formation. William of Saliceto (AD

1210–1280) unequivocally advocated that suppuration was bad for both the wound and the patient; that wounds should heal by first intention (Thurston, 2000). However, he and other such authors were attacked by established prominent practitioners devoted to the Galen approach. The development and use of gun powder and firearms in warfare would instigate an alteration in the understanding of wounds. Blades and point wounds to either the head or torso had been essentially fatal and most wound management focused on limb wounds. The advent of firearms created a dramatic change in the types of wounds inflicted upon a person, beginning in the latter half of the 14th century (Broughton et al., 2006). A primary wound was typically accompanied by the embedded shot, gunpowder and other debris transported into the wound. Combined with an associated rise in mortality, this gave rise to the notion that the wounds were poisoned by the gunpowder. One German surgeon in particular, Hieronymus Brunschwig (1450 – 1533), wrote of a "blood poisoning" caused by gunpowder, proposing that treatment should be focused on extracting this dangerous substance (Botero and Pérez, 2012). This may be where the phrase "blood poisoning" originated. It was also postured that embedded pieces of clothing and armour could be compounding the situation. However while removal of the embedded materials was aptly advocated, it was a prevalent recommendation that boiling oil be used on the wounds to counter this poisoning, a process in-line with the Galen principle of promoting suppuration advocated by the eminent Italian author Giovanni de Vigo (1460 – 1520) (Botero and Pérez, 2012). It is a reasonable interpretation that at this point in history sepsis was a considerable cause of the associated rise in mortality. The phantom "blood poisoning" was likely due to an

increase in sepsis and septic shock arising following battle further compounded by treatments involving liberal suppuration (Botero and Pérez, 2012).

It was not until the Renaissance that significant process was made. Several prominent physicians, including Paracelsus (1493-1541) the eminent Swiss physician, staunchly denounced the Galen approach and the practice of suppuration, referring to it as "repressible". An inconvenience of circumstance led to another major event which helped to debunk "Pus bonum et laudabile". During the siege of Turin, a French barber surgeon, Ambroise Paré (1509 -1590), ran out of oil for use in the treatment of wounds and due to the scarcity of supplies was forced to dress the wounds in a concoction of egg yolks, rose oil and turpentine. He wrote of his anxiety that he had doomed the soldiers treated in this manner as opposed to those he had treated previously by cauterising using hot oil. However when returning to the wounded, he found those treated with the turpentine concoction dressings fared much better than those treated with the oil:

"…beyond my expectation I found that those to whom I had applied my digestive medicament had but little pain, and their wounds without inflammation or swelling, having rested fairly well that night; the others, to whom the boiling oil was used, I found feverish, with great pain and swelling about the edges of the wounds. Then I resolved never more to burn thus cruelly poor wounded men" (Paget 2005)

Having, through obligation, challenged the medical conventions of the time, he initiated the gradual revision of theories and practices in medial wound treatment, including the resurrection of forgotten theories of the likes of Celsus. This event

could also be considered one of the earliest medical trails based upon evidence to challenge thinking of the time.

Such theories received reinforcement when Girolamo Fracastoro (1478 – 1553) formalised a theory of contagion in 1546. Fracastoro considered infection to be caused by the passage of minute bodies from person to person which could be by one of three mechanisms: direct contact, indirect contact facilitated by infected articles or items and lastly transmission from a distance (Thurston, 2000).

The Aristotelian theory that sepsis produced small animals was challenged by an Italian physician, poet and sceptic, Francesco Redi (1626-1697). In 1684, he published an experiment on the generation of maggots which would become famous. He demonstrated that pots baited with foodstuffs left open would attract flies and quickly teem with maggots. However, the same pots covered with fine gauze would attract flies but no maggots developed on the foodstuffs. Redi concluded that "rotten stuff has no function in the generation of insects other than to provide a suitable place for laying eggs at the proper time" (Majno, 1991). A verifiable challenge to Aristotelic biology was established. While this was achieved using just the human eye, around the same time the microscope was created.

Microscopes became available early in the 18th century and in 1718 Louise Joblot (1645- 1723) published his observations identifying what he called “many little fishes” or “animalcules” as a result of his studies using a microscope. Their presence was not associated with a putrid odour though which reignited debate on the Aristotelian idea of spontaneous generation of small animals (Majno, 1991).

Francesco Redi's experiments of 1684 had demonstrated that putrefaction does not produce insects or maggots, however it did not address the mechanisms of putrefaction. Coinciding with availability and continuing developments of microscopes and microscopy throughout the 18th century, new patterns were becoming evident in medical practice, expedited by the use of firearms and gunshot wounds, which were far more likely to become infected than the cleaner slash wounds of blades. This was further compounded by the rise in surgical techniques being practised and an associated rise in infection (Majno, 1991). This reached such a staggering stage of ubiquity that gangrene became known as "hospital rot". Such odious infections even merited their own categorisation; the "putrid diseases" (Majno, 1991). These included "putrid wound" and "puerperal fever". Autopsies following fatalities from such conditions revealed important insights. They sometimes revealed abscesses distal from the initial wound (Majno, 1991). This raised important questions regarding how it was possible for pus to migrate from one place to another in the body, giving rise to the theory of pyemia; pus in the blood. It is important to note that the ancient concept of miasma was still a commonly held ideal. These foul exhalations were still viewed as a formidable poison. However, there were individuals willing to combat this thinking. Francois Magendie (1783-1855) created an experiment to test this theory by sealing small animals in a barrel with a source of putrefaction separated by wire. The animals stayed there for a month being well feed, to limited adverse effect. He repeated the experiment with a dog, which did expire but upon autopsy showed no sign of putrid infection. Surprisingly, and contrary to the observations, he concluded that miasmas do kill. However, the experiments of coming years would be more rigorous.

Investigations were also carried out by injecting putrid substances into experimental animals. In 1872, Casimir Davaine (1812-1882) injected putrid blood under the skin of a rabbit and blood from the second rabbit into another and another incrementally for a total of 25 rabbits. In each instance, the lethal dose required became progressively smaller. This experiment proved to be a key event in advancing understanding of septic disease (Majno, 1991). Subsequently, the centuries old connection between sepsis and putrid exhalations began to unravel and the more modern concept of septicaemia was created. This was a bold step as the putrid blood did not exhibit a foul odour as thinking at the time would have expected and it was a commonly held belief that for blood to be putrid it would have to be "at rest" rather than moving, perhaps an idea harking back to stagnant swamps rather than flowing streams (Majno, 1991).

In-vitro studies had also taken place in the 1700's. Sir John Pringle (1707-1782) the British Army's Surgeon General encountered a great deal of what is now known to be sepsis, indeed remarking that hospitals rather than returning soldiers to health were actively a danger to the military, including the dreaded "putrid diseases". He reasoned that if there is sepsis there must be an anti-septic, not entirely dissimilarly to certain ancient Egyptian and Greek thinking, however he was the first to use the term (Majno, 1991). He devised an experiment utilising beef suspended in a salt solution as a control, which he referred to as a "standard", and observed how long the process of putrefaction took, compared to beef suspended in other substances to investigate if there was any delay or acceleration of this process (Majno, 1991). Through this process he declared that acids were antiseptic, and interestingly that nothing added appeared to accelerate the process (Majno, 1991).

In the following years significant advances were made; the concept of debridement was introduced by Pierre Joseph Desault (1738 – 1795) and advocated by John Hunter (1728 -1793), who documented the deleterious effect of devitalized and necrotic tissues, promoting their surgical removal (Vincent, 2008)

Later distinctions began to be drawn regarding an initial wound and inflammatory response and subsequent systemic progression of inflammation and sepsis. Following the battle of Waterloo, the Duke of Wellington's chief surgeon, George James Guthrie (1785-1856), observed a distinction between local inflammation and subsequent sepsis development:

"Pain, heat, redness, tumefaction of neighbouring parts constituting inflammation comes on, which speedily runs into suppuration or gangrene... fever becomes more violent and frequently ends in death in the course of a few days" (Botero and Pérez 2012).

He advocated early amputation as a mechanism to limit the systemic response, which in the pre-antibiotic era was one of very few options available. He reported mortality of 22% of patients who received early amputation compared to 37% who received later amputation (Adrie and Pinsky, 2000). However, the underlying mechanisms of the condition and indeed understanding of infection, secondary injury and systemic spreading were not yet reliably known. In the 19th century, before the appreciation and adoption of antiseptics and aseptic environments, the chances of surgical survival were not surprisingly slim at best.

Moving toward the 20th century where Rudolph Virchow (1821 - 1902) postulated and popularised the famous cell theory; that *omnis cellula e cellula* – every living cell comes from another living cell, effectively renouncing spontaneous generation and the beneficial effects of suppuration, instead establishing that cells could only originate from pre-existing cells and not from amorphous material (Botero and Pérez, 2012). His theories were further advanced by one of his students Julius Cohnheim (1839-1884), who published his studies on inflammation in 1873 which concerned the origin of white cells in pus, diapedesis and the role of blood vessels in inflammation through study of the mesentery in frogs. Such advances paved the way for investigation and understanding of chemotaxis, diapedesis, and local inflammation, that in turn provided the basis for the beginning of immunological and physical chemical studies which would ultimately shape understanding of inflammation as it is now known (Botero and Pérez, 2012).

However, the evolution of the understanding of inflammation and sepsis is not without casualties; there is the tragic story of Ignaz Semmelweis (1818 – 1865), a Hungarian obstetrician who associated a practicing physicians contaminated hands with an increased risk of puerperal sepsis. He hypothesised that "cadaverous particles" were the cause (Botero and Pérez, 2012). However though he fought for his theory it was rejected by the medical community, and he was ultimately forcibly committed to a psychiatric hospital.

Nonetheless throughout the 19th century, a body of reputable literature and evidence was gathering momentum which advocated that bacteria appeared to be responsible for sepsis. Most notably by Edwin Klebs (1834-1913) who reviewed autopsy sepsis

samples finding bacteria in almost every case; however he believed them all to be the same type of organism which he labelled *Microsporon Septicum* (Botero and Pérez, 2012). Combined with ongoing works in the study of microbiology at the time, this represented a significant step forward in the understanding of sepsis, however much was still to be done to understand the mechanisms by which bacteria related to the condition (which is still going on today).

It would not be possible to write about putrefaction and historical understanding of sepsis without mentioning Pasteur. Louis Pasteur (1822-1895) published his first work on putrefaction in 1863. He declared that putrefaction was determined by ferments of the genus Vibrio (terminology and nomenclature regarding bacteria were yet to be resolutely defined), once again raising the debate over Aristotelian spontaneous generation. Pasteur's cornerstone argument was that putrefaction took place due to bacteria present in the air, prompting a scientific dispute which involved many experimental demonstrations, including at the top of Mont Blanc in search of the purest air in which to conduct experiments. His arguments were reinforced by John Tyndall (1820 –1893), a physicist who required pure air in his own experiments on vapours, who demonstrated that in a box of air which contained no suspended particles, culture medium would remain sterile (Majno, 1991). Pasteur was able to provide several important conclusions; that air is filled with microbes ready to develop and that putrid liquids can be sterilised by heating and robustly proposed that contagious diseases may be caused by microscopic organisms (Botero and Pérez, 2012). Robert Koch (1843-1910), a German doctor, advanced the thinking into practical medicine. He was concerned about bacterial growth in wounds due to medical procedures. Following experimentation involving administering putrid

substances to animals, he concluded that bacteria were not present in the blood or tissue of healthy animals but were present in that of all sick animals (Botero and Pérez, 2012). This was promptly linked to humans by the Scottish physician Alexander Ogston (1844-1929), who established a relation between bacteria and human sepsis, even detailing staphylococcus and streptococcus, finding them in the blood and pus of septic patients (Botero and Pérez, 2012).

At a similar time, the English surgeon Joseph Lister (1827 – 1912) had been working to prevent infections in open fracture, lesions and surgical procedures, albeit without real empirical evidence for his concerns but rather astute observations. He noted that fractures where the skin remained intact healed generally without sepsis or infection but were common in open fractures postulating , in a manner not entirely dissimilar to Pasteur, that this could be caused by exposure to air, which Lister referred to as "disease dust" (Thurston, 2000). His suspicion was confirmed by the publication of Pasteur's works by which time Lister had already begun advocating the use of phenol or carbolic acid to prevent infection during surgery. Despite some initial criticisms, eventually, due to the increased survival of his patients recovering from surgery without complications, his work became adopted practice. From 1880 onwards wound care literature stressed the importance of skin cleansing, removal of foreign matter, bone splinters, and irrigation with carbolic acid (Broughton et al., 2006). In later years, Carl Reyher (1846 – 1899) combined antiseptic techniques along with careful mechanical cleaning, giving shape to debridement as it is known today and which was further advanced by Antoine Depage (1862–1925) (Broughton et al., 2006). Such antiseptic approaches were effective but a more optimal solution was sought and an aseptic environment became an area of study. Sir William McEwan

(1848-1924), who had been a student of Lister, was a proponent of this approach, boiling everything to be used in surgery. Other practitioners also pursued aseptic options, such as the German surgeon Ernst von Bergman (1836–1907) who introduced steam sterilization for instruments in 1886 (Thurston, 2000). However, such approaches were not universally adopted by 1889. Insufficient concern for sterile conditions was the norm with rubber gloves and surgical masks only being reluctantly adopted in the early 20th century. However, gradually as the evidence became overwhelming, these practices became the standard accompanied by the development of laminar flow enclosures by Sir John Charnley (1911–1982).

Significant steps were made in the early 20th century. Ilya Metchnikoff (1845-1916) won the Nobel prize in 1908 for the discovery of phagocytosis and establishing the theory that the purpose of inflammation was to bring phagocytic cells to an injured area to engulf bacteria. It was identified that macrophages arose from transformed tissue and migrating monocytes in 1926 by F. J. Lang, who also proposed endothelial cell elongating and differentiation into "fibroblasts" at wounds (Broughton et al., 2006). Cytokines were revealed in the middle of the 19th century with the discovery of endogenous pyrogen which is now known as interleukin-1 (more on this in section 2.6), nerve growth factor, and interferon (Cannon et al., 1990). In the 1960s, mediators produced by lymphocytes that affected many immune functions were described and named lymphokines (which subsequently became the interleukins). In the years which followed, the actions of tumour necrosis factor were described and clinical trials investigating the effect of monoclonal antibodies used to block interleukin receptors for the prevention of T-cell proliferation as treatment for graft-versus-host disease were underway. Gradually throughout the 1980s and 90s,

identification and characterization of a range of inflammatory mediators advanced with the identification of dozens of interleukins, cytokines, chemokines, growth factors, and more (Broughton et al., 2006).

Thus, understanding of the condition was advanced from the ancient Egyptians to the 20th Century. Terms were still being used interchangeably at this point to describe sepsis and its sequelae; pyaemia, sapremia, purulent infection, putrid infection, septicemia, surgical sepsis and traumatic fever, were all utilised (Botero and Pérez, 2012). To establish clear terminologies and definitions of the condition as it is now understood, the discussion moves to contemporary understanding of the condition and its pathophysiology.

1.1.2 Contemporary Definitions

Contemporary understanding of the condition has advanced significantly from the broad criteria offered by Celsus. The significance of antiseptics and aseptic environments has now long been established. However mortality from infection and sepsis is still possible, despite antibiotics, life support and intensive care. The notion emerged that the hosts own inflammatory and immunological responses themselves could be at least in part contributing to this phenomenon.

The clinical condition of sepsis is primarily attributable to systemic inflammation. The inflammatory response itself is part of the immune response to some insult. In the conventional response, the site of injury, be it insult or infection, is subject to the inflammation in the body's attempt to "heal" at the afflicted site. Localised to the site, these changes are beneficial, complimenting the inflammatory cascade with the coagulation cascade. This process is in large part mediated by cytokines, both pro-inflammatory and subsequently anti-inflammatory. However in the progression of sepsis, the expression of such cytokines becomes unmitigated leading to a systemic progression of an dysregulated inflammatory response, which can progress in a number of different manners. It is this unbridled inflammatory progression and subsequent complications which constitute the condition known as sepsis today. However, the current understanding of the condition and its management are comparatively recent knowledge, and still the subject of much investigation.

Many authors comment on the variability of inciting incidences of inflammation and subsequent systemic inflammatory response. In recent history, sepsis was regarded as

a hyperinflammatory response to an invading pathogen, in the worst cases resulting in multiple organ failure and death. However, such theories were contradicted by evidence demonstrating that in only 50% of cases was bacteraemia detected (Rittirsch et al., 2007, Buras et al., 2005). Accordingly sustained, and ongoing, research and investigation have led to a major shift in the way investigators view the problem of sepsis over the last 20 to 30 years. Such investigation led to several major consensuses and the establishment of defined terminology at particular points in time. Definitions and associated recommendations for therapy have altered and are still the subject of much debate.

Roger C. Bone conducted a large body of work attempting to advance understanding of the condition, its variations and classifications. In 1980s and 1990s, he and colleagues produced a significant body of material on the subject, both in terms of definitions (discussed here) and the underlying pathophysiology (discussed in chapter 2.0). At the time, there was a considerable lack of consensus about the definition of sepsis and its sequelae, which led to widely variant reports of mortality associated with septic shock range from as low as 10% to as high as 90% (Roger C. Bone, 1991). In an attempt to overcome this problem, Bone proposed a framework for defining levels of infection from uncomplicated bacteremia through to refractory septic shock, demonstrated in Table 1.

Disorder	Requirements for Clinical Diagnosis
Bacteremia	Positive blood cultures
Sepsis	Clinical evidence suggestive of infection *plus* signs of a systemic response to the infection (all of the following): • Tachypnea (respiration > 20 breaths/min [if patient is mechanically ventilated, > 10 L/min]) • Tachycardia (heart rate > 90 beats/min) • Hyperthermia or hypothermia (core or rectal temperature > 38.4 °C [101 °F] or < 35.6 °C [96.1 °F])
The sepsis syndrome **(may also be considered incipient septic shock in patients who later become hypotensive)**	Clinical diagnosis of sepsis outlined above, plus evidence of altered organ perfusion (one or more of the following): • $Pao_2/F\backslash o_2$ no higher than 280 (in the absence of other pulmonary or cardiovascular disease) • Lactate level above the upper limit of normal • Oliguria (documented urine output < 0.5 mL/kg body weight for at least 1 hour in patients with catheters in place) • Acute alteration in mental status • Positive blood cultures are not required
Early septic shock	Clinical diagnosis of sepsis syndrome outlined above, *plus* hypotension (systolic blood pressure below 90 mmHg or a 40 mmHg decrease below baseline systolic blood pressure) that lasts for less than 1 hour and is responsive to conventional therapy (intravenous fluid administration or pharmacologic intervention)
Refractory septic shock	Clinical diagnosis of the sepsis syndrome outlined above, plus hypotension (systolic blood pressure below 90 mmHg or a 40 mmHg decrease below baseline systolic blood pressure) that lasts for more than 1 hour despite adequate volume resuscitation and that requires vasopressors or higher doses of dopamine (> 6 µg/kg per hour)

Table 1: A Uniform System for Defining the Spectrum of Disorders Associated with Sepsis. Adapted from Roger C. Bone (1991). Reprinted with the permission of American College of Physicians, Inc. license Number 3846971042332.

A further problem was that none of the disorders listed in this table was a reportable disease, further diluting the reliability of epistemological data at that point. Bone's group had previously concluded that in the incidence of sepsis syndrome without shock had a mortality of 13%, the sepsis syndrome presenting with shock had a

mortality of 28%, and shock developing after the sepsis syndrome had a mortality of 43% (Cai et al., 2010) .

While such definitions are useful practically for clinicians, as they permit classification of patients and can be used to inform treatment, the authors note that sepsis, the sepsis syndrome, and septic shock are not discrete entities, but terms to delineate increasingly severe stages of the same disease and result from the same pathophysiological processes, which are discussed further in chapter 2.0. Roger C. Bone (1991) proposed a hypothetical model to demonstrate this sequential progression, demonstrated in Figure 4.

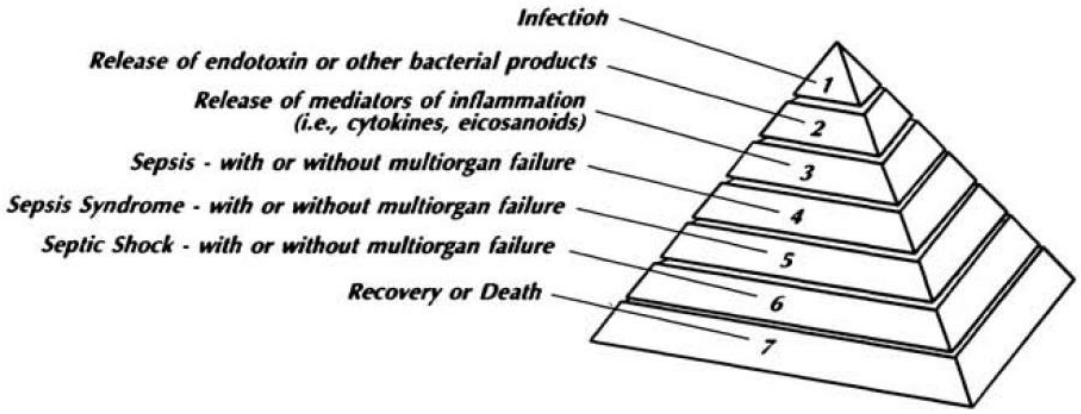

Figure 4: Model of Sepsis (Roger C. Bone, 1991). The mediators of sepsis can be shown to produce an expanding sequence of events according to intensity or dose of the original insult. Reprinted with the permission of American College of Physicians, Inc. license Number 3846971042332.

Roger C. Bone (1991) also notes in his work that other variables must be considered in any discussion of the role mediators in sepsis, such as the clinical status of the patient before sepsis, the length of illness, and any innate variations in patients'

ability to secrete mediators, which can all be factors of influence and perhaps even causation.

Systemic Inflammatory Response Syndrome (SIRS)

A key event, which still impacts upon the modern understanding and formalisation of sepsis, arguably the cornerstone of most contemporary theories on sepsis, was a consensus conference chaired by Roger C. Bone, in 1991 convened by the American College of Chest Physicians (ACCP) and the Society of Critical Care Medicine (SCCM). The aim of this conference was: "to provide a conceptual and a practical framework to define the systemic inflammatory response to infection, which is a progressive injurious process that falls under the generalized term 'sepsis' and includes sepsis-associated organ dysfunction as well" (Bone et al., 1992). It was intended that such terms would provide clarity to those working in the field both in the clinical and research settings. These will be discussed but it is important to note that even at the time those involved were aware of the limitations of such definitions and as such made the important recommendation early on that they recognised further studies would be required to validate the proposed concepts, phases and measures.

Although these definitions have been contested, they were fundamental in characterising the condition. So much so that recently one of the leading authors at the time brought their relevance back to the fore in a special edition of publications for the journal "Virulence" titled "Systemic inflammatory response syndrome (SIRS) Where did it come from and is it still relevant today?" (Balk, 2014). The outcomes of the 1991 conference had such a significant effect on how the condition is characterised and treated that they are a critical consideration of this project.

A significant outcome of the 1991 conference was the widespread introduction of the terminology "Systemic Inflammatory Response Syndrome", SIRS, and accompanying criteria. These definitions are still currently employed in the clinic. This terminology was established as a direct response to confusion at the time caused by use of the terms "sepsis" and "septic syndrome" when referring to incidences of non-infectious inflammatory conditions. It was recommended that the terminology used in relation to sepsis and its sequelae be standardised to avoid confusion and focus understanding and future work:

"By standardizing terms, such as sepsis, the ability to compare protocols and evaluate therapeutic interventions is significantly improved. The following definitions should be used as general guidelines in the design of future investigations into potential new diagnostic and treatment modalities" (Bone et al., 1992).

The consensus of Bone et al. (1992) involved the consideration, based on clinical evidence, that sepsis and SIRS may not only be attributable to an overactive inflammatory response by the immune system responding to a pathogen but that the immune system itself is somehow compromised in sepsis and SIRS (Riedemann et al., 2003, Coussens and Werb, 2002). Mechanisms of such a theory are still the source of much research but recent hypotheses propose that sepsis moves through different phases with alternating periods of amplified inflammation and amplified immune suppression (Xiao et al., 2006, Shimaoka and Park, 2008). Wherein the molecular response is divided between the pro-inflammatory mediators in the acute phase and counter-inflammatory mediators in the latter phase, this will be discussed in more detail in section 2.7, ultimately in development of sepsis regulated

expression of the appropriate mediators is lost, resulting in an exaggerated and dysfunctional inflammatory response (Buras et al., 2005, Doi et al., 2009, John A. Kellum et al., 2007). Roger C. Bone (1991) referred to this as a state of "metabolic anarchy" in which the body can no longer control its own inflammatory response.

Subsequent dramatic changes occur in the overt physiology of afflicted patients. It is from these clinical features that the consensual definitions of sepsis and SIRS are established. The accepted diagnostic criteria, outlined by the American College of Chest Physicians and Society of Critical Care (Bone et al., 1992, Dellinger et al., 2008), are summarised by Silva et al. (2008) in Table 2.

Sepsis Definitions

1	**Systemic Inflammatory Response Syndrome (SIRS):** Two or more of the following: a) temperature (core) > 38.3ºC or < 36ºC; b) heart rate > 90 beats/min; c) respiratory rate > 20 breaths/min, $PaCO_2$ < 32 mm Hg or need for mechanical ventilation; d) WBC count > 12.000/mm^3 or < 4.000/ mm^3 or > 10% immature forms (bands).
2	**Sepsis:** Defined as SIRS associated with suspected or confirmed infection. Positive blood cultures are not necessary.
3	**Severe Sepsis:** is sepsis complicated by a predefined organ dysfunction.
4	**Septic Shock:** is cardiovascular collapse related to severe sepsis despite adequate fluid resuscitation. Hypotension is: systolic blood pressure (SBP) < 90 mm Hg, mean arterial pressure (MAP) < 65 mm Hg or a reduction of > 40 mm Hg on baseline SBP.
5	**Organ dysfunction** criteria are a) hypoxemia (PaO_2 /FiO_2 ratio < 300); b) acute oliguria (urine output < 0.5 ml/kg/h for 2 h) or creatinine > 2.0 mg/dL; c) coagulopathy (platelet count < 100.000, INR > 1.5 or pTTa > 60 s); d) ileus; e) plasma bilirubin > 4 mg/dL).

Table 2: Sepsis Definitions summary table (Silva, Passos et al. 2008) based on consensus from (Bone et al., 1992). Table reproduced under CC BY-NC 4.0.

These classifications afford clinicians the opportunity to begin support for the condition early by identification through combinations of values and observations of typical symptoms (Bone et al., 1992). Importantly, the values indicated are envelopes of values rather than any absolutes, for instance, either a rise above 38.3ºC or a fall below 36ºC, highlighting the variant progression of sepsis and the variety of systems involved.

The clear establishment of the terminology for a systemic inflammatory response syndrome, SIRS, and its associated clinical manifestations (as detailed in Table 2) was the first of four recommendations made as a result of the 1991 consensus conference characteristics to establish a diagnosis of SIRS:

"The term sepsis in popular usage, implies a clinical response arising from infection. It is apparent that a similar, or even identical, response can arise in the absence of infection. We therefore propose the phrase systemic inflammatory response syndrome (SIRS) to describe this inflammatory process, independent of its cause" (Bone et al., 1992).

This was a cornerstone in the understanding of the condition and the first major consensual attempt to stratify it. Critically, it was recognised that the condition could prevail without the source of the condition arising from an infection. Accordingly, it is now established in the healthcare profession that where a systemic inflammatory condition is present without a suspected infection it is referred to as SIRS and referred to as sepsis where infection is the causation. A key visualisation of the

interrelationship of SIRS, sepsis and associated afflictions was presented in the published paper following the conference, demonstrated in Figure 5.

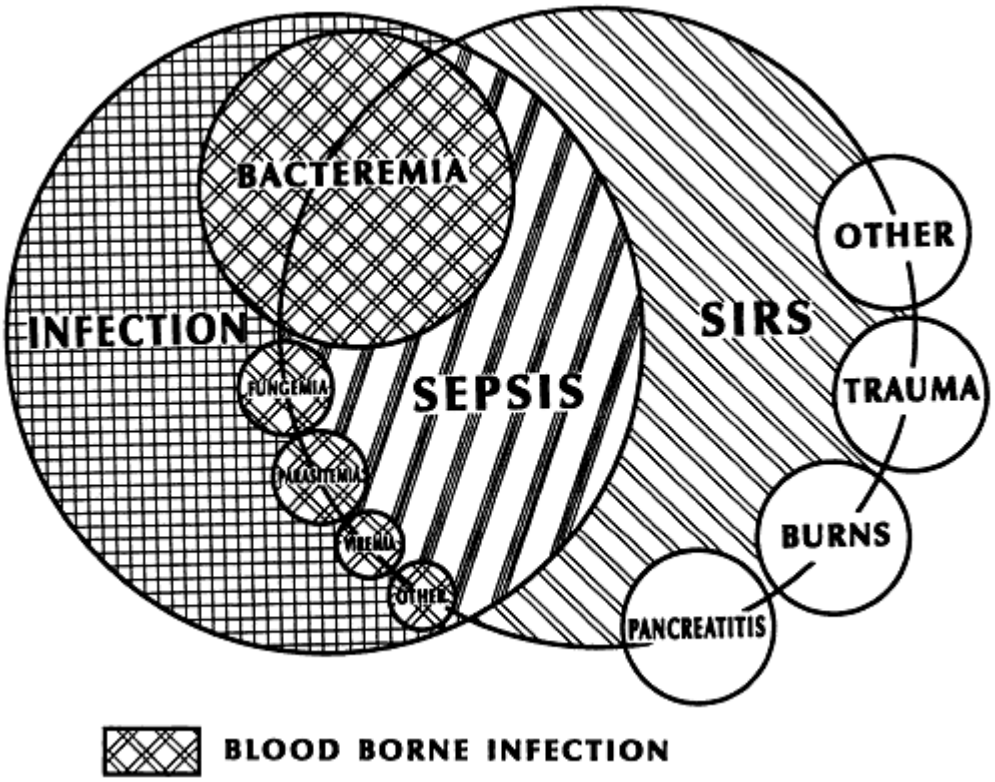

Figure 5: The interrelationship between systemic inflammatory response syndrome (SIRS), sepsis, and infection (Bone et al., 1992). Figure reproduced with permission from Elsevier licence number 3830831501325.

The second recommendation clarified the distinction of the term sepsis as the systemic result of a confirmed infectious process (however was this later changed to be only suspected rather than confirmed). The third recommendation further defined some other terminology which had at the time become common healthcare diction but that required established clarity, these are detailed in Table 3.

Definitions Associated with Sepsis

Infection	Microbial phenomenon characterized by an inflammatory response to the presence of microorganisms or the invasion of normally sterile host tissue by those organisms.
Bacteremia	The presence of viable bacteria in the blood.
Systemic inflammatory response syndrome (SIRS)	The systemic inflammatory response to a variety of severe clinical insults. The response is manifested by two or more ofthe following conditions: 1. temperature >38°C or <36°C 2. heart rate >90 beats per minute; 3. respiratory rate >20 breaths per minute or $PaCO_2$, <32 mmHg; and 4. white blood cell count >12,000/cu mm, <4,000/cu mm, or >10% immature (band) forms
Sepsis	The systemic response to infection, manifested by two or more of the following conditions as a result of infection: 1. temperature >38°C or <36°C 2. heart rate >90 beats per minute; 3. respiratory rate >20 breaths per minute or $PaCO_2$, <32 mmHg; and 4. white blood cell count >12,000/cu mm, <4,000/cu mm, or >10% immature (band) forms
Severe sepsis	Sepsis associated with organ dysfunction, hypoperfusion, or hypotension. Hypoperfusion and perfusion abnormalities may include, but are not limited to lactic acidosis, oliguria, or an acute alteration in mental status.
Septic shock	Sepsis-induced with hypotension despite adequate fluid resuscitation along with the presence of perfusion abnormalities that may include, but are not limited to, lactic acidosis, oliguria, or an acute alteration in mental status. Patients who are receiving inotropic or vasopressor agents may not be hypotensive at the time that perfusion abnormalities are measured.
Sepsis-induced hypotension	Systolic blood pressure <90 mm Hg or a reduction of 40 mm Hg from baseline in the absence of other causes for hypotension.
Multiple organ dysfunction syndmme(MODS)	Presence of altered organ function in an acutely ill patient such that homeostasis cannot be maintained without intervention.

Table 3: Definitions established at the 1991 consensus conference. Adapted from Bone et al. (1992). Table reproduced with permission from Elsevier licence number 3846501019977.

Indeed, as part of this particular recommendation it was also put forth that the commonly used term "septicaemia" be abolished. Citing that the term did not describe the spectrum of the condition satisfactorily, which created confusion in the clinic, medical literature and further created difficulties in interpretation of data.

However, it stands as testament to how prevalent use of the word septicaemia had become that it is still part of everyday language, despite no formal attempt to reinstate it in medical diction since this proposed abolishment in 1991. A gauge perhaps of how sprawling and unwieldy terminology had become at that point relating to the condition, necessitating such consensus conferences.

The fourth recommendation, and of significant note, was that:

"Sepsis and its sequelae represent a continuum of clinical and pathophysiologic severity. The degree of severity may independently affect prognosis" (Bone et al., 1992).

A critical outcome of the conference was the acknowledgement in the rational of establishing these terms that there is a "continuum of severity" (Bone et al., 1992). The group further noted "definable phases exist on that continuum which characterize populations at increased risk of morbidity and mortality." (Bone et al., 1992). This was the case both in terms of infection derived sepsis and non-infectious systemic inflammation, which incorporate bacteraemia and septic shock within the continuum. In the worst cases, the condition progressed to organ dysfunction or multiple organ dysfunction, to be defined as "severe sepsis" or "sepsis with multiple organ dysfunction". Until this point, many made use of the term "septic syndrome"

to describe this phase of the septic process, including Bone in his own earlier work (Figure 4). However, much like "septicaemia", the term had become so indiscriminately utilised to describe a wide variety of inflammatory states that it had become vague and confusing; it was recommended that the term "septic syndrome" be eliminated.

Multiple Organ Dysfunction Syndrome (MODS)

In addition to the refinement and consensual recommendations regarding sepsis and SIRS at this conference, another key sequelae of sepsis was the subject of classification; the morality associated with the condition. Generally, mortality in sepsis is due to the sequential and progressive failure of key organs or organ systems. Until this point, such prognoses had been indeterminately referred to as "progressive or sequential organ failure, multiple organ failure, and multiple systems organ failure" (Bone et al., 1992). Similar to the way in which a variety of variations of the terminology had become common diction in healthcare for sepsis, so too had it been for the dysfunction of organs. Commentary was made that typically it was reported as multiple organ failure which was binary in nature, either an organ had failed or had not. However the dysfunction of organs in an interrelated manner is a mortality indicator. A key criticism of such terminologies was their overstatement of "failure". It was argued instead that dysfunction was a more suitable terminology as more indicative of the continuum of altered function which could ultimately lead to failure but which in itself was still a progression. It was also commented upon that it was entirely possible for such dysfunction to develop without any central infectious foci and could indeed be instigated as a result of the systemic inflammatory response.

Accordingly, several recommendations were made regarding the definition of multiple organ dysfunction with the intention of providing a basis upon which to build in future studies of dysfunction not only in the case of sepsis but in critical illnesses in general.

It was recommended was that organ dysfunction should refer to "a phenomenon in which organ function is not capable of maintaining homeostasis" (Bone et al., 1992). This was also recommended to be considered as a continuum, in this instance a continuum of change over time. It was also a part of this recommendation that multiple organ dysfunction syndrome (MODS) is indeed a syndrome: "a pattern of multiple and progressive symptoms and signs that are thought to be pathogenetically related" expanding upon this with the following points:

Multiple Organ Dysfunction Syndrome (MODS)	
1	MODS describes a continuum of organ dysfunction, although specific descriptions of this continuous process are not currently available.
2	The recognition of early organ abnormalities must be improved so that treatment can be initiated at earlier stages in the evolution of this syndrome.
3	Changes in organ function over time can be viewed as an important element in prognostication. When applied to MODS, existing measures of illness severity provide only a snapshot in time of this dynamic process, and are generally without reference to the natural course of the disease.
4	MODS is subject to modulation by numerous factors at varying time periods, both interventional and host-related.

Table 4: MODS points of recognition. Summarised from Bone et al. (1992)

In the instance where MODS develops as the result of a defined insult or infection to which the dysfunction can be attributed, this is referred to as primary MODS. In the instance where dysfunction occurs as an indirect result, as a consequence of a host response to insult, this is referred to as secondary MODS (Bone et al., 1992). The distinction is visualised in Figure 6. It is therefore more common to encounter secondary MODS as the sequelae of a systemic inflammatory response, importantly:

"Secondary MODS develops, not in direct response to the insult itself, but as the consequence of a host response, and is identified within the context of SIRS" (Bone et al., 1992).

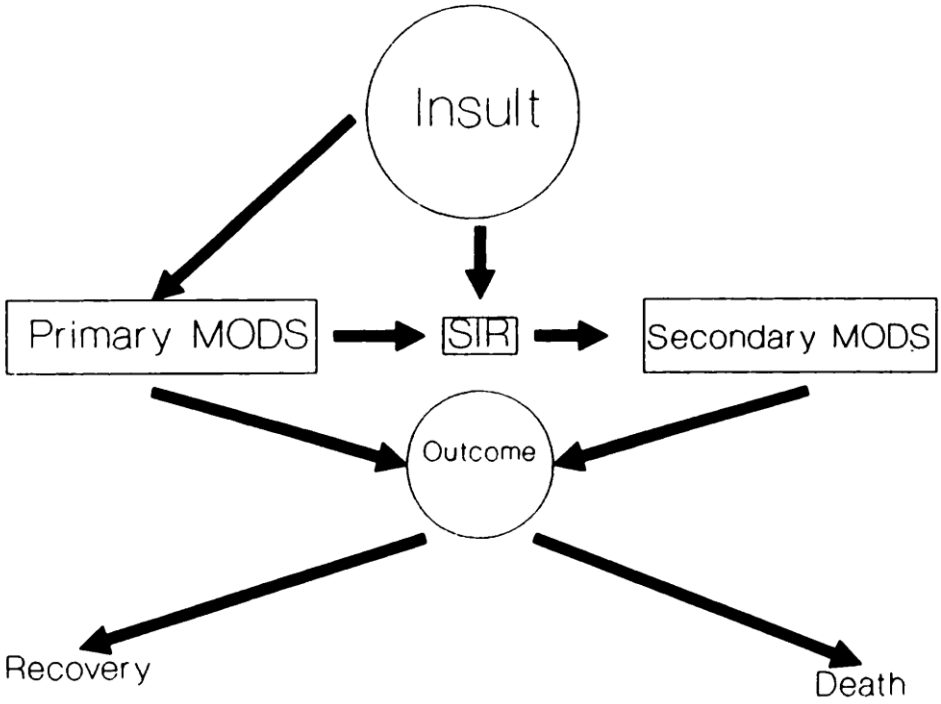

Figure 6: The different causes and results of primary and secondary multiple organ dysfunction syndrome (MODS) (Bone et al., 1992). Figure reproduced with permission from Elsevier licence number 3830831501325.

It was however documented that any such criteria were likely to be the subject of continual refinement as understanding of both individual and system level organ dysfunction progressed.

The definitions put forward at the consensus conference were established with the objective of ensuring disambiguation and improving understanding with the ultimate intention that this would lead to improved therapeutic management.

Subsequently, SIRS entered formalised medical diction along with the recommendations made at the conference. However, as was appropriately acknowledged by the participants at the time, these were to be the subject of refinement and revision. Since the early 2000s, a method of stratification for sepsis has been pursued building upon the work of Bone et al. Debate arose around the clinical impracticalities of SIRS, to such an extent that another major consensus definitions conference took place in 2001. The SIRS definition came under fire during the 2001 SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference (Levy et al., 2003). The SIRS definition was criticised for its vague nature identifying too broad a range of ICU patients. The key outcomes of this conference were a refinement of definitions and also the proposal of a new prognostic model of the sepsis continuum, PIRO. This new proposal was centred around the idea of adopting a concept analogous to the oncological Classification of Malignant Tumours, TNM, (Tumors/Nodes/Metastases) staging notation system. The TNM system had been successfully developed and was already in wide clinical use at the time.

A round table expert discussion which took place at the 23rd International Symposium on Intensive Care and Emergency Medicine in Brussels in 2003 (Angus et al., 2003) was a key point in discussion within the expert clinical community proposing and debating the suitability and potential use of the PIRO system in the stratification of sepsis. It was a major point of discussion that sepsis bears many similarities in terms of the variety of responses, comorbid conditions and various pathogenic pathways which many cancers do.

The PIRO concept incorporates; predisposition, infection or insult, response (of deleterious nature) and organ failure. Its principal purpose is to stratify patients based on their predisposing conditions, nature and extent of the initial insult or infection, nature and magnitude of the host response and degree of resultant or concomitant organ dysfunction (Rosolem et al., 2010).

Opal (2005) undertook a literature summary covering five years of publications, the five years since the concepts formalisation in early 2000s, including a consensus opinion of experts in the field of sepsis and septic shock. This study concluded that PIRO is an innovative methodology enabling a re-examination of the pathophysiological events that underlie septic shock, which were formalised into the basic elements of PIRO identified in Table 5.

Basic Elements of PIRO

P	Predisposing Factors	Innate: Genetic polymorphisms and deficiencies of immune response genes affecting innate immune response, coagulation system, complement receptors, Toll-like receptors and intracellular signaling Acquired: Burns, trauma, acquired immune deficiencies
I	Infection	Site, quantity, intrinsic virulence, and local vs. systemic infection due to specific microbial pathogens
R	Response	Differential responses based on hyperresponsiveness vs. hyporesponsiveness—immunosuppression; response modifiers such as alcohol, age, sex, nutritional status, diabetes, other preexisting diseases, and physiologic status of host
O	Organ Dysfunction	Number, severity, and pattern of organ dysfunction in response to systemic infection; primary vs. secondary organ injury; and organ injury due to sepsis vs. pre-existing organ dysfunction

Table 5: Basic Elements of PIRO (Opal, 2005). Table reproduced with permission from Wolters Kluwer Health, Inc. licence number 3846510229554.

However, Opal further states that whether or not such a system will ultimately provide a more clinically useful stratification than the general terms sepsis, severe sepsis, or septic shock remains to be seen; commenting that while the TNM classification has been invaluable for some neoplasms, it has been of limited use for others (Opal, 2005). It is possible that in the sepsis setting PIRO may be useful for particular aspects of the condition but that may not necessarily translate to clinical utility. Ultimately, the PIRO approach does present an appealing option. However, the methodology is yet to be definitively evaluated (Opal, 2005).

In addition to the promotion of the PIRO stratification model, the 2001 conference reviewed the strengths and weakness of the 1992 definitions, identified potential improvements and lastly investigated ways to improve the clinical utility of diagnosis of sepsis. It was acknowledged that due to the nature of sepsis and its sequelae that there is no "gold standard" and that the success of any definitions and diagnostic criteria can only truly be assessed by whether or not clinicians are able to make improved decisions and improve therapeutics based upon their use (Levy et al., 2003). It was agreed that the SIRS criteria were correct in so far as the recognition that a systemic inflammatory response may be instigated by a great number of insults, not limited to infection. However, the 1992 criteria were so broad in nature that that they were in practice non-specific and could be applied to a number of other conditions.

Despite the debate, the definition for sepsis however was largely upheld stating:

"As in 1992, we define sepsis to be the clinical syndrome defined by the presence of both infection and a systemic inflammatory response" (Levy et al., 2003).

It was noted that any planned revisions be with a mind to improving clinical relevance for anyone conducting research, studies or clinical trails; but that the criteria should not be so cumbersome that clinicians would not readily adopt or apply them. It was further noted that criteria had to be grounded in the "day-to-day reality" for clinicians providing care. The group concluded that:

"…few, if any, patients in the early stages of the inflammatory response to infection are diagnosed with sepsis via four arbitrary criteria. Instead, the clinician goes to the bedside, identifies a myriad of symptoms, and regardless of an evident infection declares the patient to 'look septic'" (Levy et al., 2003).

The group further concluded that the definition of severe sepsis remain unchanged. Despite the discussed misgivings on stratification of the condition, the overall findings of the conference were that no new definitions were necessary. However continual review should always be considered and that ultimately the diagnosis rests with the practising clinician.

Definitions and terminology used to describe sepsis

Term	Definition	Comments
Systemic inflammatory response syndrome (SIRS)	Temperature >38.5°C (101.3°F) or <36°C (96.8°F) Tachypnea (>20 breaths/min) Tachycardia (<90 beats/min) White blood cell count >12,000 or <4000 cells/mm^3 or <10% immature forms	May be caused by infectious and noninfectious etiologies; clinical features attributable to release of inflammatory mediators into the circulation
Sepsis	SIRS caused by an invasive infection	Caused by viral, bacterial, fungal, or parasitic pathogens; bloodstream infection need not be present
Multiple organ dysfunction syndrome (MODS)	Major organ dysfunction from sepsis	A major determinant of outcome in sepsis
Severe sepsis	Sepsis accompanied by perfusion abnormalities and organ dysfunction (CNS, renal, pulmonary, hepatic, hematologic, or metabolic)	The terms ‘‘sepsis syndrome’’ or ‘‘septicemia’’ are often used to describe what is now referred to as severe sepsis
Septic shock	Severe sepsis with hypotension not responsive to an adequate fluid challenge; sometimes referred to as ‘‘endotoxic’’ shock or ‘‘toxic’’ shock	Systolic blood pressure >90mmHg or mean arterial pressure <65mmHg despite adequate fluid resuscitation
Bacteremia or fungemia	Detection of viable bacteria or fungi in the bloodstream	Transient bacteremia without clinical symptoms can occur; bacteremia may or may not be present in sepsis

Table 6: Definitions and terminology used to describe sepsis from (Opal, 2007). Table reproduced with permission from Elsevier licence number 3846510628051.

The definitions are frequently referred to in more recent literature on the subject and are still used in clinical practice. These are best summarised by (Opal, 2007) in

Table 6, accompanied by the assertion that despite their simplistic and vague nature, they are proven as invaluable in identifying the condition and that they are still the cornerstone of contemporary practice regarding sepsis and its sequelae. Throughout this body of research and thesis, these are the definitions adhered to regarding sepsis and its sequelae. However its limitations are acknowledged. For brevity in reporting, the condition will be referred to as “sepsis” unless otherwise stated in discussion relating to a particular stage of the continuum.

Compensatory Anti-inflammatory Syndrome (CARS)

As has been discussed throughout this chapter in the late 1980s and early 1990s, the term systemic inflammatory response was established by Roger C. Bone and colleagues. The term was introduced in order to highlight the key involvement of the inflammatory components in sepsis and to ensure both appreciation that the condition could be of an endogenous nature as well as exogenous and that it could lead to abnormal inflammation in organs remote from the initial insult.

At the time, the prevailing theory in pathophysiology of sepsis was that the condition was due to an uncontrolled hyperinflammatory response, dominated by pro-inflammatory cytokines. The theory was that the condition could be attributed to an overzealous pro-inflammatory response, a so-called "cytokine storm" (Adib-Conquy and Cavaillon, 2009) of pro-inflammatory cytokines. On that basis, various therapeutic strategies were derived and trials conducted. However, they failed to demonstrate any meaningful improvements in therapy of the condition. In light of this information, it appeared that the mechanisms underlying the condition were also due for a revision in a time period where the definitions of the overt condition were also undergoing revision.

It was beginning to be understood that beyond an overexuberant pro-inflammatory response, the immune system itself appeared to be altered in the progression of the sepsis. High susceptibility to nosocomial infections in ICU patients appeared to correspond with an altered immune status (Adib-Conquy and Cavaillon, 2009).

Considering this new information in 1997, Bone proposed a new hypothesis for the progression of sepsis:

"We have understood that a massive inflammatory reaction underlies both SIRS and MODS, but now we must understand that this reaction is only half the picture. It is now clear that quite rapidly after the first pro-inflammatory mediators are released, the body mounts a compensatory anti-inflammatory reaction (CARS) to the initial pro-inflammatory response" (Roger C. Bone et al., 1997).

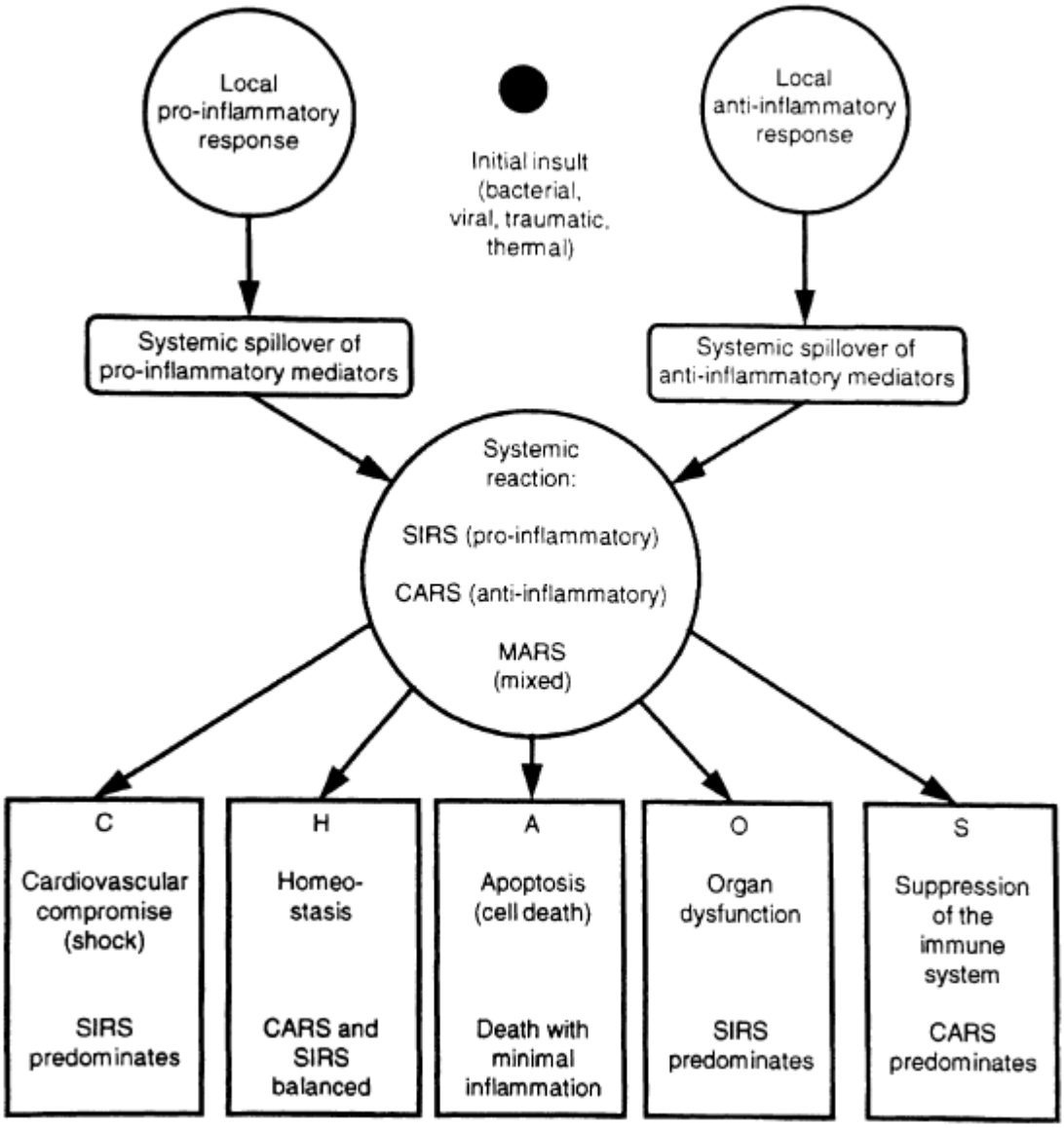

Figure 7: New concepts for the clinical sequelae of sepsis, SIRS, CARS and MARS (Roger C. Bone et al., 1997). Figure reproduced with permission from Elsevier licence number 3830840867177.

At this time, Roger C. Bone et al. (1997) proposed both the concepts and terminology of compensatory anti-inflammatory response syndrome (CARS) and mixed antagonists response syndrome (MARS). Adding them to the existing set of definitions to revising the initial pro-inflammatory response theory to state that "If balance cannot be established and homeostasis is not restored, a massive pro-

inflammatory reaction (SIRS) or a compensatory anti-inflammatory reaction (CARS) will ensue. A range of clinical sequelae may then follow." (Roger C. Bone et al., 1997). It was also proposed that a mnemonic "CHAOS" be used to aid the practicing clinician accompanied by a visualisation of the new concept for the clinical sequelae of sepsis, Figure 7. Crucially, this proposition that a variety of pathophysiological events may follow an initial insult, dominated by either pro-inflammatory, anti-inflammatory or indeed a mix of both responses, is a cornerstone of much contemporary debate and research in sepsis, which will be discussed further in section 2.7.

1.2 Mortality & Clinical Burden

Sepsis and Systemic Inflammatory Response Syndrome (SIRS) currently represent a major healthcare concern. As a condition, sepsis is commonplace, expensive and often fatal. It was reported in 2006 that in Europe it affects 3 people in every 1,000 and kills at least 135,000 each year, approximately the same number of deaths caused by lung cancer in the EU, costing approximately €7.6 billion (Hunter, 2006). In the USA, it is responsible for as many fatalities as acute myocardial infarction, developing among 900,000 people each year in the United States, a third of whom die when the condition is complicated by acute organ dysfunction (Angus et al., 2001). The demand for a therapeutic intervention in the treatment of sepsis is profound.

It has been argued that as modern medicine advances, new techniques could be increasing the risk of patients developing the condition. This has been attributed to the increasing use of indwelling catheters, chest drains, monitoring equipment, surgery involving increased endotracheal intubation and use of prolonged mechanical ventilation (Yoshihiko Kanno et al., 2003). In addition, general advances in medical care have ensured longer life spans for the elderly and immunocompromised or immunodeficient patients, groups which remain at increased risk (Roger C. Bone, 1991).

Despite these figures, the management and therapy of sepsis remains a major clinical challenge largely due to the fact that the condition is so complex. Currently, management still revolves around support of organ function and prevention of

complications until recovery occurs (Singer, 1998). The development and progression of sepsis is multifactorial, affecting the cardiovascular, immunological and endocrine systems of the body (Buras et al., 2005), and the underlying pathophysiology is still the subject of much research and debate, along with its resulting clinical symptoms and effects (Rittirsch et al., 2009, Riedemann et al., 2003). Sepsis typically arises as a complication in patients with severe trauma, burns, blood loss or patients having recently undergone major surgery, most notably surgeries involving extracorporeal circulation (Angus et al., 2001). As a result, sepsis is arguably the most common condition encountered in the intensive care unit, arising as a complication in approximately 30 to 40% of intensive care unit (ICU) admissions (Jean-Louis Vincent, 2006)

Angus et al. (2001) published one of the most robust epidemiological studies on sepsis of the 20 years, reviewing 1995 data from hospital discharge records, census information and the Centre for Disease Control. Their goal was: "to determine the incidence, cost, and outcome of severe sepsis in the United States" (Angus et al., 2001). A summary of their key findings is presented in

Table 7 below. The study concluded that the condition was frequent and costly, both in terms of resources and financially. They reported that 215,000 estimated national deaths as a result of sepsis represented 9.3% of all deaths in the United States in 1995, equivalent to the number of fatalities due to acute myocardial infarction, resulting in an economic burden of nearly $16.7 billion nationally, with an average cost per patient of $22,100 (Angus et al., 2001).

Factor	Measure
Sample size (n)	6,621,559
Sepsis cases	192,980
Estimated national cases	751,000 cases (3.0 cases per 1,000 population and 2.26 cases per 100 hospital discharges), of whom 383,000 (51.1%) received intensive care and an additional 130,000 (17.3%) were ventilated in an intermediate care unit or cared for in a coronary care unit. Incidence increased >100-fold with age (0.2/1,000 in children to 26.2/1,000 in those >85 yrs old).
Mortality	Mortality was 28.6%, or 215,000 deaths nationally (estimated national figures) and also increased with age, from 10% in children to 38.4% in those >85 yrs old. Women had lower age-specific incidence and mortality, but the difference in mortality was explained by differences in underlying disease and the site of infection.
Length of Stay (LOS)	The average LOS (Length of Stay) and cost per case were 19.6 days and $22,100. Nonsurvivors had a similar LOS (19.9 vs. 19.4 days, p , .005) but cost considerably more ($25,900 vs. $20,600, p , .0001) than survivors. LOS varied little with the number of organ systems in which acute dysfunction developed (range, 18.5–22.8 days), but average costs increased from $19,500 for those with acute dysfunction in one system to $32,800 for those with dysfunction in four or more systems.
Cost	The average costs per case were $22,100, Annual total costs of $16.7 billion nationally. Costs were higher in infants, nonsurvivors, intensive care unit patients, surgical patients, and patients with more organ failure.
Projected increase	The incidence was projected to increase by 1.5% per annum

Table 7: Key epidemiological conclusions summarised from Angus et al. (2001)

Angus et al. (2001) also demonstrated the number of incidences and percentage mortality by age group, demonstrated in Figure 8. It was observed that mortality was highest for those with some form of pre-existing condition, those in ICU care, and those with more than one organ failure. Additionally, mortality was slightly higher amongst men. The greatest variance was amongst those aged 25–30. Differences

were attributable to differences in age, underlying comorbidity, and site of infection. Ultimately, although the chances of developing sepsis differed as identified, after adjusting for these differences, the likelihood of dying from sepsis was the same (Angus et al., 2001).

Based upon their investigation, Angus et al. (2001) projected that the number of sepsis cases would rise 1.5% per annum, resulting in 934,000 and 1,110,000 cases by the years 2010 and 2020 in the U.S. which is faster than the anticipated population growth, due in part to the high incidence of sepsis in elderly patients coupled with an aging population. (Angus et al., 2001). However, Angus et al's estimates were later challenged by Wenzel and Edmond (2001) who stated that their estimates may overstate the incidence by a factor of two to four.

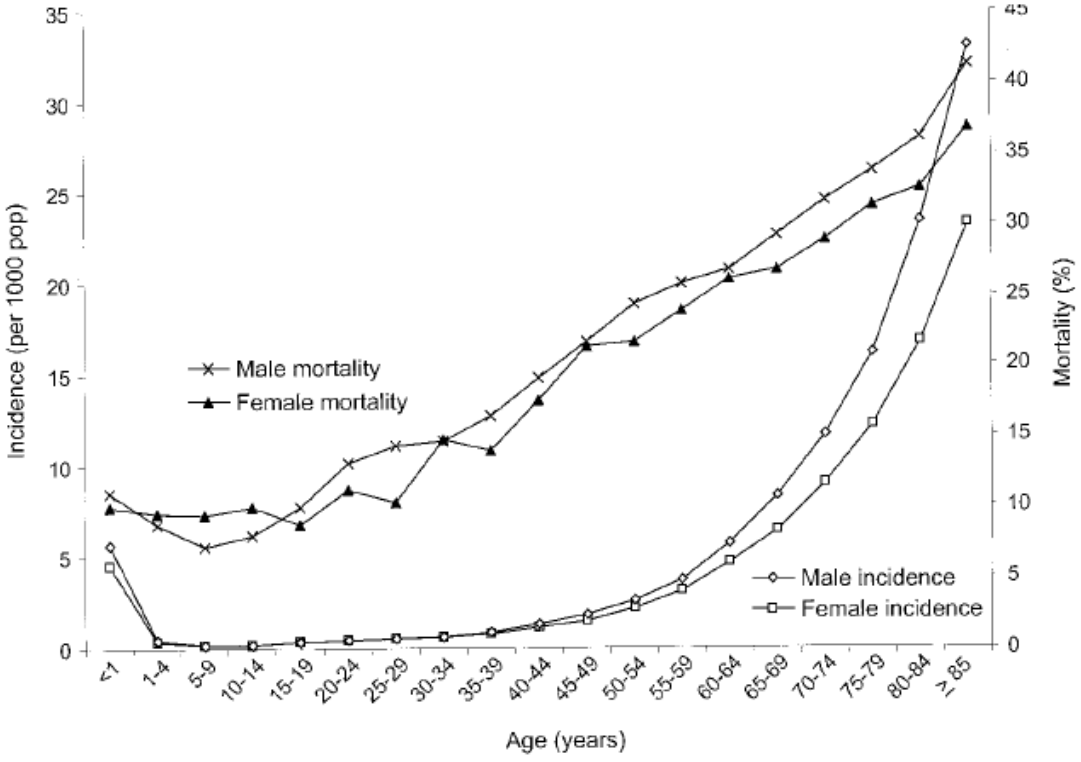

Figure 8: Sepsis incidence and mortality against age (Angus et al., 2001). Figure reproduced with permission from Wolters Kluwer Health, Inc. licence number 3846970739916.

This was followed by an epidemiological study of sepsis in the US from 1979 to 2000 by Martin et al. (2003) utilising data from acute care hospitals. Their aim was:

"…to provide a broad characterization of sepsis for use in epidemiologic estimates, as well as to identify specific groups with an altered propensity for sepsis" (Martin et al., 2003).

This study concluded that sepsis was the main cause of death in the non-coronary ICU, noting that up to 35% of patients admitted to such units were diagnosed with the condition. Their key outcomes are presented in Table 8:

Factor (over 22 year period)	Measure
Overall data sample size	750 million hospitalisations
Incidences of Sepsis	10,319,418
Annualised increase in incidence of sepsis	8.7% from about 164,000 cases (82.7 per 100,000 population) to nearly 660,000cases (240.4 per 100,000 population)
Sepsis due to fungal organisms (increase over 22 year period)	Increase by 207% (with gram-positive bacteria becoming the predominant pathogens after 1987)
Total in-hospital mortality	rate fell from 27.8 percent during the period from 1979 through 1984 to 17.9 percent during the period from 1995 through2000, yet the total number of deaths continued to increase.
Length of hospital stay (days)	Decreased from 17.0±8.5 in the period 1979-1984 to 11.8±2.6 in the period 1995–2000

Table 8: Key outcomes of epidemiological study summarised from Martin et al. (2003).

In response to the prediction by Angus et al. (2001) that the incidence of sepsis would rise by 1.5% per year, and contrary to Wenzel and Edmond (2001), Martin et al. (2003) argue that this may be a substantial underestimation. However they did not offer their own projection for this particular measure instead stating that further investigation is required to confirm changes in sepsis incidence and mortality. However, there are limitations to such national estimates based upon point prevalence studies as has recently been commented upon by Mayr et al. (2014), who state that the assumptions can only extrapolate information based upon the conditions relative at a specific point in time and that over a time course if therapies or management of the condition improve resulting in increased duration of the illness and associated LOS, then prevalence may actually increase while based upon point information incidence would appear to fall (Mayr et al., 2014). As an alternative, they argue the case for cohort studies, stating that if a sufficient duration of study is applied, more valid results can be interpreted eliminating factors such as seasonal

variance. Further citing the inherent flaws of extrapolating ICU incidence to population incidence, as not all sepsis cases are brought to ICU and indeed that such cases are generally the treated cases. In a follow up work by two lead authors of the 2001 epidemiology work, Walter T Linde-Zwirble and Derek C Angus, in 2004 they also put forward that:

"*Importantly, the availability of ICU services may well determine the number of treated cases of severe sepsis, and it seems clear that these studies are reporting the treated incidence, not the incidence, of severe sepsis. In the future, we must focus on whether all severe sepsis should be treated, and, consequently, what level of ICU services is optimal*" (Linde-Zwirble and Angus, 2004).

A matter further compounded by the fact that there is a high degree of variance in the classifications and eligibility considerations for ICU admission between countries, resource availability and healthcare insurance considerations (Mayr et al., 2014).

There is a theme throughout literature published on the matter that in the incidence of sepsis the cumulative burden and magnitude of organ failure, including the number of organs in failure and the degree to which they are failing, is the key precursor to mortality (Mayr et al., 2014). Additionally, due to predominantly aging populations, microbial endurance, increasing use of indwelling motoring equipment and medical devices the incidence of sepsis is likely to continue to increase, however the rate at which it will do so is still the subject of debate. As Botero and Pérez (2012) state:

"*We have a permanent armed race in which our invaders will always have an advantage over us and we find ourselves unrelentingly urged to innovate from the*

biomedical sciences. In conclusion, maybe sepsis imposes new challenges of great magnitude in the following years that can allow us, and force us to open new ways to develop new paradigms in this century's medicine… at least that is what the costly lessons of the past teach us" (Botero and Pérez, 2012).

1.3 Chapter 1 Summary Points

The following key points have been established in this chapter:

- Sepsis describes a condition involving a continuum of severity, ranging from systemic inflammation to septic shock, the definitions of which have been established.

- Sepsis is one of the major clinical challenges facing intensive care units across the globe, and the greatest cause of intensive care mortality.

- Sepsis incurs significant cost in the healthcare field.

- The current understanding of the condition is comparatively recent knowledge and is still an active area of research.

2.0 Pathophysiology & Treatment

The scale of the clinical challenge along with the morbidity and mortality was established in chapter 1.0 As has been discussed in section 1.2, sepsis represents a major global healthcare concern creating a profound demand for novel interventional therapies. Until the relatively recent endeavours to establish consensus definitions for sepsis and its sequelae, discussed in section 1.1.2, this matter has been compounded by conflicting terminology and reports. Sepsis remains a prominent clinical challenge and is still the subject of much research and debate, particularly regarding the pathophysiology, as Hunter (2006) states:

"*This might be partly because severe sepsis is not really a disease, but rather a syndrome describing a systemic response. Invading pathogens release endotoxins into the bloodstream, which, in turn, activate various immunological and coagulatory responses that can go awry, leading to sepsis and septic shock*" (Hunter, 2006).

Such convolution in terminologies and diagnostic criteria is only relatively recently being clarified, shedding light on the pathophysiology of the condition. The varying routes of development are still the subject of much debate and while certain key theories are becoming increasingly prominent, they are not conclusively accepted. As a result, current therapy still revolves around supporting physiological function, as will be discussed further in section 2.8. Indeed as Huttunen and Aittoniemi (2011) put it:

"*it is difficult to see the forest for the trees when considering the pathogenesis of this condition*" (Huttunen and Aittoniemi, 2011).

A broad overview of the central phenomenon of sepsis is provided by Huttunen and Aittoniemi (2011). As can be seen there are many overlapping systems involved and their progression is still the subject of much debate and ongoing research. This section will review contemporary understanding of the pathophysiology of the condition and various theories thereof.

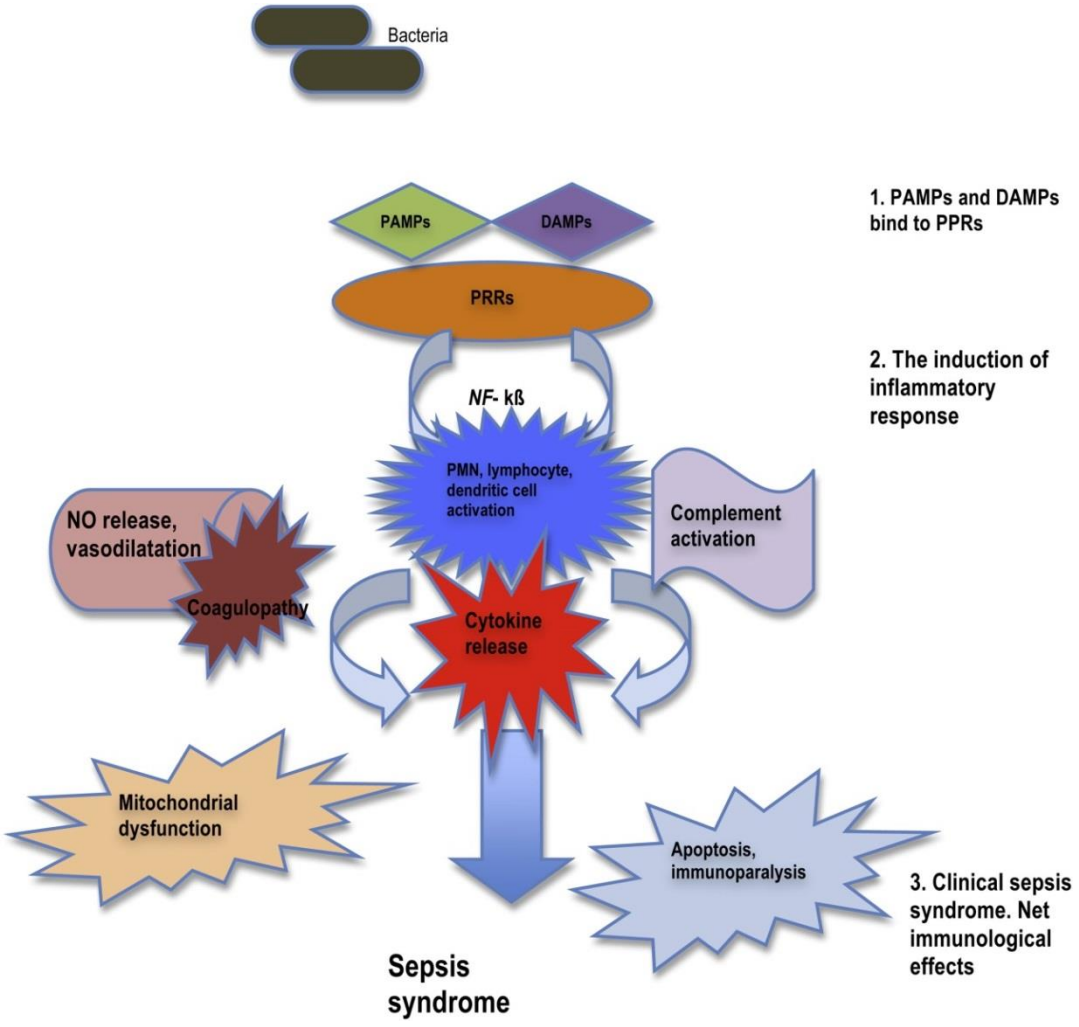

Figure 9: Central phenomena in sepsis pathogenesis (Huttunen and Aittoniemi, 2011). Figure reproduced with permission from Elsevier licence number 3830821135329.

2.1 Initiating causes

A variety of initiating agents or events has been documented as causations of sepsis and its sequelae. Gram-negative and gram-positive organisms, viruses and fungi have all been implicated as instigators of the condition (Huttunen and Aittoniemi, 2011). It has been established that sepsis is a clinical syndrome characterized by the presence of both infection and systemic inflammation. Following the 2001 SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference (Levy et al., 2003), this infection may be either confirmed or suspected. Systemic Inflammatory Response Syndrome (SIRS) presents a similar clinical picture and outcome but is triggered by non-infectious stimuli, such as trauma, thermal injury or cardiopulmonary bypass surgery (Levy et al., 2003).

It has been reported that 90% of infectious cases are due to gram-positive (mainly staphylococci and streptococci) or gram-negative bacteria (predominantly Escherichia coli, Pseudomonas aeruginosa and Klebsiella species) (Treacher and Alun Brown, 2009). Vincent and Abraham (2006) reported that the most common infection associated with sepsis is pneumonia (approximately 40%), followed by intraabdominal infection (20%), catheters and primary bacteremias (15%), and the urinary tract (10%), with the remainder attributable to fungal or parasitic infection or without an identifiable initiating agent. They further noted that roughly similar numbers of gram-positive and gram-negative organisms are associated with sepsis. Although Daniel G. Remick (2007) states that while the causes of sepsis are multifactorial and may include virtually any infectious organism, recently, gram-

positive infections have been documented to be more frequent than gram-negative infections.

Accordingly, it can be said that sepsis and SIRS encompass multiple signs and symptoms that emanate from the host's response to invading microorganisms. The condition results from some nidus of infection or insult that triggers the release of inflammatory mediators and induces cellular dysfunction in the affected host (Sagy et al., 2013). Initiating and progressing from some insult, which could be infection, fungal or trauma, perhaps a local infection at a burn wound, an infected catheter tip or pulmonary infection, to identify a few possibilities. The spread of microbes can then further potentiate systemic inflammation. Pathological alterations of metabolic, cardiovascular, pulmonary, renal, gastrointestinal, and coagulation systems ensue due to the dysregulated immune system (Sherwood and Traber, 2012).

Treacher and Alun Brown (2009) raise the important question that since the majority of bacterial infections are resolved and cleared by the host without major complications, why do the same circumstances ultimately result in organ failure for others? Clinicians often know the aetiology and target of the insult, but remain uncertain as to the nature of the intervening events in sepsis. Many authors, Treacher and Alun Brown (2009) included, propose that there is a staged progression to organ failure, demonstrated in Figure 10.

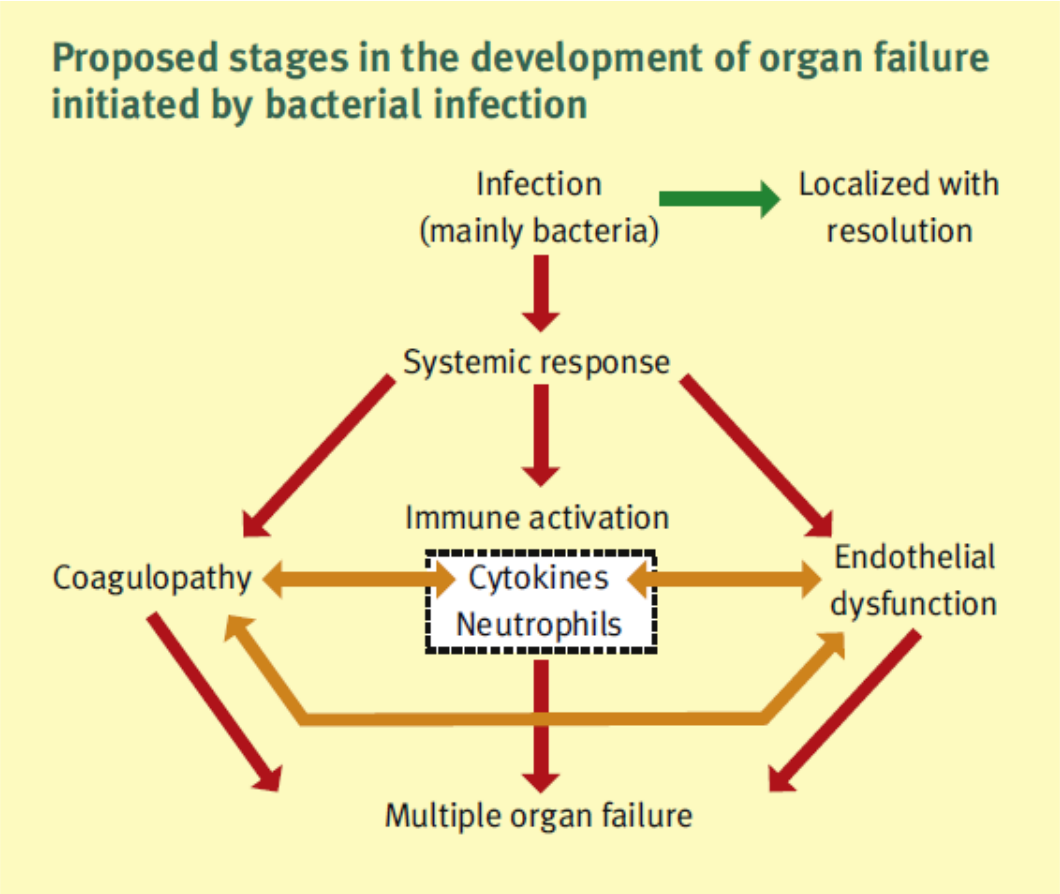

Figure 10: Proposed Stages in the Development of Organ Failure Initiated by Bacterial Infection (Treacher and Alun Brown, 2009). Figure reproduced with permission from Elsevier licence number 3830850237855.

2.1.1 Bacterial Sepsis

Severe sepsis and septic shock are the primary causes of multiple organ dysfunction syndrome, which is what ultimately leads to the mortality of patients with sepsis having resulted from the host response to insult, be it gram-positive or gram-negative (Ronco et al., 2004). Depending on the gram positive or negative nature of the initiating insult, different signalling events and subsequent expression and release of inflammatory mediators, such as the cytokines, arise. This would suggest that the host immune responses are pathogen specific and mediated by various sets of pathogen associated molecular patterns (PAMP) and pattern recognition receptors (PRR) (Bochud and Calandra, 2003).

Gram-Negative & Gram-Positive Endotoxin Release

The outer membrane of gram-negative bacteria is composed of endotoxin which interact with the host during gram-negative sepsis. (Huttunen and Aittoniemi, 2011). The release of endotoxins, such as lipopolysacharides (LPS), is commonly expected to occur during gram-negative infections (Sagy et al., 2013). While gram-positive bacteria do not have LPS in their cell wall, peptidoglycan and lipoteichoic acids (LTA) can have similar effects. Additionally, some gram-positive bacteria produce exotoxins (e.g. staphylococcal toxic shock toxin), discussed in the following subsection (Cohen, 2009). Both have an effect on macrophage function and result in production of mediators, both of these toxins are PAMPs recognised by PRRs. These particular PAMPs are recognised respectively by the PRRs toll-like receptors TLR-2 and TLR-4; these receptors, along with the co-receptor CD-14, recognize the

aforementioned toxins as they adhere to the macrophages' walls. While LPS requires a LPS-binding protein (LBP) before it is recognized by the macrophage's TLR-4, LTA, on the other hand, adheres directly and independently to the macrophage's TLR-2, Figure 11 (Sagy et al., 2013).

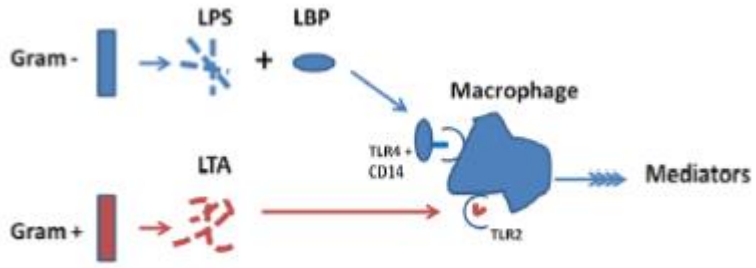

Figure 11: The effect of endotoxin on macrophage release of mediators of inflammation (Sagy et al., 2013). Figure reproduced with permission from Elsevier licence number 3830850374828.

The majority of gram-negative cases of sepsis occur in the lung, abdomen, bloodstream, or urinary tract and are due to Enterobacteriaceae, such as E coli and Klebsiella species, Pseudomonas aeruginosa is the third commonest cause (Bochud and Calandra, 2003).

Gram-Positive Exotoxin (Superantigen) Release

Endotoxin is not present in gram-positive bacteria. However, gram-positive cells produce potent exotoxins, also referred to as superantigens. The best known examples are the toxic shock syndromes caused by toxic shock syndrome toxin-1 (TSST-1)-producing strains of Staphylococcus aureus and the pyrogenic exotoxins from Streptococcus pyogenes (Cohen, 2002). Superantigens are capable of activating T-lymphocytes and trigger production of the pro-inflammatory cytokines interleukin-2 (IL-2) and interferon gamma (IFN-γ). These cytokines are key

mediators required for differentiation and proliferation of T cells to become "effector" T cells with enhanced immunological memory. Additionally their functionality includes anti-viral, immunoregulatory, and anti-tumour properties, as well as activating inducible nitric oxide synthase and promoting leukocyte migration. They perpetuate further release of additional pro-inflammatory cytokines from macrophages, including IL-1 and tumour necrosis factor alpha TNF-α, which in turn are important stimulants for generating an adequate inflammatory response to infections, Figure 12 (Sagy et al., 2013). Cytokine involvement in the sepsis will be discussed further throughout section 2.6.

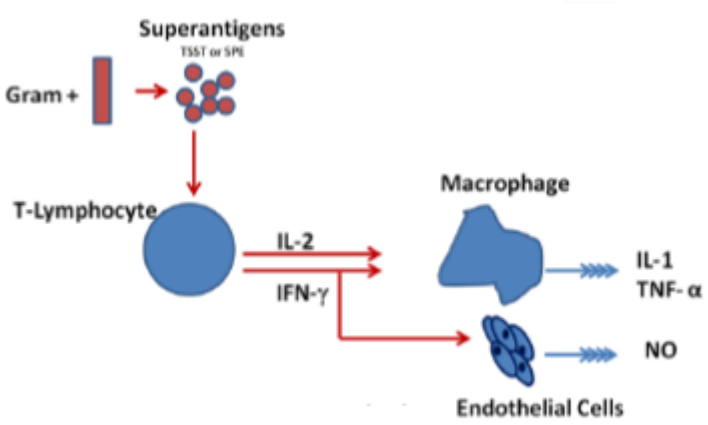

Figure 12: Effect of exotoxin (superantigen) on release of mediators of inflammation (Sagy et al., 2013). Figure reproduced with permission from Elsevier licence number 3830850374828.

2.2 Signalling Pathway

The innate immune system is the host's frontline defence against invading pathogens. Potential threats are detected and numerous defensive mechanisms are launched. Additionally innate immune activation is also critical for subsequent activation of acquired immunity (Takeuchi and Akira, 2010). Exposure of surface-expressed receptors on innate immune cells, primarily macrophages, dendritic cells and neutrophils, to conserved motifs results in the initiation of the inflammatory response, which in turn results in the secretion of inflammatory cytokines IL-1b, IL-6 and TNF-a, and many different chemokines (Williams et al., 2011).

The principal sensors of the system are Pattern Recognition Receptors (PRRs). PRR's are expressed on the surface of innate immune cells (Figure 13) (Bochud and Calandra, 2003). PRRs recognize conserved motifs, known as Pathogen-Associated Molecular Patterns (PAMPs) that are expressed by pathogens but are not present in vertebrate animals (O'Brien, 2012). PAMPs are surface molecules, such as endotoxin (lipopolysaccharide), lipoproteins, outermembrane proteins, flagellin, fimbriae, peptidoglycan, peptidoglycan-associated lipoprotein, and lipoteichoic acid; and internal motifs released during bacterial lysis, such as heat-shock proteins and DNA fragments (Annane et al., 2005). As these molecules are common to pathogenic, non-pathogenic, and commensal bacteria, some authors such as Annane et al. (2005) and Cohen (2002), suggest that "microbial-associated molecular patterns" or "microorganism-associated molecular patterns" would be a better term. PRRs recognise these motifs which in turn stimulate signal transduction pathways that

produce a broad array of cytokines, chemokines, and other inflammatory mediators to sequester and eradicate the invading organism (O'Brien, 2012).

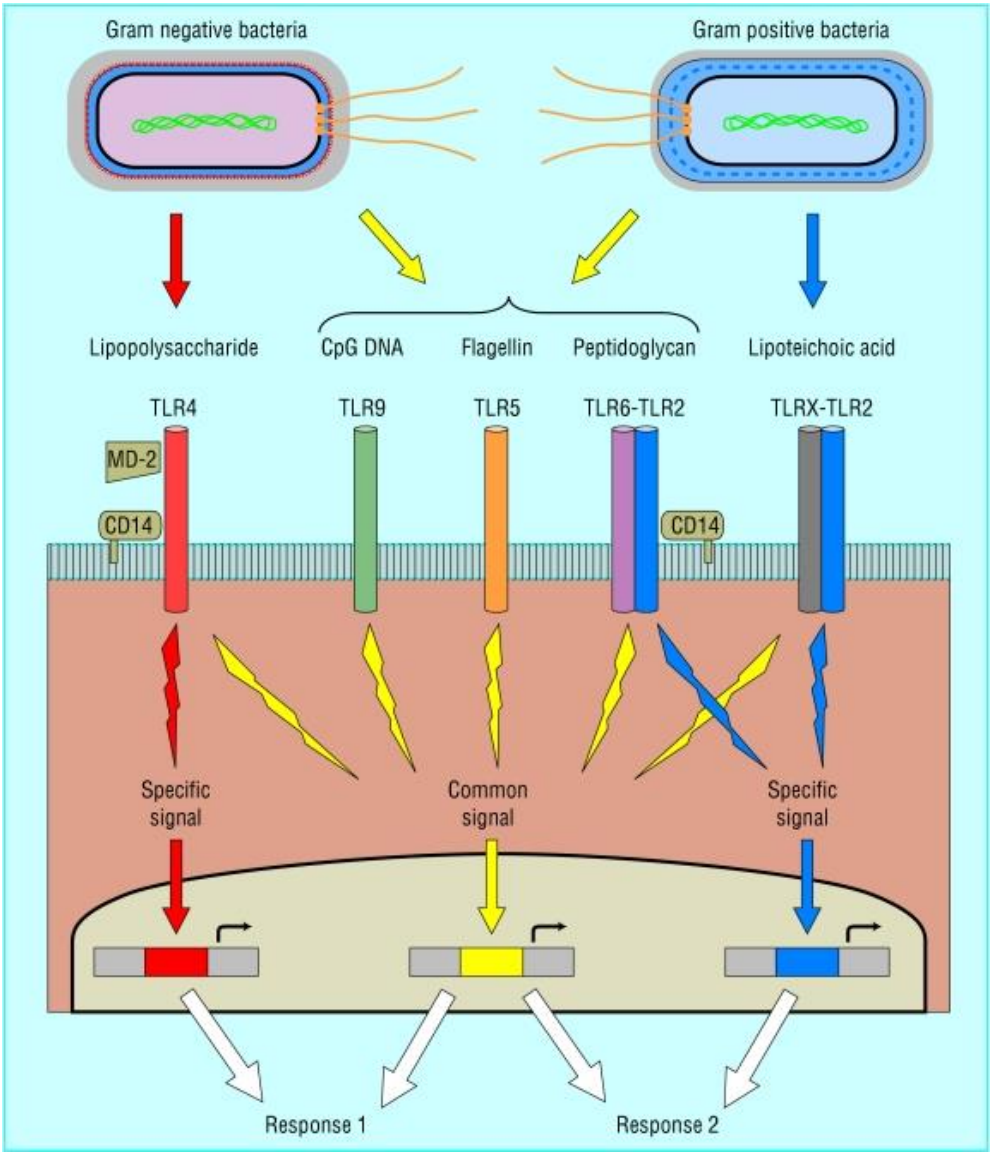

Figure 13: Interaction between bacterial products and pattern recognition receptors (PRR) expressed on immune cells (Bochud and Calandra, 2003). Figure reproduced with permission from BMJ Publishing Group Ltd licence number 3830850720055.

However, recognition of PAMPs alone would only provide reaction to exogenous microbes when, as is certainly the case in sepsis, endogenous host microbes can also

present a significant threat. It was established by Matzinger (1994) that the immune response evolved to respond to trauma and injury by recognition of and reaction to internal danger signals, rather than simply by discerning between self and non-self molecules. These signals include intracellular proteins or fragments thereof, DNA, and even inorganic crystals, which are expressed or released after host tissue injury and can be considered the endogenous equivalents of PAMPs. These molecules are termed “alarmins” or Damage-Associated Molecular Patterns (DAMPs) (Namas et al., 2012, van der Poll and Opal, 2008). Accordingly, regardless of whether an insult is endogenous or exogenous, the host is able to respond to insult and the defence mechanisms are activated.

PRRs recognise the specific structures, or motifs, of microorganisms of DAMPs or PAMPs . Four families of PRR are currently known, categorised by their cellular location: Toll-like receptors (TLRs); nucleotide oligomerization domain (NOD) leucine-rich repeat proteins (NLRs); cytoplasmic caspase activation and recruiting domain helicases, such as Retinoic Acid-Inducible Gene (RIG)-I-like receptor helicases (RLRs); and C-type lectin receptors (CLRs) expressed on dendritic and myeloid cells (Namas et al., 2012). It is important to note that these PRRs are expressed not only in specific innate immune cells, macrophages, dendritic cells and neutrophils, but also in other cells, such as epithelial cells, endothelial cells, and fibroblasts, which are not specialised immune cells but which also contribute to innate immunity (Takeuchi and Akira, 2010). Recognition of PAMPs or DAMPs by PRRs results in upregulated transcription of genes involved in inflammatory responses. These genes encode, depending on the activated PRRs, inflammatory cytokines, chemokines and antimicrobial proteins, proteins involved in the

modulation of PRR signaling, and many others proteins still to be characterised (Takeuchi and Akira, 2010). The two PRRs most commonly associated with sepsis are TLRs and NLRs. (Wiersinga et al., 2014). Toll-like receptors (TLR), which respond to endogenous cellular factors that are produced or released following tissue trauma, and NOD-like receptors (NLR) that sense endogenous and exogenous ligands to cause activation of inflammasomes (Sherwood and Traber, 2012).

2.2.1 TLRs:

TOLL-like receptors are capable of recognising and responding to a large number of microbial organisms, including gram-negative and gram-positive bacteria, Mycoplasma spp., fungi, viruses, parasites, as well as bacterial flagellin (O'Brien, 2012). They get their name as they are homologues of the Drosophila protein Toll first discovered in the fruit fly. Engagement of TLRs elicits signalling cascades via the activation of transcription factor nuclear factor κB (NF-κB) (Shimaoka and Park, 2008, Treacher and Alun Brown, 2009). Activated NF-κB translocates from the cell cytoplasm to the nucleus, binds to gene promoter regions and induces activation of an array of genes which encode acute-phase proteins, pro-inflammatory cytokines (such as TNF-a, IL-1b and IL-6), inducible nitric oxide synthase (iNOS), coagulation factors, and enzymatic activation of cellular proteases (Huttunen and Aittoniemi, 2011, Sherwood and Toliver-Kinsky, 2004), demonstrated in Figure 14.

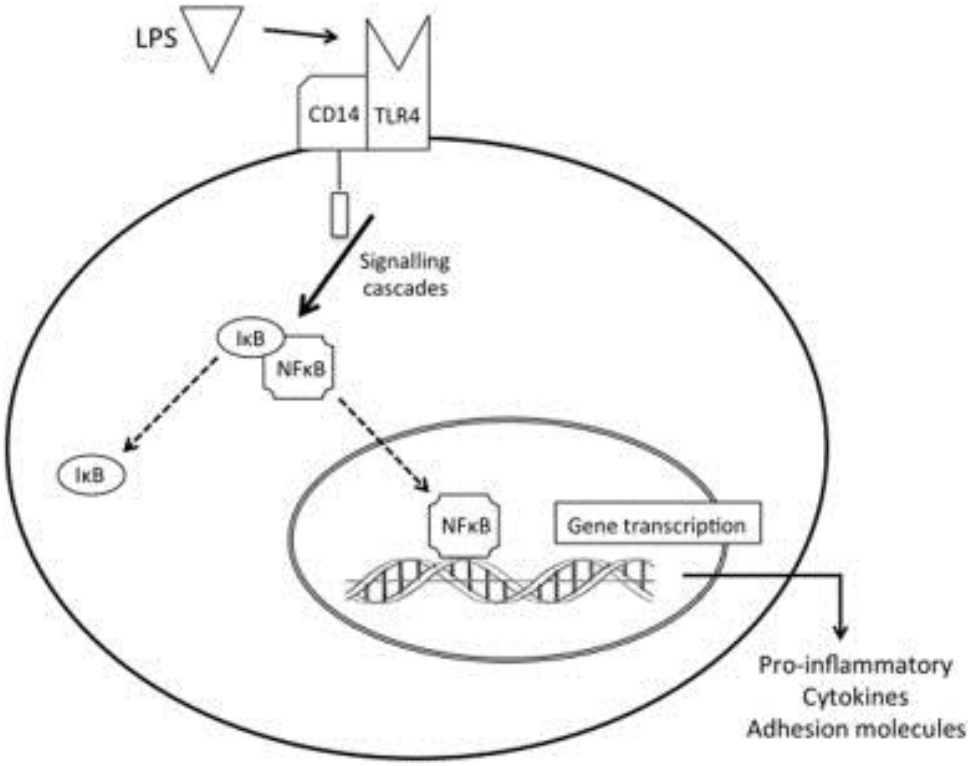

Figure 14: TLR Signalling and NFκB activation (O'Brien, 2012). Figure reproduced with permission from Elsevier licence number 3830851026719.

TLRs are single-spanning transmembrane proteins characterised by an extracellular leucine-rich repeat domain and a cytoplasmic toll-interleukin-1 receptor (TIR) domain that shares considerable homology with the interleukin-1 receptor cytoplasmic domain (van der Poll and Opal, 2008, Annane et al., 2005). There are 13 human and murine TLRs known to date, this family of receptors signals via 4 adaptor proteins (O'Brien, 2012): myeloid differentiation primary-response protein 88 (MyD88); TIR-domain-containing adaptor protein (TIRAP); TIR-domain-containing adaptor-protein inducing interferon β (TRIF); and TRIF-related adaptor molecule (TRAM). All of which work to respond to various conserved microbial motifs (van der Poll and Opal, 2008).

As a successful insult resolution hinges upon a self-limiting response, TLR signalling is controlled by several negative regulators. In the cytoplasm, these include MyD88 short (MyD88s), interleukin-1 receptor-associated kinase-M (IRAK), Toll-interacting protein (TOLLIP) and at the cell surface they include single immunoglobulin interleukin-1R-related molecule (SIGIRR). All of which serve to negatively control the TLR signalling cascade. (Annane et al., 2005, van der Poll and Opal, 2008)

2.2.2 NLRs:

While TLRs detect pathogens at either the cell surface or in lysosomes or endosomes, a second PRR sensing system recognises pathogens that invade the cytosol. This family of cytoplasmic pathogen sensors are the NOD-like receptors (NLRs). NLRs sense endogenous and exogenous ligands to cause activation of inflammasomes, demonstrated in Figure 15 (Sherwood and Traber, 2012, van der Poll and Opal, 2008, Schroder and Tschopp, 2010).

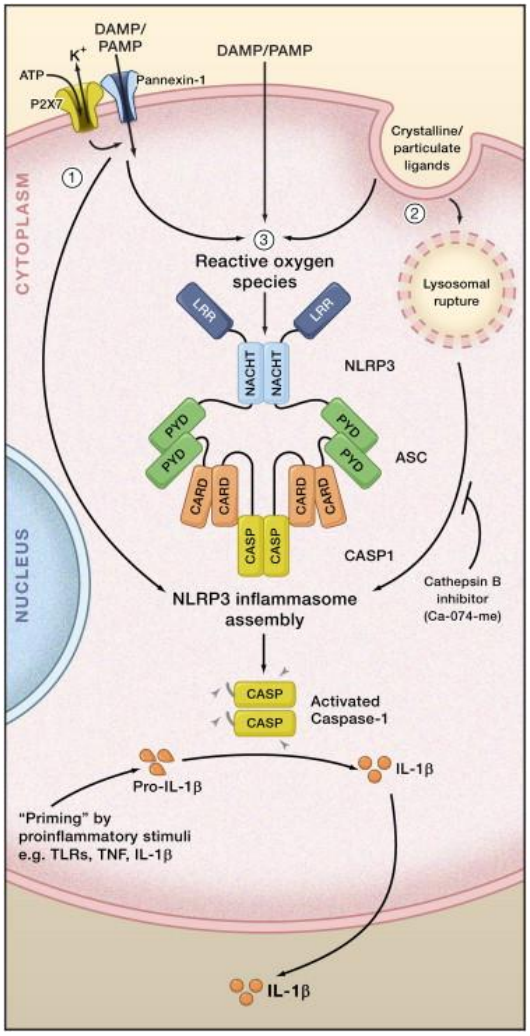

Figure 15: Inflammasome Activation (Schroder and Tschopp, 2010). Figure reproduced with permission from Elsevier licence number 3830851170183.

The NLRs consists of a central nucleotide-binding and oligomerization (NACHT) domain, which is commonly flanked by C-terminal leucine-rich repeats (LRRs) and N-terminal caspase recruitment (CARD) or pyrin (PYD) domains (Takeuchi and Akira, 2010, Schroder and Tschopp, 2010). NLRs are further subcategorized based on differences in the NACHT N-terminal domains; the NODs, the NLRPs (also referred to as NALPs) and the IPAF subfamily, consisting of IPAF and NAIP (Schroder and Tschopp, 2010, Wiersinga et al., 2014).

Nucleotide-binding oligodimerisation domain (NOD) proteins provide surveillance for PAMPs or DAMPs in the cytoplasm by recognition of common fragments of bacterial cell wall breakdown, for example diamino-pimelate from gram-negative bacteria is the ligand for NOD1, and muramyl dipeptide is the ligand for NOD2.(Schroder and Tschopp, 2010, Wiersinga et al., 2014, van der Poll and Opal, 2008). Once a ligand is sensed they oligomerize and recruit RIP2 via CARD-CARD interactions. Assembly of NOD1 and NOD2 signalosomes ultimately culminates in the activation of the NF-kB transcription factor, which drives pro-inflammatory gene regulation (Schroder and Tschopp, 2010).

However unlike NODs, which harbour protein-binding motifs, such as CARDs, NLRs harbouring a pyrin domain or a BIR domain in their N terminus are not involved in the transcriptional activation of inflammatory mediators and are components of the inflammasome that regulates caspase-1 activation (Takeuchi and Akira, 2010). The largest NLR group consists of 14 members which have an N-terminal pyrin domain (PYD) they accordingly named “NLRP” (previously called “NALP”) of which ASC (apoptosis-associated speck-like protein containing a

caspase activation and recruiting domain) serves as the central adaptor molecule. Several members of the NLR-family, including NLRP1, NLRP3 (also known as cryopyrin), and NLRC4, can assemble multimolecular complexes termed inflammasomes in response to various activators, including both endogenous danger signals, such as double-stranded DNA and uric acid crystals, as well as exogenous pathogen-derived molecules, such as viral RNA or bacterial peptidoglycans, leading to caspase activation (Wiersinga et al., 2014). Caspase 1 is an enzyme responsible for the secretion of three interleukin-1 family members implicated in host defence against infection: interleukin 1β, interleukin 18, and interleukin 33. The potential deleterious or advantageous role of caspase 1 and its pro-inflammatory products resembles the bimodal roles of TLR2 and TLR4 as a part of the early warning system against microbial invasion, even though they also contribute to the initiation of sepsis (van der Poll and Opal, 2008). Activation of inflammasomes during sepsis can amplify inflammatory responses. The consequence thereof, whether beneficial or detrimental, depends on the extent and duration of inflammasome activation (Wiersinga et al., 2014).

It should be noted that intact microbial pathogens are usually composed of a number of PAMPs, which activate multiple PRRs. Moreover, different PRRs may recognize the same PAMP. Accordingly TLRs, in concert with other PRRs, orchestrate both pathogen-specific and cell type-specific host immune responses to fight infections (Kawai and Akira, 2011). The outcome of PAMP recognition by PRRs depends upon the nature of both the responding cell and the invading microbe. However, signal transduction from these receptors converges on a common set of signaling modules,

often including the activation of the NF-kB and AP-1 transcription factors that drive cytokine production (Schroder and Tschopp, 2010).

Whether initiated by endogenous or exogenous means, the signalling pathways result in activation of transcription factors and subsequent expression of numerous effector molecules, including cytokines, which have an essential role in orchestrating the host response to insult (Bochud and Calandra, 2003). This recognition and subsequent signalling events of the immune response must maintain an adequate and balanced response to ensure a successful resolution, as stated by Wiersinga et al. (2014):

"*The immune response to sepsis can be seen as a pattern recognition receptor-mediated dysregulation of the immune system following pathogen invasion in which a careful balance between inflammatory and anti-inflammatory responses is vital*" (Wiersinga et al., 2014).

2.3 The Inflammatory Response

Under conventional circumstances, the inflammatory response is a critical supportive process which attempts to “heal” some insult. It is initiated by the previously described process of exposure of innate immune cells, primarily macrophages, dendritic cells and neutrophils, to PAMPs or DAMPs which secrete inflammatory cytokines, such as IL-1b, IL-6 and TNF-a, and chemokines (Williams et al., 2011). This is broadly demonstrated in Figure 16. However it is also the critical process in sepsis, as elements of the response drive the physiological alterations that become manifest as the systemic inflammatory response syndrome (Daniel G. Remick, 2007). An effective and regulated inflammatory response should result in resolution of an insult without unnecessary damage to the host systems.

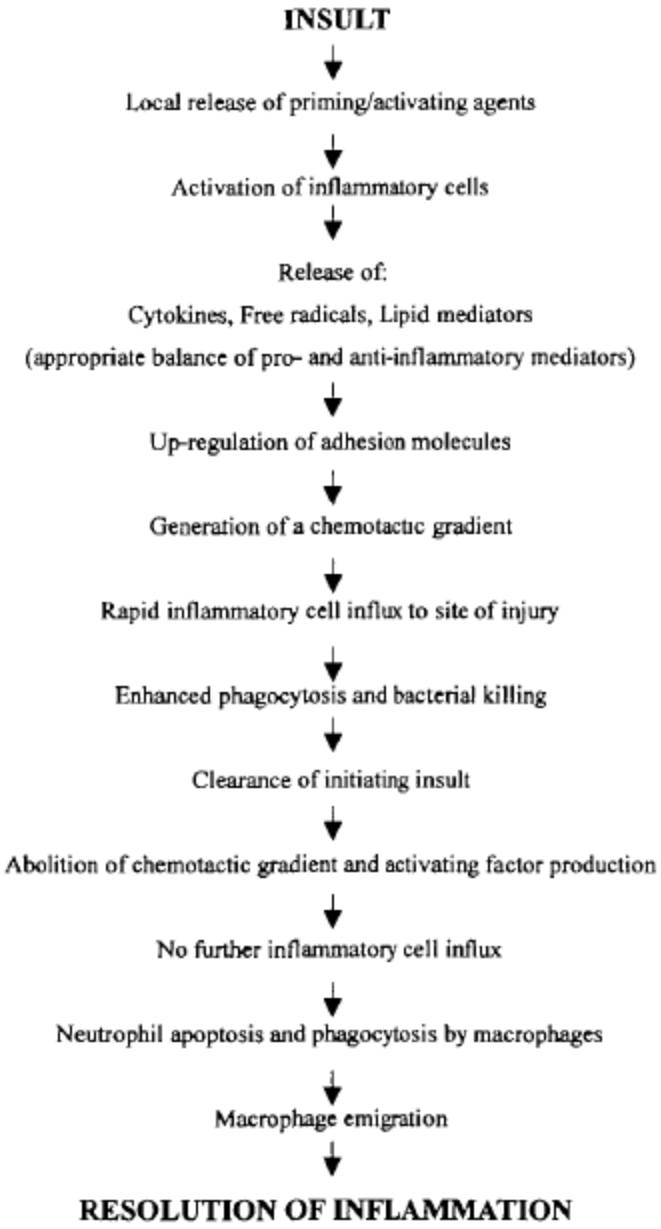

Figure 16: The normal inflammatory response. Although shown as a clear sequence, in reality many of these processes act in concert (Bellingan, 1999). Figure reproduced with permission from Elsevier licence number 3830851383411.

It is worth highlighting that the inflammatory process outlined in Figure 16 by (Bellingan, 1999) is demonstrated as a linear, easily followed incremental process. However in reality, the progression is multifaceted, overlapping and variant, this is of particular importance as many theories on sepsis progression are also of a non-linear

nature; the key difference is that in the instance of conventional inflammation the process is a self-limiting, protective response of the body in which the various mediators are appropriately activated, controlled and deactivated.

Following the initiating insult, pathogens trigger the intracellular sequence of events identified in Figure 16 via their microbial-associated molecular patterns when they bind to phagocytic cells through pattern recognition receptors, which activates the inflammatory response involving immune cells, epithelium, endothelium, the neuroendocrine system and crucially the release of inflammatory cytokines (Annane et al., 2005, Treacher and Alun Brown, 2009).

This is most commonly recognisable in the case of an epithelial injury, which presents with the familiar heat, redness, swelling and pain of inflammation, or in the more traditional nomenclature; calor, rubor, tumor and dolor (Bellingan, 1999). The inflammatory cells, a cover-all expression common in the literature to describe a wide plethora of cells involved in the mediation and propagation of inflammation, are the key agents of this process. The inflammatory cells are circulating leukocytes (including neutrophils, monocytes and lymphocytes), macrophages, dendritic cells, mast cells and eosinophils (Bellingan, 1999).

Activation of these inflammatory cells involves the migration of leukocytes from the venous system to sites of damage as most infections, such as pneumonia or peritonitis, occur initially in the tissue and not in the bloodstream (Coussens and Werb, 2002). This process of recruitment and migration is referred to as extravasation of circulating leukocytes and is essential in bring inflammatory cells

and pathogens into contact. Although circulating neutrophils and monocytes inevitably come into contact with the endothelium, binding to this layer is limited in their quiescent state (Bellingan, 1999). This extravasation process of recruiting leukocytes from within the vasculature to sites of injury or infection is governed by molecular mechanisms mediated by both cytokines and adhesion molecules (Tsokos, 2007).

A key element is cytokines, which are host-produced, pleomorphic immunoregulatory peptides. The initial insult triggers the production of pro-inflammatory cytokines (discussed in more detail in section 2.6), which in turn promote leukocyte-endothelial-cell adhesion, activation of clotting, and generation of numerous secondary inflammatory mediators, including other cytokines, prostaglandins, leukotrienes, and proteases. In addition, they also trigger production of subsequent anti-inflammatory compounds and cytokines, which should function as negative feedback for the inflammatory process once appropriate (Wheeler and Bernard, 1999).

Some of these cytokines have chemotactic activity, referred to as chemokines. These chemokines promote the migration of cells towards a chemical stimulus along a concentration gradient and are responsible for leukocyte traffic (Huttunen and Aittoniemi, 2011, Treacher and Alun Brown, 2009).

The recruitment, extravasation and endothelial transmigration of leukocytes, specifically neutrophils, are facilitated by a complex intercellular communication.

However, it follows several distinct steps demonstrated in Figure 17 and outlined as follows:

1. Margination: The process of neutrophil movement from the central bloodstream to the periphery of the vessel. This phenomenon is facilitated by stasis following fluid exudation at the insult site and physical forces involved in the erythrocyte–leukocyte interactions, a process facilitated by cell surface expression of selectins and their ligands (Vincent, 2008).
2. Rolling: Once close to the site, a weak adhesive interaction, or "tether" develops between neutrophils and vascular endothelial cells; a low-affinity adherence and the shear stress of passing erythrocytes causes the neutrophil to "roll" along the endothelium (Vincent, 2008).
3. Adhesion: rolling concludes with a strong adhesive interaction between a neutrophil and endothelial cell, a high-affinity adherence facilitated by cell surface integrins and their ligands (Sherwood and Toliver-Kinsky, 2004).
4. Diapedesis: Once firmly adhered, a neutrophil is required to move through the endothelium to enter the extravascular inflammatory environment. The exact mechanisms of this process are not fully understood, namely if the leukocytes transmigrate through the junctions between adjacent endothelial cells or directly through a single endothelial cell (Tsokos, 2007).
5. Chemotaxis: Leukocytes require a chemoattractant gradient to complete transmigration. This is facilitated by chemoattractants; soluble molecules which orientate cell migration in the direction of increasing concentration, a process referred to as chemotaxis (Vincent, 2008).

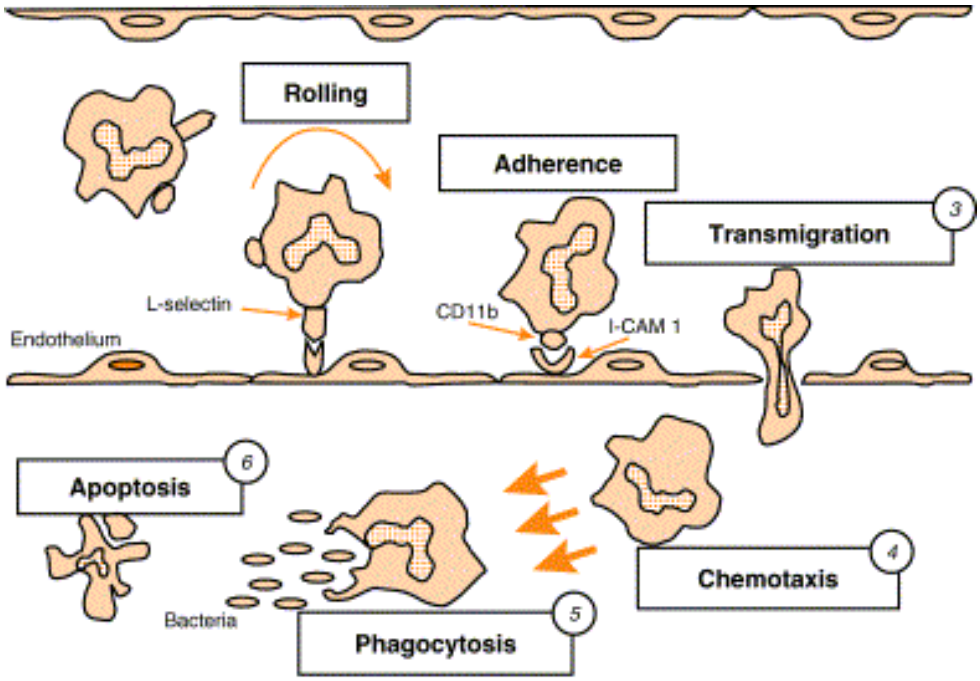

Figure 17: Mechanisms of neutrophil rolling, adherence, diapedesis and chemotaxis (Sherwood and Toliver-Kinsky, 2004). Figure reproduced with permission from Elsevier licence number 3830860286571.

This stepwise process involves activation of leukocyte and endothelial cell expressed adhesion receptors and their ligands when stimulated from their quiescent state by cytokines. The weak adhesive interactions involved in tethering and rolling are mediated by members of the selectin family of adhesion molecules; L- P-, and E-selectin, which are named after the cell type in which they were discovered (endothelium, platelet, and leukocyte). These are the key elements in facilitating rolling along the endothelium. It is worth noting that certain L-selectins and their ligands may both be expressed by activated leukocytes, which can result in

leukocyte-leukocyte interaction, also termed secondary tether, resulting in strings of rolling leukocytes (Kelly et al., 2007, Coussens and Werb, 2002).

Rolling leukocytes are in intimate contact with the vascular endothelium which enables them to sense signals from endothelium bound chemokines which in turn stimulates them to adhere firmly to its surface. Once they have been stimulated by cytokines, leukocyte-expressed integrins bind to members of the immunoglobulin superfamily expressed on the endothelial cell surface which serve as ligands (Tsokos, 2007, Williams et al., 2011). This adherence leads to a reduction of rolling velocity, sometimes referred to as slow rolling or crawling, and subsequently to firm adhesion on the endothelium (Williams et al., 2011).

Firm adherence is a necessary precursor for diapedesis, or transmigration, across the endothelial cell barrier and subsequent chemotaxis, which is mediated by the action of integrins and their ligands (Sherwood and Toliver-Kinsky, 2004). The extract mechanisms of diapedesis are the subject of ongoing research and debate. The prevailing theories are that leukocytes either transmigrate through intercellular junctions between adjacent endothelial cells, transcellularly directly through an endothelial cell or a combination of both. However, the intercellular route is thought to be the predominant mechanism (Sadik et al., 2011). Indeed, the endothelial cells have a critical role in regulating vascular permeability and have been implicated in the sepsis progression as permitting capillary leakage.

Various proteins have been implicated in the emigration process including, but not limited to, endothelial cell surface adhension molecules (Williams et al., 2011, Kelly

et al., 2007). Most of which are localized to the endothelial cell–cell junctions, which might explain why neutrophils transmigrate preferentially at the cell–cell junctions (Williams et al., 2011). The importance of junctional versus transcellular migration is the subject of current debate, including theories that certain molecules can act as "gatekeepers" (Kelly et al., 2007).

The process of transmigration is a multistep, sequential process, with each step dependant upon the previous one. These cellular processes are governed by molecular interactions between receptors and their ligands expressed on neutrophils (Vincent, 2008). The process is no simple feat. Indeed, one author drew the comparison of this process to attempting to escape a raging river (Kelly et al., 2007); the force of the flow making it difficult not to be swept away, and once at the bank encountering a slippery surface upon which it is difficult to gain purchase, such is the battle migrating neutrophils face, although they have an evolved sequential practice to facilitate the process. The end result of this chain of events is the migration of neutrophils from the intravascular compartment and into the interstitium at sites of infection or injury (Sherwood and Toliver-Kinsky, 2004).

It should be noted that endothelial cells do not merely serve as targets during systemic inflammation. They can actively contribute to the ongoing inflammatory process. They can be stimulated by endotoxin or cytokines to express adhesion molecules such as E-selectin, which will interact with corresponding ligands on PMNs to facilitate rolling of these cells along the endothelium (Sherwood and Traber, 2012). Indeed, the endothelium plays a key role in the progression of sepsis.

In normal homeostasis, the principal circulating inflammatory cells are neutrophils and monocytes. In their conventional quiescent state, they have a life-span limited by apoptosis (programmed cell death) of approximately 24 hours. However following insult, they are transformed into highly active phagocytes with a greatly enhanced capacity to release mediators, enzymes and reactive oxygen intermediates (ROI) (Bellingan, 1999). Neutrophils can exist in a variety of functional states associated with different patterns of membrane expression becoming activated by inflammatory mediators (Vincent, 2008). In the activated state, they can produce reactive oxygen metabolites, known as the "respiratory burst", and destructive proteolytic enzymes. In addition to the activated state, neutrophils can also be "primed" to produce an exaggerated response to an activating stimulus; an amplification of the neutrophil respiratory burst. A neutrophil moving from a quiescent state to a primed state does not directly activate the respiratory burst but rather potentiates the neutrophil response to a subsequent stimulus (Vincent, 2008). This primed state can be initiated by priming agents, including cytokines, and has been implicated in multiple organ failure (Vincent, 2008). A key point to note is the redundancy and synergy of the mechanisms involved in this process, leading to heightened surface receptor expression.

The leukocyte "first responders", as it were, to insult are the innate immune cells, primarily neutrophils. Neutrophils are the first leukocytes to be recruited to a site of insult followed by monocytes. Monocytes subsequently differentiate into macrophages in tissues and upon activation have a profound effect in the local microenvironment (Williams et al., 2011, Coussens and Werb, 2002) Macrophages engulf, kill and digest micro-organisms and subsequently present their foreign

antigen to lymphocytes and engender highly specific adaptive immune responses (Bellingan, 1999). However, the function of activated macrophages goes beyond their phagocytic role; as mentioned, they invoke an adaptive immune response by presenting antigen, promote the resolution of inflammation by tissue debridement and stimulation of fibrosis and angiogenesis. They are also the main progenitors of other inflammatory cells, including cytokines, enzymes, lipid mediators, complement, coagulation mediators, matrix components, cytokines and colony stimulating factors (Coussens and Werb, 2002) (Bellingan, 1999). As Bellingan (1999) put it:

"*macrophages can be regarded as generals co-ordinating an army of neutrophils and other activated cells*" (Bellingan, 1999).

Neutrophils are produced in the billions daily as part of normal function but once they have fulfilled their role they go into apoptosis, as previously mentioned their half-life is approximately 24 hours. This is necessary to limit the number of neutrophils present in the tissues. However following insult, delayed apoptosis, has been demonstrated, which has been reported to last up to 3 weeks (Hietbrink et al., 2006). This alteration of the normal apoptosis of leukocytes has been implicated in sepsis.

In the normal homeostatic inflammatory response, the insult, infection or otherwise, is cleared along with the inflammatory cells, which following apoptosis return to their quiescent state as a circulating population in the host. However if the inflammatory response is not sufficient, there is a risk of overwhelming sepsis. In the

worst cases, an unregulated response can lead to systemic inflammation and consequent multiple organ damage (Bellingan, 1999). The sepsis milieu transforms this normally beneficial dynamic into a double-edged sword wherein the invading pathogen itself can be directly toxic and destructive to tissues, but much of the pathology associated with sepsis is attributed to host response (O'Brien, 2012).

2.4 The Coagulation Response

It has been established that the host response to insult involves various systems, the central theme being the inflammatory response. However, the coagulation and complement systems are also key elements.

Haemostasis and its maintenance, require careful balance and response to insults. Coagulation, the ability of blood to clot, and platelet-mediated primary haemostasis have evolved as important defence mechanisms against bleeding (Dahlbäck, 2000). When considering blood coagulopathy, this involves interaction and balance of vascular, cellular, and plasma components (Levy et al., 2013). Following insult, the ability to form a clot at the appropriate site and obtain haemostasis is a vital survival mechanism, the vascular effects of which include vasoconstriction, expression of procoagulant factors, including tissue factor (TF), and loss of the normal anticoagulation pathways (Levy et al., 2013), (Moran and Viele, 2005).

A variety of components contribute to this process of coagulation and clotting, which interact together with local and systemic factors, the pathways of which are often redundant, converging, and counterintuitive (Levy et al., 2013), (Moran and Viele, 2005). The process is facilitated by a variety of coagulation proteins, known as "factors" which have similarities in sequence, structure, and function, to a certain extent. The proteins are generally members of the serine protease family, which act by cleaving downstream proteins from molecules, thus also cleaving the bond. Cleavage of a specific bond or a sequence of bonds is needed to activate the circulating inactive pro-proteins also known as zymogens. One factor in the chain

often activates another, resulting in a change to both its form and its function, an "a" suffix used to indicate the activated form (Moran and Viele, 2005). These reactions occur on activated phospholipid membranes and in some cases are accelerated by the presence of cofactors (factors VIIIa and Va) (Aird, 2003). Additionally, there are regulatory and other proteins that are essential to clotting. Antithrombin III, protein C, protein S, thrombomodulin, and von Willebrand factor (vWF) are the five major proteins involved in clotting (Moran and Viele, 2005).

In normal haemostatic conditions, the endothelium provides an antithrombotic surface. Endothelial cells express anticoagulants that prevent inappropriate activation of coagulation on the cell membrane and repel platelets from the vessel wall. This is maintained through the production of nitric oxide (NO), prostacyclin (PGI2), tissue plasminogen activator (tPA), heparan sulfate, antithrombin III, protein C, and endothelium-derived relaxing factor (EDRF). All of which promote blood fluidity, inhibit platelet activation, fibrin formation, and provide vascular patency (Schouten et al., 2008b, Moran and Viele, 2005, Levy et al., 2013).

However in the event of insult to a vessel, the cells undergo changes that can either transform them from an anticoagulant state to a procoagulant state or cause them to become detached to expose the vessel wall. Coagulation promoters including, tissue factor (TF), are released or exposed to provide a thrombotic surface. This "release reaction" from activated platelets, where they secrete or discharge the contents of their granules, including additional platelet activators, initiates the coagulation system (Dahlbäck, 2000, Moran and Viele, 2005).

Receptors on platelets bind to the damaged blood vessel by forming a bridge with von Willebrand factor (vWF) to propagate platelet adhesion. Once adhered, they undergo surface receptor changes that result in platelet aggregation. Subsequently, they expose factors that provide a substrate for activation of the coagulation cascade and formation of an early haemostatic plug (Levy et al., 2013).

The clotting cascade occurs in a stepwise manner, where a series of zymogens are converted (activated) to enzymes that advance and amplify the cascade (Moran and Viele, 2005). The cascade consists of an intrinsic pathway, which is composed of factors circulating in the blood, and an extrinsic pathway composed of factors extrinsic to the circulation pathway (Moran and Viele, 2005), demonstrated in Figure 18.

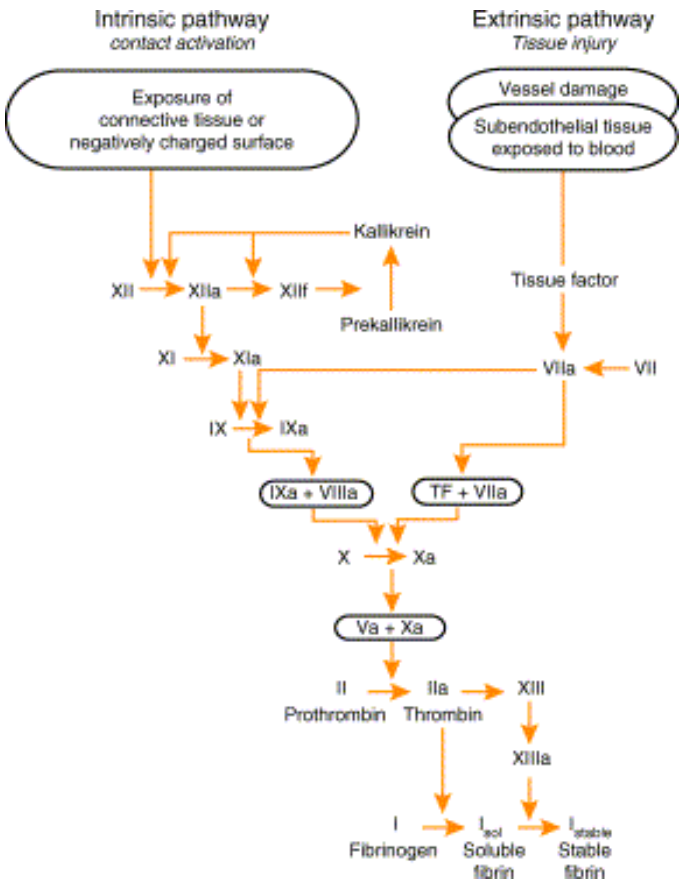

Figure 18: The coagulation cascade (Sherwood and Toliver-Kinsky, 2004). Figure reproduced with permission from Elsevier licence number 3830860286571.

It is known that generation or exposure of TF at the site of insult is the key physiological event which triggers the cascade. The primary initiating pathway is the extrinsic system, which is activated by TF, or TF-like substances, at the site of the tissue injury. Factor VII is activated in this fashion, which subsequently activates factors IX and X. The intrinsic system serves to enhance or amplify the extrinsic system (Dellinger, 2003, Moran and Viele, 2005). The intrinsic (or contact) system is activated by the exposure of blood to a negatively charged surface. Both systems converge at the activation of factor X, which converts prothrombin to thrombin. Thrombin, in turn, converts fibrinogen from a soluble plasma protein to an insoluble

fibrin clot and activates factor XIII which stabilizes the clot. This part of the cascade is referred to as the common pathway (Figure 18) (Moran and Viele, 2005, Johari and Loke, 2012).

Thrombin is the key effector enzyme performing a variety of critical functions in addition to being the final enzyme of the coagulation cascade. As a result, thrombin is the target of most anticoagulants. Its production occurs in two "waves" of differing magnitude. During the initiation phase, small amounts of thrombin are generated, preparing the cascade for the second larger thrombin "burst" (Johari and Loke, 2012). It is worth noting that the classic view of the clotting cascade is not completely accurate. It is now know that the pathways are not independent of each other and that certain deficiencies in each cannot be compensated for in the unaffected pathway (Moran and Viele, 2005).

The cascade is self-amplifying, which could result in excessive clotting if allowed to progress unchecked. However, along with the initial signals for clot formation comes another set of factors that regulate the process (Moran and Viele, 2005). Each pathway is regulated either by inhibition of enzymes or modulation of the activity of cofactors (Dahlbäck, 2000). As has been previously mentioned, under normal haemostatic conditions, anticoagulants are continuously active as a default on the endothelial surface. Coagulation is controlled by three major anticoagulant proteins (Figure 19): two circulating enzyme inhibitors, antithrombin (AT) and tissue-factor-pathway inhibitor (TFPI); as well as a clotting initiated inhibitory process, the protein C pathway involving activated protein C (APC) (Schouten et al., 2008b, Moran and Viele, 2005).

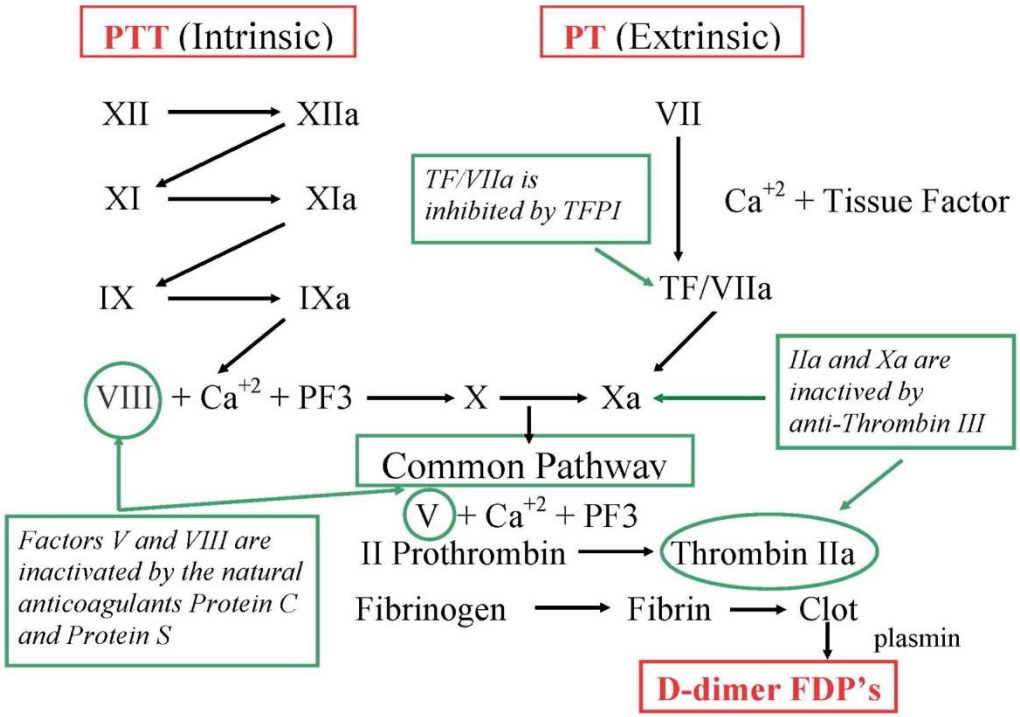

Figure 19: Physiological downregulation of coagulation (Johari and Loke, 2012). Figure reproduced with permission from Elsevier licence number 3830860459968.

TFPI is an endothelial-cell-derived protease inhibitor that blocks the activity of factor Xa when bound to factor-VIIa–tissue-factor complex (van der Poll and Opal, 2008, Dahlbäck, 2000). Most of the enzymes generated during activation of coagulation are inhibited by the serine-protease inhibitor antithrombin, previously called antithrombin III (AT-III) (Dahlbäck, 2000). Antithrombin inhibits factor Xa, thrombin, and factor IXa, as well as factor-VIIa–tissue-factor complex. (van der Poll and Opal, 2008). On its own, antithrombin is a comparatively inefficient inhibitor, but heparin and the heparin-like molecules present on endothelial cells stimulate its activity, which is the basis for the use of heparin as a therapeutic anticoagulant. (Dahlbäck, 2000)

The protein-C system regulates coagulation by the action of activated protein C (APC) which can cleave the phospholipid-membranebound cofactors factors Va and VIIIa, which results in inhibition of the coagulation system (van der Poll and Opal, 2008). It is activated on the surface of intact endothelial cells by thrombin that has bound to the membrane protein thrombomoduli, in this manner it is possible for thrombin to express both procoagulant and anticoagulant functions depending on the context under which it is generated. The functionality of APC has been controversially explored as a treatment modality in sepsis, which will be discussed further in section 2.9.

Haemostasis and regulation of coagulation are further controlled by the fibrinolytic system (Figure 20), in which plasmin is the key enzyme which acts to degrade fibrin clots (Schouten et al., 2008b). Plasminogen, a precursor molecule, binds fibrin and tissue plasminogen activator (tPA) and is converted to the active proteolytic plasmin which can cleave the polymerized fibrin strands, releasing fibrin degradation products; it can cleave fibrinogen, and a variety of plasma proteins and clotting factors (Moran and Viele, 2005). Physiological regulation of fibrinolysis is governed by endothelial cells that secrete both serine protease plasminogen activators (tPA and urokinase-type plasminogen activator) and plasminogen activator inhibitors 1 and 2 (PAI-1 and PAI-2) (Johari and Loke, 2012, Moran and Viele, 2005).

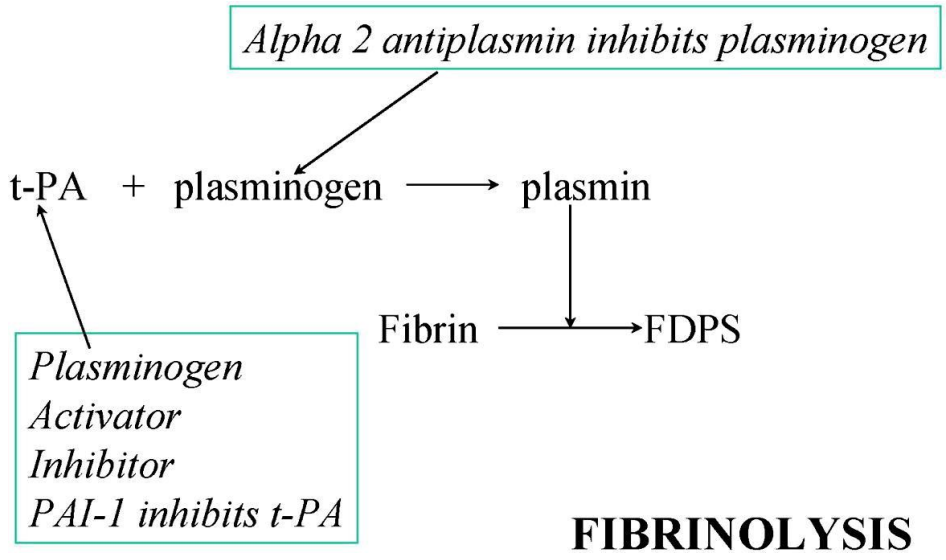

Figure 20: The fibrinolytic system (Johari and Loke, 2012). Figure reproduced with permission from Elsevier licence number 3830860459968.

Nitric oxide, prostacyclin, and TXA2 also modulate vascular and platelet reactivity, and all contribute to limiting clot formation (Moran and Viele, 2005).

There is a tendency to view sepsis all too often as a dysregulation of inflammation alone. While this may be the central perpetrator, dysregulation of coagulation is also a key factor in sepsis progression. There is significant evidence that sepsis should also be regarded as a condition of dysregulated coagulation, as demonstrated by Esmon et al. (1999) in Figure 21.

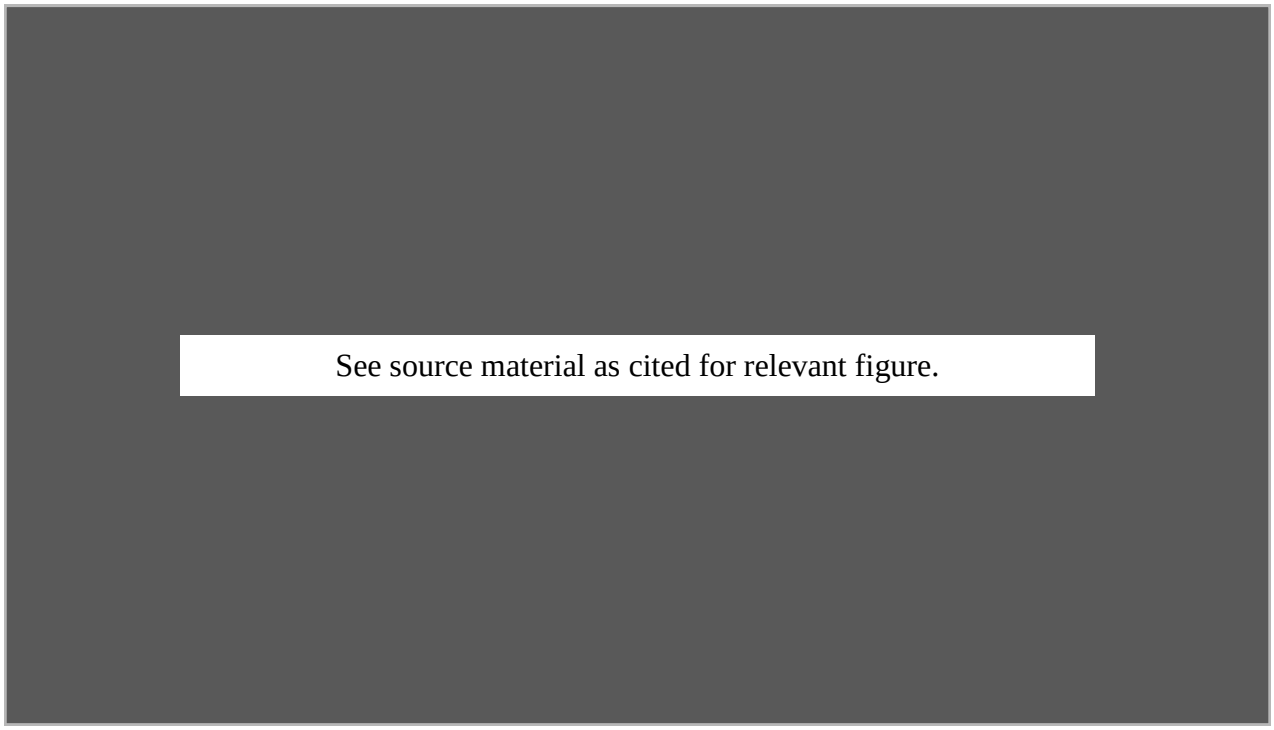

Figure 21: Progression of inflammation-coagulation autoamplication loop (Esmon et al., 1999).

In sepsis, coagulation is activated and additional anticoagulation and fibrinolysis are down-regulated. The endothelium plays a role in this by expressing TF and other procoagulant elements along with down-regulation of the coagulation regulatory proteins, such as thrombomodulin, all of which combine to lead to a procoagulant state in which thrombogensis is possible (Schouten et al., 2008b). As sepsis progresses, this can lead to the generation of microthrombi which can have grave consequences, leading to disseminated intravascular coagulation (DIC) (Semeraro et al., 2012), a situation with such poor prognosis it is synonymous among practising clinicians as “Death is Coming”.

2.5 The Complement Response

As has been established, several key components of the immune response are triggered following an insult to the host. The complement system is a central mechanism of innate immunity, activated immediately in such situations in order to control the replication of intruding pathogens (Rittirsch et al., 2012). The complement system is composed of plasma and membrane-bound self-assembling proteins (receptors and regulators), which function to purge foreign substances, including microorganisms, foreign bodies and apoptotic cell debris from the host, either by opsonisation and direct lysis or by mediating leukocyte function in inflammation and innate immunity (Nilsson et al., 2007).

The system acts as an enzymatic cascade through various protein–protein interactions. Complement activation results in increased vascular permeability, attraction of leucocytes and their immobilization at the site of inflammation, enhanced phagocytosis and cell lysis (Haeney, 1998, Tsiotou et al., 2005, van der Poll and Opal, 2008). There are three pathways in the complement system: classical, alternative, and lectin, demonstrated in Figure 22. The classical pathway (CP), so called because it was the first pathway to be defined (Sherwood and Toliver-Kinsky, 2004), is triggered by the formation of antigen–antibody complexes but can also be triggered by bound C-reactive protein (CRP) and endogenous element, DAMPs, released from damaged cells (Nilsson et al., 2007). The alternative pathway (AP), discovered after the classical pathway, is triggered directly by foreign microbial surface molecules including man-made biomaterials, that bind the complement component C3 and serve as a platform for the activation of complement proteins

(Sherwood and Toliver-Kinsky, 2004, Nilsson et al., 2007) The lectin pathway (LP) is activated by mannose-binding lectin, which interacts with microbial glycoproteins and glycolipids (Sherwood and Toliver-Kinsky, 2004).

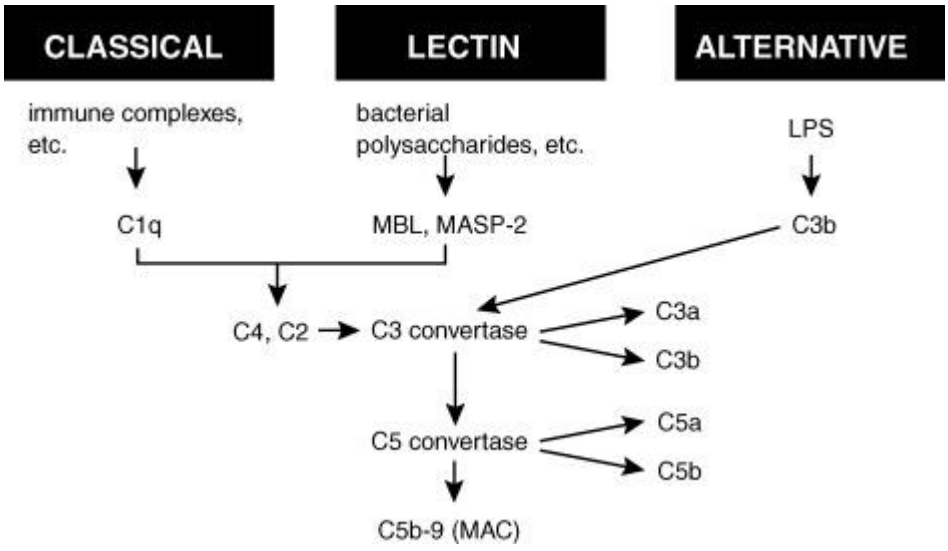

Figure 22: The three pathways of complement activation (Ward, 2008). Figure reproduced with permission from Elsevier licence number 3830860934288.

Not entirely dissimilar to the coagulation system, several complement components exist as inactive precursors and their activation involves proteolytic cleavage of the component into two or more fragments. Once cleaved, the larger, or major, fragment has both a binding site for attachment to cell membranes or the initiating immune complex and an enzymatic site for cleavage of the next component in the sequence. The minor fragments also perform critical roles such as chemotactic activity (Haeney, 1998). A single enzyme molecule is capable of activating many substrate complement components, resulting in stepwise amplification of the process. Similar to coagulation again, this process is deleterious if unchecked. In normal homeostasis,

activation of complement is regulated by the coordinated action of soluble as well as membrane-associated regulatory proteins (Kirschfink, 2001).

All three pathways, regardless of initiator, converge to complement protein C3 and are followed by a common cascade (C5-9), resulting in the deposition of a membrane-attack-complex on targets and the release of chemoattractants (C3a and C5a) for inflammatory cells (Charchaflieh et al., 2012). The point of convergence is the assembly of the ‘‘C3 convertase’’, which cleaves C3 into a small (C3a) and a large (C3b) fragmentation product (Figure 22) (Ward, 2008).

The major fragment C3b is a vital opsonic product that binds to the surface of bacteria and other microbes, to facilitate recognition by phagocytic cells and promote phagocytosis (Sherwood and Toliver-Kinsky, 2004) (Ward, 2008). In addition, C3b forms a proteolytic complex with other complement components to cause the cleavage of C5 into C5a and C5b (Sherwood and Toliver-Kinsky, 2004) The split products C3a and C5a act as potent anaphylatoxins (Rittirsch et al., 2012), which serve as chemotactic factors for neutrophils, and C5a also alters vascular permeability at the site of inflammation (Sherwood and Toliver-Kinsky, 2004).

C5a is an extremely active pro-inflammatory peptide, involved in almost all inflammatory processes and has been heavily implicated in sepsis. It reacts with high affinity receptors (C5aR and C5L2) on phagocytic cells, which triggers the synthesis of cytokines, chemokines, reactive oxygen species, and adhesion molecules, with the subsequent infiltration of myeloid cells, mostly neutrophils, into the area of injury (Dinarello, 2010, Ward, 2008).

C5b binds to the microbial surface and facilitates the formation of the membrane attack complex (MAC), which causes pore formation in target cells (including bacteria as well as nucleated and nonnucleated cells), resulting in cell lysis. It is also known to activate endothelial cells to release pro-inflammatory chemokines, such as IL-8 and chemokines (Ward, 2008, Sherwood and Toliver-Kinsky, 2004).

Importantly, and a key consideration in sepsis, is that elements from outwith the complement cascade can cleave complement components into biologically active complement products. In particular, thrombin, the key element in coagulation, which is known to increase in sepsis, can cleave proteins outside the coagulation pathway. Thrombin can cleave complement components C3 and C5 without the involvement of their complement precursors C3 or C3b (O'Brien, 2012). Furthermore, proteases from PMN and macrophages can cleave C5 as well (Rittirsch et al., 2012), demonstrating the degree of overlap and redundancy involved in the host response to insult.

2.6 Cytokines

A key element of host defence against any form of insult, infection or otherwise is cytokines, which are host-produced, pleomorphic immunoregulatory peptides. The initial insult triggers the production of pro-inflammatory cytokines. These cytokines promote leukocyte-endothelial-cell adhesion, activation of clotting, and generation of numerous secondary inflammatory mediators, including but not limited to other pro-inflammatory cytokines, prostaglandins, leukotrienes, proteases and importantly anti-inflammatory compounds and cytokines which should function as negative feedback the inflammatory process once appropriate (Wheeler and Bernard, 1999). Following insult, cells of the immune system increase production and secretion of these critical mediators, which act on many other cell types to modulate the host's immune response. Under normal haemostatic circumstances, cytokines act locally which restricts their effects to neighbouring cells. Such appropriate local effects benefit the host. However, cytokines are soluble molecules that can circulate systemically and in circumstances of prolonged or excessive circulation, such as in cases of chronic or severe infection, the excessive release of pro-inflammatory cytokines overwhelms the host's defenses and contributes to the morbidity and mortality of sepsis (O'Brien, 2012).

Cytokines can be categorised as either pro-inflammatory or anti-inflammatory. Following some insult, activated cells, principally macrophages, produce pro-inflammatory cytokines as part of the generalised inflammatory response to eliminate pathogens and promote wound healing. Once no-longer required, these pro-inflammatory cytokines should then be downregulated by release of anti-

inflammatory cytokines. Pro-inflammatory cytokines include tumour necrosis factor (TNF)a, interleukin-1 (IL-1) and anti-inflammatory cytokines include IL-10 and IL-1 receptor antagonists (RA). It has been argued that some possess both activities, e.g. IL-6 (Treacher and Alun Brown, 2009). In sepsis, however, these normally beneficial host defences appear to be unable or insufficient to eliminate the infectious agent and overstimulation of the host's immune effector cells occurs, known anecdotally as the "cytokine storm". This overwhelming systemic pro-inflammatory reaction is frequently followed by an overactive compensatory anti-inflammatory mediator release. When the balance between pro- and anti-inflammatory response is lost, immunological imbalance and massive systemic inflammation results (Tsokos, 2007). There now follows a discussion of the key cytokines implicated in sepsis and its sequelae.

Tumour Necrosis Factor – α (TNF-α)

TNF-α is a critical mediator in inflammation and the progression of early sepsis, responsible for the familiar hypotension and fever. At the nidus of an insult or infection, TNF-α initiates the immune response that activates the various cellular defences discussed earlier to ensure an infection or insult is addressed and subsequently repair tissue. It is a potent activator of neutrophils and mononuclear phagocytes, and also serves as a growth factor for fibroblasts and as an angiogenesis factor (Sherwood and Traber, 2012).

TNF was the first mediator to be implicated and investigated in the context of sepsis and has been considered as a potential marker of the conditions progression. It has

been referred to as the "prototypical pro-inflammatory cytokine" (Sherwood and Toliver-Kinsky, 2004). In sepsis, TNF-α can provoke neutrophil-mediated tissue injury, as it enhances the expression of adhesion molecules, ICAM-1 and VCAM-1, and chemokines in endothelial cells. Additionally. TNF-a increases the permeability of endothelial cells their pro-coagulated activity (Pape et al., 2007). By up-regulating integrin adhesiveness, TNF-α activates neutrophils and promotes extravasation. Extravasated neutrophils lead to tissue damage through the release of oxygen free radicals and proteases. Tissue damage can lead to distal organs, such as the lungs, liver and gut. In addition, TNF-α amplifies inflammatory cascades in an autocrine and paracrine manner by activating macrophages/monocytes to secrete other pro-inflammatory cytokines, particularly IL-1 and IL-6 (Sherwood and Traber, 2012, Shimaoka and Park, 2008). Another important effect of TNFα is its ability to induce apoptosis of a variety of cell types, which may be one mechanism by which it induces tissue injury at high systemic concentration. It is known that its stimulation can be down regulated by interleukins (Pape et al., 2007).

Interleukin-1 (IL-1)

IL-1 is a term used to describe a family of similar proteins, including IL- 1α and IL-1β (Schulte et al., 2013). The IL-1 family of cytokines is produced primarily by mononuclear phagocytes following activation of the so-called "inflammasomes", discussed earlier, which initiate cleavage and release of IL-1 family cytokines (Latz, 2010). IL-1 shares many functional similarities with TNF-α resulting in similar physiological effects as identified above, indeed at their initial discovery they were collectively identified together as "endogenous pyrogenic mediators" (Galley and

Webster, 2008). They are frequently described as synergistically acting mediators, leading to increased permeability and coagulation ability of the endothelium, as well as to increased levels of adhesion molecules on the endothelium, leading to PMN activation and transmigration (Lenz et al., 2007). Once it was discovered that this macrophage derived "endogenous pyrogen" could augment T lymphocyte responses, namely their activation, the name interleukin was applied (Dinarello, 1991).

IL-1 consists of two similar proteins; IL- 1α and IL-1β, both of which act upon the same receptor. However IL-1β is generally associated with higher concentrations in circulation during sepsis. IL-1 remains comparatively poorly understood. Data on IL-1 in SIRS are sparse and no conclusions can yet be drawn (Jaffer et al., 2010). Both TNF-α and IL-1β are involved almost immediately following insult, acting to induce further release of pro-inflammatory cytokines, increase the concentration of neutrophils in circulation, increase chemotactic response, amplify phagocytosis, increase endothelial permeability and importantly decrease the apoptosis ratio (Hietbrink et al., 2006). All of which leads to the increased extravasation described earlier. Despite similar effects to TNFα important differences exist between the functions of IL-1 and TNFα. Most notably, IL-1 does not induce tissue injury or apoptotic cell death by itself, but can potentiate the injurious effects of TNFα (Sherwood and Traber, 2012).

The IL-1 family is the only group of cytokines which have a known, naturally occurring, antagonist; the IL-1 receptor antagonist (IL-1ra) which binds to the same receptors as IL-1 but do not result in activation of the receptor. Such action would

suggest as control function in the immune response to insult (Sherwood and Traber, 2012, Lenz et al., 2007).

Interleukin-6 (IL-6)

IL-6 is a pivotal mediator in sepsis. However, its classification as either a pro-inflammatory or anti-inflammatory mediator is the subject of debate, as it has been reported to demonstrate properties of both. It remains unclear whether or not IL-6 is a direct agonist or antagonist of sepsis. It induces release of cortisol, IL-10, IL-Ra and hepatic acute-phase proteins and accordingly is considered by many as an anti-inflammatory cytokine (Philippart et al., 2009). However, it must be acknowledged that controversy exists in this area. Some investigators believe that IL-6 serves as a marker of injury, whereas others believe that IL-6 may be responsible for the altered pathophysiology (Daniel G. Remick, 2007).

IL-6 arises early in the progression of events following insult, although not as promptly as TNF-α and IL-1 it has a longer half-life. Its secretion from a variety of cell types, including monocytes and macrophages, endothelial cells, adipocytes, B- and T-cells (Pape et al., 2007), is initiated by TNF-α and IL-1. IL-6 induces secretion of acute-phase proteins, C-reactive protein (CRP), from the liver a proliferation and a differentiation of both B- and T-lymphocytes (Pape et al., 2007).

Controversy arises when considering the pro-inflammatory activities IL-6 appears to mediate, such as induction of tissue factor, activation of neutrophils, proliferation and differentiation of T cells, enhancement of natural killer cell activities, and maturation of megakaryocytes. Conversely IL-6 is also implicated in anti-

inflammatory mediatory activities, including the attenuation of TNF-α and IL-1 activity and inducing acute phase hepatic protein synthesis, which down regulate the inflammatory response (Jawa et al., 2006). Regardless of its mechanism of action, it is generally considered to be one of the best markers of the progression of sepsis (Philippart et al., 2009).

Interleukin-12 (IL-12)

IL-12 performs key functions by facilitating adaptive immunity, it acts in concert with IL-15, IL-15 and IL-18 to induce IFN-γ production by Natural Killer (NK) cells. IFN- γ itself is a cytokine involved in amplification of the inflammatory response, most notably stimulating secretion of other cytokines, phagocytosis and macrophage respiratory burst. IFN-γ then potentiates macrophage inflammatory function, including further IL-12 production. In this way, acting in concert IL-12 and IFN- γ amplify both innate and adaptive immunity by establishing a positive feedback loop potentiating inflammation (Sherwood and Toliver-Kinsky, 2004).

IL-12 also has an effect on adaptive immunity, IL-12 causes activation of T-cells and promotes the differentiation of T-cells to the Th1 phenotype, which is characterized by enhanced mononuclear phagocyte responses. Thus, IL-12 links early, nonspecific, and later, specific immune responses (Schulte et al., 2013).

Interleukin-10 (IL-10)

Monocytes and lymphocytes produce IL-10, which functions to inhibit production of monocyte/macrophage derived TNF-α, IL-6 and IL-8 (Tsukamoto et al., 2010).

There is increased production of IL-10 in the latter stage of sepsis and it is one of the anti-inflammatory cytokines believed to contribute to CARS or "immunosuppression" (Shimaoka and Park, 2008). Excessive production of IL-10 and an amplification of its action to mitigate pro-inflammatory cytokines and decrease the number of circulating lymphocytes and monocytes could render the host susceptible to lethal community-acquired infections, such as meningococcus, possibly due to increase apoptosis of such cells (Treacher and Alun Brown, 2009).

Interleukin-8 (IL-8)

IL-8 plays a critical role for numerous physiological functions as a neutrophil chemotactic cytokine, a key element in inflammation. It functions in this role alongside other chemotaxins to effect leukocyte, and subsequent neutrophil, migration to a site of insult and additionally to induce expression of surface adhesion molecules (Tsiotou et al., 2005). Produced by macrophages, IL-8 provides a critical role in the host defence. However, much like the other cytokines discussed here, when inappropriately or excessively expressed, they can contribute to a deleterious inflammatory response (Sherwood and Traber, 2012).

Cytokines are key mediators in inflammation and the progression of sepsis. Their involvement largely determines the magnitude of the innate response. They are principally produced by cells of the immune system in response to insult, which function to regulate immune and inflammatory reactions. Their production and action, as has been previously mentioned, should be self-limiting, however as they are soluble proteins, they can circulate systemically and in cases persist in

circulation. Their effects are pleiotropic and redundant, meaning that a single cytokines has multiple functions and they are redundant, meaning that multiple cytokines can affect the same, or similar, functions. For this reason, the blockade of a single cytokine will often have limited effects on the overall inflammatory response (Sherwood and Toliver-Kinsky, 2004) In order to improve patient survival, it may be necessary to identify the optimum balance between opposing cytokines, so that infection is controlled without compromising organ function. Due to the complexity of cytokine interactions, this task will be difficult to achieve in the near future (Treacher and Alun Brown, 2009).

It is a worthwhile observation that comparisons to "language" and "communication" are frequently employed to describe the role of cytokines in the progression of sepsis throughout the literature (Giannoudis, 2003), a particularly apt comparison when considering the distal nature of many of the pathophysiological alterations from the initiating insult in sepsis. It would not be possible for such effects to take place without some form of "communication" or "signal" to transmit the affliction. In this research, it is proposed that if this communication is interrupted or disrupted it may be possible to mitigate the progression of the condition.

2.7 Theories of Pathophysiological Progression

It has been established in the discussion so far that sepsis is a systemic response to some insult and symptoms are produced by host defence systems rather than by invading pathogens. (Schouten et al., 2008a). In response to some insult, a multifactorial network of chemical signals initiates and maintains a response designed to 'heal' the afflicted tissue (Coussens and Werb, 2002). It is when dysregulation of this process occurs that complications arise. This concept is demonstrated in Figure 23. Sepsis and SIRS describe a dysregulated inflammatory state represented by uncontrolled levels of inflammatory mediators with development of multi-organ dysfunction syndrome (MODS) and multi-organ failure (MOF) (Rittirsch et al., 2007, Bone et al., 1992).

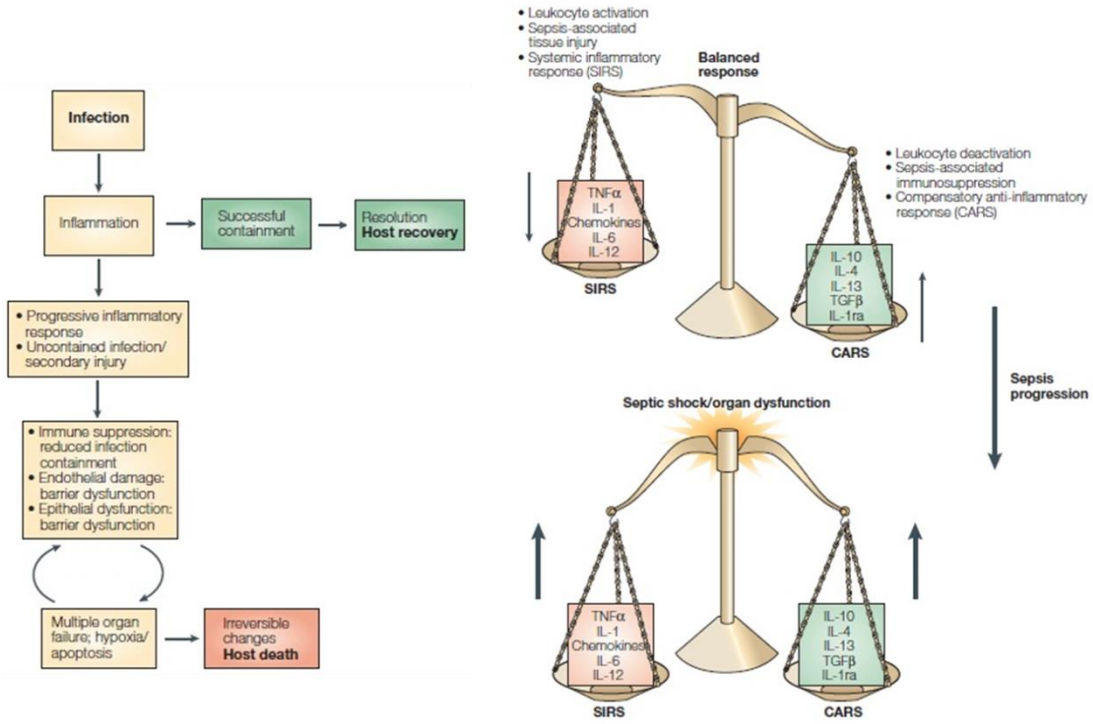

Figure 23: Sepsis pathogenesis. The host inflammatory response can be viewed as a balanced response between pro-inflammatory mediators (SIRS) and anti-inflammatory mediators (CARS) adapted from Buras et al. (2005). Figure reproduced with permission from Macmillan Publishers Ltd licence number 3830861186053.

In the course of the last 20 to 30 years, understanding of the nature of the sepsis condition has advanced significantly. However the nature of the progression of sepsis is still the subject of much debate and ongoing research, including how appropriate therapeutic interventions may be made to impede or halt this progression. This section will discuss the key theories of this progression.

2.7.1 The Two (or more) Stages

Sepsis and non-infectious systemic inflammation were traditionally viewed as an excess of pro-inflammatory mediators exclusively, however, understanding has evolved beyond this. The inflammatory process involves a series of events that can be elicited by numerous stimuli, for example, infection agents, ischemia, antigen–antibody interactions, chemical, thermal or mechanical injury (Rotelli et al., 2003). The response has been theorised to progress sequentially from being predominantly governed by the pro-inflammatory mediators in an acute phase, and the counter-inflammatory mediators in a later phase with associated physiological progression, as demonstrated in Figure 24. The pro-inflammatory mediators include tumour-necrosis factor-α (TNF-α) and interleukin (IL)-1, IL-6 and IL-12, which activate the host immuno-inflammatory system. They initiate an inflammatory cascade mediated by several cell lines and involving the complement, coagulation, and fibrinolytic systems (Joseph E. Parrillo, 1993, Vriese et al., 1999). These should then be deactivated through the expression of counter-inflammatory mediators, including IL-1 receptor antagonist (IL-1ra), IL-4, IL-10 and IL-13 (Vriese et al., 1999). Ultimately, in the development of septic shock, regulated expression of SIRS and CARS mediators is lost, resulting in an exaggerated and dysfunctional inflammatory response (Buras et al., 2005, Doi et al., 2009, John A. Kellum et al., 2007). This loss of regulated expression is demonstrated in Figure 23. The resultant defects include diminished expression of important cell surface antigens, dysregulated cytokine production, alterations in antigen-presenting ability, and accelerated apoptosis (Reddy et al., 2001). When sepsis progresses and propagates MODS and MOF, the

mortality becomes high, ranging from 30-80% depending on the number of failed organs (Baue et al., 1998), representing a serious health care issue.

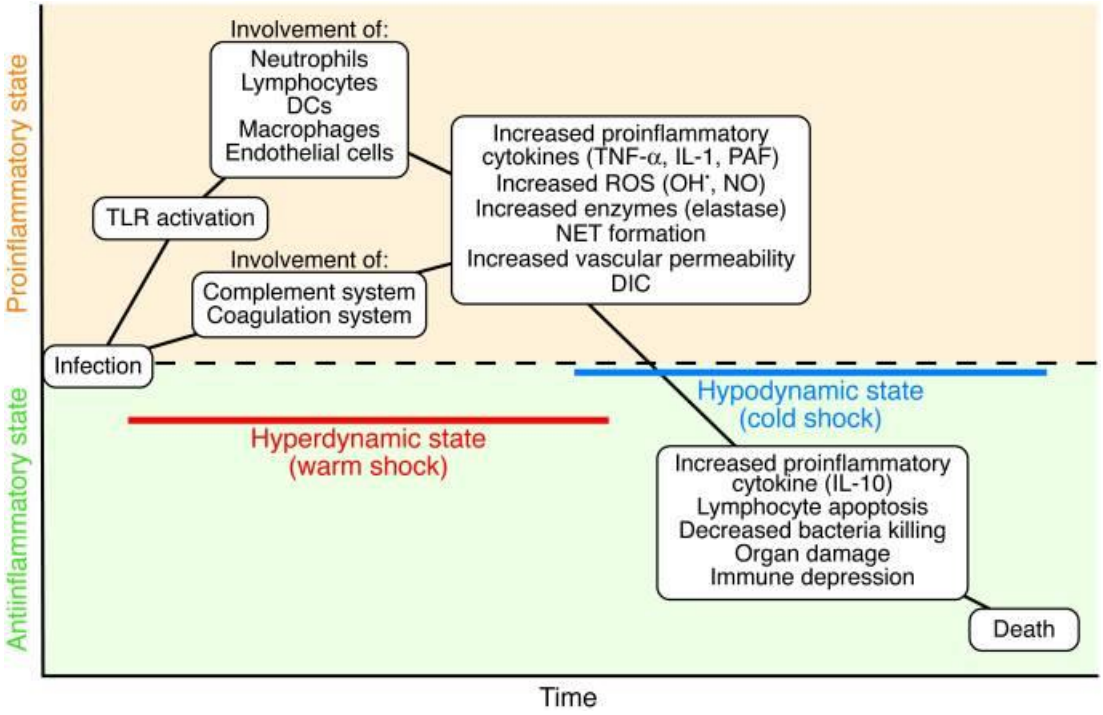

Figure 24: Simplified clinical course of sepsis. Progression of disease is complex, nonlinear, and varies from one patient to another (Doi et al., 2009). Figure reproduced with permission from American Society for Clinical Investigation licence number 3846980214877.

The consensus of Bone et al. (1992) involved the consideration, based on clinical evidence, that sepsis and SIRS may not only be attributable to an over-active inflammatory response by the immune system responding to a pathogen but that the immune system itself is somehow compromised in chronic sepsis and SIRS (Riedemann et al., 2003, Coussens and Werb, 2002). Mechanisms of such a theory are still the source of much research but recent hypotheses propose that sepsis moves through different phases, with alternating periods of amplified inflammation and amplified immune suppression (Xiao et al., 2006, Shimaoka and Park, 2008), with a

postulated shift toward an anti-inflammatory immunosuppressive state in the later phases of sepsis (Xiao et al., 2006).

Anti-inflammatory responses and even immune paralysis have been detected during chronic infection, giving rise to the concept that the systemic inflammatory and compensatory anti-inflammatory response syndromes could occur simultaneously. Such research indicates that an unbalanced relationship between the two syndromes is thought to initiate a cascade of cellular signalling events, ultimately producing severe effects in distant organs (Reddy et al., 2001).

These two distinct but not mutually exclusive phases have been the subject of much investigation and have subsequently been defined by many names. However, they are generally typified as the systemic inflammatory response (SIRS), associated with the acute condition (sometimes referred to as hyperdynamic state, or "warm shock") and the compensatory anti-inflammatory response (CARS), associated with the chronic condition (sometimes referred to as hypodynamic state, or "cold shock") (Buras et al., 2005, Richard S. Hotchkiss and Irene E. Karl, 2003, Doi et al., 2009).

It has been suggested that endothelial damage in sepsis results from persistent and repetitive inflammatory insults, which result in such damage that downregulation can no longer occur (Roger C. Bone, 1991). As was mentioned in chapter 1.0, Roger C. Bone (1991) refers to this as a state of "metabolic anarchy" where the host cannot adequately control its inflammatory response. Some authors have also argued the case for an intermediary stage, a subacute phase wherein the chain of events would occur in three distinct phases: an acute one characterized by local vasodilatation and

increased capillary permeability, a subacute phase characterized by infiltration of leukocyte and phagocyte cells and a chronic proliferative phase, in which tissue degeneration and fibrosis occur (Rotelli et al., 2003).

It has also been documented that endothelium dysfunction has a major role to play. Under normal conditions, the endothelium provides for an anticoagulant surface, a property that is lost in sepsis (Schouten et al., 2008a). Some haemodynamic studies indicate that the basic pathogenic mechanism in sepsis is an increased vasopermeability, presumably caused by endothelium damage, in combination with vasodilatation (Hack et al., 1989).

It has been established that a variety of aberrant processes take place in sepsis, typically involving disruptions to the inflammatory, coagulation and complement systems, rather than the previously held belief that it was simply a hyperinflammatory state alone. Instead, it appears there is far more heterogeneity in the progression of the condition, involving both hyper and hypo immune responses (Daniel G. Remick, 2007). Indeed, a major question still exists as to whether or not patients are predominately hyperinflammatory, immunocompromised or some alternating combination of both, which will ultimately dictate what therapeutic techniques, or combination of techniques, are utilised or develop to improve patient outcome. Improved understanding of the pathophysiology will help to direct future management of the condition (Russell, 2006).

2.7.2 The “Two Hit” Theory

It has been proposed that the condition may progress in a “two hit” fashion. It can be considered that the magnitude of some insult alone is sufficient to trigger an overwhelming inflammatory response leading to SIRS and possible subsequent CARS, but it is also possible that the condition progresses as a result of a heightened susceptibility, induced by the initial insult, to a subsequent secondary insult, a concept demonstrated in Figure 25.

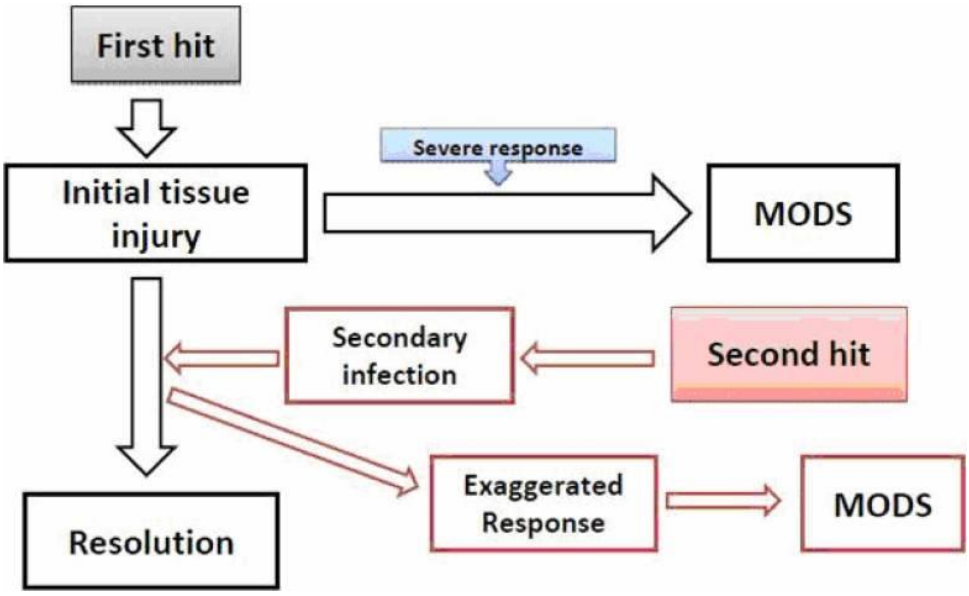

Figure 25: The "one-hit" and "two hit" paradigm of traumatic injury (Namas et al., 2009). Figure reproduced under CC BY-NC 3.0.

The concept is well documented in the context of polytrauma and post-traumatic injury, where a patient dies from in one of three time periods either an initial highly drastic trauma such as massive head injury or severe haemorrhage, or subsequent death due to hypoxia or hypovolemia reasonably soon after the initial insult while treatment is administered, or lastly, considerably later, due to the high risk of

developing immunological dysfunction and sepsis or SIRS leading to organ or multiple organ dysfunction (Lenz et al., 2007). When considering the concept in the specific context of sepsis and systemic inflammation, progression to organ dysfunction can be attributed to “one hit”, an initial massive insult, such as organ injury, hypoxia, hypovolaemia or head trauma, which causes the classic hyperinflammatory response and shock associated with a heightened systemic inflammatory response (SIRS) sufficient enough to result in remote organ or multi-organ dysfunction. Or it can be attributed to a “second hit”, which describes an initial insult resulting in a limited inflammatory response, which can be resolved by the host. However, this leaves the host vulnerable, perhaps with a heightened responsiveness, to a secondary inflammatory hit, such as infection, ischaemia, reperfusion or operations which can further augment the inflammatory immune response in a magnified nature which can lead to organ or multiple organ dysfunction at a later stage (Namas et al., 2009) (Lenz et al., 2007). This potential for a deleterious second hit often forms the rational for a practicing clinician decision on how to advance therapy for polytrauma patients. Hyperstimulation of the inflammatory system, either by a single or multiple “hits”, is considered by many to be the key element in the pathogenesis of ARDS and MOF (Giannoudis, 2003).

While the pathophysiology of the “two hit hypothesis” is the subject of ongoing investigation, inflammatory cell priming (discussed in section 2.3) is thought to play a key role. Amplification of circulating levels of cytokines prime macrophages leading to a heightened response if a second insult is involved (Sherwood and Traber, 2012).

2.7.3 Parallel or Sequential Progression

The theories discussed so far have all considered the progression of sepsis in a relatively linear nature progressing from an acute to latter phase. However, it has been proposed that it is not linear but rather constantly changing and that it requires close observation (Xiao et al., 2006). Accordingly, many researchers assert that the pro- and anti- inflammatory syndromes operate in parallel rather than in strict sequence, as demonstrated in Figure 26.

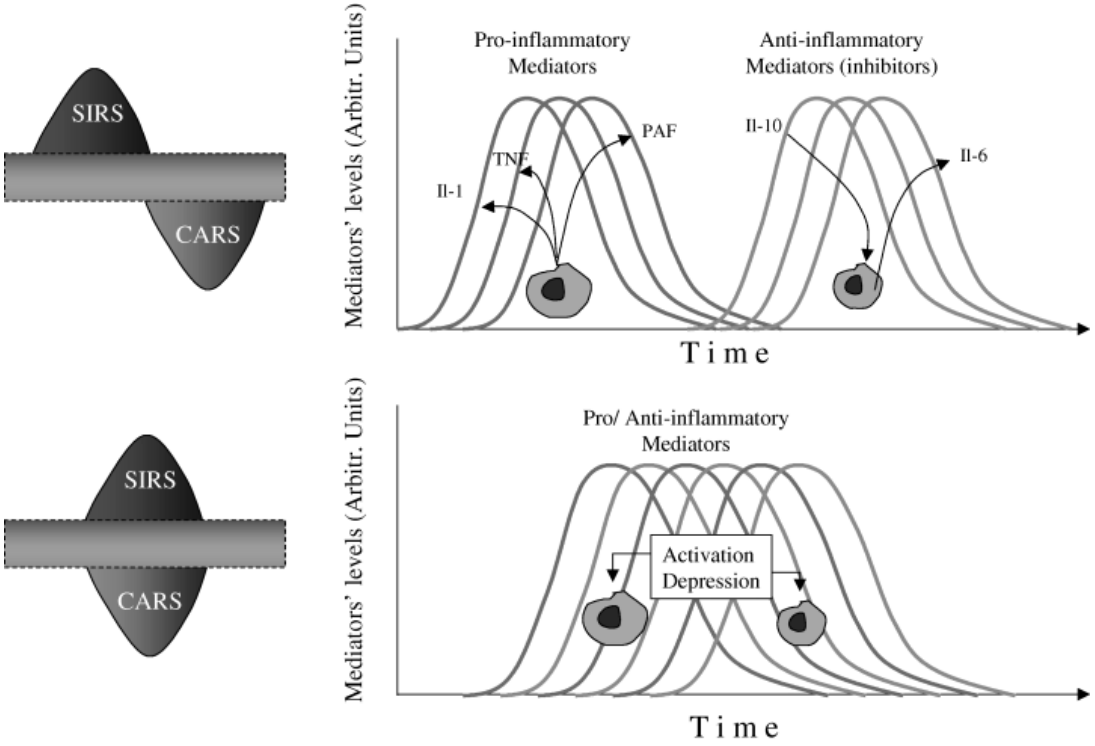

Figure 26: In the sequential theory, peaks of inflammatory mediators are followed by peaks of anti-inflammatory mediators. In the parallel theory a mixture of pro- and anti-inflammatory mediators coexists (Ronco et al., 2003). Figure reproduced with permission of John Wiley and Sons licence number 3831881054092.

The argument for parallel progression arises from the thinking that it is too simplistic to describe the sepsis progression as simply increased or decreased expression of

various mediators and it is not representative of clinical observations. If the sequential theory is adhered to, then targeted therapies could be administered at definitive stages. However such attempts have not met with success. If the parallel theory is adhered to, then there is an element of chance involved in applying targeted therapies at specific time points, as at any given stage it could be either effective or deleterious. It may be possible that ultimately mortality results from either early pro-inflammatory complications or later anti-inflammatory complications. However it is possible that both are continuously battling for control of a dysregualtated response to insult, in which case a less selective treatment modality, while broader, may represent a greater opportunity for clinical effectiveness.

2.8 Current Treatment Approaches

Since the time of the Egyptians and ancient Greeks, discussed in chapter 1.0, effective therapeutics have been sought for sepsis. However, the search has yielded only incremental advances disproportionate to the scale of the condition and the tireless efforts of many clinicians and researchers. Indeed, the majority of current therapeutic approaches is supportive rather than interventional. Previous and current therapeutic approaches include: antibiotics, activated protein C, corticosteroids, vasopressors and early goal-directed therapy. All have met with such disappointing impact that sepsis has become infamous as a "graveyard" of therapeutic development. Early recognition is key in order for practising clinicians to prevent the often rapid progression from sepsis or SIRS to MODS.

2.8.1 Detection & Diagnosis

A key challenge for clinicians in employing any therapy for sepsis is acutely identifying the condition early. Indeed, it is stated as a belief of the Surviving Sepsis Campaign (SSC) committee that "*the greatest outcome improvement can be made through education and process change for those caring for severe sepsis patients in the non-ICU setting and across the spectrum of acute care*" (Dellinger et al., 2013a). Vanzant and Schmelzer (2011) in their article aimed directly at improving early detection of sepsis provide a staging system of recognition, influence by the definitions discussed earlier in chapter 1.0. This system is displayed in Table 9:

Recognising the stages of sepsis

Stages of Sepsis	Signs and Symptoms
Sepsis	SIRS criteria Suspected or confirmed infection
Severe sepsis	Elevated serum lactate level (>2 mmol/L) Profound hypovolemia that responds to fluid resuscitation Signs of tissue hypoperfusion in the following: • Brain - delirium • Heart and circulation - hypotension, tachycardia, mottled skin, and capillary refill >3 s • Blood - thrombocytopenia (platelet count <100,000 platelets/mL) • Lungs - increased respiratory rate and hypoxia • Kidney - decreased urine output (<0.5 mL/kg for at least 1 h) and elevated blood urea nitrogen and creatinine • Gut - decreased motility, decreased bowel sounds, and anorexia • Liver - increased bilirubin and liver enzymes
Septic shock	Profound hypovolemia, hypoperfusion, and hypoxia Hypovolemia does not respond to fluid resuscitation Multiple organ failure Usually fatal

Table 9: Recognition stages of sepsis adapted from Vanzant and Schmelzer (2011). Table reproduced with permission from Elsevier licence number 3846980761020.

It is additionally suggested that there are four suitable methods for the detection of sepsis in the clinic: the SIRS/Sepsis criteria, serum lactate levels, the 10 vital indicators of body function, and cognitive changes (Vanzant and Schmelzer, 2011). As has been discussed in chapter 1.0, early sepsis is considered to be compliant with the SIRS criteria plus a confirmed or suspected infection. Additionally, Vanzant and Schmelzer (2011) report the success of various scoring based tools derived from this approach in detecting sepsis early successfully.

Another potential criterion, serum lactate, is produced by anaerobic metabolism and is a marker of tissue hypoperfusion. It helps to identify inadequate oxygen delivery and has prognostic value for patients in septic shock (Griffiths and Anderson, 2009).

Rising serum lactate levels are considered useful, as they are indicative of cellular hypoxia, rapidly reported, and can be collected from venous blood, making sampling easy.

The validity of using physiological parameters as thresholds for diagnostic purposes, for sepsis or any other condition, should always be considered not as absolutes in their own right but in relation to a patient's individual baseline. As previously mentioned in chapter 1.0, assessment at the bedside was a critical point of discussion and agreement by Levy et al. (2003). Funk et al. (2009) suggest an additional ten symptoms for assessment at the bedside, as follows:

- Temperature changes
- Increased pulse
- New or changing pain
- Changes in respiratory rate
- Decreased systolic and mean arterial blood pressures
- Level of consciousness changes (lethargy or anxiety)
- Capillary refill greater than 3 seconds
- Urinary output less than 30 mL/h
- Changes in ScVO2 (measured via blood gas analysis)
- SaO2 < 90%. (oxygen saturation [arterial])

It is suggested that changes in two or more of these parameters require immediate attention.

Delirium can frequently be the first sign of sepsis. At the bedside, the Confusion Assessment Method (CAM), a standardized tool that uses patient behaviour and standardized questions to assess the features of delirium and differentiate it from dementia, is usually employed to assess cognitive function (Vanzant and Schmelzer,

2011). In conclusion, Vanzant and Schmelzer (2011) recommend a combination of, SIRS screening, vital indicators of body function, serum lactate measurement, and use of CAM.

Ultimately, limited by present technology, there is still an overall sensation that despite improved understanding of the condition, sepsis diagnosis still requires much clinician training, judgement and can arguably be regarded as more of an art than an exact science; reinforcing the assertion discussed earlier in chapter 1.0 by (Levy et al., 2003) that any diagnosis should be grounded in the day-to-day reality for bedside clinicians and assessing whether or not they "look septic". Numerous sepsis awareness campaigns are underway around the globe, including the dissemination of the Surviving Sepsis Campaign guidelines and bundles, the "know your sepsis six" campaign in the NHS in the UK.

2.8.2 Antibiotics and Source Control

In management of the sepsis condition, particularly when considering severe sepsis or even septic shock, time is a critical factor. It is generally agreed that early, or even pre-emptive, administration of broad-spectrum antibiotics is essential in the management of sepsis. Adequate and timely antimicrobial treatment is a cornerstone for survival in patients with bacteremia and sepsis (Huttunen and Aittoniemi, 2011).

Ideally, the nature of the initiating insult should be determined to direct this treatment. Accordingly, samples should be taken from a variety of sources to facilitate this, bearing in mind the so-called "big-five" of sepsis in the search for causation; lungs, abdomen, urine, wounds and catheters (Vincent, 2008). Additionally, high quality wound stewardship is of great importance, such as the drainage of abscesses, removal of necrotic tissue and the removal of any foreign materials as much as is possible. As mentioned in chapter 1.0, the increased use of indwelling medical devices is a key suspect in the prevalence of sepsis. If deemed appropriate administration of two or more antibiotics may be used to broaden the antibacterial spectrum, treat polymicrobial infections and could act synergistically (Zimbler and Campbell, 2004). However, microbial resistance has become a significant issue for the modern clinician and an ever increasing rise of resistant pathogens is causing infections. As such, use of antimicrobial treatment has become complicated and indeed concerns of over prescription abound. At the same time, the clinical approach to broad-spectrum antimicrobials as first-line choice should be critical in view of the increasing antimicrobial resistance associated with the use of these agents (Huttunen and Aittoniemi, 2011).

Should the sepsis source control require a more dramatic surgical intervention than the removal of a cannula, a skilled approach is required, often image guided. It is generally advocated that radiological and surgical procedures to control the source of an infection should be undertaken only after stabilization, although resuscitation should proceed as rapidly as possible. Although the often serious complications associated with sepsis present serious compromise of haemodynamic stability, as such interventional source control can be a difficult route to treatment with the most dramatic interventions reserved for use only in cases where the benefit significantly outweighs the risk (Zimbler and Campbell, 2004).

2.8.3 Haemodynamic Therapy

Shock is the alteration of tissue perfusion, with a reduction in the delivery of oxygen and other nutrients to the tissues, causing cellular, and then organ, dysfunction (Zimbler and Campbell, 2004). Sepsis is associated with alterations in tissue perfusion, reduction in oxygen and nutrient delivery, which can ultimately lead to cellular and organ dysfunction. Haemodynamic therapy is targeted towards restoring tissue perfusion and normal cellular function, and consists of two components; fluid resuscitation and use of vasoactive agents (Vincent, 2008).

Hypoperfusion is a major component of the clinical progression of sepsis, which should be countered with fluid resuscitation as soon as sepsis is implicated in the patient. It is suggested that this is undertaken before ICU or even hospital admission (Raghavan and Marik, 2006). Successful volume replacement should result in increased cardiac output and improved oxygenation and directed towards targeted specific endpoints based upon of blood pressure, heart rate, urine output and central venous pressure (Zimbler and Campbell, 2004). Such resuscitation should be patient specific and should continue to be administered as long as there is haemodynamic improvement based upon the targeted endpoints (Schorr et al., 2014).

Until comparatively recently, it was stated that the choice of fluid used for resuscitation was of little significance, crystalloid or colloid, as there is no evidence that either one was superior to the other (Griffiths and Anderson, 2009). The fluid of first choice has typically been unbalanced crystalloids, most commonly conventional saline; however, this has become a source of significant debate and research. First

choice of crystalloids alone has been challenged as capillary permeability is increased in sepsis, which is the key element at play in increasing the likelihood of tissue or pulmonary oedema. Crystalloids can compound this problem by further increasing the interstitial volume without sufficient volume being restored intravascularly potentially along with decreased pH and hyperchloremia (Dellinger, 2014). The alternative use of colloids, such as albumin, however also presents concerns regarding colloid osmotic pressure. Although the debate and research continue in an attempt to arrive at definitive recommendations, it is universally acknowledged that fluid resuscitation is vital in sepsis (Raghavan and Marik, 2006).

Patients in severe sepsis or septic shock may not respond appropriately to fluid resuscitation alone. This may be due to the dramatically reduced vascular resistance in sepsis resulting in persistent hypotension and hypovolemia despite fluid resuscitation. At this point, the use of vasopressors is pursued. As with fluid resuscitation the use of vasopressors is directed towards targeted specific endpoints based upon blood pressure and perfusion. The first choice vasopressor is norepinephrine, which results in vasoconstriction and an associated rise in the mean arterial pressure (MAP), preferable to dopamine which also increases MAP but is associated with more pronounced effects on heart rate and oxygen consumption (Raghavan and Marik, 2006).

2.8.4 Activated Protein C

Given the variety of host systems involved in the progression of sepsis and the significant crosstalk between them, immunomodulatory therapies are a considerable area of investigation in the search for sepsis therapies.

It was established in earlier sections that coagulation and inflammation are key elements within sepsis. Within the coagulation system when thrombin is coupled with thrombomodulin, it converts protein C to its activated from; activated protein C (APC), which advances fibrinolysis by inhibiting plasminogen activator inhibitor-1 (PAI-1) and thrombin activatable fibrinolysis inhibitor (Kylat and Ohlsson, 2012), as demonstrated by (Bernard et al., 2001) in Figure 27. APC acts by several mechanisms to prevent injury in the microvasculature associated with sepsis, it does so by inhibiting factors Va and VIIIa exerting an antithrombotic effect (Green et al., 2005, Kylat and Ohlsson, 2012). Additionally, APC is known to have an anti-inflammatory action by inhibiting leukocyte adhesion to selectins and subsequently blocking pro-inflammatory cytokine release (Green et al., 2005, Kylat and Ohlsson, 2012)

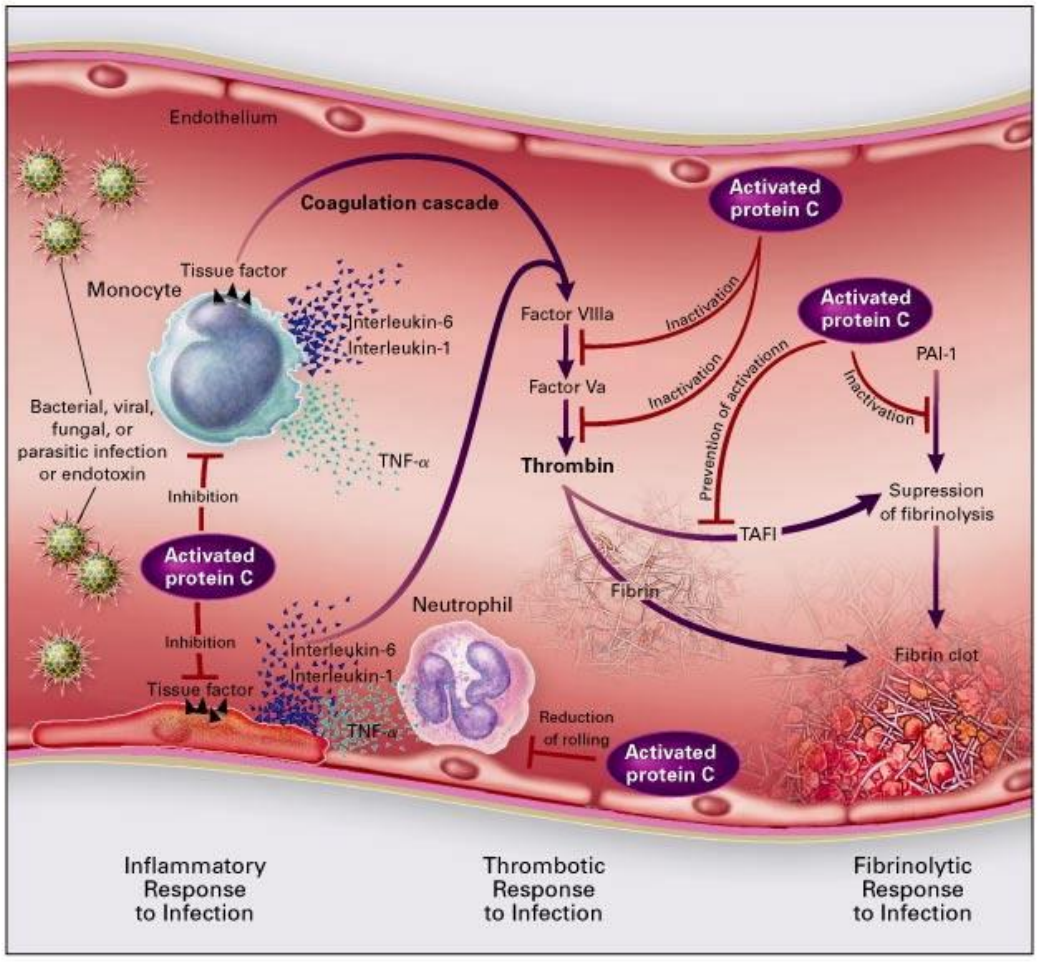

Figure 27: Proposed actions of activated protein C (Bernard et al., 2001). Figure reproduced with permission from Bernard et al. (2001), Copyright Massachusetts Medical Society.

The half-life of APC is short and in sepsis conversion to the activated form is compromised shifting the homeostatic balance towards systemic inflammation, increased coagulation and ultimately organ dysfunction. Administration of APC has been considered a valid area for study as a potential sepsis therapy by limiting vascular injury and thrombosis. A controversial immunomodulatory drug, recombinant human activated protein C (rhAPC), marketed as Drotrecogin-alfa

(activated) or Xigris® by Eli Lilly & Co. (Indianapolis, IN), was developed in an attempt to address septic shock.

APC was put to the test in 2001 in a controlled clinical trial 'Protein C Worldwide Evaluation in Severe Sepsis' (PROWESS), where it was found to decrease mortality in those sepsis patients with a low risk of bleeding: 24.7% compared to 30.8% in the placebo group (Bernard et al., 2001, Shimaoka and Park, 2008). Upon publication of these results, there was considerable excitement as it appeared APC could become the first treatment for sepsis successful in phase III clinical trial and subsequently a viable treatment option. This led to Food and Drug Administration approval for the use of APC as a therapeutic intervention. However, the approval of APC was controversial, with half of the Food and Drug Administration panel voting to require a confirmatory trial (Daniel G. Remick, 2007).

In the first set of sepsis management bundles published by the Surviving Sepsis Campaign (SSC), more on the SSC in the next section, in 2004, use of APC was advocated and accordingly it became clinical practice. However, the enthusiasm began to fade quickly as it became reported that investigation in specific patient sub-groups demonstrated that APC was only by measurable benefit in the sickest patients, those with severe sepsis and a high risk of death (APACHE Score equal to or more than 25). The subsequent clinical trial which was requested, the Administration of Drotrecogin Alfa (Activated) in Early Stage Severe Sepsis (ADDRESS) trial, involved patients with a lower mortality risk. At an interim analysis of the trial, it was found that there were no significant benefits or effectiveness in lower risk patients and that there was an associated increased risk of

bleeding complications (Abraham et al., 2005). The trial was terminated early as a direct result. Following these developments, many hospitals restricted the use of recombinant human activated protein C to the most severe cases of sepsis with multiple organ failure and the highest risk of mortality exclusively. Serious debate arose regarding whether the high cost of the treatment and its bleeding risks justified its use (Treacher and Alun Brown, 2009).

A subsequent revision of the Surviving Sepsis Campaign guidelines in 2008 (Dellinger et al., 2008) only suggests (rather than making a clear recommendation) that recombinant human activated protein C be used in patients with severe sepsis, a high risk of death and no contraindications (Vincent, 2008). The hotly contested debate continued and additional warnings were mandated for the prescribing information indicating that it should not be used in patients with recent surgery and single organ dysfunction. The European Medicines Agency stipulated that Eli Lilly conduct a new trial in order to continue use in any fashion, the Prospective Recombinant Human Activated Protein C Worldwide Evaluation in Severe Sepsis and Septic Shock (PROWESS-SHOCK) trial, which would be a multicentre, randomised, placebo-controlled trial with 28-day mortality as the primary outcome measure (Vincent, 2008).

The PROWESS-SHOCK trial did not confirm the findings of the original PROWESS trial. However interestingly the findings did not indicate that there was an association with any increased incidence of severe adverse events (Xigris 1.2% vs. placebo 1.0%). However, 28- day mortality was not statistically different between patients receiving Xigris (26.4%) compared to placebo (24.2%). As no demonstrable effect

was concluded, the drug was withdrawn from the market by Eli Lilly in 2011 (Green et al., 2012). There was widespread fallout in the medical and pharmaceutical professions, as one author put it:

"*At best, we have been treating the sickest septic shock patients with an expensive medication that has no benefit, and at worst we have wasted money and research resources on a potentially harmful drug. There are many lessons to be learned here for all of us*" (Green et al., 2012).

2.8.5 Early Goal-Directed Therapy and the Surviving Sepsis Campaign

Most modern treatment for sepsis is centred around a bundled early goal directed therapy (EGDT) with a pivotal onus on a timely delivery of these bundles; the so called "golden hours". This approach is widely referred to as the "Rivers method" throughout literature on the matter, after Emanuel Rivers M.D, the principal investigator on a seminal study on the use of EGDT for sepsis (Rivers et al., 2001):

"*The transition to serious illness occurs during the critical "golden hours," when definitive recognition and treatment provide maximal benefit in terms of outcome. These golden hours may elapse in the emergency department, hospital ward, or the intensive care unit*" (Rivers et al., 2001).

It was stated that many forms of early assessment criteria being used failed to promptly detect the condition, instead it was advocated that a more definitive goal-orientated resuscitation strategy should be employed. A significant element of the

logic to this approach was that the majority of patients with the condition would be arriving by way of the emergency department and accordingly it would be pertinent to begin goal directed treatment early rather than letting the so-called "golden hours", where recognition and treatment provide maximum benefit, elapse before the patient is admitted to ICU. Their early therapy involved a set of therapies referred to as bundles that included starting antibiotics quickly and aggressive fluid replacement, red blood cell transfusion and haemodynamic management (Vanzant and Schmelzer, 2011). This approach of time fenced goal directed therapy is best visualised by Annane et al. (2005) in Figure 28 The study concluded that there were significant benefits related to patient outcome from this approach.

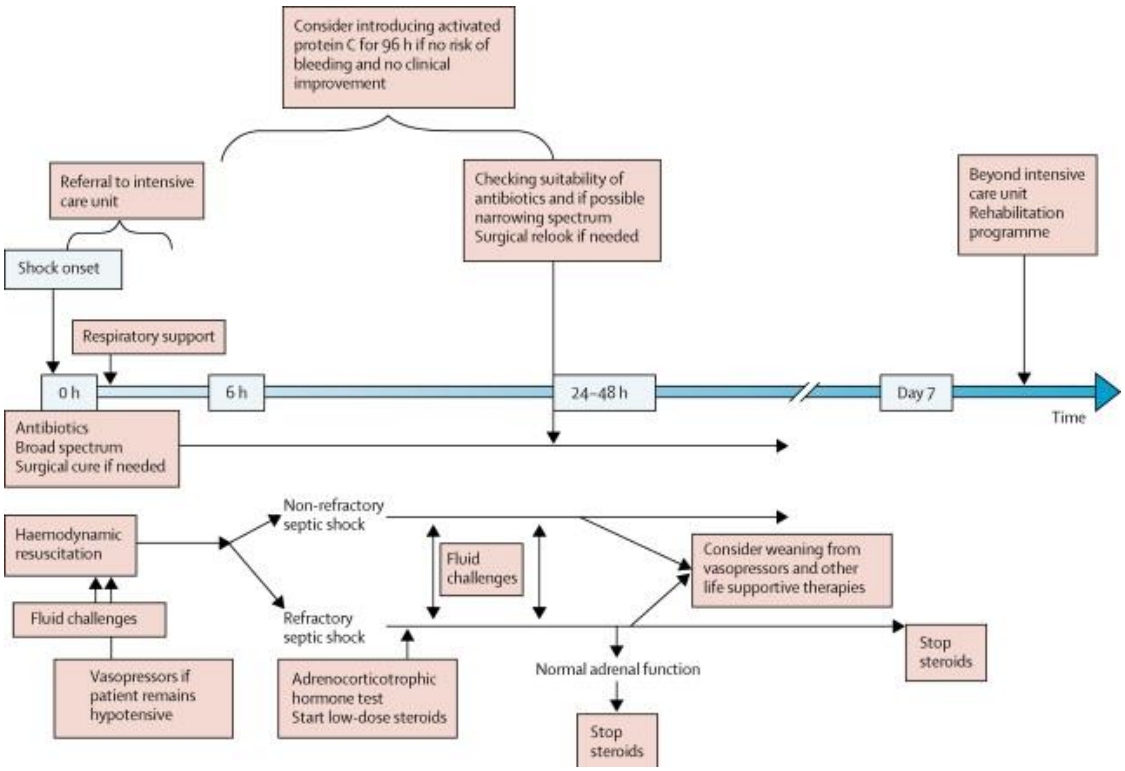

Figure 28: Principles of treatment in septic shock (Annane et al., 2005). Note that this process diagram includes the recommendation for use of activated protein C as it was produced in 2005 before APC was withdrawn. Figure reproduced with permission from Elsevier licence number 3830871204845.

The reported success of the study and its approach led directly to widespread initiatives to reduce sepsis related mortality utilising this approach. In 2004, an international group of experts was assembled to develop consensus guidelines for sepsis and septic shock treatment, these guidelines were to be based upon both expert opinion and research data. This endeavour became known as the Surviving Sepsis Campaign (SSC) (Vanzant and Schmelzer, 2011). The "Surviving Sepsis Guidelines for Management of Severe Sepsis and Septic Shock" were first published in 2004 (Dellinger et al., 2004), revised in 2008 (Dellinger et al., 2008), and recently revised again and published in 2013 (Dellinger et al., 2013b, Dellinger et al., 2013a, Schorr et al., 2014).

The Surviving Sepsis Campaign (SSC) represented an international effort to improve patient outcome in relation to sepsis and SIRS. It was launched by the European Intensive Care Society and the International Sepsis Forum in October 2002 with the "Barcelona Declaration" to improve survival in severe sepsis. As mentioned, it has undergone several significant revisions, the most recent, published in 2013. Arguably, their principal function is the production of treatment guidelines which are the product of an explicit, systematic approach to the evaluation and synthesis of available information on a particular clinical topic. The reliability of which is stratified on the basis of the extent to which all relevant evidence is sought for evaluation; the quality of the available evidence; and the rigour of the evaluation process used (Vincent and Marshall, 2008). Since the 2008 SSC published guidelines, the campaign has employed the use of the Grades of Recommendation Assessment, Development and Evaluation (The GRADE Group) to demonstrate the categorization of the quality of evidence for each recommendation made and the

strength of that recommendation. Overall, the core areas of the Surviving Sepsis Campaign guidelines have been crystallised into resuscitation and management 'bundles' (Poulton, 2006) as while the more detailed guidelines are an important body of knowledge, they are of little use at the bedside where time is of the essence, as stated by Dellinger (2014):

"*Simply put, guidelines are not enough. In order to change bedside behaviour, protocols and performance improvement programs with audit and feedback are needed. Early screening and hospital based performance improvement programs are now recommended by the Campaign*" (Dellinger, 2014).

Accordingly following the publication of each set of guidelines, they are additionally converted to "sepsis bundles" adopting the principles of EGDT, which provide measureable quality indicators to be achieved at specific time points. The most recent 2012 bundles are demonstrated in contrast to the original 2004 bundles by Barochia et al. (2013) in Table 10, notably APC is no longer mentioned.

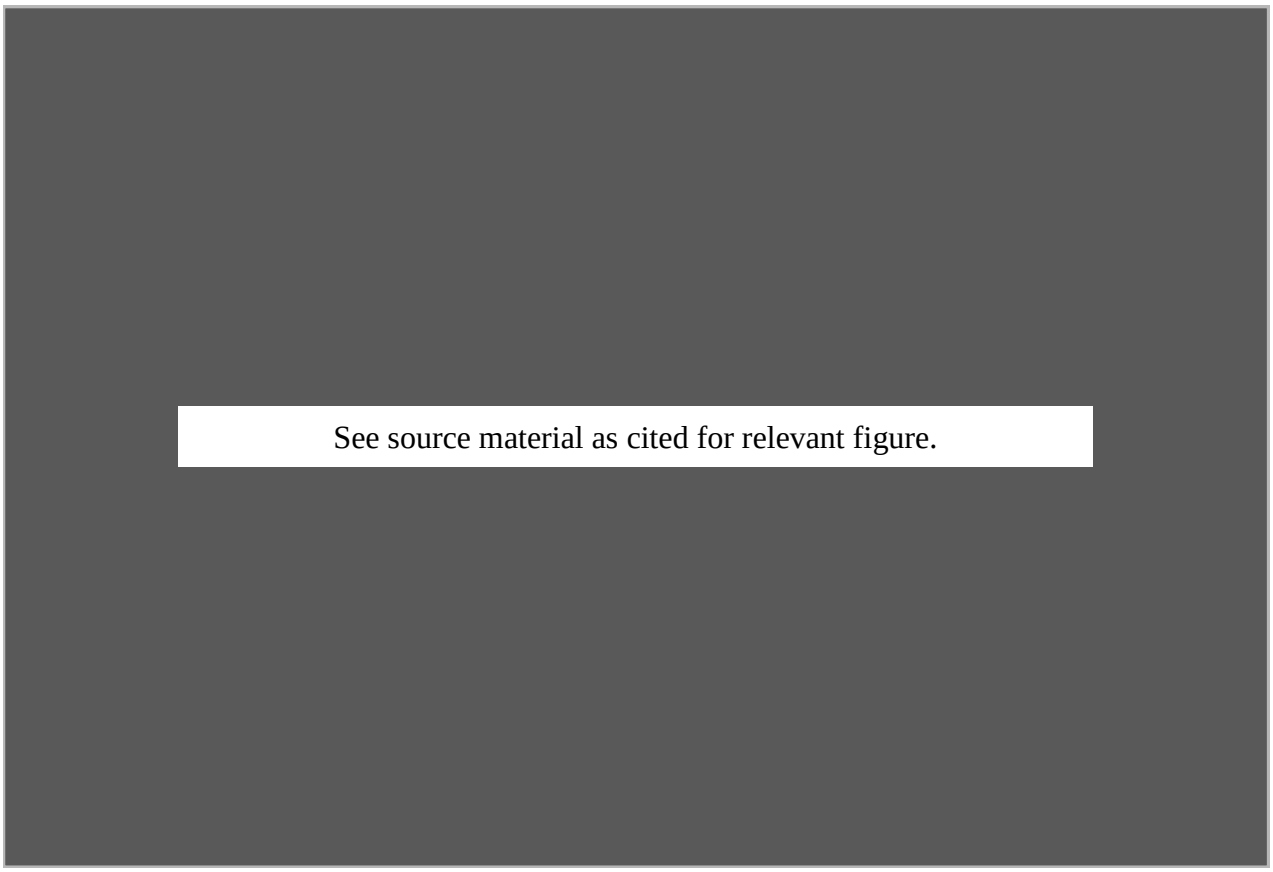

Table 10: Original and revised Surviving Sepsis Campagn (SSC) Sepsis Bundles (Barochia et al., 2013)

It is worth noting that the EGDT approach and SSC is not without its critics. One of the chief critics of the EGDT approach points out that the original study conducted by Rivers was a small, unblinded, single-centre study (Marik, 2015). Despite this, EGDT has since become the standard of care around the world; endorsed by major organizations and formed the basis of the resuscitation bundles of the SSC. However, a number of concerns exist regarding the basis, conduct and analysis of the study (Marik, 2015).

Critics question the validity of CVP for assessing haemodynamic response to fluid challenge and arguing that a majority of the patients treated by the EGDT approach

were fluid overloaded. Additionally, it is argued that advocating use of dobutamine is likely to be dangerous, particularly for patients with abnormal left ventricular function, and challenging the legitimacy of the evidence and analysis involved in the most recent SSC guidelines. Indeed regarding the pivotal 2001 EGDT study by Rivers, Marik (2011a) raised concerns about undisclosed conflicts of interest and called into question the validly of the data published on that study. Ultimately concluding that:

"*Accumulating data suggest that the major components of the 6-h sepsis resuscitation bundle lack high-quality evidence. Early identification and treatment with appropriate antibiotics remains the most important elements in the treatment of patients with sepsis. Patients with sepsis should be resuscitated according to an individualized physiological-based approach. A conservative fluid strategy and the early use of norepinephrine may further improve the outcome of this common disorder. Like much in medicine, 'a less aggressive approach may be more*'" (Marik, 2015).

The discussion abounds from many sides, although the SSC authors are quick to the defence, pointing out that the guidelines represent recommendations which support but cannot replace the clinician's decision making capability when presented with a patient's unique set of clinical variables. (Dellinger et al., 2013a). The most severe form of the argument within the on-going debate is that the SSC is, or at least was, part of an advanced and aggressive marketing strategy for Eli Lilly's commercialised APC, Xigris. However, following the withdrawal of Xigris, the most recent publications by the group have been keen to disassociate the two. However, perhaps

the most revealing fact is that Eli Lily partially funded the initial 2004 SSC development of clinical guidelines (Marik, 2011b). The debates around the matter are frequent in the literature and are likely to continue as various endeavours are made to address sepsis and its sequelae.

2.9 Developmental Therapies & Treatment Approaches

Numerous other therapies at various stages of development are currently being explored for sepsis. Most are predominantly focused upon down-regulating the host immune response to insult. However, the complexity of the host systems involved and their interactions make the development of pharmacological treatments difficult. Newer adjunctive and less specific treatment modalities represent an area of intense research worldwide (Tsiotou et al., 2005).

2.9.1 Corticosteroids

Glucocorticoids are frequently used as therapy for chronic autoimmune diseases, used for gout, osteoarthritic joints and tendonitis (Dinarello, 2010). They are pleiotropic in nature and while their exact mechanism of action is not fully understood, they are recognised as promoters of haemodynamic stability and reduced inflammation (Angus, 2011). While their anti-inflammatory action may be due to several mechanisms, a key avenue is thought to be the reduction of gene expression for cytokine inducement, as they bind to a cell's cytoplasmic steroid receptor, which translocates to the nucleus and is recognised by specific DNA sequences, the outcome of which is suppression of transcription by opposing activation of transcription factors AP-1 and NF-kB, which are responsible for the encoding of nearly all proinflammatory cytokines (Dinarello, 2010). Glucocorticoids reduce expression of cytokine induced gene transcription of COX-2, inducible nitric oxide synthase, and intracellular adhesion molecule-1 (ICAM-1). Additionally, glucocorticoids increase expression of genes encoding anti-inflammatory molecules,

such as IL-10 and the IL-1 type 2 decoy receptor (Dinarello, 2010). This led to therapeutic experimentation involving high doses of the drugs. However, analysis revealed that little or no benefit was evident and that their use in septic patients use was associated with increased mortality (Batzofin et al., 2011).

However, a second rational for the use of corticosteroids in septic shock arose; relative adrenal insufficiency. An adequate adrenal response is essential to survival in critical conditions, and sepsis is associated with relative adrenal insufficiency, accordingly the hypothesis arose that use of a stress dose or low dose of corticosteriouds could mitigate this while avoiding the negative effects of high doses (Andersen et al., 2009).

However, the use of corticosteroids remains controversial. A trail conducted in 2002 investigating the use of hydrocortisone and fludrocortisone in septic shock (Annane et al., 2002) found that treatment with low doses of these steroids significantly reduced the risk of mortality in patients with septic shock and relative adrenal insufficiency without increasing adverse events. However, this was followed by a larger trial, The Corticosteroid Therapy of Septic Shock (CORTICUS) trial, involving many of the same investigators, (Sprung et al., 2008), which did not reproduce similar outcomes, although it did indicate that the approach may improve the haemodynamic situation and improve the rate of recovery in patients who did recover. Corticosteroids are not recommended in the treatment of sepsis or septic shock in instances where fluid resuscitation and vasopressors are sufficient to regain haemodynamic stability. Only in instances where this is not achieved and the patient is in septic shock is the use of corticosteroids to be considered (Schorr et al., 2014).

2.9.2 Extracorporeal Blood Purification

The complexity of the pathophysiology of sepsis and its sequelae, involving numerous mediators and interacting pathways, has ensured that a wide variety of therapeutic approaches has met with limited success if not outright failure. Attempts made to target specific components within sepsis progression have been unsuccessful, including immumodulatory drugs. In response to this situation, various approaches are being investigated which are directed at the inflammatory response at the core of the sepsis progression. Extracorporeal blood purification (EBP), in a variety of mechanisms and configurations, could present such an approach.

Types of EBP

Apheresis is the nomenclature applied to extracorporeal blood purification (EBP); the process of removing blood from the host and utilising some apparatus to separate particular constituents and subsequently return the blood to circulation. EPB can be conducted on either an intermittent or continual basis and can operate by one of three mechanisms: convection, diffusion and adsorption by the filtering membrane (Ronco et al., 2004), with the possibility of use of any combination of these, depending on patient need (Panagiotou et al., 2013). Additionally such approaches may be utilised in circuits which provide separation of plasma from whole blood to be returned to circulation after undergoing some therapy or replaced by a substitute, a process referred to as plasmapheresis (plasma exchange) (Stegmayr, 2005). Technologies have also been developed which allow for adsorption of constituents from a column perfused by whole blood (haemoperfusion) (Stegmayr, 2005). A variety of combinations of these techniques is available and has been investigated for their potential use as a therapy in sepsis.

Diffusion involves a form of solute transport across a semipermeable membrane generated by a concentration gradient. The degree of solute clearance is governed by the molecular weight of the solute, concentration gradient, temperature and surface area, thickness and pore size of the membrane (Panagiotou et al., 2013, Venkataraman et al., 2003). Smaller solutes, urea, creatinine, and electrolytes, are cleared efficiently by diffusion, and as a solute's molecular weight increases diffusivity decreases. By introducing countercurrent dialysate flow, a circuit can

accomplish diffusive clearance by maximizing the concentration gradient between blood and dialysate (Venkataraman et al., 2003).

Convection refers to a process where solutes passively follow a fluid flow along with the movement of solvents (ultrafiltration) across a highly permeable membrane, which takes place in response to a positive transmembrane pressure gradient, referred to as "transmembrane pressure", which determines the rate of ultrafiltration (Panagiotou et al., 2013). In this context, clearance relies on the ultrafiltration rate and sieving characteristics of the membrane and solute, and to a lesser extent on the molecular size of the solute. Such approaches are classified based on their ultrafiltration coefficients as either "high-flux" and "low-flux" membranes, where high-flux membranes have a higher filtration rate (Venkataraman et al., 2003).

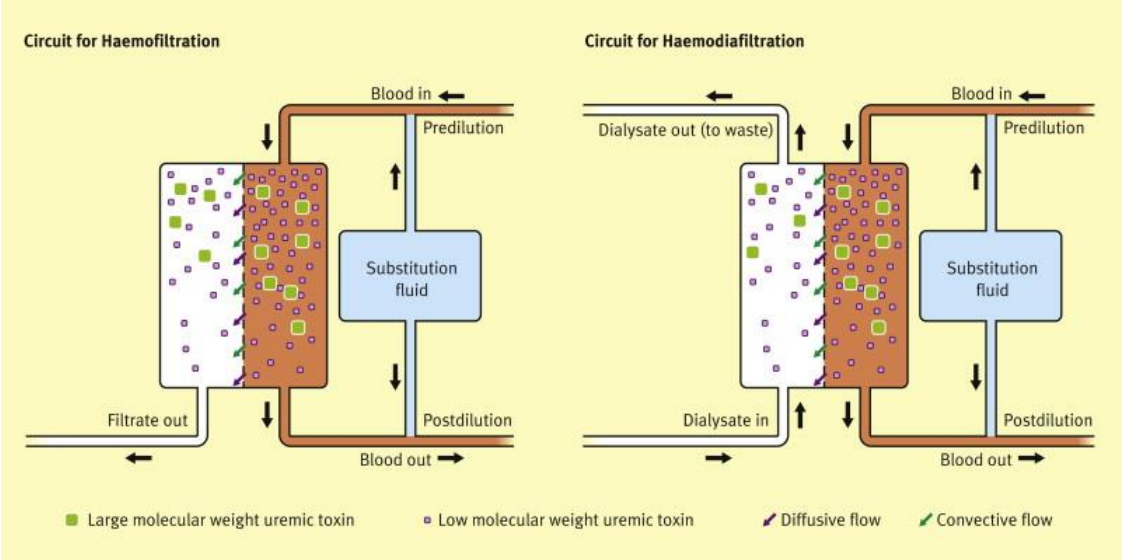

Figure 29: Types of EBP: Haemofiltration and Haemodiafiltration (Shaheen et al., 2009). Figure reproduced with permission from Elsevier licence number 3830880002989.

Adsorption describes the process of deploying an adsorbent in an extracorporeal circuit, which will attract and bind solutes through a variety of forces, such as hydrophobic interactions, ionic interaction, hydrogen bonding and van der Waals interactions (Panagiotou et al., 2013). Adsorbents had previously been limited to an extremely non-selective nature. However, newer techniques for manufacture have led to refinements in the available adsorbents, allowing for a greater degree of specificity (Panagiotou et al., 2013).

Terminology regarding EBP depends upon the site of access, either arteriovenous or veno-venous, and the mechanism of clearance. If a purely diffusive approach is adopted, it is referred to as "haemodialysis", in instances of purely convective clearance, then "haemofiltration" is used, if a combination of both is adopted, the term "haemodiafiltration" applies (Venkataraman et al., 2003), demonstrated in Figure 29.

EBP as a Treatment Modality for Sepsis

The concept of EBP as a therapeutic approach has existed for many years, the first reported use in dogs was in 1914 and humans in 1926 (Stegmayr, 2005). The use of a continuous circuit for a haemofiltration was first proposed as a method of treatment for diuretic-unresponsive fluid overload in the 1970s (Kramer et al., 1977). Throughout the 1970s, debate abounded for the range of applications of EBP, particularly in the treatment of acute poisoning. Charcoal had long since been used as an adsorbent media, perhaps as far back as the ancient Greek civilisation discussed in chapter 1.0, due to its porous nature it was a logical choice for such applications. However, such approaches quickly gathered a fickle reputation as they were associated with numerous deleterious adverse effects, including thrombocytopenia embolism, marked thrombocytopenia, leucopenia and fibrinogen loss but perhaps most significantly the threat of charcoal embolism (Vale et al., 1975). In the mid 1970s, Vale et al. (1975) proposed the use of charcoal coated with a synthetic hydrogel to overcome these issues. Their proposal involved the use of a haemoperfusion circuit, as demonstrated in Figure 30.

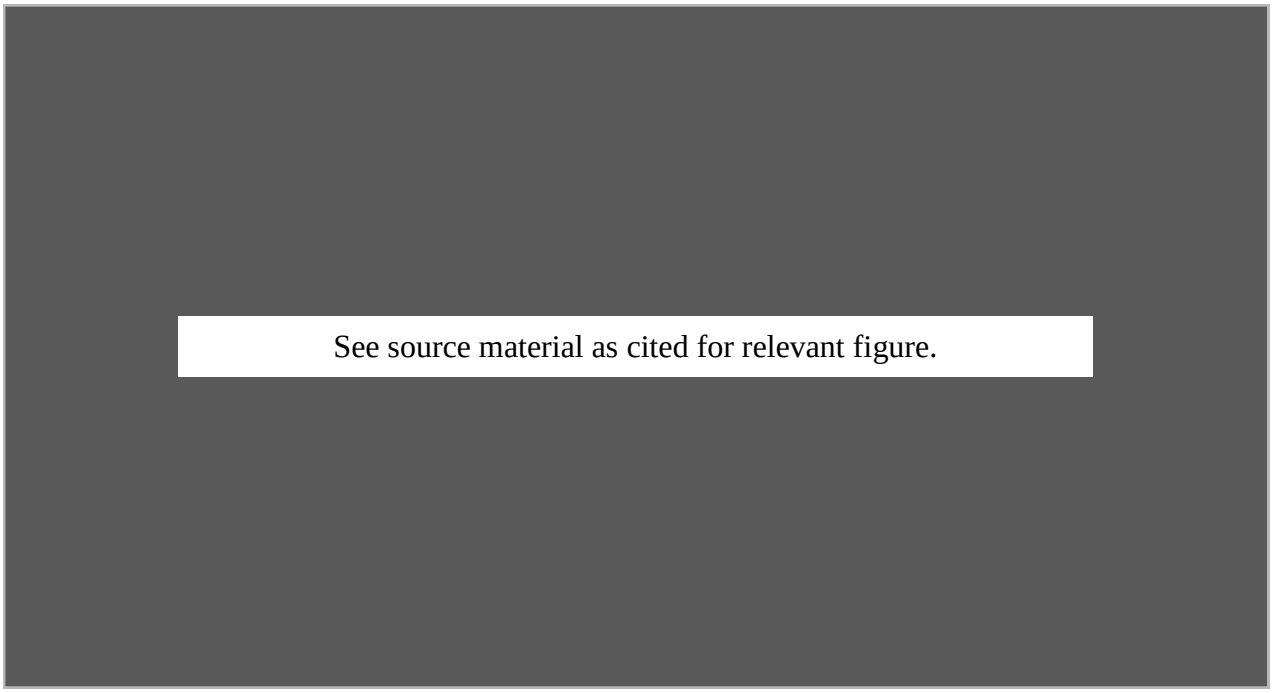

Figure 30: Diagram of haemoperfusion circuit (Vale et al., 1975)

It was tested upon a number of patients and concluded to be a success. Their use for such applications began to become more commonplace. However, the risk of side effects still persisted.

It has been established that severe sepsis and septic shock represent states of systemic inflammatory response and immunocompromise facilitated by various mediators. The concept of extracorporeal blood purification as a therapeutic option in this setting is to mitigate the dysregulated expression of these mediators and return the host to a state of homeostasis (Rimmele and Kellum, 2011). Previous investigations which focused upon the mitigation of single components met with little or no beneficial effect; accordingly the concept of non-specific removal of a boarder range of mediators has emerged as a predominant theory.

When considering the specific context of EBP for sepsis, several lines of investigation have been pursued. Impaired renal function, or in the worst cases failure, is an almost obligatory element of septic multiple organ dysfunction syndrome (Schefold and Jörres, 2010). While this critical organ function itself must be addressed in the clinic, acute renal failure (ARF) appears to exert negative additional immunomodulatory properties in severe sepsis, apparently caused by both cellular dysfunction in the uraemic environment and reduced tubular degradation of certain inflammatory mediators (Schefold and Jörres, 2010). Previously, attention had focused on EPB use for replacing clearance function of the failing kidney frequently encountered in sepsis. However, recently, there has been a shift towards renal support (rather than replacement) and indeed towards a more comprehensive use of EBP for sepsis and multiple organ dysfunction therapy in general, in an attempt to mitigate the progression of the condition and lengthen the “golden hours” for clinical intervention (Panagiotou et al., 2013).

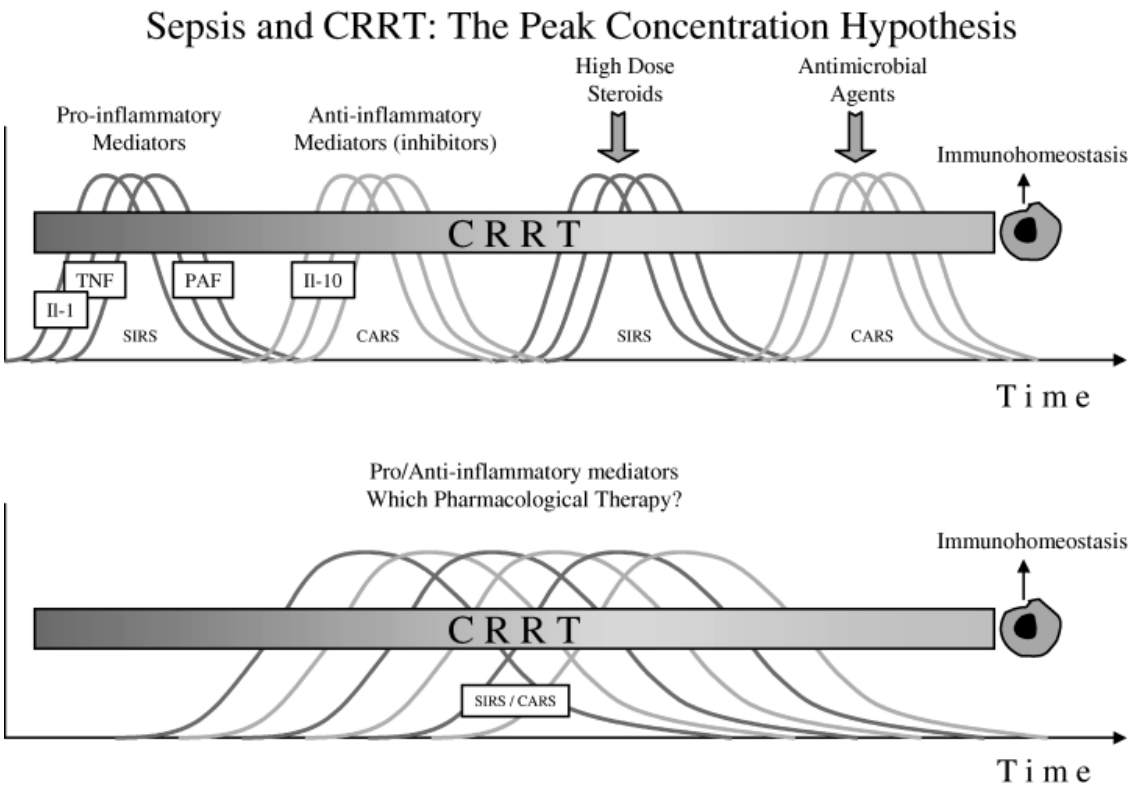

Figure 31: In both the sequential and parallel theories the concept of the peak concentration hypothesis suggests a nonselective control of the peaks of mediators possibly mitigating the progression of the condition and returning to normal homeostatsis (Ronco et al., 2003). Figure reproduced with permission of John Wiley and Sons licence number 3831881054092.

Three key theories and lines of investigation are frequently cited in the literature on the use of EBP for sepsis, beginning with the work of Ronco et al. (2004) and their "peak concentration hypothesis", which arose from their theories regarding sequential progression of sepsis discussed earlier in section 2.7.3. It was proposed in this work that by eliminating the peaks of dysregulated circulating mediators, the sepsis progression could be moderated by the use of EBP continuous renal replacement therapy (CRRT). It was argued that a key criticism of continuous EBP, its lack of specificity, could in fact be its greatest asset in this context. Its unspecific removal of soluble mediators regardless of their nature may present a valid treatment modality (Ronco et al., 2003), a concept visualised in Figure 31. This was countered

by concerns that even if the peaks could be cut off in circulating blood that would not provide any effect on the mediators of the tissue compartment. This was built upon by Honore and Joannes-Boyau (2004) in their "threshold immunomodulation hypothesis", which concentrates on the heterogeneity and cross-talk of the inflammatory networks, including the redundancy of pleiotropic mediators, asserting that even if one or two are mitigated inflammation may still progress. However, they argue that if a less selective filtration approach is adopted, it may be possible to interrupt the inflammatory process and additionally by depleting the mediators in the circulation redistribute those from the tissue compartment to circulation, where they will subsequently be removed, although the mechanism by which this would occur remains unexplained (Schefold and Jörres, 2010). Honore and Joannes-Boyau (2004) state that such a process would continue up to a "threshold point" where inflammatory cascades would be halted preventing subsequent organ injury and dysfunction. Both of these theories are concerned with the elimination of circulating mediators. A hypothesis put forward by Di Carlo and Alexander (2005) focuses instead on "washout" of cytokines referred to as the "mediator delivery hypothesis". They propose that by infusion of high volumes of replacement fluid, it is possible to drive a dynamic interstitial circulation to deliver mediators from the tissue compartment, first to circulation and then to multiple sites to be metabolized, scavenged or cleared, this could be facilitated by the host, in the liver or kidneys, or by external means such as a haemofilter (Di Carlo and Alexander, 2005). Most recently, a theory has been put forward by Peng et al. (2010) stating that EBP can act to restore immune function by regulating numbers, functionality and expression of surface markers of inflammatory cells; monocytes, neutrophils and lymphocytes.

Further, they suggest that haemoadsorption specifically could effectively act to reprogramme leukocytes. This theory would also mean that by removing mediators from plasma upon return to the circulation a concentration gradient would exist resulting in increased trafficking from infected tissue to circulation; a “cytokinetic” model to explain improved outcome even though observable plasma cytokine levels may remain constant, those eliminated being replaced by those removed from circulation (Rimmele and Kellum, 2011).

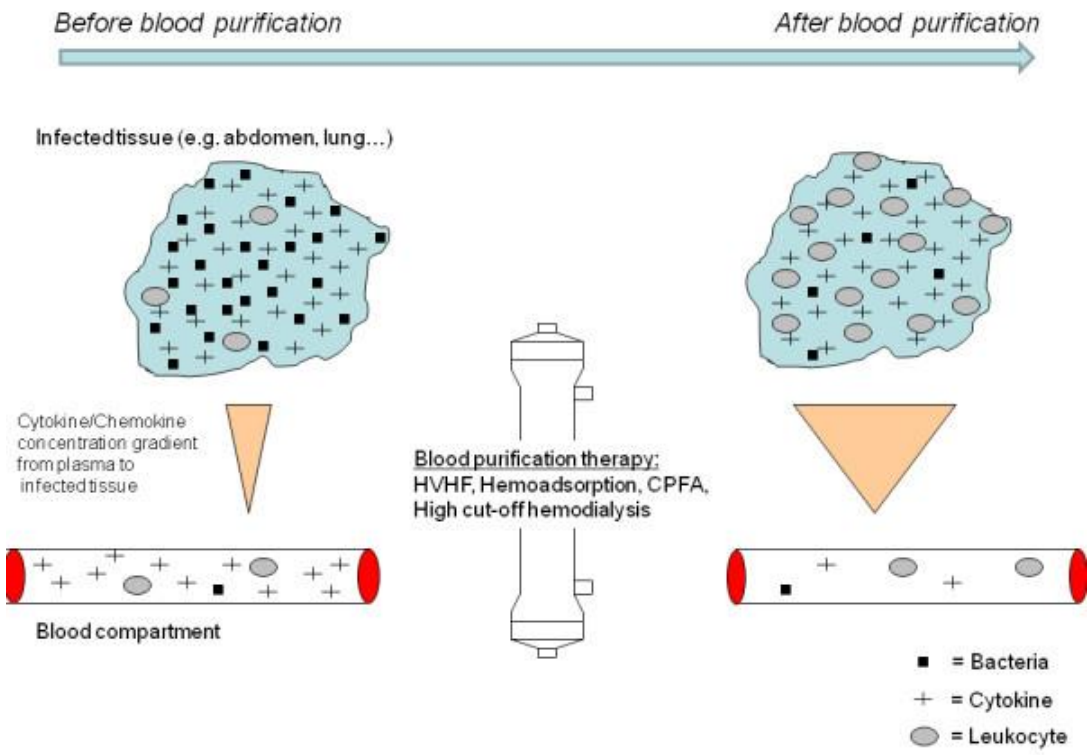

Figure 32: The "cytokinetic" model (Rimmele and Kellum, 2011). Figure reproduced under CC BY 4.0.

While the mechanics of these theories is still the subject of debate and research, it is considered that a reconciliation of the theories provides rational for further experimentation in the use of EBP as a treatment modality for sepsis.

Investigations are ongoing involving various combinations of the mechanisms and modalities described. It has not yet been conclusively established which form of clearance is preferable in the sepsis setting. However, the use of either diffusive or convection based transport alone remains unproven in sepsis and accordingly investigative focus in the community has shifted toward high ultrafiltration and adsorption technologies in an effort to enhance clearance of middle and high molecular weight solutes (Andersen et al., 2009).

Ronco et al. (2000) conducted a randomized control trial in critically ill patients with ARF, comparing low (20 ml kg^{-1} h^{-1}), medium (35 ml kg^{-1} h^{-1}) and high (45 ml kg^{-1} h^{-1}) ultrafiltration rates. They reported that while mortality among the critically ill patients was high, increase in the rate of ultrafiltration improved survival significantly. Survival for the low rate was 41%, medium rate 57% and high 58%. Further noting that within the subgroup of septic patients, a trend between ultrafiltration rate and survival was observed, even above 35 ml kg^{-1} h^{-1}, supporting their concept of a ‘‘minimal septic dose’’ of this size leading them to recommend the use of ultrafiltration prescribed according to patient’s bodyweight and reaching at least 35 ml kg^{-1} h^{-1} (Ronco et al., 2000). The terminology “septic dose” being proposed in comparison to the established terminology “renal dose” of haemofiltration (20 ml kg^{-1} h^{-1}) in the treatment of acute renal failure in (Ratanarat et al., 2005))

However, the HVHF approach of Ronco et al. (2000) is continuous in nature and it is a very resource and labour intensive process. Accordingly a revised approach was proposed by Ratanarat et al. (2005); a discontinuous "pulse HVHP (PHVHF)", which provides interval based provision of HVHF, typically 6 to 8 hours per day followed by conventional continuous venovenous haemofiltration (CVVH). A 28-day mortality study was conducted in sepsis patients. The results indicated a patient mortality of 46.7%, as compared with a rate of 72% predicted for these patients without use of the approach. It was reported that PHVHF was well tolerated and appeared to offer many of the benefits of continuous HVHF while avoiding some of its drawbacks. It was concluded that PHVHF is a feasible and promising modality that can safely be performed on both a daily and prolonged basis, which may represent a beneficial adjunctive treatment for sepsis (Ratanarat et al., 2005).

In 2002, Kellum and Dishart (2002) conducted an investigation into the use of haemofiltration adsorbents to mitigate the circulating levels of cytokines in rats. They sought to differentiate the clearance due to adsorbance from the clearance due to haemofiltration. As the mechanisms involved in the immunomodulation observed with the use of haemofiltration are not completely understood, it is important to distinguish between the action of haemofiltration and adsorption. Ultimately, this will influence further developmental therapies as if the primary beneficial effect is due to adsorption, then the surface area should be maximised in further endeavours (they suggest beads rather than filaments may be an suitable method of achieving this), whereas if it is mainly by sieving in the haemofilter, then the ultrafiltration rate should be increased (Kellum and Dishart, 2002). Their study was conducted by inducing sepsis in rats through cecal ligation and puncture (CLP) and measuring

plasma concentrations of IL-6 and TNFα by ELISAs at hourly intervals as a measure of circulating mediators and markers of sepsis. Their results indicated that the inflammatory response induced by CLP appeared to be mitigated in their haemofiltration experimental circuit regardless of whether or not it was infused with ultrafiltrate, indicating that the primary clearance was attributable to adsorption of circulating mediator substances to the filter membrane and stating that future studies of haemofiltration in sepsis might be more successful if steps are taken to maximize adsorption Kellum and Dishart (2002). However, the authors also noted that the clinical relevance of the outcomes is not conclusive, as unlike therapies designed to target specific mediators, haemofiltration has the theoretical advantage of being at once selective and nonspecific. Haemofiltration can only be considered selective in that it will mitigate circulating mediators in relation to their plasma concentration. However they can be considered non-selective in that they remove multiple substances, not just specific mediators

Experiments have also been conducted using "high-cut off" haemofilters, those with an increased effective pore size in an effort to maximise cytokine removal (Morgera et al., 2004). In this case, a polyflux haemofilter with a cutoff point of approximately 60 kd was used. While this approach was effective in clearing cytokines, it was also associated with a larger degree of essential proteins, including albumin (Andersen et al., 2009) Attempts have been made to address these issue by coupling plasmafiltration with adsorption (CPFA). In these applications plasma is separated and passed through a sorbent to eliminate the presence of mediators and is then returned to the blood component before being passed through a haemofilter for renal replacement therapy of a particular configuration and ultimately returned to

circulation, as demonstrated in Figure 33. Investigations of this approach have reported success and promise (Formica et al., 2007). This format of combining adsorption with haemofiltration as both an adjunctive and supportive therapy holds significant promise for future development in the sepsis setting.

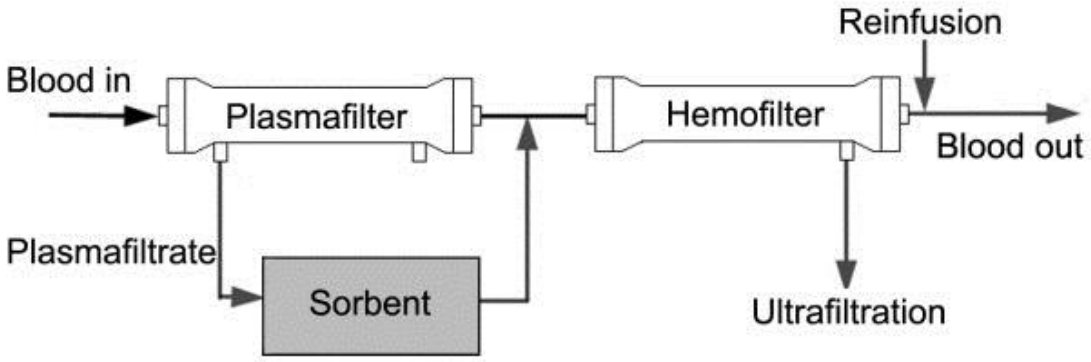

Figure 33: Coupled Plasma filtration adsorption circuit (Rimmele and Kellum, 2011). Figure reproduced under CC BY 4.0.

The effective clearance of adsorbents allows for removal of medium to high molecular weight exceeding the cut-off of conventional synthetic high-flux haemofilters (Rimmele and Kellum, 2011).

It has become apparent that adsorption is arguably one of the best methods to achieve high mediator clearance giving rise to the investigation of 'haemoperfusion' as a technique in which a sorbent is placed in direct contact with blood in an extracorporeal circuit (Venkataraman et al., 2003). Nonselective adsorbents including charcoals and resins have been investigated for this purpose as they attract solutes through a variety of forces and they generally provide good adsorbent capacity and present large surface areas. Previously, poor biocompatibility prevented

experimentation with such adsorbents as they were frequently associated with numerous side effects. However, recent advances in manufacturing processes has permitted the use of various biocompatible coatings. By manipulating the porous structure of solid phase sorbents, it is possible to increase the selectivity of nonspecific adsorbents for particular solutes (Winchester et al., 2003). In the context of high-molecular-weight adsorption characteristics of sorbents, it is possible to target larger molecules that exceed the molecular weight cutoff of synthetic high-flux dialysis membranes (Venkataraman et al., 2003). Such approaches make sorbents an promising area for investigation of a treatment modality in sepsis. Sorbents have been applied in different treatment modalities, including haemoperfusion and haemoperfusion coupled with haemodialysis or with plasma filtration (Venkataraman et al., 2003).

EBP approaches have perhaps most frequently been utilised in the clinic in Japan, where approaches, including the use of a polymyxin B-immobilized haemoperfusion cartridge (Toraymyxin®, PMX) and continuous haemodiafiltration (CHDF), have been applied to treat patients with sepsis (Sakata et al., 2006). Polymyxin B is an antibiotic which binds to endotoxin, such as LPS involved in sepsis. However, it also has severe neuro and nephrotoxic properties. To ameliorate these properties, it is bound and immobilised to polystyrene fibres and was launched as a class III medical device in 1994 (Toraymyxin; PMX-F; Toray Industries, Japan) (Andersen et al., 2009). It has since been deployed to more than 60,000 patients, principally in Japan. A review conducted by Cruz et al. (2007) investigated studies reporting upon its use. They concluded that the trials involved were not of a sufficiently rigorous nature, citing issues with bias and blinding which may present exaggerated positive findings

but that overall mortality was lower with use of the device than the predicted mortality without its use. However, a recent large multicentre, randomized controlled trial (Early Use of Polymyxin B Hemoperfusion in Abdominal Sepsis (EUPHAS) trial) conducted in Italy, involving many of the same investigators, concluded that polymyxin B haemoperfusion added to conventional therapy significantly improved haemodynamics and organ dysfunction and reduced 28-day mortality in a targeted population with severe sepsis and/or septic shock from intra-abdominal gram-negative infections (Cruz et al., 2009).

Figure 34: Polymyxin B-immobilized haemoperfusion cartridge, Toraymyxin (Sakata et al., 2006). Figure reproduced with permission from Elsevier licence number 3831890165985.

The polymyxin B-immobilized haemoperfusion cartridge is not the only device developed for this application. In 2006, a Swedish design, the Alteco LPS Adsorber (Alteco Medical AB, Sweden) was launched (a class IIa medical device). In an early trial, it was found to reduce levels of mediators, improve haemodynamics and improve patients survival (Kulabukhov, 2008). However in a larger subsequent controlled trial between 2010 and 2012, no significant difference was noted and the trial was terminated early as interim analysis showed a low probability of significant findings. It was concluded that there was no identifiable clinical benefit on the addition of the device to conventional therapy in patients who suffered from intra-abdominal sepsis with shock (Shum et al., 2014).

2.10 Chapter 2 Summary Points

The following key points have been established in this chapter:

- Sepsis and the associated continuum of severity can be initiated by a wide variety of initiating insults which propagate various pathways of progression.
- Pathways of progression are often intertwined and redundant.
- Key central mediators of the progression are common to these pathways, most notably cytokines.
- Sepsis arises when regulation of the mediators of these pathways becomes dysregulated.
- Key pathophysiological systems are affected by sepsis progression, including inflammation, coagulation and complement systems.
- Dysregulation of these systems results in severe potentially fatal consequences for the host.
- Cytokines are broadly either pro-inflammatory or anti-inflammatory in their function.
- The progression and associated levels of circulating cytokines is currently debated to occur in a number of ways, notably either in sequence or in parallel, presenting either a hyper or hypo immunological state.
- Current treatment centres around supportive therapy.
- Numerous experimental therapies have met with limited success.
- Extracorporeal blood purification presents an active area of research as a potential treatment modality.
- Various extracorporeal blood purification modes are being investigated, adsorption based haemoperfusion in particular may present a beneficial treatment modality.

3.0 Thesis Objectives

Sepsis and Systemic Inflammatory Response Syndrome (SIRS) are commonplace, arise frequently as complications of a great number of medical procedures and are often fatal. The aim of this research is to evaluate a potential treatment option for the management of sepsis, utilising an adsorbent. In order to achieve this overall aim, a series of objectives was met: an extensive literature review was conducted to establish current contemporary theory and practices in both the progression and the treatment of sepsis. Based upon the lessons learned from this literature review, work was carried out to establish a robust and valid *in-vivo* animal model relevant to the clinical human sepsis condition. Once completed, this model was used as the basis against which to evaluate the efficacy of the adsorbent. A device capable of suspending the adsorbent in an extracorporeal circuit was designed and produced. The sepsis model was then used to create a miniaturised extracorporeal haemoperfusion treatment circuit which incorporated the designed device preloaded with the adsorbent. This provided a means to evaluate the effectiveness of such a treatment modality.

Aim: To evaluate the use of an adsorbent as an interventional therapy for sepsis.

In order to achieve this aim the following objectives were pursued:

- To carry out an extensive review of literature in order to establish current contemporary thinking and research in the sepsis condition.

- To develop a clinically relevant model, indicative of the physiological symptoms and mediators, of the sepsis condition.

- To develop a miniaturised extracorporeal circuit for evaluation of haemoperfusion utilising an adsorbent.

- To develop a test device capable of deploying the proposed adsorbent in the miniaturise extracorporeal circuit for use with the induced sepsis model.

- To evaluate the effect of the developed integrated device upon the target symptoms and mediators of the condition.

3.1.1 Statement of Hypotheses

Following rigorous review of published work in the area of sepsis it has been established that a suitable method for developing understanding of the progression of the condition is valuable. It is further proposed that the network of signals mediating the chain of events in sepsis is a potential window for interventional therapy in the development of a medical device. It is hypothesised that:

1. A valid analogous model of early sepsis can be created which will incorporate a miniaturised extracorporeal circuit for the testing of adsorbent (or other extracorporeal intervention) as a potential therapy for the condition.

2. The deployment of an adsorbent in an extracorporeal haemoperfusion circuit will mitigate or halt the progression of the condition, extending the window for interventional therapy.

3.1.2 Proposed Methodologies

This investigation took in a variety of activities in order to characterise the validity of the deployment of the adsorbent as a potential therapy in the clinical setting. In order to achieve the overall aim and objectives identified above, and to ensure compliance with all necessary requirements relating to animal use in experimentation, a phased approach was adopted. It was initially planned that three phases would be required, although ultimately 4 were necessary, demonstrated in Figure 35.

Phase 1 would address the basic feasibility of the model to ensure an adequate time course could be maintained under anaesthesia and that it would be possible to dynamically measure key physiological parameters and take iterative blood samples for subsequent analysis.

Phase 2 would provide reliable control data and establish an induced sepsis-like condition and data thereon.

Phase 3 would develop a miniaturised extracorporeal circuit to permit a modular testing environment for deployment of the adsorbent and its impact upon the identified induced-sepsis progression. It was initially planned to establish a brief control period in each rat, followed by an induced-sepsis period before finally deploying the adsorbent within the extracorporeal circuit. It became apparent through testing that such an approach was not feasible or suitable which will be discussed.

Following the unsuccessful phase 3, a 4^{th} phase was developed. Phase 4 involved the use of three separate groups of rats, all of which ran using the miniaturised

extracorporeal circuit, the first provided control data of normal rat physiology while using the extracorporeal circuit without any adsorbent, the second provided induced-sepsis data, while using the extracorporeal circuit without any adsorbent, and the third provided data on the induced-sepsis rat while using the extracorporeal circuit and deployment of the adsorbent.

Figure 35: Flowchart of experimental phases

4.0 Concept Development

As has been discussed in chapter 1.0, sepsis is a major health care concern. It is proposed that deployment of an adsorbent in an extracorporeal haemoperfusion circuit could present a viable treatment modality to mitigate progression of the condition. Any approach to investigating the use of a novel therapy for sepsis must begin with an accurate model of sepsis. However, the complex nature of the condition, its progression and resulting physiological effects, discussed throughout chapter 2.0, mean that any new therapeutic approach must address the intricate and heterogenic nature of the systemic immune responses characterized as the sepsis condition, which cannot be adequately replicated *in vitro* (Nemzek et al., 2008, Kucharewicz et al., 2008). As such, an *in vivo* model will be developed and utilised.

4.1 Design of Control & Sepsis Models

The model developed will have to perform two overall primary functions:

1. Provide a stable control model
2. Provide a clinically relevant model of the sepsis condition

The requirements for which are as follows in Table 11:

Requirements for Sepsis and Control Models

Requirement	Comment/Detail
Comparable/ symptomatic of the clinical condition (in humans)	It is a key requirement that the model be indicative of the clinical presentation and meets the clinical diagnostic criteria of SIRS/Sepsis in order to accurately evaluate the effectiveness of any subsequently deployed therapy.
Repeatable	The model must be readily repeatable in order to ensure that the condition can be accurately replicated for iterative experimentation and subsequent evaluation.
Modular	The model must be modular in the sense that any points of attachment, equipment utilised and automated readings taken are of a standard fashion. Bespoke, unique or difficult to replace components should be avoided where possible to increase the repeatability of the model and to ensure complete transparency in any subsequent studies. The principal exception to this consideration will be the adsorbent test device.
Represent Control	The model developed must not only represent the condition of but must also represent a stable control model. Any model of the condition will be exactly that; a model, in order to accurately evaluate any interventional therapy a valid control version of the model must also be developed. This model should maintain a stable, minimally affected, representation from which all the same readings and considerations will be taken for the purposes of comparison to the induced sepsis condition.
Present an opportunity for iterative testing of a potential intervention	Closely tied to the requirements that the model must be repeatable and modular, it must also present an opportunity to deploy a developed interventional therapy, in the format of a miniaturised extracorporeal circuit.
Cost effective	Financial considerations must be made regarding the model, therapies applied, components, tools and consumables. It is intended to run several experiments for the purposes of evaluation. Accordingly this is a requirement which bears careful consideration.
Provide tangible and meaningful output data	It must be possible to make accurate and dynamic readings of key parameters, ideally dynamically for subsequent review and analysis, the model must not only be clinically relevant, an experimenter must be able to record it as such.
Facility and regulation compliant	Any animal work conducted must fully comply with UK and institution legislation.

Table 11: Design requirements for Sepsis and Control Models

The ideal model should accurately reproduce the clinical effects of the condition, as has been discussed throughout chapters 1.0 and 2.0, particularly the inflammation and cardiovascular parameters; the vasodilation, hypotension, increased cardiac output, and similar response to treatment seen in patients with septic shock (Hollenberg et al., 2001, Nemzek et al., 2008). A number of existing models each represents the condition with varying degrees of success in particular features of the condition. Unfortunately, no one model accurately recreates all features of the condition (Hollenberg et al., 2001). Each model of inflammation produces a different pattern of response; the mediators of the inflammatory process may differ in each case (Lam and Ferrell, 1991) or indeed be a wildly variant combination of mediators differing in their effect patient to patient, model to model.

Furthermore, the use of any animals in laboratory work is strictly governed and as such a number of practical, legal and ethical responsibilities must be considered in the design of any such laboratory work. Accordingly while there are a number of potential animal models which can be used to replicate sepsis, they must be selected with their suitability for the site where the work will be carried out in mind. At the University of Strathclyde Bioengineering facility, animal work may be carried out by appropriately trained and licensed personnel under the jurisdiction of the UK Home Office, however no living animal may be housed overnight. Accordingly, any animal model selected must not only replicate clinical sepsis but must also be valid and present such characteristics within the normal working hours. This is borne in mind as a practical consideration in the design of any experiment involving animal work. A statement of compliance relating to the animal work conducted is included in the

appendix and a copy of the personal licence for the experimenter following training and assessment at the University of Cambridge is also included.

As has been established in chapters 1.0 and 2.0, dramatic changes occur in the host's cardiovascular system in the initial "hyperdynamic" response, seen as early sepsis progresses. As this progresses to the "hypodynamic" state of septic shock, further complications can arise involving various organ systems leading to poor prognosis and mortality. In addition, inflammation and vascular collapse can lead to gastrointestinal dysfunction, compromised gut barriers, and bacterial translocation (Richard S. Hotchkiss and Irene E. Karl, 2003).

Based on this information, early in the model it would be expected to see a rise in temperature, indicative of so-called "warm shock" followed by a fall indicative of later stage "cold shock". The first stage is also characterised typically with low systemic vascular resistance, SVR, and an increased cardiac output (CO) (Baue et al., 1998, Buras et al., 2005). As the time course progresses, CO should decline without change in the SVR producing the hypodynamic later phase (Baue et al., 1998, Buras et al., 2005). Many studies overall report that in septic patients, the dominant clinical findings are systemic vasodilatation, elevated CO, low blood pressure and decreased peripheral vascular resistance (Court et al., 2002, Thijs et al., 1990, Langenberg et al., 2005). As stated by Hunter (2006):

"*Clinical symptoms of the condition are typified by high or dangerously low blood temperature, palpitations, cool blue extremities and low blood pressure. In its most severe form, sepsis develops into acute septic shock as a result of an extreme fall in*

blood pressure, which starves the major organs and leads to characteristic symptoms, such as blackening of the extremities" (Hunter, 2006).

In order to characterise the progression of the condition along the continuum of severity, these symptoms will also be investigated by means of Doppler laser imagery to assess perfusion in the model.

In order to ensure the model was highly repeatable and cost effective while maintaining validity in relation to human clinical sepsis, rodents were selected, specifically adult male Sprague-Dawley albino rats (480g-590g). The most widely recognised methods of inducing sepsis can be classified according to their initiating agent: exogenous administration of a toxin (such as lipopolysaccharide (LPS), endotoxins or zymosan); or alteration of the animal's endogenous protective barrier (e.g., cecal ligation and puncture (CLP), or colon ascendens stent peritonitis (CASP) (Buras et al., 2005, Doi et al., 2009).

Each of these methods represents its own relative merits and caveats. From a practical point of view, an intraperitoneal (IP) LPS-induced model was deemed the most appropriate for this project. As a major endotoxin in gram-negative infection, lipopolysaccharide stimulates the expression of a variety of pro-inflammatory mediators that organize inflammation and tissue damage (You et al., 2009, Doi et al., 2009). It also represents the only suitable method given the timescale of the project, as LPS has been documented to cause the rapid induction of cytokines, which reach high levels, peaking by 1.5 hours to 4 hours and then beginning to decline (Remick et al., 2000). Such cytokines, induced by LPS injection, have been proven to exhibit

kinetics similar to those after CLP but at a significantly higher level (Villa et al., 1995).

The LPS method is not without its caveats since human patients are generally not only exposed to gram-negative organisms exclusively but also gram-positive organisms or indeed to both in a more gradual way (Villa et al., 1995). The experimental model of cecal ligation and puncture (CLP) in rodents, which resembles the clinical situation of bowel perforation and mixed bacterial infection of intestinal origin, has been argued to be a more realistic model of sepsis with clearer parallels to the clinical situation (Buras et al., 2005). This is acknowledged in the design of the present experiment. However, the same authors, who advocate the CLP method, also acknowledge that LPS models of sepsis serve as a valid preliminary testing ground for therapeutic agents and as a model of septic shock for the first screening of protective therapies prior to human clinical trials (Buras et al., 2005, Villa et al., 1995).

Furthermore, LPS induced models present greater, more accurate, repeatability and have been translated to larger animals for the study of systemic and organ-specific haemodynamics (Doi et al., 2009). Since the LPS model does not require surgery, it also has benefits with regard to cost and presents fewer concerns with regard to animal welfare than models that do (Nemzek et al., 2008).

For these reasons, an intraperitoneal injection of LPS to induce sepsis was selected . While the case for other models can be argued, for the purposes of this project, the model was deemed suitable. Experimentation with other models may represent

opportunities for investigation at a later date, given the considerations to be met by the model (including timeline) LPS proved most suitable.

The animal handling perspective of the experiments, including anaesthetic, were built upon previously conducted and verified approaches utilised by staff in the Bioengineering unit at Strathclyde University (Gourlay et al., 2001, Gourlay et al., 2002). Further details on the procedures utilised in this project are described in section 4.5

4.2 Experimental Approach

The experimental setting in the artificial organs lab in the department of Bioengineering at the University of Strathclyde is demonstrated in Figure 36.

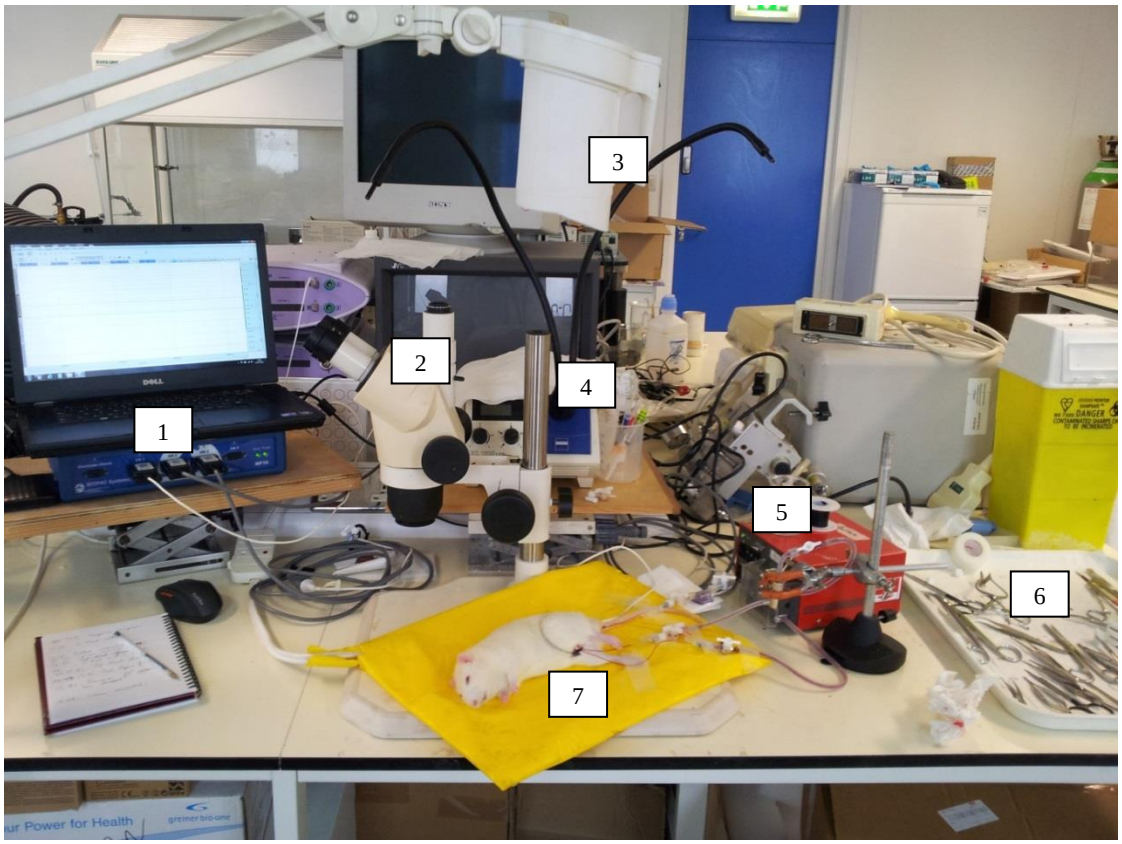

Figure 36: Photograph of the experimental environment in the artificial organs laboratory at the University of Strathclyde Bioengineering Unit. Clockwise from left; 1 laptop and BioPac data logging equipment, 2 microscope (rotated out of position for picture), 3 surgical overhead lighting, 4 goosenecked point surgical lighting, 5 roller pump, 6 surgical instrumentation, 7 experimental animal

In order to assess the status of the animal models and evaluate the efficacy of any applied therapy, relevant physiological parameters and mediators of sepsis must be recreated in the model. Once the model presenting these characteristics was

produced, it was possible to apply the adsorbent in an extracorporeal haemoperfusion circuit and assess the impact of the adsorbent on these factors.

The cytokine mediators of the condition are a key area of interest, which are investigated by means of enzyme-linked immunosorbent assay (ELISA). However of the various cytokines involved in the sepsis condition, it is necessary to select a limited number which will serve as key markers of the condition along with collecting physiological data. This is largely a practical consideration, as it is not financially feasible to run analysis on every one involved or, more pertinently, physically possible to draw more than 1 ml of blood at hourly intervals in the experiment. It was therefore necessary to select two cytokines involved in the condition and its progression. Those selected were TNFα and IL-6.

TNFα and IL-6 were selected as they are widely considered to be an early mediator by several authors. One such study reported early peak presentation in 37 of 40 sepsis patients during the acute phase of the condition (Pierre Damas et al., 1992). Furthermore they have been reliably used for other animal based investigations into sepsis (Kellum and Dishart, 2002). Of particular note, is an early peak in TNFα generally followed by a rapid return to lower values. While it is no longer considered the central mediator as it once was due to the ubiquitous association between TNFα and sepsis, it is still arguably one of the most, if not the most, indicative markers of the condition. For these reasons, its presence and levels were analysed. IL-6 is widely regarded as a reliable marker of sepsis and the progression of the condition (Philippart et al., 2009).

In order to assess the physiological state of the animal's heart rate, mean arterial blood pressure and body temperature were recorded. In phase 1, as it was a preliminary pilot study by nature, this was performed with available laboratory equipment, for phase 2 and all subsequent phases alternative and more reliable equipment allowing for dynamic data logging was utilised. Full details of all equipment and methods applied for phases 1, 2 and 3 are discussed in section 4.5.

4.3 Extracorporeal Haemoperfusion Circuit

Development of a suitable extracorporeal circuit and a device within which to suspend the adsorbent were developed. Following phases 1 and 2, a further set of experiments, phase 3, would be carried out involving a group of LPS-induced sepsis models employing the adsorbent as a therapy. This was intended to form the basis of further study and evaluation of the deployment of the adsorbent. However, this proved challenging and required significant revision as will be discussed.

In the development of the miniaturised extracorporeal circuit numerous iterative issues were encountered and overcome to produce a successful operational extracorporeal adsorbent circuit, these will be discussed throughout section 4.5. The final circuit for phase 3 involves drawing blood from the caudal artery, drawn by a roller pump passed through a column preloaded with adsorbent before being returned to circulation via the femoral vein. Through this process physiology (heart rate, body temperature and mean arterial blood pressure) is monitored via a cannulation in the femoral artery, iterative blood samples were also drawn through this cannula from which two cytokine markers for the condition were also investigated (TNFα and IL-6).

In extracorporeal applications blood flow rate is generally maintained between 120ml/min and 240 ml/min for adult human patients, although it can be significantly higher in certain applications (Ronco et al., 2000). When scaling this for rats it should be considered in terms of blood volume circulating per minute.

For an average adult human male (70kg mass and 5.5l blood volume) utilising the safer lower rate of 120ml/min this can be considered in terms of blood volume as a percentage of blood circulating extracorporeally per minute which is 2.18% of the total blood volume circulating per hour, roughly $1/40^{th}$ of the blood volume.

This can be used to inform the flow rate which should be pursued as a target in a miniaturised circuit. A 0.5kg rat has a blood volume of 30-32ml, accordingly if 2.18% of blood is to be circulated per minute a target of at least 0.6976ml/min should be pursued.

4.3.1 Adsorbent

It was discussed in section 2.9 that haemoperfusion as a mechanism of EPB in the therapy of sepsis is a potential area of development. Whole blood would be circulated in an extracorporeal circuit to perfuse a device featuring suspended adsorbent of a porous structure, which would adsorb and entrap mediators circulating in the blood before it is returned to the host circulation. Charcoal and carbons have long been established as adsorbents with medical applications however any naturally occurring charcoal derived from a natural carbonisation process is unpredictable in the creation of its structure resulting in highly variable porosity and geometry. Additionally, as has previously been mentioned, approaches utilising such materials were associated with poor biocompatibility. These issues were addressed by coating the adsorbents with biocompatible substances. However, this had a significant reductive impact upon their adsorbent capability (Mikhalovsky, 2003).

Figure 37: Polymer precursor which undergoes a process of carbonisation which results in a material with a porous structure which can be tightly controlled and tailored to the requirements of the material. Pre-carbonisation on the left and post-carbonisation on the right, note the significant shrinkage involved

Recent improvements in manufacturing processes to produce adsorbents however present new opportunities, Figure 37. There are a number of ways to control the process of carbonisation of various materials, by construction of a polymer precursor, by controlling temperature and duration of temperature treatment, the atmosphere in which the carbonisation takes place and lastly the curing time. All of these variables can be controlled in order to produce a predictable pore structure. Additionally, options are now available to begin the process with an improved biocompatible material in the form of a polymer precursor all of which can result in finished carbon products, which address the issues associated with poor biocompatibility of previous adsorbents, while the process of carbonisation creates a suitably porous adsorbent for use. The adsorbent which will be utilised for this investigation is a mesoporous carbide-derived carbon based ceramic which is derived from a polymer precursor and produced under the following controlled conditions, this material has demonstrated potential for use in adsorption from blood in previous works (Yachamaneni et al., 2010).The adsorbent was produced by Mast Carbon Ltd (Guildford, Surrey). This material has demonstrated cytokine adsorbent potential in previous studies related to cardiopulmonary bypass. Demonstrated in Figure 37 is an example of the material in a monolith structure both before and after the process of carbonisation. In order to maximise the available surface area and in line with various authors' observations in the literature (Kellum and Dishart, 2002) beads will be utilised. The beads utilised have a diameter of 100-250 μm. Owing to their mesoporous nature, they present a surface area of approximately 2,000m^2/g. A volume of 1ml of beads (a mass of 1g) was to be suspended to provide the haemoperfusion in the extracoporeal circuit. A

diagram of the adsorbent bead and action is provided in Figure 38, an image of a group of beads in Figure 39.

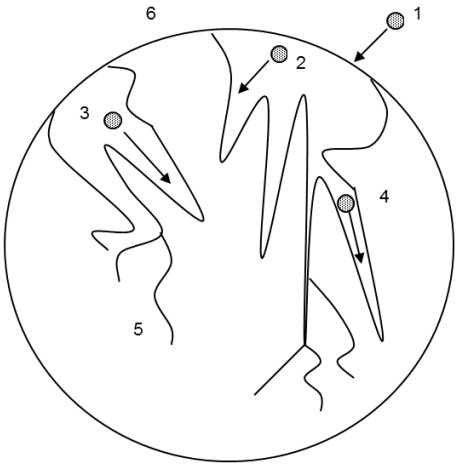

Figure 38: A schematic diagram of a porous adsorbent; 1 Molecule of adsorbate, 2 Diffusion of molecule from the solution to the surface, 3 Diffusion of molecule into mesopores, 4 Diffusion of molecule into micropores, 5 Super- and ultra- micropores, 6 External surface.

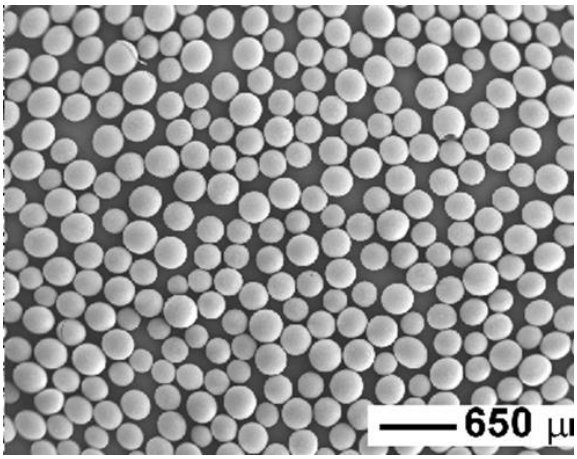

Figure 39: Image of produced adsorbent beads

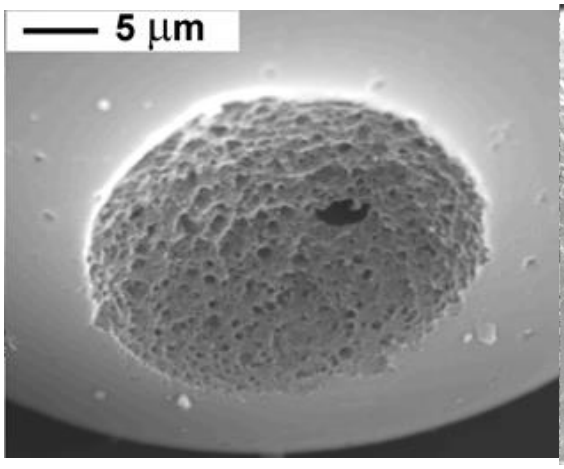

Figure 40: Image of a puncture in a coated adsorbent bead demonstrating the purous structure within

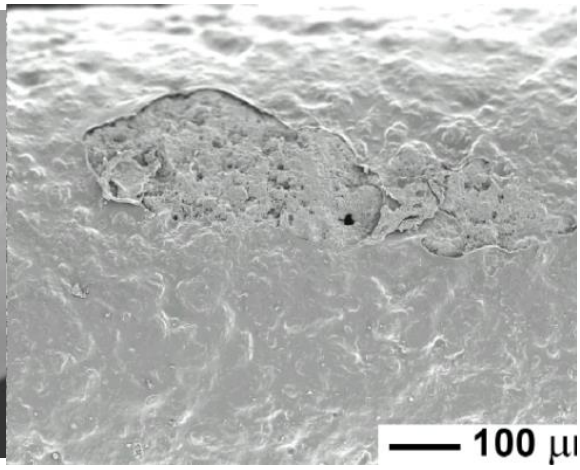

Figure 41: Image of a bead which has the surface scratched

4.3.2 Design of Adsorbent Device

In order to be able to expose circulating whole blood to the surface area of the adsorbent beads, a device had to be created to ensure maximum contact and also to ensure that no beads could escape into the circulation. In order to achieve this, a column design, demonstrated in Figure 42 and Figure 43, was developed. The column is composed of two sections or halves, which join and close with a tight screw fit and an O-ring seated in the top of the upper section. The column has an inlet and outlet and a central spindle, perforated with 1 mm holes along its length. 1ml of adsorbent is placed in to a "tea bag" sock made of woven polyester with a 40μm pore size produced by Pall Europe Ltd (Portsmouth, Hampshire) which provides a method of securely suspending the 100-250 μm beads, while providing a permeable membrane for the circulating blood. The "tea bag", which is filled with adsorbent is then tied shut around the central spindle using sutures and shut into the column. This ensures that the blood must diffuse out from the spindle across the adsorbent's surface area before leaving the column to return to circulation. Figure 45 provides images of the finished device and suspension "tea bag" membrane. The finished device was produced, based layout drawings, from stainless steel by the technical staff in the Bioengineering Unit at the University of Strathclyde. Full layout drawings of the device are available in the appendix.

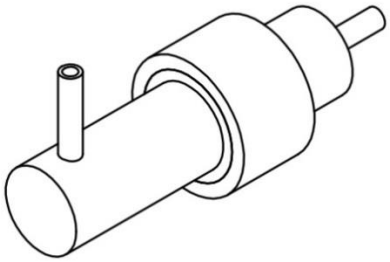

Figure 42: Design of Assembled Column to House Adsorbent In-Line Miniaturised Extracorporeal Circuit

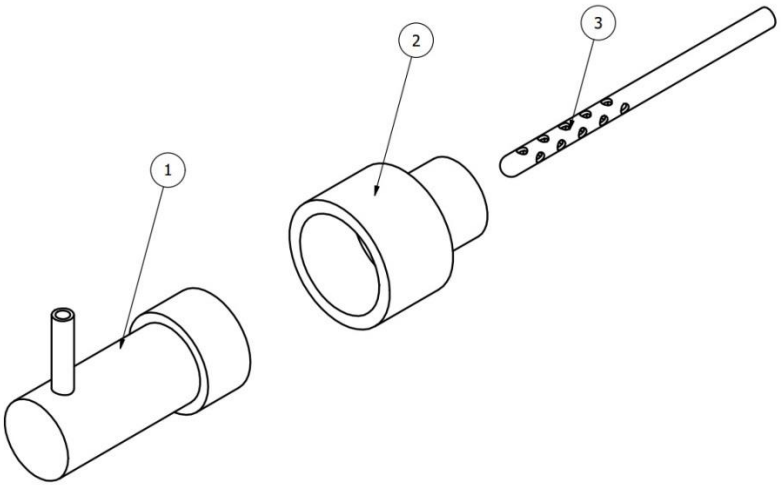

Figure 43: Assembly Sequence of Column to House Adsorbent In-Line Miniaturised Extracorporeal Circuit, 1 is the lower section of the device, 2 the upper section and 3 the central spindle.

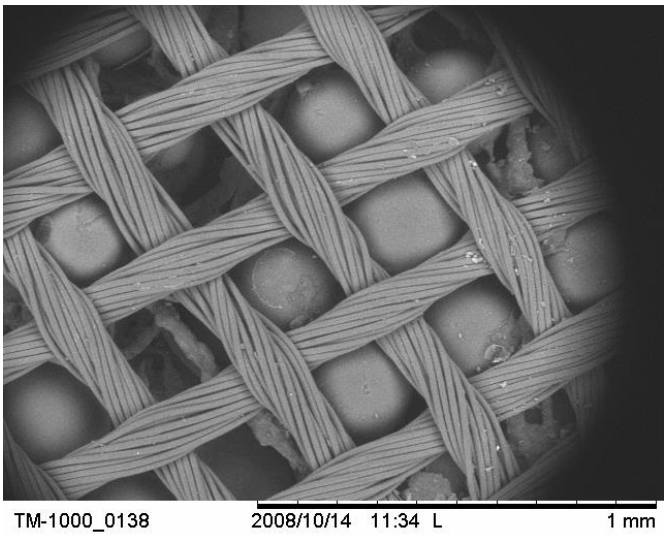

Figure 44: Scanning Electron Microscope (SEM) image of adsorbent beads trapped within suspension membrane

Figure 45: Clockwise from upper left, adsorbent column ready to be loaded with adsorbent, empty "tea bag" ready to be filled and secured to column spindle with surgical thread, loaded adsorbent "tea bag" primed with heparinised saline, assembled adsorbent column, full adsorbent “tea bag” following experimentation.

4.3.3 Design of Extracorporeal Circuit

Several iterations of the circuit were carried out before a successful and stable circuit was developed, details of which will be provided through this section. The objective of each iteration of experiments was to establish the impact of the adsorbent on the systemic condition in order to extrapolate impact on the clinical condition in humans and thus assess the potential for an interventional therapy. However, ultimately in phase 3, the data indicated that the circuit and protocol were not suitably refined to provide meaningful insight into the effect of the adsorbent.

Pump Selection

A variety of pumps has been utilised for diverse medical applications for many years. Most notably, pumps are utilised in extracorporeal circulation where they assist in therapeutic endeavours, ranging from haemodialysis to extracorporeal membrane oxygenation (ECMO). In order to achieve evaluation of the thesis hypothesis, it is required that selection of an appropriate pre-existing pump solution be utilised in the extracorporeal circuit for our application.

The pump selected must be reliable, controllable, minimise adverse reaction and achieve a suitable flow rate. Pumps used for extracorporeal applications fall into one of two categories; pulsatile and non-pulsatile. Pulsatile pumps have generally been utilised in implantable longer term applications, such as provision of bridge to transplant applications, their intention being to replicate the naturally occurring pulsatile nature of the heart and cardiovascular system by means of a diaphragm operating in a positive displacement manner. Previous applications include

ventricular assist devices (VADS). Early iterations of these devices were associated with risk of blood trauma and thrombosis. While these issues were addressed in later models, pulsatile pumps by their nature require a large size to displace adequate blood volume with each stroke and accordingly they are increasingly falling out of favour for such applications in lieu of the second category of pumps, non-pulsatile pumps.

The most commonly utilised non-pulsatile pump for medical applications is the roller, or peristaltic, pump. Roller pumps also act in a positive displacement manner. Fluid in a flexible tube is displaced from an intake to an outtake by rotational action of a pump head with a number of sprung rollers which compress the part of the tubing they contact while rotating about an axis, such as demonstrated in Figure 46. As the head rotates, the rollers compress the part of the tubing they contact to the point of occlusion and as they move round.

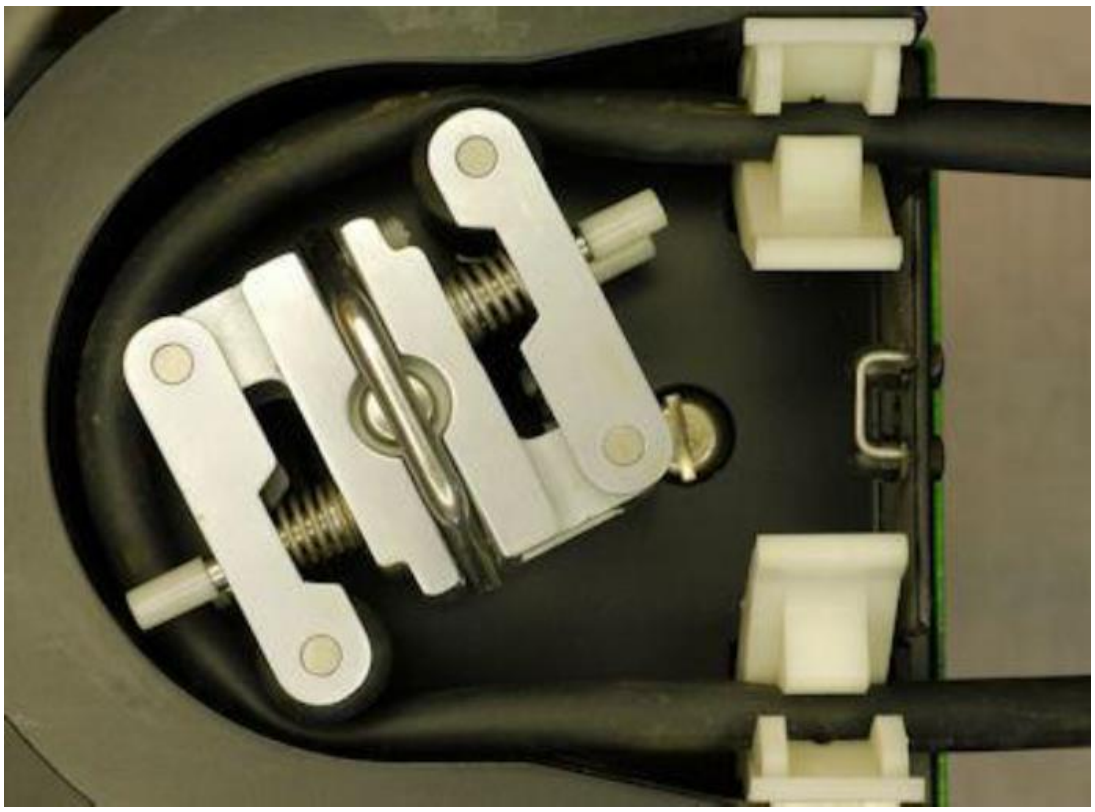

Figure 46: Waston-Marlow roller pump. Taken from http://www.watson-marlow.com

The roller pump is commonly utilised in extracorporeal therapies including cardiopulmonary bypass, haemodialysis and ECMO procedures. Advantages for using a roller pump are that the fluid, in this case blood, only comes into contact with the material the tubing is made of and a high degree of control is possible by varying the degree of occlusion of the rollers against the tubing, the number of rollers featured and the pump speed. In designs which feature a single roller, a pulsatile flow is created, although this practice has fallen out of favour. In arrangements featuring two or more rollers, full occlusion of the tubing at any given point in the rotation can be achieved.

However, roller pumps in medical applications are not without their flaws. The degree of occlusion will have implications for blood trauma, as the degree of haemolysis is linked to the degree of occlusion. Designs featuring multiple rollers are also available however they are generally not applied in extracorporeal applications as an increase in rollers is also associated with an increase in haemolysis, and lastly the continual stress placed upon the material of the tubing results in gradual breakdown of the material and indeed spallation.

Other non-pulsatile options exist, such as centrifugal and axial flow pumps, which are attractive due to reductions in size, improved performance and power requirements. However, they can be associated with high sheer stresses, resulting in increased haemolysis and additionally expose blood to more moving parts and foreign surfaces and geometries. Accordingly, for the purposes of this project roller pumps were utilised.

Design of Extracorporeal Circuit Protocol

The designed column containing adsorbent was to be placed in an extracorporeal circuit to investigate the impact of the adsorbent upon the sepsis rat model previously developed in phase 2 of experimentation. The objective of phase 3 is to provide a means to evaluate the suitability of the adsorbent as a haemoperfusion treatment modality for sepsis, assessing the effectiveness of the therapy against the induced sepsis model.

The model established in phase 2 of experimentation (Section 4.5.2) was deemed indicative of the inflammatory and physiological parameters seen in patients (Hollenberg, Dumasius et al. 2001; Nemzek, Hugunin et al. 2008).

Phase 3 was designed in such a way that each animal which was utilised for testing would provide as much information as possible. It was intended that each animal would provide a portion of time as control information, a second portion of time as induced sepsis information and a third portion of time as haemoperfusion therapy information as follows:

Time	Set-up
0-30 minutes	Control information, animal connected to extracorporeal circuit but with no LPS induced sepsis and without incorporation of haemoperfusion.
30-90 minutes	At 30 minutes LPS was administered by intraperitoneal injection.
90-180 minutes	At 90 minutes the haemoperfusion device was incorporated into the circuit.

Table 12: Experimental time points and set-ups in experimental phase 3.

As in phase 2, sepsis was induced by means of an intraperitoneal injection of lipopolysaccharide (LPS) (Sigma-Aldrich, Dorset) at a dose of 50mg/kg. Regrettably, the process was far from straightforward. Details of the challenges encountered are discussed in section 4.5.

4.4 Phases 1, 2 & 3

Phases 1, 2 and 3 are presented in this section, discussion follows in section 4.5. Phase 4 follows separately in chapter 5.0.

4.4.1 Phase 1: Pilot Study

The purpose of phase 1 was to establish a rat control model capable of remaining stable for 6 hours, while dynamically monitoring physiological parameters and taking iterative blood samples. It was also sought to preliminarily evaluate the use of LPS as a method of inducing sepsis.

Methods

Sepsis was induced by intraperitoneal injection of lipopolysaccharide. Key physiological characteristics were monitored and recorded over a six hour time course, in order to establish the suitability of the model. Heart rate, body temperature and mean arterial blood pressure, and perfusion in the periphery were measured. In this initial pilot study a single animal was used. It should be stressed that the onus of the pilot study was to establish "proof of concept" of the model and its clinical relevance to the human condition it seeks to replicate. This was later scaled up in in phase 2 of experimentation detailed in section 4.5.2. The experimental set-up is illustrated in Figure 47 and Figure 48.

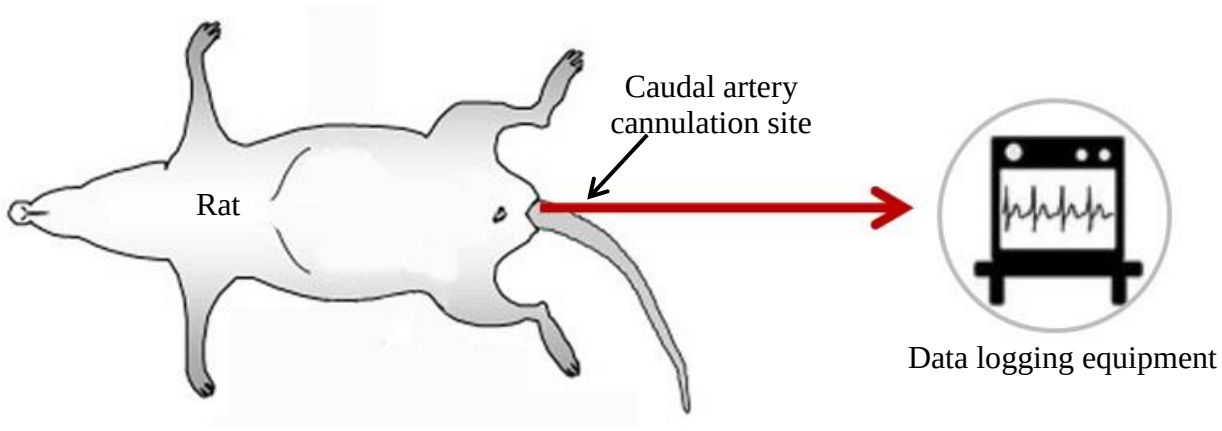

Figure 47: Phase 1: Pilot Study. Experimental Set-up Diagram.

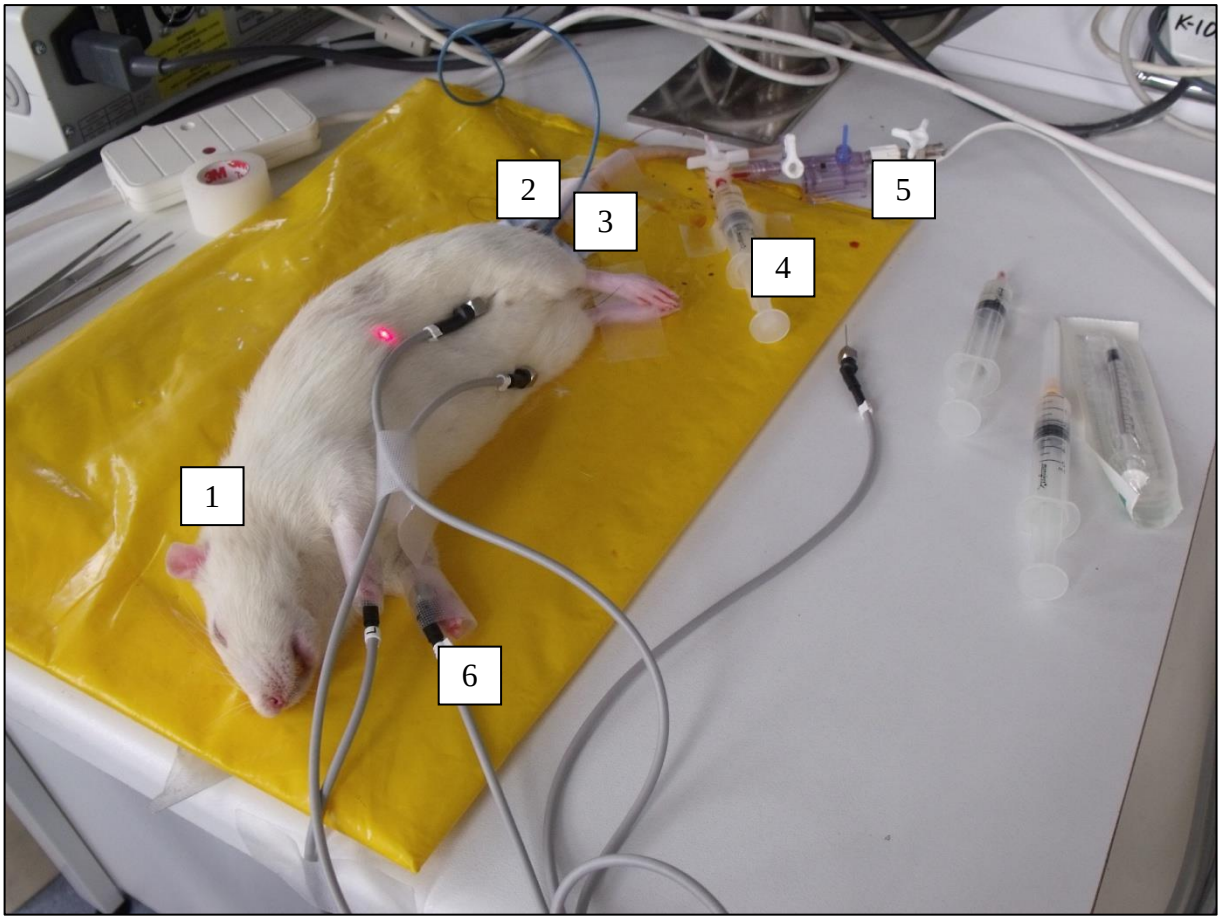

Figure 48: Phase 1: Pilot Study. Experimental set-up. Clockwise from left; 1 Sprague-Dawley rat, 2 rectal temperature probe, 3 caudal cannulation site, 4 anticoagulant flush syringe, 5 pressure transducer, 6 ECG needle electrodes.

It is reiterated that phase 1 was a pilot study to establish feasibility of further phases. The animal was anaesthetized with inhalation of 2ml isoflurane (Abbott Laboratories Ltd Maidenhead) and subsequently injected intraperitoneally with an anaesthetic mix containing 1ml Hypnorm (VetaPharma Ltd, Leeds), 1ml Hypnovel (Roche Products Ltd, Hertfordshire) and 2ml sterilised water for injection (W. & J. Dunlop Ltd, Dumfries) used as a dissolvent. For this first animal, an initial dose of 1.5ml of anaesthetic was used. Subsequent preparations of this 4ml anaesthetic mix were prepared pre-emptively should they be required, over the time course.

Depth of anaesthesia was monitored and determined by spontaneous movement, twitching, increased respiratory rate, and increased work at breathing, movement of extremities upon stimulation of plantar surface, and tail and toe pinch. Based on early observations following the initial 1.5ml anaesthetic dose some piloerection, a brisk whisker motion in this case, was observed. Accordingly before any further procedures were carried out, a further 1ml of anaesthetic was administered, which induced a suitable depth of anaesthesia. Constant level of anaesthesia was maintained throughout the experiment by supplemental doses of 0.75 ml as needed (approximately once every hour). The animal was situated upon a heat mat and tethered in the supine position using surgical tape.

The intention was to use the heat mat to maintain body temperature in a normal range of 37.5 to 38.5 C throughout the time course. Temperature was monitored using a digital rectal thermometer, TC-08 thermocouple, and associated PicoLog data logging software (Pico Technology, Cambridgeshire) at five minute intervals. Temperature results will be discussed further in section 4.5.1 but it is worth

observing that the animal appeared to be below a normal temperature range at the start of the experiment. Additionally, it was found that use of the heat mat was causing hyperthermia in the rat, accordingly it was switched off following some brief experimentation, which will be discussed further in section 4.5.1.

In order to measure mean arterial blood pressure, the caudal artery in the tail was cannulated with a catheter and Edwards TruWave Pressure Transducer (Edwards Lifesciences, Berkshire) and monitored, the resultant BP reading was recorded every five minutes. In order to ensure proper placement, the catheter was advanced into the artery after verifying it was in the vessel, filling with blood as it does. The catheter was subsequently secured with surgical tape. Only then was it connected to the pressure transducer. This same catheter was used to collect blood samples of 1ml at 60 minute intervals. In order to prevent coagulation in this system, the transducer was connected by means of a three-way tap valve to a store syringe containing 0.5ml of heparin (Sigma-Aldrich, Dorset) and 5ml of saline (Sigma-Aldrich, Dorset).

In order to prevent coagulation of the samples, EDTA (ethylene diaminetetra-acid) (Sigma-Aldrich, Dorset) was added to each sample tube before the blood sample was deposited therein. The sample was then separated by centrifuge at 5000rpm for 10 miuntes using a Sorvall Pico centrifuge (Kendro Laboratory Products, Langenselbold Germany). The separated plasma was stored at −80°C. In this phase, the samples were not used for any analysis but provided key information in establishing that this volume of blood and sample frequency was suitable. Regarding the safe volume of blood samples, this volume falls within recommended limits, which state that for an adult male of approximately 500g, the average blood volume is 30-32ml and that a

maximal value of 30-40% may be safely sampled over a 24 hour period without adverse effect (Tuffery, 1987, Suckow et al., 2006). However, it is advisable to replace the lost fluid intermittently. This was done through the use of a plasma expander solution (Sigma-Aldrich, Dorset) halfway through the time course at 3 hours.

In order to determine heart rate (HR), the animals were connected to an electrocardiograph monitor system, GE Marquette Solar 8000 Patient Monitor (GE Healthcare, Berkshire). Utilizing a standard limb electrode arrangement in the bipolar limb lead system of Eindhoven, placing the electrode needles subcutaneously in the appropriate arrangement (Suckow et al., 2006). The animals were initially restrained in the supine position following cannulation, although this had to be altered, which will be discussed further below. The electrodes were attached: right arm (RA) electrode on the right forelimb; left arm (LA) on left forelimb; right leg (RL) on right hindlimb; and left leg (LL) on left hindlimb. Utilising this equipment in phase 1, there was not the facility to data log the ECG dynamically, instead observational readings had to be taken once each hour (prior to the hourly blood sample to ensure minimal disruption) by waveform occurrence in a given time interval to establish the HR in beats per minute (BPM). Mean arterial blood pressure was also recorded based upon observation of this system.

In order to investigate blood flow distribution, perfusion was measured using a Doppler laser imager, Moor LDI2-IR (Moor Instruments Instruments Ltd, Devon). The measurement of outcome units are arbitrary perfusion units (PU), best illustrated by the completed scans demonstrated in Table 13, discussion of these results will be

covered in the next section. From a practical perspective, this process was challenging to complete, although the animal was restrained and sedated, small movements either by the animal or the experimenter altered the positioning of limbs and location in relation to the scanner, for instance when administering additional anaesthetic or collecting blood samples.

It should at this point be noted that it was necessary to run two animals in this phase as the first expired a little after 2 hours into the time course. The cause appeared to be some form of pulmonary difficulty, which began approximately 90 minutes into the experiment, the animal's breathing became laboured and was accompanied by a marked rasping during inhale. This was accompanied by a steady decline in mean arterial blood pressure, eventually with a BP 38 mmHg down from an average of 70 mmHg. The animal's respiration was being moderately encouraged by experimenters through chest tapping before eventual demise. It was hypothesized that the pulmonary difficulty was either a complication of the particular mode of anaesthetic or a postural asphyxia due to the restrained supine position of the animal during the experiment, which appeared more likely. Subsequently, another control animal was utilized but was maintained in a right lateral position whenever possible, with the exception of the initial set up, cannulation and the hourly perfusion scans. This further complicated the process of obtaining laser perfusion scans as the animal had to be turned from the lateral to supine positions, which also added an element of risk by potentially dislodging the caudal cannula. For a survival perspective however, this proved much more successful requiring the euthanasia of the animal at the end of the 6 hour time course. Animals were euthanized by a cardiac injection of potassium chloride (JM Loveridge Ltd, Southampton) inducing cardiac arrest and death. This

observation of the restrained postural effect on the animal survival was a key observation and shaped the course of further animal studies in the later phases. The experimental set-up is illustrated in Figure 47 and Figure 48.

The results are best illustrated plotted on graphs, which are shown in Figure 49, Figure 50 and Figure 51. The results of the Doppler scans for perfusion are shown in Table 13. It should again be stressed that this project was concerned with the feasibility of developing an animal model. To that end, establishing a functional control model is considered a valid result.

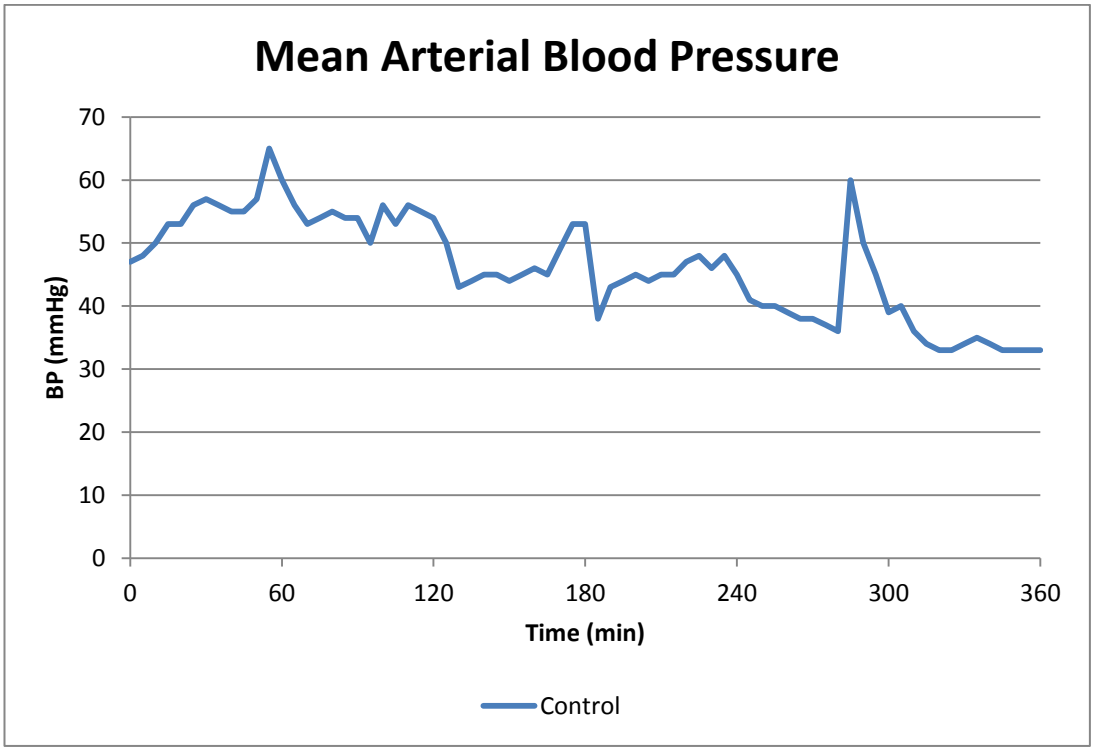

Figure 49: Phase 1: Pilot Study. Mean arterial blood pressure over 6 hour time course, mmHg against minutes elapsed.

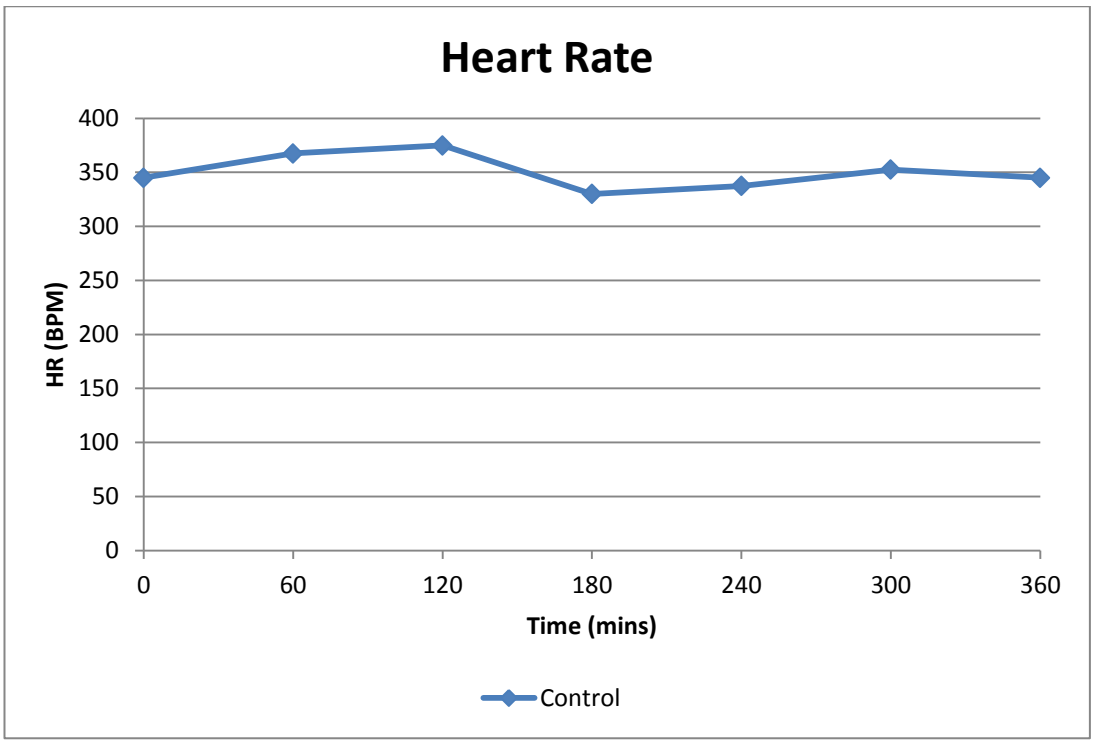

Figure 50: Phase 1: Pilot Study. Heart Rate over 6 hour time course, beats per minute against minutes elapsed.

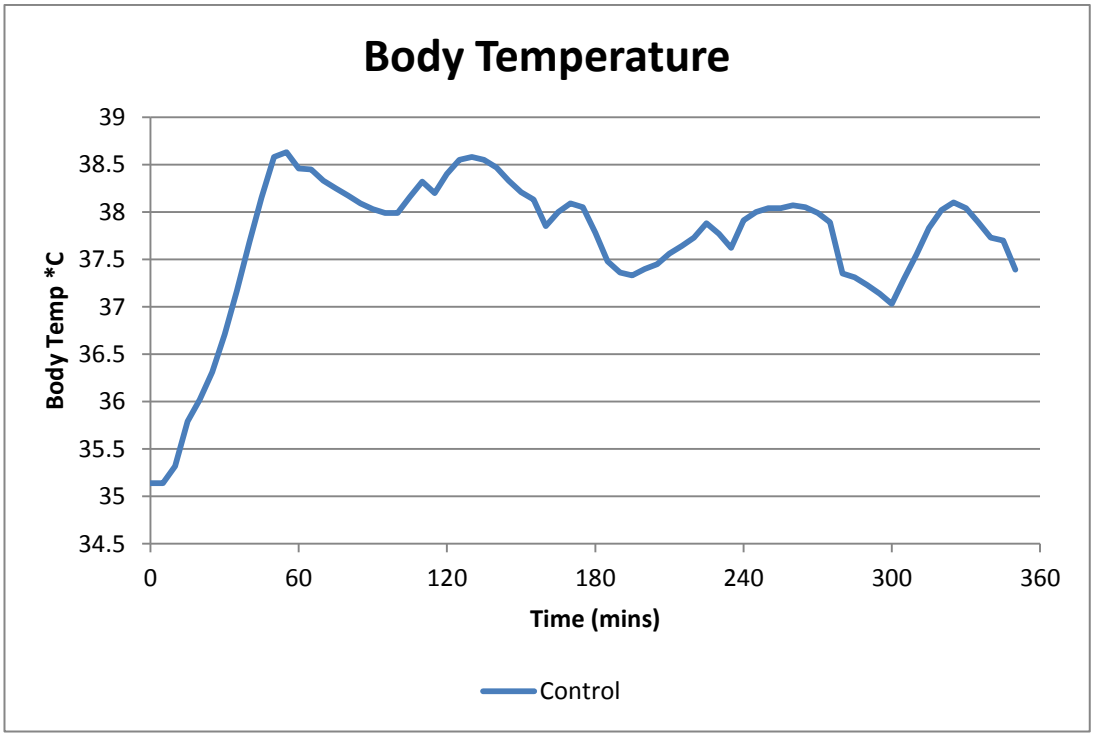

Figure 51: Phase 1: Pilot Study. Body Temperature over 6 hour time course, °C against minutes elapsed.

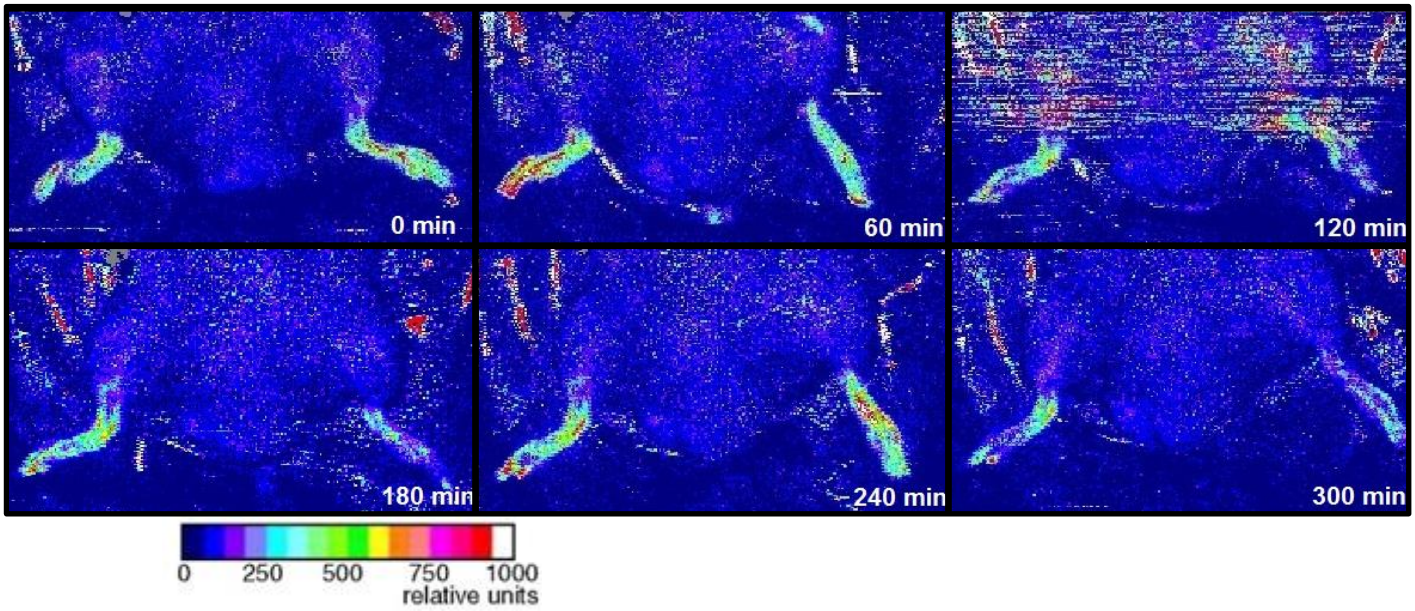

Table 13: Phase 1: Pilot Study. Results of the Doppler perfusion study scans of the control model including scale demonstrating areas of poor to good perfusion, note the inconsistencies in limb position and the poor acquisition at 120 minutes. The scale is relative units from the Doppler scanner blue indicates poor perfusion and red indicated good perfusion.

4.4.2 Phase 2: Control & Induced-Sepsis

The purpose of this phase was to build upon phase 1 and generate a reliable model analogous to human sepsis in the rat, which could be used to subsequently evaluate the efficacy of an adsorbent in mitigating its progression in phase 3.

Methods

Sepsis was induced by intraperitoneal injection of lipopolysaccharide. Key physiological characteristics were monitored and recorded over a six hour time course, in order to establish the suitability of the model. Heart rate, body temperature and mean arterial blood pressure, were measured. Two cytokine mediators for the condition were also investigated, TNFα and IL-6, the rational for this selection has been discussed in section 4.2. Several models were completed of which three control and three LPS-induced sepsis models were analysed. The objective of the study was to develop a repeatable, accurate model of the clinical human sepsis condition. The data gathered would appear to indicate that the model fulfils this purpose, presenting an indicative physiological response. The experimental set-up is, from a diagrammatic point of view, the same as in phase 1, demonstrated in Figure 47.

Animals were anesthetized in the same manner as those in phase 1, discussed in section 4.4.1. It was quickly discovered in the phase of experiments that the initial dose was in most cases insufficient to maintain a suitable depth of anaesthesia while a cannula was placed, similar to phase one but the limited sample size prevented any significant conclusions be drawn at that point. This was due to the location of the vessel being cannulated, the caudal artery's close proximity to the caudal nerve.

While great care was taken to ensure the caudal nerve was not disturbed, it is still a highly sensitive area. On occasion rats twitched mildly in reaction to the operation occurring, indicating no significant discomfort but making it difficult to place the cannula. Supplying a supplementary dose of anaesthetic mitigated this response. The practice led to an increase in the dose of initial anaesthetic administered in the first instance. The optimal initial dose of anaesthetic was 2ml and on one occasion 3ml were necessary. Constant level of anaesthesia was maintained throughout the experiment by supplemental doses of 0.75 ml as needed (approximately once every hour, although this varied slightly animal to animal). Care was taken to ensure that neither hyper nor hypovolemia could occur, although it should be noted this was conducted in an ad-hoc manner. Any fluids administered following the initial dose of anaesthesia were monitored to ensure they fell between blood drawn each hour and equalled approximately the same volume.

Temperature was monitored continuously using a digital rectal thermometer, Rectal Temperature Probe SS7L (Linton Instrumentation, Norfolk). Based upon the observations in phase 1, it was opted to place the animals on the heat mat with no power and monitor their temperature. Similar to phase 1, it was found that the animals regulated their own body temperature amply, even in periods following collection of blood samples, which has often been commented upon in literature as directly related to a drop in body temperature. However this was only over a 6 hour time course. Comment cannot be offered on how this parameter might change over a longer time course. As now no external factors were influencing body temperature, it was possible to record and observe the differences in the important parameter between the control and sepsis models.

Blood samples were collected and stored in the same manner as phase 1, discussed in section 4.4.1. It was established in phase 1 that this volume and frequency were suitable. As previously mentioned, the liquid volume was replaced by the volume received from the injection of anaesthetic and intermittent flushing of the cannula line with mildly heparinised saline.

In order to determine cardiovascular parameters the animals were connected to a data acquisition system, BioPac MP36 Data Acquisition Unit BSLBSC-W/-M, featuring a number of sensors; Blood Pressure Transducer SS13L, Monopolar needle electrodes EL452 and a Rectal Temperature Probe SS7L (Linton Instrumentation, Norfolk). This equipment replaced a combination of pieces of equipment which had been used in phase 1, producing greater reliability and enabling dynamic data logging.

It was also intended that blood flow distribution would be investigated; perfusion would be measured using a Doppler laser imager, Moor LDI2-IR (Moor Instruments Instruments Ltd, Devon). However, numerous practical issues arose in the utilisation of this equipment for this projects experimental work. As has been mentioned, the lengthy scan time created inconsistencies in the captured data due to animal movement, and inconsistencies in placement in relation to the scanner further compounded this issue. Additionally, concerns were maintained relating to the observed early mortality in phase 1 due to pulmonary difficulty associated with the supine position. Perhaps most importantly however, were the difficulties associated with moving the animal in and out of position for the scan. On several occasions, the cannula became dislodged in this process of moving the rat into position for the first

or second scan. The issues associated with the adverse events were serious. As the caudal artery is a prominent artery, bleeding was fast and difficult to halt. Additionally, the process of replacing the cannula was extremely difficult at a site subsequently obscured by blood. It was concluded that the risk to reward ratio of continuing to undertake this action was not justified; for the benefit of one unreliable, albeit valuable, measurement the risk was the loss of other reliable cardiovascular measurements. Accordingly, the decision was taken not to persevere further with acquisition of this measurement. However, it would be a recommendation for future work that newer, or perhaps entirely alternative, equipment may enable a more suitable and reliable measurement of perfusion.

This completed the procedures carried out for the control animals. For the sepsis model, following the set-up outlined above, the animal was injected intraperitoneally with 50 mg/kg Escherichia coli lipopolysaccharide endotoxin (*E.Coli* type 0111:B4; Sigma-Aldrich, Dorset) in 2ml sterile pyrogen-free saline to induce sepsis. This dose was based upon literature investigations of other sepsis animal studies.

A further outcome from phase 1 had been that when rats were restrained in the supine position, they could expire due to some form of positional asphyxia. It had initially been hypothesised that this was due to the animal being actively restrained in the supine position, tethered using surgical tape, with its limbs attached to the mat, this unnatural position seemed to cause some description of pulmonary congestion. Accordingly, the first control animal in the most recent series of experiments was maintained in the supine position but crucially untethered. However, this animal still expired prematurely with the same observations, it appeared that this would occur

whether the rat was actively restrained or not. Subsequently, the experimental protocol was altered so that only when perfusion scans were being performed did the animal lie in the supine position. However when the decision was made not to proceed with the perfusion measurement, there was no longer any need to position the animal in the supine position once initial cannulation had been completed. All subsequent animals were maintained in a lateral position and the pulmonary difficulty presented no further issues.

The animal was euthanized at the end of the time course. Animals were euthanized by a cardiac injection of potassium chloride (JM Loveridge Ltd, Southampton) inducing cardiac arrest and death.

It was mentioned that blood samples were collected at hourly intervals. The plasma from these blood samples was then tested for the presence and levels of the cytokines by ELISA. Quantitative "plate-yourself" sandwich enzyme immunoassay technique kits were obtained for IL-6 (BD Biosciences, Oxford) and TNFα (eBioscience, Hatfield). Both kits were suitable for reliable use with serum samples and the assays were carried out according to the protocols provided by the manufacturer. Each kit was prepared by coating with the supplied antigen and overnight incubation then washed. The samples were then added, washed again and lastly a colour reactive enzyme added which fluoresced when a stop solution was added. The intensity of this fluorescence was measured and could be used to quantify the levels of each cytokine present in each sample. These assays were linear in their calibration.

It should be noted however that once the process was started it became apparent that there was insufficient serum sample to run the samples in duplicate. Duplicate runs are generally considered to be best practice, if one well became compromised in some manner it could be compared to its duplicate well. However as has been mentioned earlier, 1ml of blood drawn at hourly intervals, while suitably safe, is a comparatively large volume given the frequency of sampling. However, there appeared to be no issues when the samples were run in single, the results can be seen in Figure 55 and Figure 56.

Results are best plotted on graphs shown in Figure 52, Figure 53 and Figure 54, results of ELISAs are presented in Figure 55 and Figure 56. 3 control animals and 3 sepsis induced animals are represented; the results plotted are the average of those animals with standard deviation at hourly intervals.

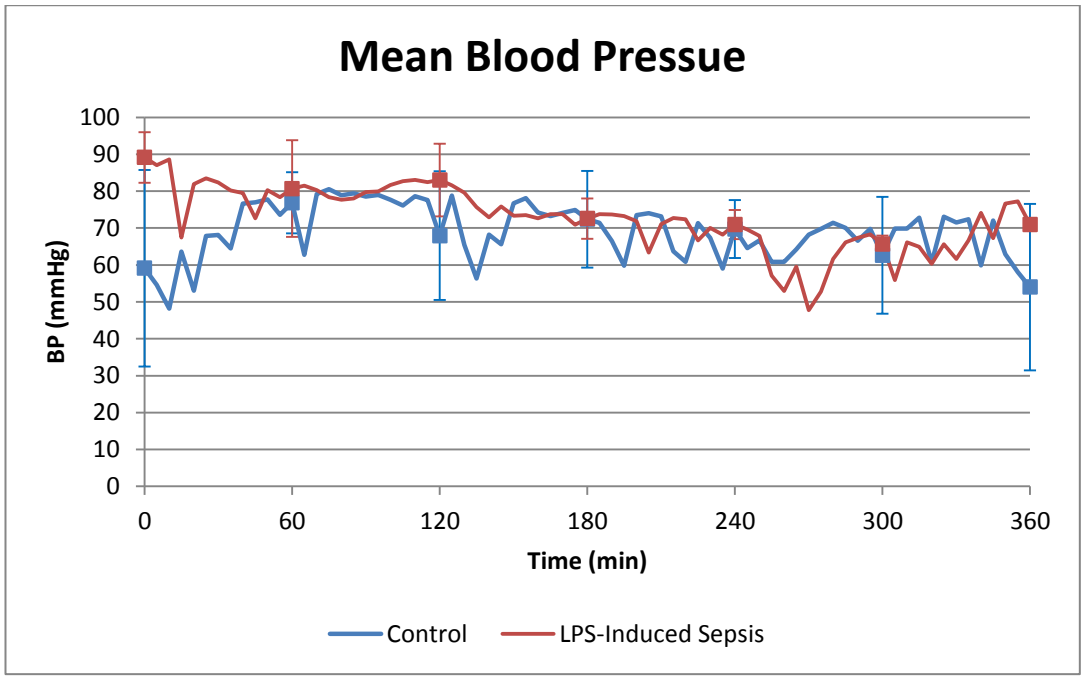

Figure 52: Phase 2: Sepsis & Control Models. Mean arterial blood pressure over 6 hour time course, mmHg against minutes elapsed.

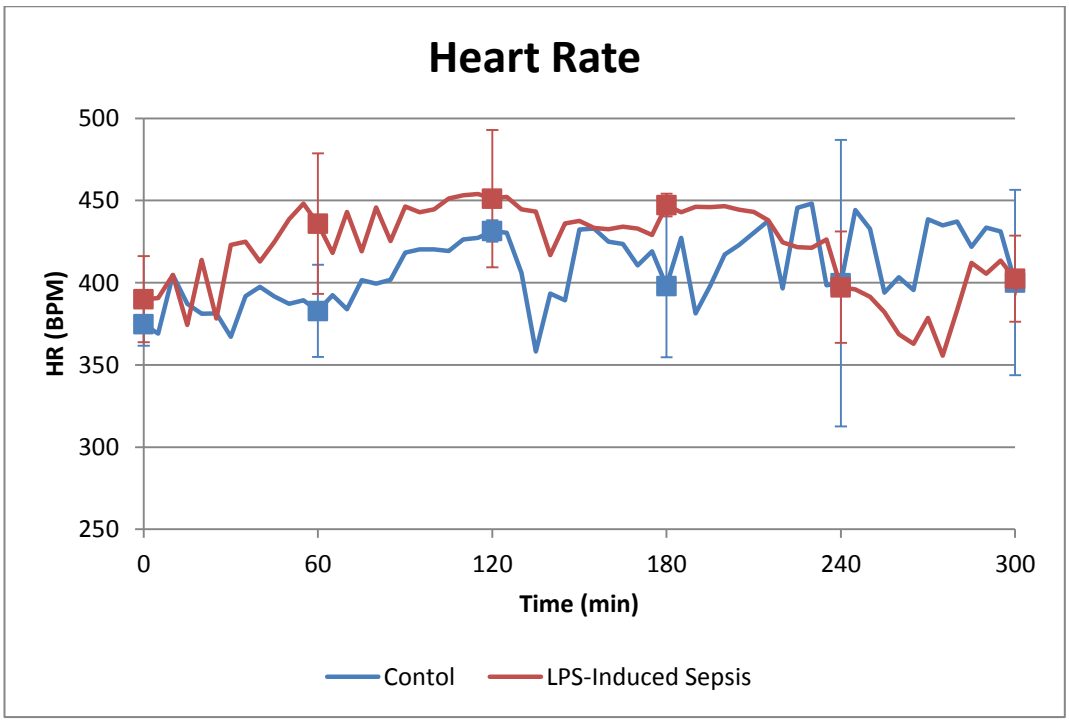

Figure 53: Phase 2: Sepsis & Control Models. Heart Rate over 6 hour time course, beats per minute against minutes elapsed.

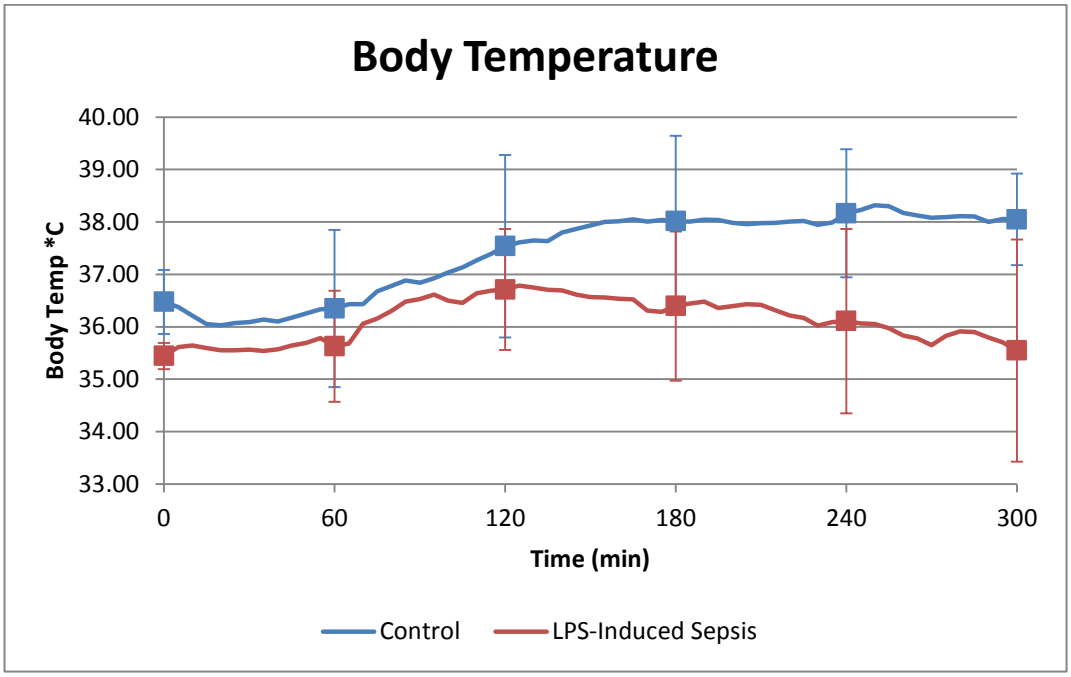

Figure 54: Phase 2: Sepsis & Control Models. Body Temperature over 6 hour time course, ºC against minutes elapsed.

The results of the ELISA tests are displayed below. As these were not monitored continuously and instead at hour long intervals, they are displayed as mean results from each group of 3.

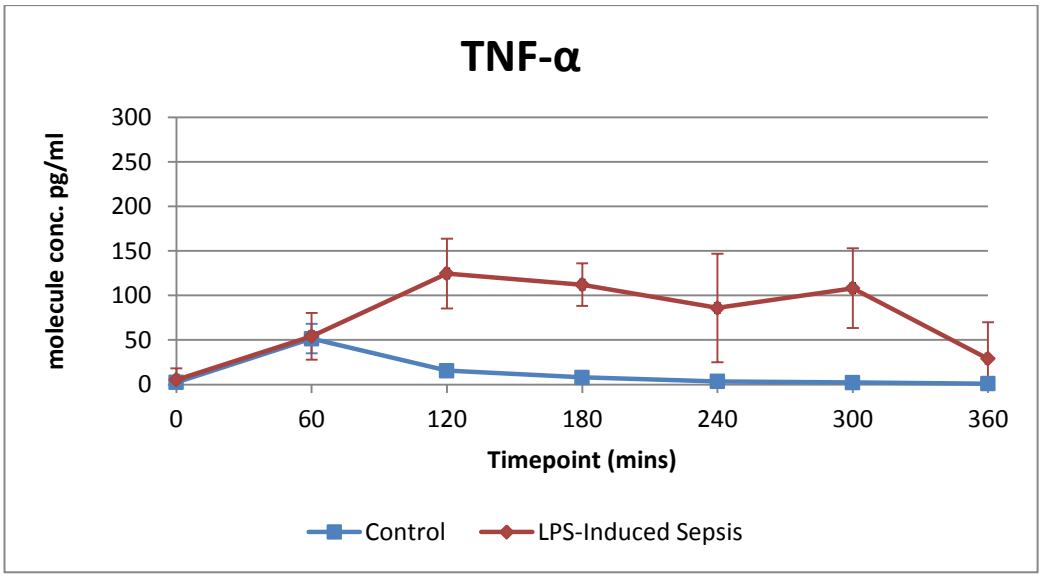

Figure 55: Phase 2: Sepsis & Control Models. Mean and Standard Deviation TNFα ELISA Results, molecule concentration pg/ml against minutes elapsed

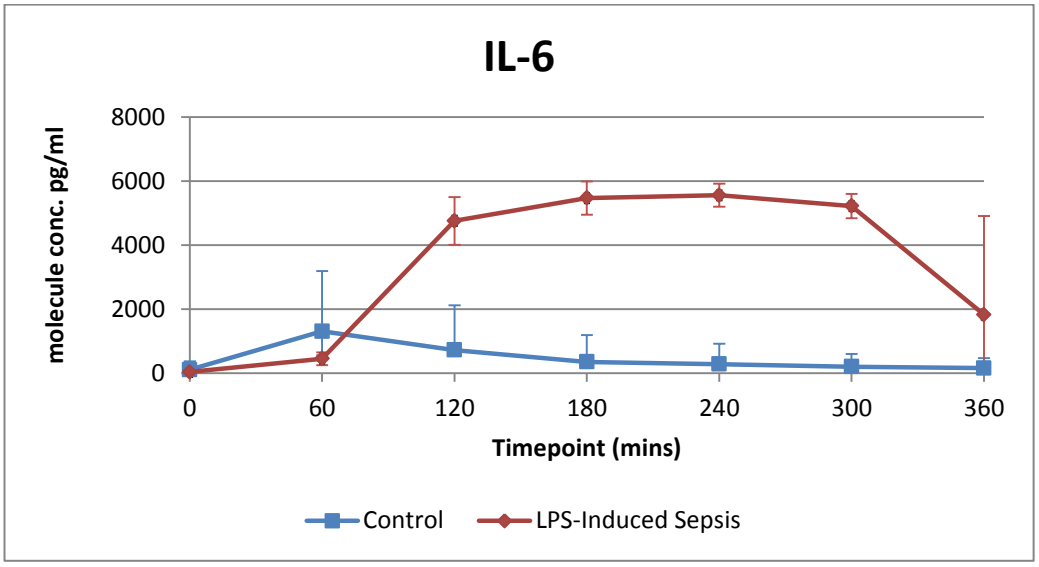

Figure 56: Phase 2: Sepsis & Control Models. Mean and Standard Deviation IL-6 ELISA Results, molecule concentration pg/ml against minutes elapsed

4.4.3 Phase 3: Extracorporeal Haemoperfusion Study Results

The purpose of phase 3 is to provide a means to evaluate the suitability of the adsorbent as a haemoperfusion treatment modality for sepsis, assessing the effectiveness of the therapy against the induced sepsis model.

Methods

The extracorporeal circuit was run for the first 30 minutes without any additional substances administered. Then LPS was used to induce sepsis and the progression was monitored for 60 minutes. The adsorbent column was then introduced and the condition progression was monitored for a further 90 minutes where possible. Key physiological characteristics were monitored, heart rate, body temperature, mean arterial blood pressure. Two cytokine markers for the condition were also investigated, TNFα and IL-6. The purpose of the study was to evaluate the efficacy of the adsorbent when deployed as haemoperfusion in this circuit. Set-up is demonstrated in Figure 57.

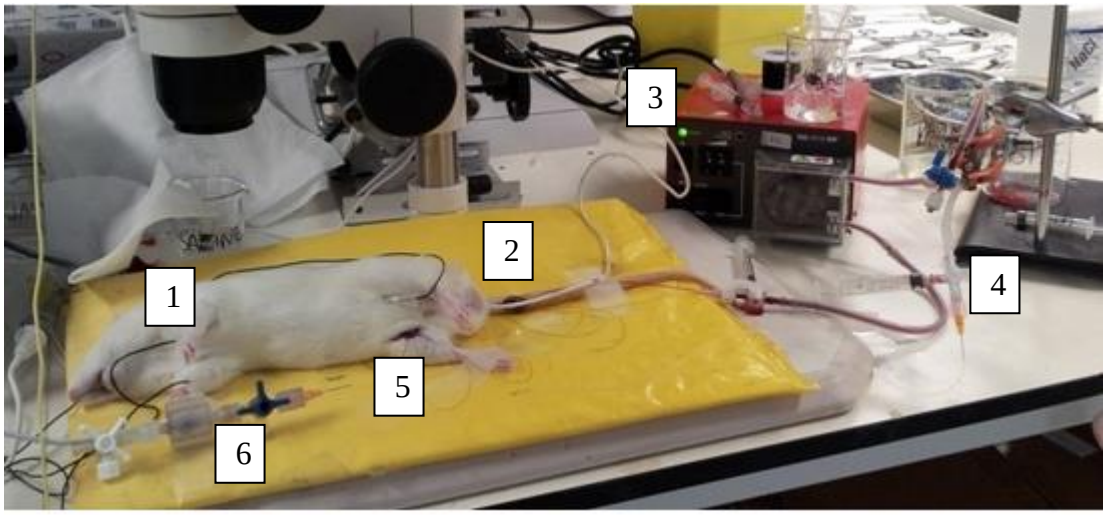

Figure 57: Phase 3: Extracorporeal Study. Extracorporeal circuit experimental layout. Clockwise from left; 1 Sprague-Dawley rat, 2 caudal cannulation site, 3 roller pump, 4 surgical tubing and anticoagulant flush syringe, 5 femoral cannulation site, 6 pressure transducer.

The same anaesthetics and equipment were utilised in phase 3 as in phases 1 and 2. Any alterations or additional equipment are explicitly stated in this section. The process of conducting phase 3 was far from straightforward. Initially, it was hoped to create as simple and minimally invasive a circuit as possible. The initial hope was to cannulate the caudal artery in the tail for use as the draw side of the circuit and the caudal vein for the return side, as demonstrated in Figure 58.

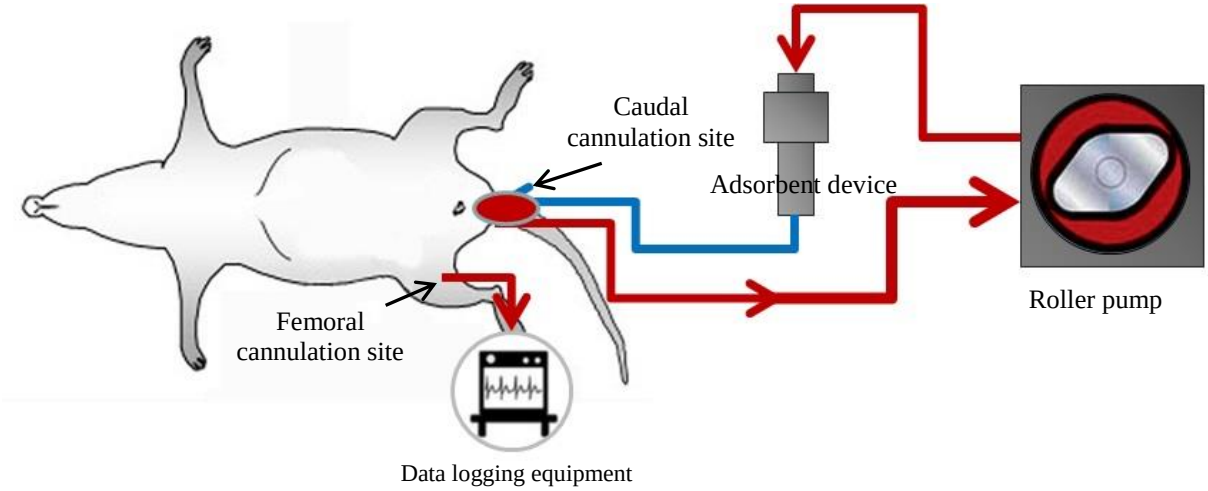

Figure 58: Simplified diagram of initial phase 3 experimental extracorporeal circuit.

This would provide a minimal number of surgical sites and also minimise ischemia or other potential complications associated with an extracorporeal circuit as it is both draw and return at the end of the rats circulatory system. Cannulation of the caudal artery had already been achieved in previous experiments in phases 1 and 2 for drawing blood samples and monitoring physiological parameters, which proved to be comparatively straightforward using a "stretched down" 5 Fr cannula.. The process of "stretching down" involves mildly heating the cannula tip and teasing it manually to stretch out the material and reduce the diameter at the tip, it is difficult to therefore provide an exact finished diameter but all measurements indicated an average of approximately 3 Fr. Due to short supplies of 3 French cannula in the laboratory, this was necessary as the manufactured finish 3 French cannulas would be required for the caudal vein. All cannulas had cut bevelled tips at this point. Cannulation of the caudal vein proved impossible. Initially, it was thought that the cannula may be too large for the vein but after multiple attempts on a few occasions it was placed successfully, albeit with extreme difficulty. Unfortunately on all of these successful

placements, after mere minutes the cannula tore through the vessel wall. This was thought to be for one of two reasons, the bevelled tip presented too much of an edge or that the material was too rigid to flex sufficiently in step with the vein, or perhaps a combination of the two. Due to these complications and the amount of time and resource being utilised, it was elected to make use of the femoral vein for the return side, which presented a more durable vessel. This revised experimental set-up is demonstrated in Figure 59.

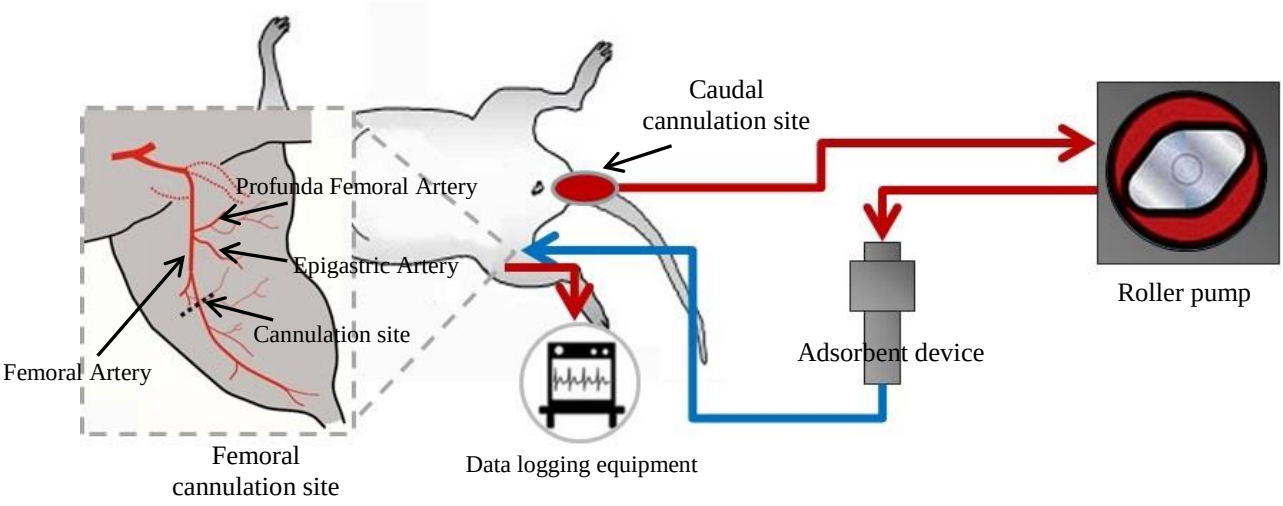

Figure 59: Simplified diagram of revised phase 3 experimental extracorporeal circuit.

Making use of the femoral vein provided several advantages over the caudal vein, at least in theory, principally it is a larger and thicker walled vessel. Additionally, the surgical site is the same as that to be used for access to the femoral artery, which would be cannulated for use of the cardiovascular sensing equipment. However in practice, the surgery, by comparison to the caudal site alone, is significantly more complex and time consuming. That said in initial attempts it was still vastly more promising than the caudal vein had been. Iteratively some problems were encountered and sequentially overcome.

In the first attempts despite utilising a stronger vessel, the cannulas were still puncturing the vessel wall, on one occasion even the stronger caudual artery was torn. At this point, it was concluded that the stretching down method of reducing the cannula diameters was stiffening the material too much, subsequently some new cannulas were obtained (Harvard Apparatus, Kent), this resolved the torn vessel issue. Concurrently cannulating the femoral artery for attachment of sensors also proved difficult, this was also resolved by the new cannulas. With the cannulas successfully placed and sensing equipment connected, the basis of the circuit was achieved. The cannulas were connected to surgical tubing and three way-taps for inclusion of syringes, which would be used to draw blood samples, administer any fluids (I.V. administration of heparin and also eliminate any air bubbles which propagated in the circuit for any reason. Initially, the circuit had to be proved fit for purpose before involvement of the adsorbent. It was sought to successfully run the circuit for 90 minutes as a pilot before refining a protocol for experimental procedure. This took some refining to achieve.

In the first circuit trials, it was possible to achieve the circuit with the pump running but the circuit was becoming occluded around 30-40 minutes into the time course. Upon investigation, it was revealed that the cannula in the caudal artery, the draw side, was occluding as the vessel wall was being drawn over the bevelled tip. This was resolved by making use of a rounded tip cannula preventing the occlusion.

Subsequently, the circuit was running for a longer time period but air bubbles were appearing 50-60 minutes into the time course. At first, it was hypothesised that the level of heparinisation was not sufficiently maintained. Subsequently, top-up doses

of heparin were administered approximately each half hour but the air bubbles persisted. A practical failure mode analysis was undertaken, deconstructing the circuit and assessing each individual component in the circuit in turn, it was eventually revealed that there was a very small puncture in the tubing in the pump housing. This is a known issue in the use of roller pumps, as previously mentioned in section 4.3.3. Over time, the action of the rotating sprung heads compressing the tubing against the housing wall abrades the material to breaking point. The tubing was replaced and the circuit ran for the intended 90 minutes smoothly.

At this point, it was deemed that the circuit could go ahead to include the adsorbent in the haemoperfusion column. As was established in both experimentation phases 1 and 2 (section 4.4.1 and 4.5.2 respectively), presentation of the mediating cytokines is evident after one hour and the physiological changes can be tracked dynamically via data logging equipment. However, it should be noted that it was only possible to achieve a flow rate of 0.2ml/min, less than the target flow rate. It was decided that rather than run control and experimental groups at this point, it would be more valid and efficient to utilise one rodent and run the circuit, unaltered for 30 minutes, in order to assess any changes in parameters due to the blood being circulated extracorporeally, then administer the LPS to induce sepsis, run for a further 60 minutes to take effect and collect data then introduce the haemoperfusion column containing the adsorbent and run for 90 minutes to collect data on the effects, a total experimental time of 3 hours. The column was preloaded with adsorbent and all elements of the circuit were primed with a priming mix of 50% saline and 50% gelofusane. It was intended to increase the frequency of blood sampling from once every hour to once every 30 minutes. Blood samples were collected from the draw

side via a syringe and three-way tap before the adsorbent column in the circuit to ensure the observed level of mediators was indicative of the circulating blood rather than after the column. It would become established that this was not a suitable protocol.

Several rats were run in this fashion, they met with varying degrees of success. Heparinisation and fluid balance are concluded to be the principal issues. Due to the time course of these experiments, it was sought to acquire blood samples every 30 minutes. In order to obtain sufficient blood to run ELISA analysis for two cytokines, 1ml of blood is required at each sample. Drawing 1ml of blood every 30 minutes is pushing the limit of blood volume to be extracted safely according to the literature on the matter and this may also have been a factor in the varying success of the experiments. In each model, drawing blood became increasingly difficult to achieve to the point that it was apparent that if the experiment were to run for a longer time course it would not be possible to withdraw samples after an estimated 2-3 hours.

Heparinisation was carried out in an ad-hoc manner, it was initially estimated that administration of 1ml heparin I.V. would be sufficient for this 3 hour time course, but it soon became apparent this was insufficient and subsequent doses were required, approximately 0.5 to 0.75 ml each hour. A photograph of the circuit in operation is included in Figure 57.

At the end of the time course, the animals were euthanized by intravenous administration of potassium chloride (JM Loveridge Ltd, Southampton), inducing cardiac arrest and death.

Results are best plotted on graphs shown in Figure 60, Figure 61 and Figure 62. It should be borne in mind that with a small group the highly variable nature of animal work may have an impact upon the trends observed. 3 rats are represented; the results plotted are the mean of those animals with standard deviation at half hourly intervals.

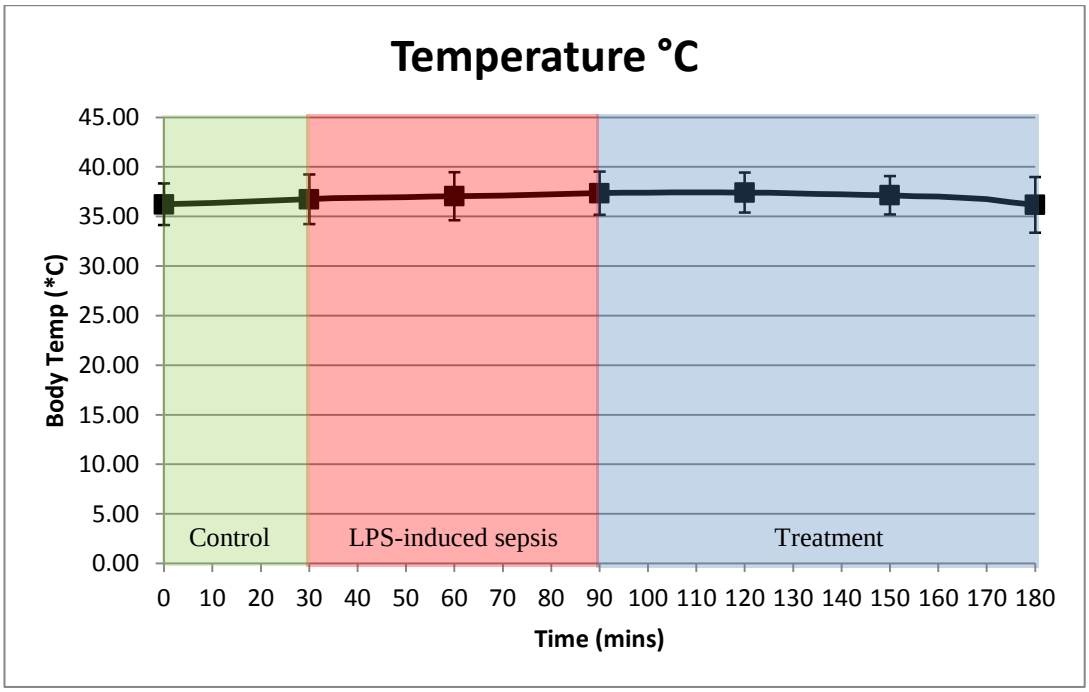

Figure 60: Phase 3: Extracorporeal Study. Body Temperature over three hour time course, degrees centigrade against minutes elapsed

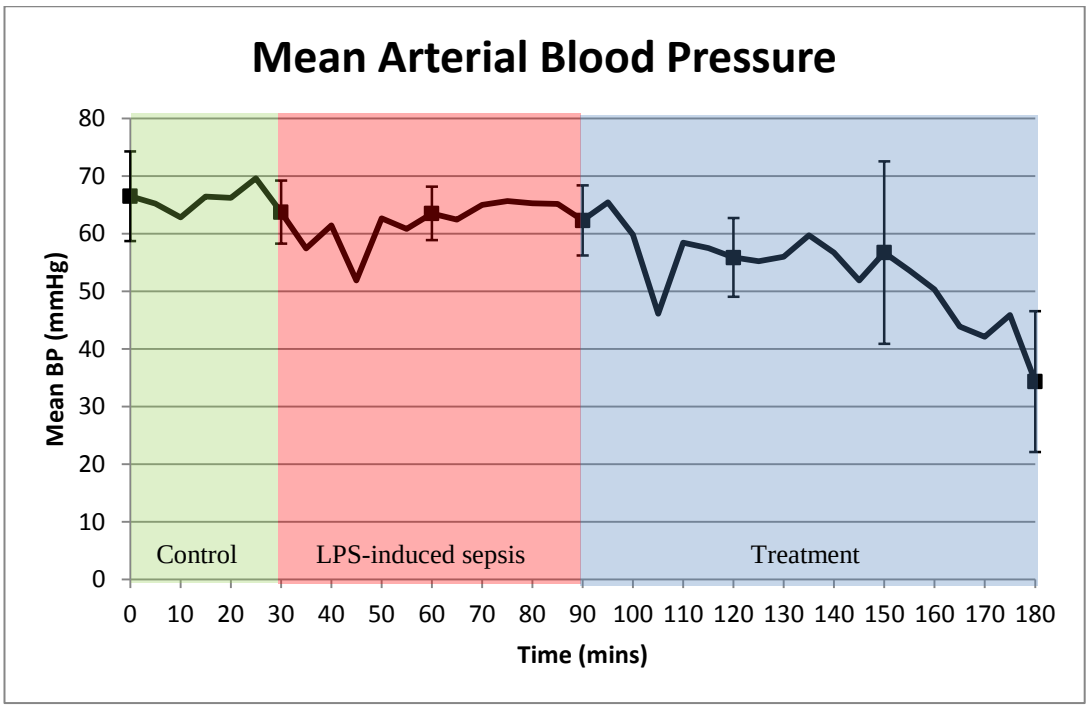

Figure 61: Phase 3: Extracorporeal Study. Mean arterial blood pressure over three hour time course, millimetres of mercury against minutes elapsed

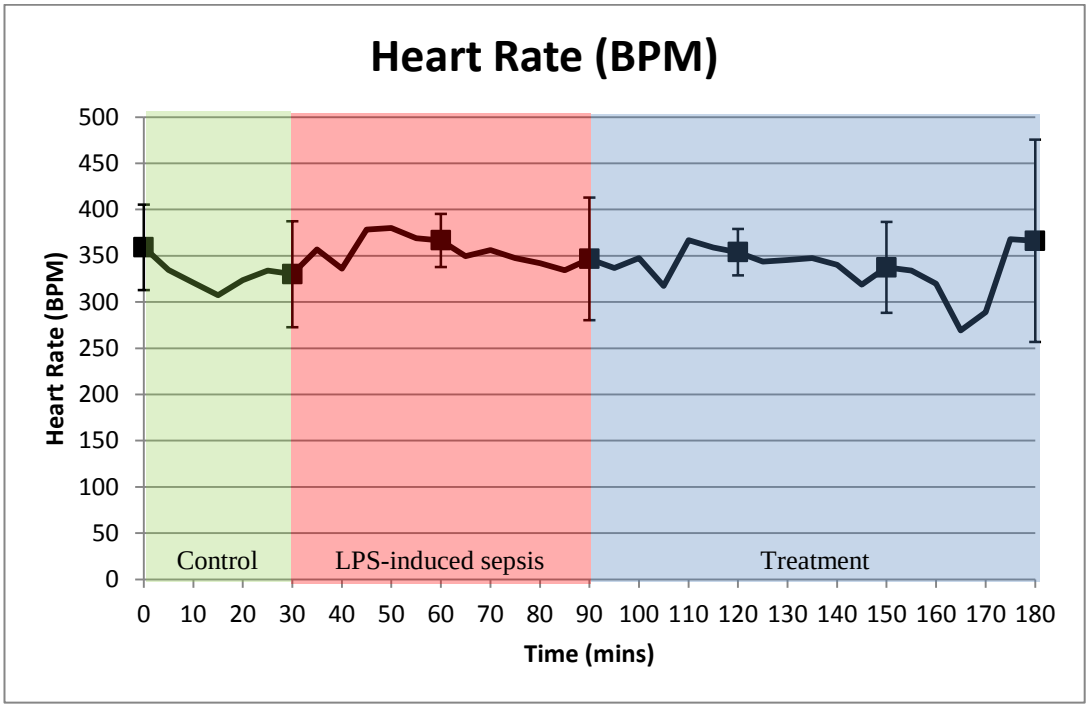

Figure 62: Phase 3: Extracorporeal Study. Mean heart rate over three hour time course, beats per minute against minutes elapsed

The results of the ELISA tests are displayed below. As these were not monitored continuously and instead at half hour long intervals, they are displayed as mean readings with standard deviations, displayed in Figure 63 and Figure 64.

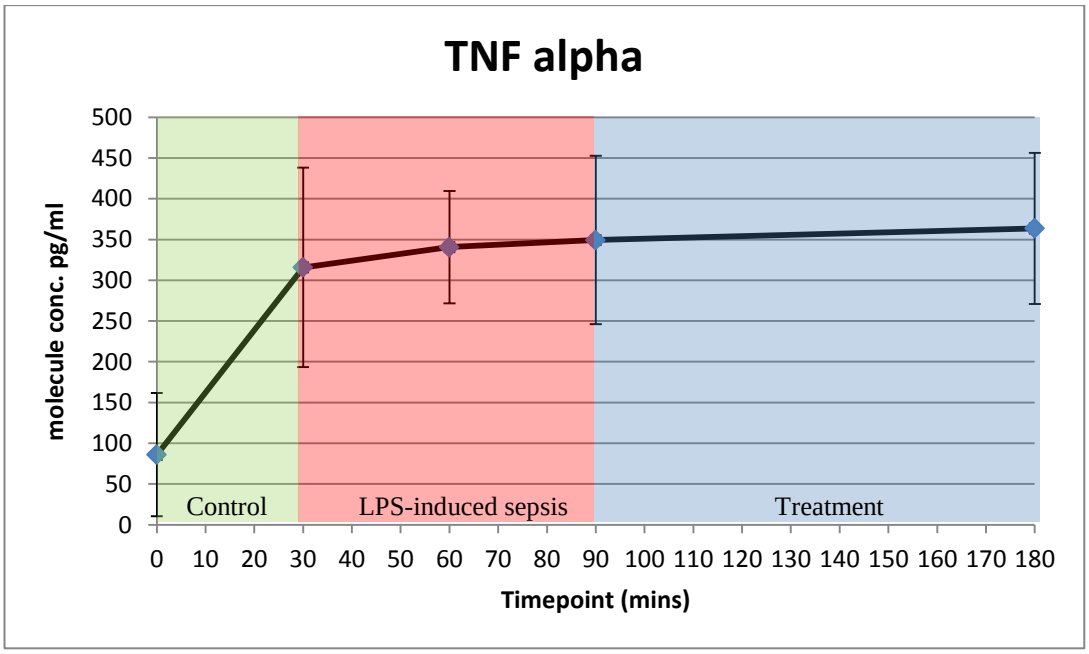

Figure 63: Phase 3: Extracorporeal Study. Mean and standard deviation TNFa ELISA results, molecule concentration pg/ml against minutes elapsed

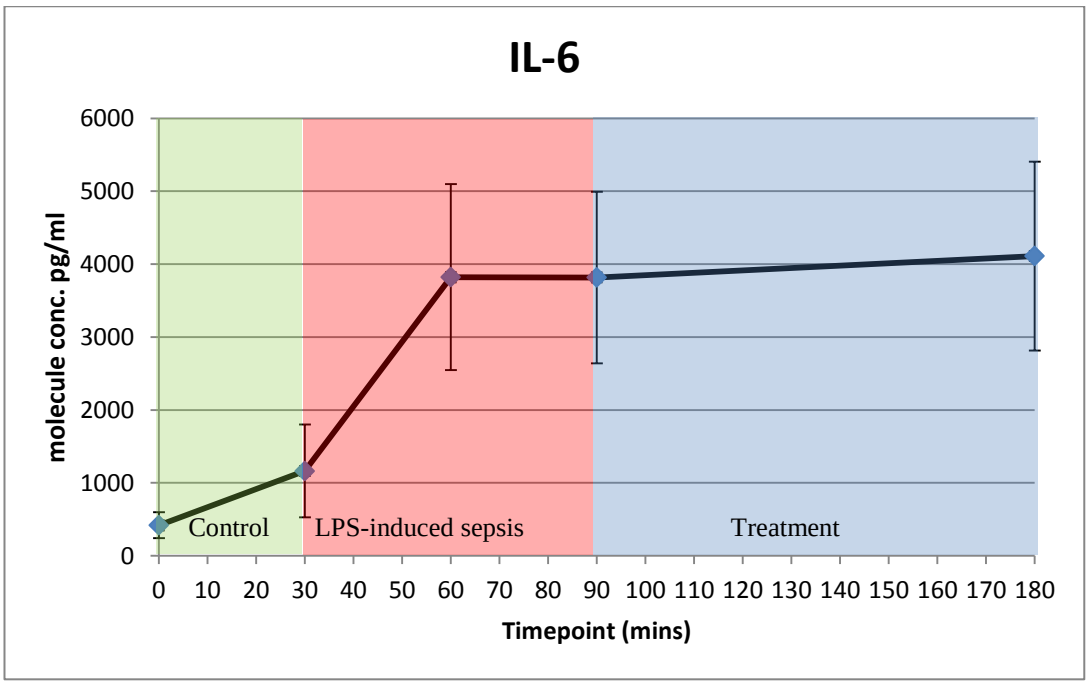

Figure 64: Phase 3: Extracorporeal Study. Mean and standard deviation IL-6 ELISA results, molecule concentration pg/ml against minutes elapsed

4.5 Discussion: Phase 1, 2 & 3

This section provides discussion on Phase 1, 2 and 3 of experimentation. The discussion will consider both experimental aspects and results. Experimental aspects and procedures will be discussed in order to address the first thesis hypothesis identified in chapter 3.0, stating that a valid analogous model of early sepsis can be created and utilised for experimentation to evaluate a miniaturised extracorporeal circuit for testing treatment modalities. Results will be discussed in order to address the second hypothesis identified in chapter 3.0 that an extracorporeal haemoperfusion circuit will mitigate or halt the progression of the sepsis condition, extending the window for interventional therapy.

4.5.1 Phase 1: Pilot Study. Discussion of Results

At the conclusion of phase 1, a stable control animal model and experimental procedure lasting 6 hours that provided a means to monitor key characteristics had been established, albeit with elements for refinement. The observed readings displayed in Figure 49, Figure 50, Figure 53 and Table 13 can be discussed in relation to the pathophysiology of the model. It should first be stated that there can be significant alterations between individual animals, each will feature its own baselines of particular variables within a generally accepted range and each will respond as individuals to any given procedure or treatment (Suckow et al., 2006, Tuffery, 1987), much the same as humans do. Additionally, as only one animal was utilised for this phase, no truly statistically significant conclusions can be drawn from phase 1, but it was used to determine whether or not the model was a valid avenue for a further investigations, particularly as it is a requirement of the UK Home Office that no animal work is carried out needlessly.

Beginning with the observed mean arterial blood pressure, the early readings (0-60 minutes) can be argued to be the individual animal equilibrating to a resting state following the set-up procedures, as with any of the variables being investigated. This initial time period could arguably vary depending on each animal's individual response to the anaesthetic, environmental temperature on the day of experimentation, or perhaps other environmental factors, for instance, the level of background noise in the laboratory depending on which pieces of equipment were being utilised by other staff. All reasonable attempts were made to ensure any such environmental factors were kept to a minimum. However, it is not possible to state

with certainty this was always completely possible. There are peaks in the readings at approximately each hour of the time course, this is attributed to blood samples being taken at these intervals. The blood samples were being collected from the same cannula as the blood pressure readings were being recorded. Accordingly around the hourly blood sample collection intervals, the readings are prone to seemingly sudden troughs or peaks. It was initially hypothesized that if the experimental layout were redesigned so that the readings were being measured from an alternative vessel cannulation, this would be reduced. However, such a significant volume of blood is being drawn that the effect on the animal's blood pressure systemically would likely still be profound. Taking the measurements from an alternative vessel may slightly mitigate this but not significantly. Further, the opening of an additional surgical sight presents more risk than the limited benefit this would present at this time.

Body temperature is a factor significant to the survival of an animal model over a given time course. It has been mentioned that the animals were maintained on a heat mat throughout the experiments. The intention was to utilise the mat in order to maintain body temperature, which was adjusted according to the dynamic monitoring of body temperature in the model. Accordingly, there are undulations in the data trend between rise and fall in body temperature reflecting these interventions. However despite these, it was found that the rat was cold at the start of the experiment. This could be attributable to a cold environment and that the rat required time to acclimatise. It has also been suggested by some authors that inhalant isoflurane, such as in the initial dose, can be responsible for low body temperature readings (Remick et al., 2005). Once placed onto the mat, the body temperature returned to a normal range quickly, but then continued to rise towards a dangerous

level at around 50 minutes, despite the mat being utilised at its lowest setting. Accordingly, the mat was turned off and the rat's body temperature returned towards a normal range. At around 100 minutes, the mat was switched on again to observe the effect; it was found to rise quickly towards a dangerous level once more. After this point, it was concluded that the rat appeared to be self-regulating body temperature reasonably comfortably and that the use of the heat mat was only pushing the rat towards hyperthermia. The mat was not turned on again, subsequent dips in temperature coincide with the timing of either blood draws or administration of fluid but the rat appear to comfortably regulate body temperature back to a normal range. In future phases, the heat mat was only utilised to provide an insulated surface on which the rat would lie, as opposed to the laboratory bench top, for the duration of the experiment, this also meant that further readings related to body temperature would be uncomplicated by any external means.

Regarding perfusion it can be seen that the model appeared to remain well perfused throughout the course of the experiment. However, the reliability of this measurement is debatable. From a practical experimental perspective, it takes a considerable amount of time for the scan to complete, approximately 5 minutes. During this time, slight movements can occur, perhaps best demonstrated in the 120 minutes image in Table 13, which prevent accurate capture. Given the difficulties associated with restraining the rat in a supine position, moving the rat into position for each scan, which in itself carries risk of dislodging the catheter, and attempting to ensure appropriate alignment is a difficult task prone to experimenter error. The placement of the limbs in relation to the scanner is not necessarily a concern in the horizontal plane but in the vertical plane the distance from the scanner is a critical

dimension and deviations in this parameter from scan to scan or worse during a scan are likely to produce erroneous results if indeed they can be successfully gathered. However, it was felt that the information would be useful in evaluation the validity of a haemoperfusion treatment modality, the intention of later phases, and at the beginning of stage 2 it was intended to persevere with this measure.

Combining the recorded information on mean arterial blood pressure, heart rate, body temperature, perfusion and observational evidence during experimentation, it is concluded that a suitably stable control model is possible and that parameters of interest can be measured. The purpose of phase 1 investigating the feasibility of a suitable control model has been completed and further study in subsequent phases can be recommended.

The first and most significant limitation of the work carried out is the size of the study. However, this was both anticipated and required. Animal work of any nature requires significant commitments of equipment, time and expense, but above all the welfare of the animals involved must be considered, not only from an ethical perspective but also as a legal requirement under the UK Home Office. As such, this study provided data which can confirm minimal distress of any form to the animals involved, while still producing a model capable of recording suitable data for the study.

The time course of six hours is a selection (based around literature indicating that cytokine expression should be maximal at six hours, which is required for subsequent experimentation). Due to legislation regarding the housing and welfare of animals in the laboratory, this is also a stipulated time course, which is acknowledged as a limitation of the experiment and beyond these time limits may not truly reflect a clinical patient timeline in the longer term progression of sepsis. It would be a recommendation for future work that longer term study be undertaken.

It was a key recommendation that before proceeding to phase 2, equipment capable of data logging for the cardiovascular parameters should be investigated. This would allow greater reliability in recording and remove experimenter error.

Key Outcomes of Phase 1

The purpose of phase 1 (in conjunction with the following phases 2 and 3) was to partially evaluate the first thesis hypothesis stated in Chapter 3.0, that a valid analogous model of early sepsis can be created which will incorporate a miniaturised extracorporeal circuit for the testing of adsorbent (or other extracorporeal intervention) as a potential therapy for the condition.

The following is a concise summary of the key outcomes of experimentation phase 1: Pilot Study

- It is possible to successfully maintain a rat model, with minimal to no severity in a non-survival experimental time course of 6 hours.

- It is possible to draw up to 1ml blood samples each hour for subsequent study.

- It is possible to dynamically monitor physiological parameters used to indicate sepsis in the condition clinical presentation.

- Blood pressure readings can be dramatically affected by hourly blood sample collection.

- Animals restrained in supine position expire due to some form of pulmonary congestion.

- Use of the heat mat resulted in hyperthermia.

4.5.2 Phase 2 Control & Sepsis Models. Discussion of Results

In phase 2 of experimentation, refinements were made based upon the outcomes from the phase 1, which will be discussed in this section. It was sought to refine the model to be comparable to the acute phase physiological symptoms of a systemic sepsis condition in humans and complete a valid run of both control and sepsis models. Sepsis was induced by intraperitoneal injection of lipopolysaccharide. Key physiological characteristics were monitored and recorded over a six hour time course, in order to establish the suitability of the induced sepsis model. Heart rate, body temperature, mean arterial blood pressure, and perfusion in the periphery were measured, although it became apparent early in the phase that the perfusion measurement would not be practically feasible or reliable. Two cytokine markers for the condition were also investigated TNFα and IL-6. Experimentation was conducted using two groups of animals; one group of three control and one group of three LPS-induced sepsis models were analysed. The objective of the study was to develop a repeatable, accurate model analogous to the clinical human sepsis condition. The data gathered would appear to indicate that the model fulfils this purpose, presenting an indicative physiological response.

In phase 2 three control models and three LPS-induced sepsis models were conducted over a 6 hour time course. The results of both physiological monitoring and ELISAs can be discussed in relation to the pathophysiology of the sepsis condition. It is also noteworthy that animals like human patients exhibit their own individual baselines and unique variations of normal physiology within a generally

accepted range (Suckow et al., 2006, Tuffery, 1987). Therefore some margin of fluxuation must be allowed for this. However, clear trends can be seen in the results.

Beginning with the observed mean arterial blood pressure readings, a clear trend can be seen in both the control and LPS-induced groups. The control group maintains a predominantly constant, if erratic, trend. This would be expected as other than the comparatively mild suppression of cardiovascular function associated with any procedure involving anaesthetic, no other factors were imposed upon the control models. The erratic nature of the readings can be attributed to the fact that the sensor used to obtain this reading is connected to the caudal artery cannula. This cannula was also used to draw blood samples, so the signal was iteratively interrupted, which is the cause of the sudden drops and odd minor peaks at approximate one hour intervals when the sample is taken and the line is flushed to be kept patent respectively. The LPS-induced group however exhibits a plateau until around 120 minutes then a steadily decreasing trend. As was discussed earlier, in accordance with an early hyperdynamic phase followed by a hypodynamic phase, an early rise in BP followed by a gradual decrease could be anticipated, the decrease is certainly visible but the early readings could only be described as steady at best. Nevertheless, the marked deviation in physiology between the LPS group and control group is notable and in keeping with documented theory.

Heart rate has been reported to become tachycardic in the hyperdynamic phase of sepsis, before becoming bradycardic in the hypodynamic phase. The trends displayed in the results for the LPS group display a rise in HR until around 120 minutes then the trend becomes a predominant decline, whereas the control group has an overall

constant trend, if very erratic. The readings are within a very similar range. Therefore, it is questionable how much validity can be read into such findings. It is possible that due to the relatively low sample size, an insufficient spread of physiological characteristics is represented and the variability between those present is cluttering the trend.

Body temperature has presented an interesting set of results. The LPS group experienced a rise in temperature before falling back to normal range, followed by a dramatic drop towards the end of the time course. This would appear to be in line with theory on a rise in temperature due to a hyperdynamic "warm shock" phase of sepsis followed by a drop in a hypodynamic "cold shock" phase. However, the control group also experienced a similar rise which continued throughout the time course. The problem is that the normal body temperature for these animals is approximately 37.5ºC, in an accepted range of 36.2 to 38.2ºC (Suckow et al., 2006). In light of this, it can only be commented that the animals appeared to be normalising their temperature over the first hour and a half. At this point in the project, it was not conclusively known as to why their temperatures started below the normal threshold, it is hypothesised that the animals required time to become acclimated to their environment. As has been mentioned in discussion of phase 1, on occasion isoflurane has been known, due to its suppressor effect on cardiopulmonary circulation, to be associated with a decrease in body temperature but only in very isolated cases, so it cannot be commented on with any degree of certainty that this could be the cause. It is therefore difficult to ascertain the behaviour of temperature changes in both groups. It could perhaps be attributed to an environmental factor in the experimental set-up, although it should be reiterated that no heat mat was utilised for the three

control and three LPS models from which results were gathered. It could be due to circulating levels of pyrogens (IL-6 is a known pyrogen) following the insult during placement of the caudal cannula or a reaction to the particular anaesthetic utilised. Overall, following the initial normalisation of body temperature, there is a clear distinction between the control and LPS groups. However owing to the unusual start of the experiment in this parameter, further study was required before a conclusion can be drawn on the matter.

Combining these results, there does appear to have been an alteration in the physiology of the LPS group compared to that of the control group, with particular onus on the approximate 120 minute mark. It would also appear that the condition of the LPS group would continue to worsen as time progressed beyond 6 hours. However, some minor issues with the experimental set up have also been identified, principally disruption of data logging signal during blood sample collection. This is acknowledged as a limitation and while options including opening an additional surgical site and cannulation of an additional vessel were considered, the associated complications would not present sufficient benefit for justification, ultimately the volume of blood being extracted will have a systemic effect upon these measurements regardless. The physiological data should now be combined with that of the ELISA.

When reviewing the information in Figure 55 and Figure 56, it is clear immediately that there is a difference between the control group and the LPS group. In the control group there is a mild rise in serum levels of both IL-6 and TNFα. However, this can be attributed to the insult caused by the placement of the caudal artery cannula before

it returns to a normal level for the remainder of the time course. In the case of the LPS induced model however, there is a rise and a sustained elevated level of both cytokines.

Both of these cytokines are considered key markers of the sepsis condition. However, the TNFα levels found in patients with other disorders are often not markedly different from those found in most patients with sepsis. (Roger C. Bone, 1991) However, there is a direct correlation between IL-6 peak serum level and TNF-α peak serum level during acute septic shock (Pierre Damas et al., 1992). While it has been established that the progression of the condition is extremely complex and still the subject of much investigation, amongst all the possible pathways, TNFα is the single most common factor independently capable of triggering the associated pathophysiology (Schetz et al., 1995). No doubt partly for this reason for a significant period of time TNFα was believed to be the central mediator of the condition. However as further work has been carried out on TNFα, it has become clear that TNFα levels are elevated in a wide variety of conditions other than sepsis, thus it can be described as a nonspecific mediator of inflammation with additional noninflammatory roles (Roger C. Bone, 1991). However, it is listed frequently as a mediator of great importance in the promotion of sepsis, acting to initiate and propagate the inflammatory cascade (Vriese et al., 1999).

It has also been documented that at the time of admission to ICU, 32 out of 37 patients presented greatly elevated levels of IL-6, which were associated with sepsis patients related to haemodynamic and biochemical parameters, as well as clinical outcome, noting that levels of IL-6 on admission appeared to be of prognostic

significance: levels were higher in septic patients who subsequently died than in those who survived (Hack et al., 1989). It has also been demonstrated that in animal models IL-6 serves as a marker of disease severity in sepsis and does modulate some physiological responses (Remick et al., 2005). However, the same experiments concluded that a complete lack of IL-6 does not alter mortality due to sepsis, again indicating that like TNFα, IL-6 alone is not a central mediator. Unlike TNFα, which exhibited in peaks early in the progression of the condition, IL-6 is consistently present at elevated levels throughout the early phase of the condition, correlating at high levels to mortality (Pierre Damas et al., 1992). The clear correlation between TNFα and IL-6 was also commented upon in this study.

It would therefore appear that in combination of the physiological events and cytokine levels, the LPS-induced model falls within the criteria of sepsis.

Key Outcomes of Phase 2

The purpose of phase 2 (in conjunction with the previously completed phase 1 and the following phase 3) was to partially evaluate the first thesis hypothesis, stated in Chapter 3.0, that a valid analogous model of early sepsis can be created, which will incorporate a miniaturised extracorporeal circuit for the testing of adsorbent (or other extracorporeal intervention) as a potential therapy for the condition.

The following is a concise summary of the outcome of experimentation phase 2: Sepsis & Control Models.

- It is possible to draw 1ml blood samples each hour for subsequent study.
- Animals kept in the supine position, even without restraint, expire due to pulmonary congestion.
- The intraperitoneal LPS dose appeared to propagate a systemic sepsis condition.
- Physiological data would appear to suggest a presentation of a sepsis condition.
- ELISA results would appear to suggest a presentation of a sepsis condition.
- Following this phase of experimentation, it is now possible to progress to evaluation of the adsorbent.

4.5.3 Phase 3 Extracorporeal Haemoperfusion Study Discussion

The objective of phase 3 is to provide a means to evaluate the suitability of the adsorbent as a haemoperfusion treatment modality for sepsis, assessing the effectiveness of the therapy against the induced sepsis model developed in phase 2. Ultimately, the approach and protocol adopted were not successful in achieving this objective. However, they did provide valuable insight for reflection and development of future work. Phase 3 will be discussed throughout this section.

The results of phase 3 are presented in Figure 60, Figure 61, Figure 62 and Figure 63. These should be considered in relation to the stages the extracorporeal circuit demonstrates in the clinical condition:

The first thirty minutes demonstrate that there is some change, which would be expected due to the inflammatory response associated with extracorporeal circulation. However, this is markedly different from the change in parameters following sepsis inducement by administration of LPS at the thirty minute mark, where a dramatically greater response is observed. By its very nature, extracorporeal circulation induces a number of physiological processes, including an activation of the inflammatory response in its own right, ahead of the administration of LPS to induce sepsis as established in phase 2. This is clearly observed in the rise of cytokines over the first 30 minutes, especially TNF-α.

However, the animals were appearing to become hypovolemic, most likely due to the volume and frequency of samples. As such, it became impossible to draw 1ml of blood at a 30 minute interval after 120 minutes and such blood samples are missing for the time point at 150 minutes.

Beginning with the observed mean arterial blood pressure readings, a clear overall trend can be seen. In the first thirty minutes, the readings fluctuate but stay within a normal range. Interestingly following administration of LPS, blood pressure drops initially then returns to a normal range. At 90 minutes after connection of the haemoperfusion column, the trend begins to steadily drop. In previous sepsis models,

LPS-induced animals exhibited a plateau until around 120 minutes then a steadily decreasing trend, which is consistent with theory on the condition. It would appear that this trend is persistent in the extracorporeal circuit too. However, the time course may not be sufficient to allow any meaningful effect. The blood pressure fall could also be linked to the low volume of blood left in the rats circulatory system at this stage in the experiment, as has been mentioned blood draws every 30 minutes is pushing the limit of recommended sampling volume, if not breaking it.

Regarding heart rate, it would be anticipated that after administration of LPS there would be a tachycardic response. While a rise is witnessed between 30 minutes and 50 minutes, it is not especially drastic and still falls within a normal classification (albeit towards the upper limit of normal) before gradually decreasing then maintaining the same trend following addition of the column before a more marked decrease then quickly returning to a higher rate, although the deviation here is wide suggesting it may have been an abnormally heightened response in just one rat.

Body temperature remained within a normal range throughout the time course, predominantly rising over the first 90 minutes then predominantly falling over the remaining 90 minutes. Little can be drawn from this observation expect that the 60 minutes following LPS administration is not ample to invoke the overt change in this physiological parameter and that the same can be said for the effect of the adsorbent in the subsequent 90 minutes. However another interesting conclusion which can be drawn settles an inconclusive point which occurred in phases 1 and 2 of experimentation (section 4.4.1 and 4.5.2 respectively). In phases 1 and 2 early in the time course, wide variances were observed in temperature readings. At the time of

review, it was hypothesised that the animals were normalising their body temperature over the first 60 to 90 minutes following transport to the laboratory and the inconsistent environmental temperature within the laboratory. The significantly longer surgical time in this newer series of experiments varied between 90 minutes to 120 minutes, allowing the animals suitable time to normalise their body temperature before data logging began, which has resulted in the low degree in variance in the body temperature readings confirming this theory.

Overall, these observations unfortunately provide no clear information as to the effect, if any, of the adsorbent on the progression of a sepsis condition in the rat. At best, it can be said that there appears to be no adverse effect due to the adsorbent. The impact of the extracorporeal circuit in the initial 30 minutes elicits its own minor inflammatory response. The 60 minute time course following LPS administration may not be sufficient to allow sepsis to fully take hold systemically and as such propagate the associated overt physiological changes. Inclusion of the column and associated 90 minute therapeutic window may not be sufficient to demonstrate what effect the adsorbent is having on the circuit and consequently the rat.

The ELISA results in Figure 63 and Figure 64 are of a similar nature to the physiological characteristics. They both demonstrate a response to blood being circulated extracorporeally with TNFa demonstrating the more aggressive response. Both also display similar value ranges to the LPS induced sepsis models in previous experiments. It can then be said that it would appear the sepsis response is taking effect but that the adsorbent has not been allowed sufficient time to have any impact on this, a similar tentative conclusion to the physiological characteristics.

It would therefore appear that the experimental set-up as it exists currently requires refinement in order to establish meaningful data on the impact of the adsorbent on the progression of the condition.

There were numerous issues encountered throughout the investigation and it was necessary to undertake significant investigation as to how these should be addressed. At this point, the most notable is fluid balance. This had so far been undertaken in an ad-hoc manner, attempting to replace, where possible, the volume of blood withdrawn with administration of other fluids between the sample collections, be it anaesthetic, the 50% saline 50% gelofusine solution or heparin,. This must be more tightly controlled.

It could also be suggested that the anaesthetic is presenting more of a cardiovascular depression than initially suspected, in which case it could be argued that an alternative method might be a valid area of investigation. If this were the case, it could be advised that a continual intubation of isoflurane might be used. This would also help to maintain a tighter control on fluid balance as the anaesthetic is administered when necessary, taking priority over fluid balance as the animal must remain unconscious and compliant for the duration of the experiment. However, isoflurane is significantly more unpredictable and can have adverse effects on body temperature, again a consideration requiring much investigation.

It could also be argued that experimentation should run with three groups to investigate the circuit, the progression of the condition and the progression of the circuit and haemoperfusion therapy. This would provide a means of separating and identifying the various effects of each situation. A further benefit to this approach would be that it would be possible to assess the impact the blood circulating

extracorporeally has on the systemic response alone as a control group. However, this would require the use of significantly more animals and requires careful consideration.

The procedure also needs to run for a longer time course, as such the level of heparinisation must be maintained appropriately to prevent blood clotting at any point in time, either a regulated dose delivered at a regular interval or some form of continual infusion should be considered.

It is clear that withdrawing a 1ml volume of blood at a 30 minute interval is simply too much. This is the most likely cause for many of the issues in contributing to the unsuccessful outcome of phase 3 completion of the time course.

Key Outcomes of Phase 3

The purpose of phase 3 (in conjunction with the previously completed phase 1 and phase 2) was to partially evaluate the first thesis hypothesis stated in Chapter 3.0, that a valid analogous model of early sepsis can be created, which will incorporate a miniaturised extracorporeal circuit for the testing of adsorbent (or other extracorporeal intervention) as a potential therapy for the condition.

Phase 3 was additionally intended to address the second thesis hypothesis, stated in Chapter 3.0, that the deployment of adsorbent in an extracorporeal haemoperfusion circuit will mitigate or halt the progression of the condition, extending the window for interventional therapy. Phase 3 provided inconclusive and incomplete information to address this and as such was not successful in achieving this purpose. However significant lessons were learned and accordingly a redevelopment and an additional phase of experimentation would be required.

The following is a concise summary of the outcome of experimentation phase 3 extracorporeal haemoperfusion study:

- The shorter three hour time course is not sufficient to provide meaningful insight into the efficacy of the haemoperfusion circuit as a treatment modality.

- It is not possible to draw 1ml blood samples at 30 minute intervals over the three hour time course.

- Animals appeared to be becoming hypovolemic due to excessive blood sampling and inadequate fluid balance.

- Heparinisation should be more closely observed to ensure coagulation is avoided in the extracorporeal circuit.

- It is not possible to adequately distinguish between control, induced-sepsis and haemoperfusion treatment modality utilising this experimental procedure.

5.0 Concept Redevelopment

Phase 3 was unsuccessful in achieving its objective. However, numerous lessons were learned and it was proposed that with refinement to the experimental procedures it would be possible to achieve the project aim of evaluating the effects of the adsorbent as a potential haemoperfusion treatment modality. A 4th phase of experimentation was proposed.

Phase 4 would utilise 3 separate groups of rats as in Table 14:

Group	Set-up
Control Group:	Rats in this group would undergo the same procedures and be connected to the same extracorporeal circuit as the other groups. However without LPS and without deployment of the adsorbent contained in the haemoperfusion device.
LPS-Induced Sepsis Group:	Rats in this group would undergo the same procedures and be connected to the same extracorporeal circuit as the other groups. They would be subject to the IP injection of LPS however without deployment of the adsorbent contained in the haemoperfusion device.
Treatment Group:	Rats in this group would undergo the same procedures and be connected to the same extracorporeal circuit as the other groups. They would be subject to the IP injection of LPS however they would additionally be subject to deployment of the adsorbent contained in the haemoperfusion device. Which was deployed in the circuit 30 minutes after the start of the experiment allowing the LPS to circulate first.

Table 14: Groups for experimental phase 4.

Each group would consist of 3 rats. The same physiological parameters and mediators would be investigated as in phases 1, 2 and 3. By utilising 3 groups, it would be possible to evaluate the effect on the model of the constituent elements of the extracorporeal circuit, the induced sepsis condition and the haemoperfusion

extracorporeal circuit. The time course would be reverted back to 6 hours as in phase 1 and 2 rather than the shorter 3 hour time course of phase 3.

The purpose of phase 4 is to provide a means to evaluate the suitability of the adsorbent as a haemoperfusion modality for sepsis. The 3 groups to be utilised provide data suitable for assessing the effectiveness of the therapy against the induced sepsis model and the extracorporeal circuit being utilised.

In phase 2, the vessels cannulated were the caudal artery to provide the draw side of the circuit and femoral vein to provide the return side. The femoral artery was cannulated to provide connection to the data logging equipment. However, the challenges encountered in phase 3 suggested that the use of the caudal artery as the draw side for the extracorporeal circuit could be inferior to alternative vessels. Accordingly, it was opted to utilise the femoral artery as the draw side and the femoral vein for the return side of the extracorporeal circuit, the data logging equipment would make use of the caudal artery. Additionally in order to ensure maximum opportunity for adequate blood flow, it was decided to locate the cannulation above the epigastic artery branch rather than below, as had previously been undertaken in phase 3. This arrangement is demonstrated in Figure 65, Figure 66, Figure 67, Figure 68 and Figure 69.

It has been mentioned in the literature that while cytokines are a very valid area of investigation, the longer term investigation of the suitability of an interventional therapy in the treatment of sepsis should not focus on these markers alone. The actual impact to particular organs and their function must also be addressed. Accordingly in

phase 4, it was proposed to undertake organ harvesting at the completion of the time course following euthanasia of the rat. A known effect of sepsis and systemic inflammation is oedema, including the build up of fluid in the extravascular environment and organs. It was intended to harvest the heart, lung, liver, kidney, section of colon, sample of muscle tissue and a sample of brain tissue. In order to assess the extent of fluid present in these organs, and tissue delta dry weight analysis was undertaken. The delta dry weight is a simple but indicative test of the presence of fluid in tissue or organ sample which has proven indicative in previous studies in the context of cardiopulmonary bypass. The process involves collecting a sample measuring the "wet weight" heating in an oven at 80°C to remove fluid and achieve a dry mass, measuring the resultant "dry weight" and calculating the percentage difference. This process was conducted in order to gain a clearer understanding of the effect of both the condition and therapy.

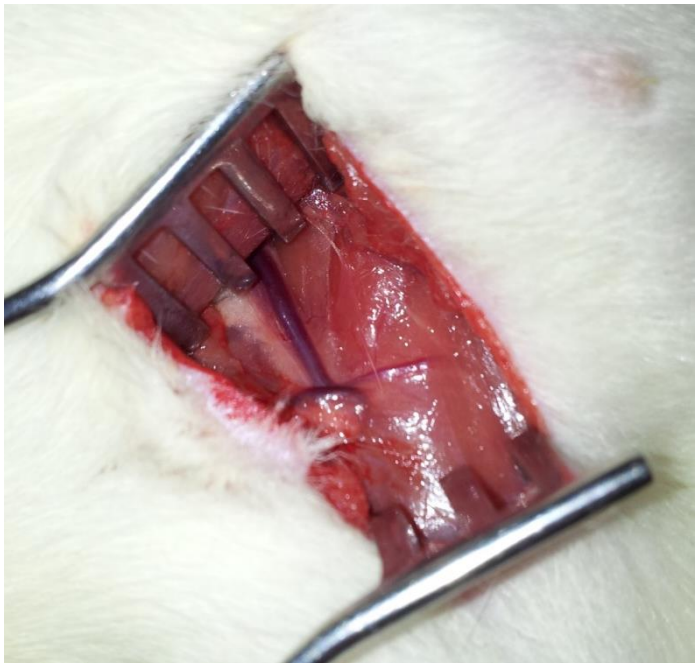

Figure 65: Photograph of femoral surgical site

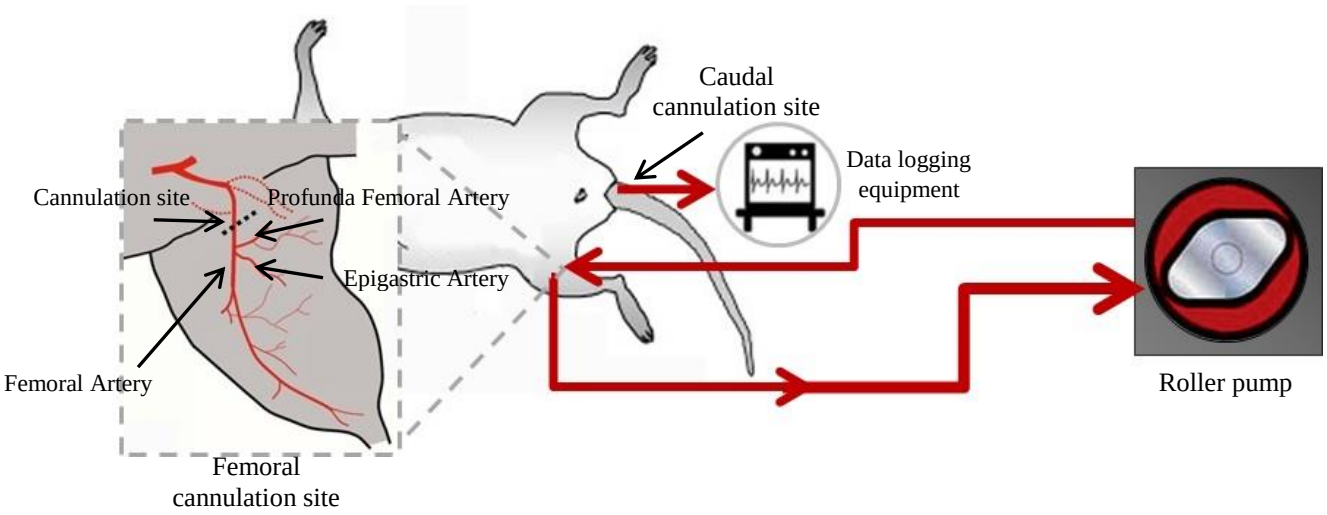

Figure 66: Diagram of experimental set-up for phase 4 control and induced sepsis groups

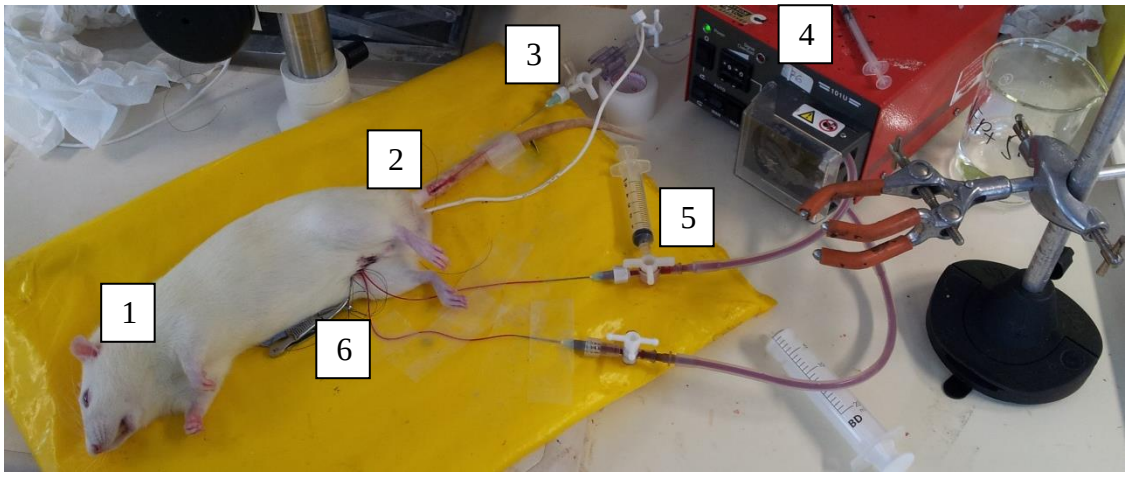

Figure 67: Photograph of experimental set-up for phase 4 control and sepsis groups. Clockwise from left; 1 Sprague-Dawley rat, 2 caudal cannulation site, 3 pressure transducer, 4 roller pump, 5 surgical tubing and anticoagulant flush syringe, 6 femoral cannulation site.

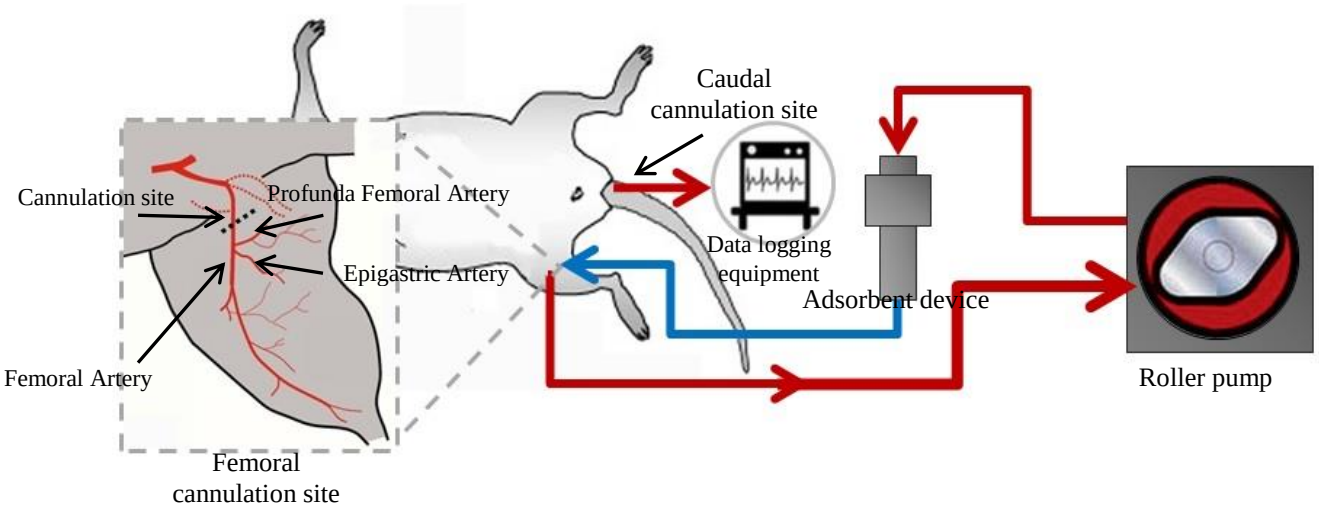

Figure 68: Diagram of experimental set-up for phase 4 treatment group

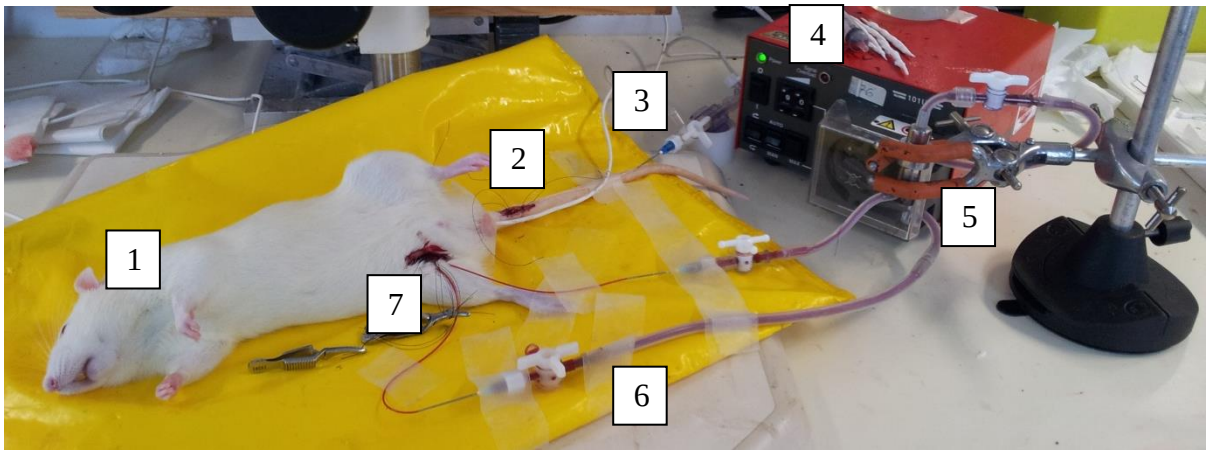

Figure 69: Photograph of experimental set-up for phase 4 treatment group. Clockwise from left; 1 Sprague-Dawley rat, 2 caudal cannulation site, 3 pressure transducer, 4 roller pump, 5 adsorbent device, 6 surgical tubing and 3-way taps for connecting anticoagulant flush syringe, 7 femoral cannulation site.

5.1.1 Methods

In the control group, no LPS or adsorbent was utilised, only the extracorporeal circuit. In the induced sepsis group, sepsis was induced by intraperitoneal injection of lipopolysaccharide. In the treatment group, the adsorbent was deployed in the column device. 3 rats were involved in each group. Key physiological characteristics were monitored and recorded over a six hour time course. Heart rate, body temperature and mean arterial blood pressure were measured. Two cytokine mediators for the condition were also investigated, TNFα and IL-6. Organ and tissue samples were gathered; heart, lung, liver, kidney, colon, muscle and brain to establish their dry weight percentage in each group at the end of the time course. The purpose of the study was to evaluate the efficacy of the adsorbent when deployed for haemoperfusion in this circuit compared to the induced sepsis model and the extracorporeal circuit.

5.1.2 Results

Results are best plotted on graphs shown in Figure 70, Figure 71, Figure 72, Figure 73, Figure 74, Figure 75, Figure 76 and Figure 77. It should be borne in mind that with a small group, the highly variable nature of animal work may have an impact upon the trends observed. 3 rats are represented; the results plotted are the mean of those animals with standard deviation at hourly intervals.

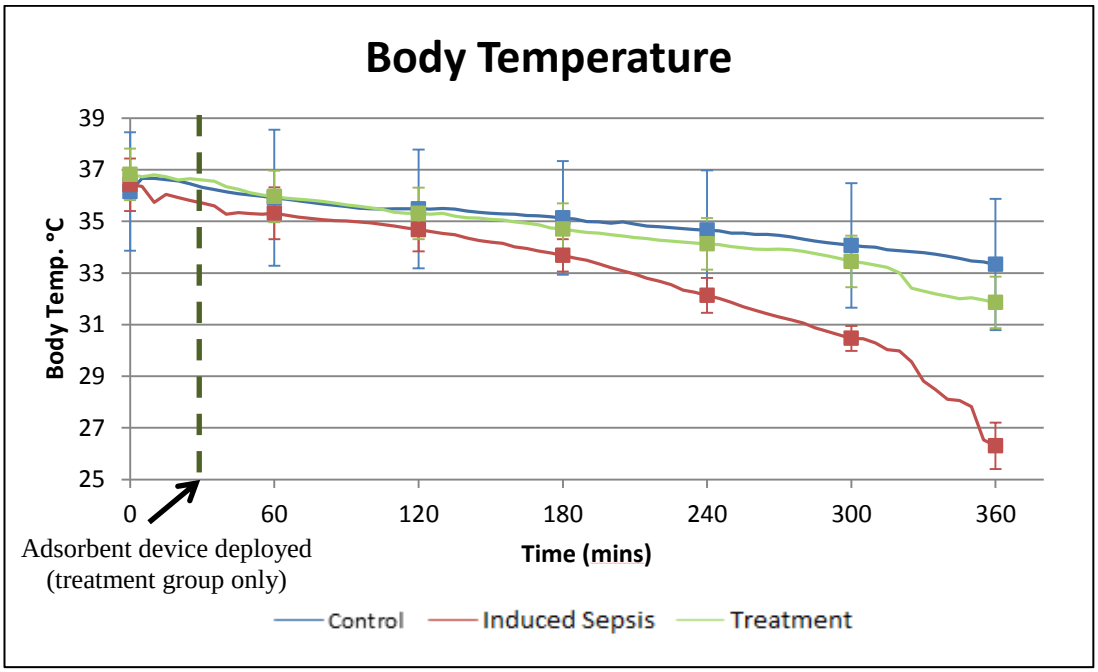

Figure 70: Phase 4: Sepsis, Control & Treatment Models. Body Temperature over 6 hour time course, ºC against minutes elapsed

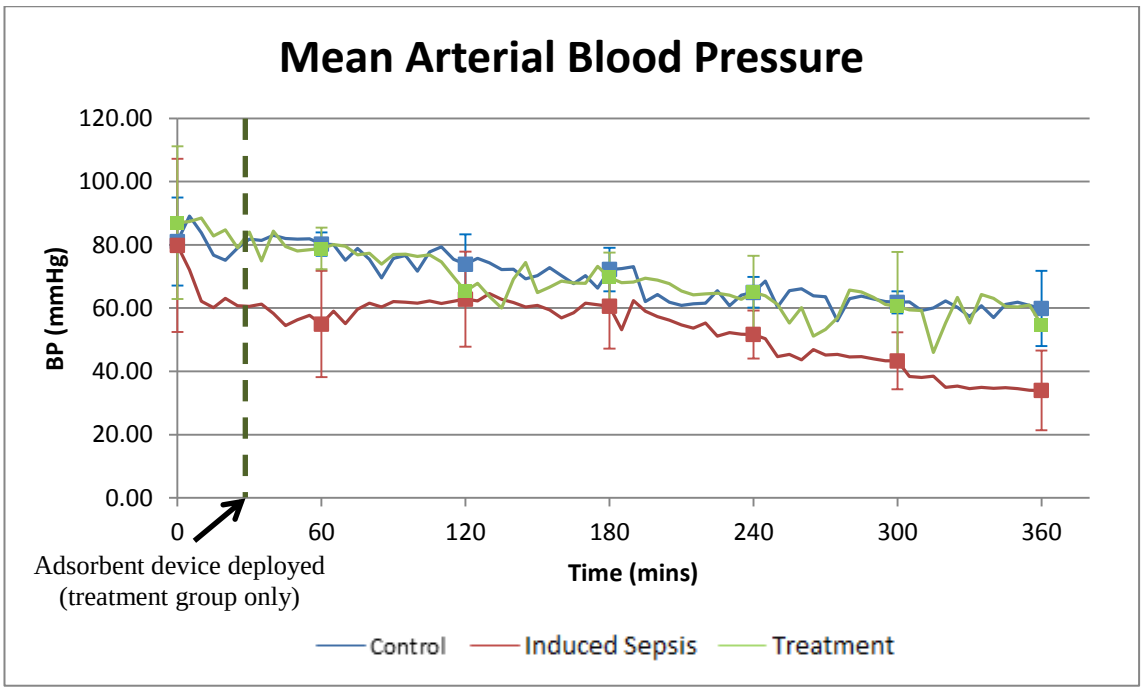

Figure 71: Phase 4: Sepsis, Control & Treatment Models. Mean arterial Blood Pressure over 6 hour time course, mmHg against minutes elapsed

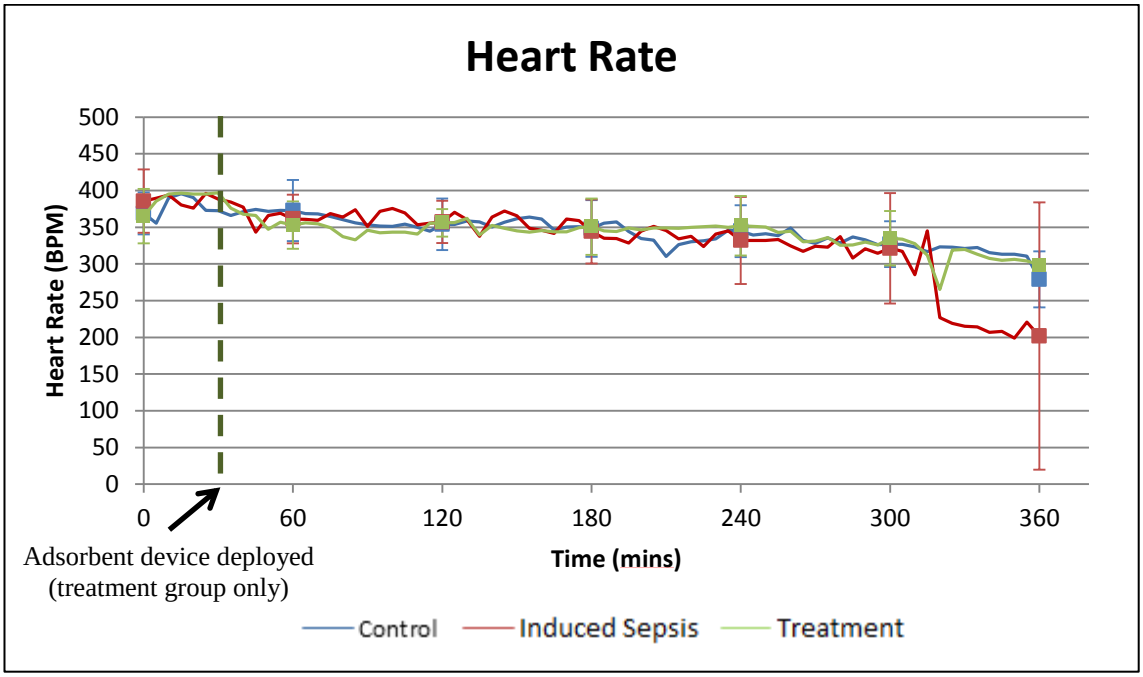

Figure 72: Phase 4: Sepsis, Control & Treatment Models. Heart Rate over 6 hour time course, BPM against minutes elapsed

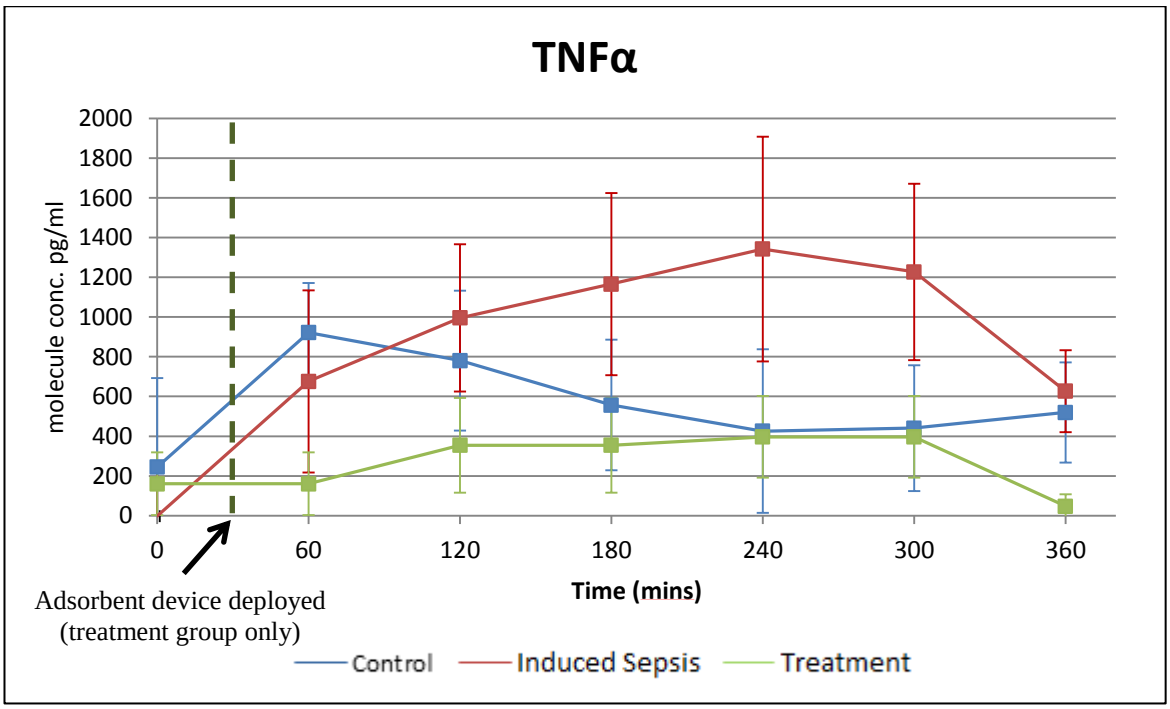

Figure 73: Phase 4: Sepsis, Control & Treatment Extracorporeal Study. Mean and standard deviation TNFa ELISA results, molecule concentration pg/ml against minutes elapsed

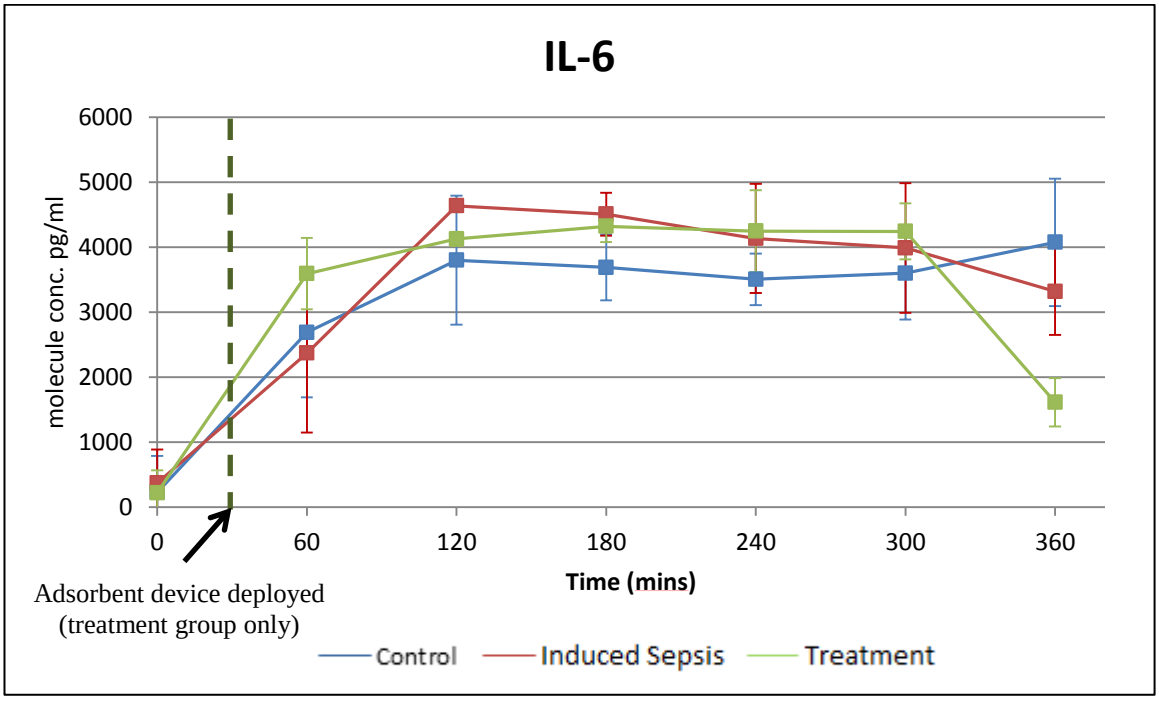

Figure 74: Phase 4: Sepsis, Control & Treatment Extracorporeal Study. Mean and standard deviationIL-6 ELISA results, molecule concentration pg/ml against minutes elapsed

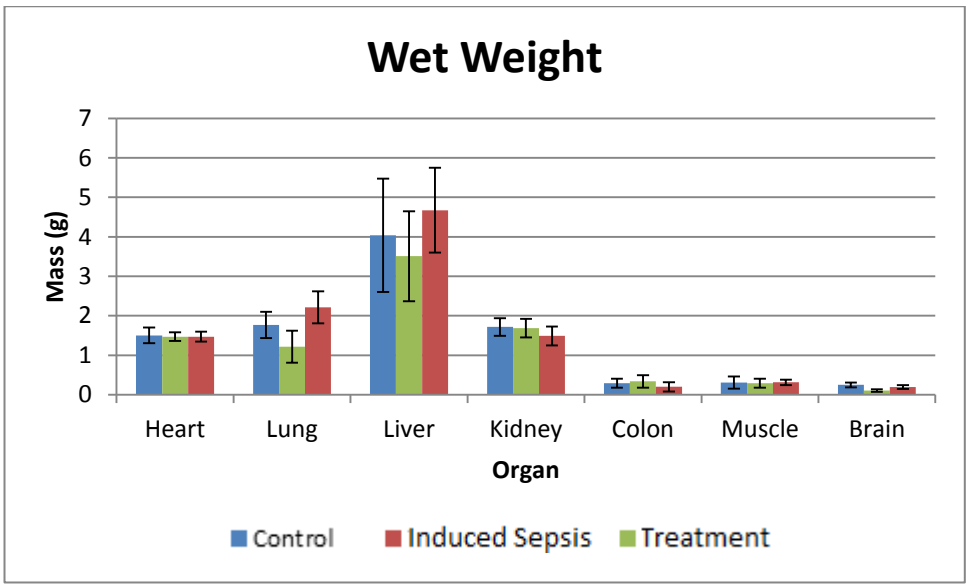

Figure 75: Phase 4: Sepsis, Control & Treatment. Harvested Organs Wet Weight

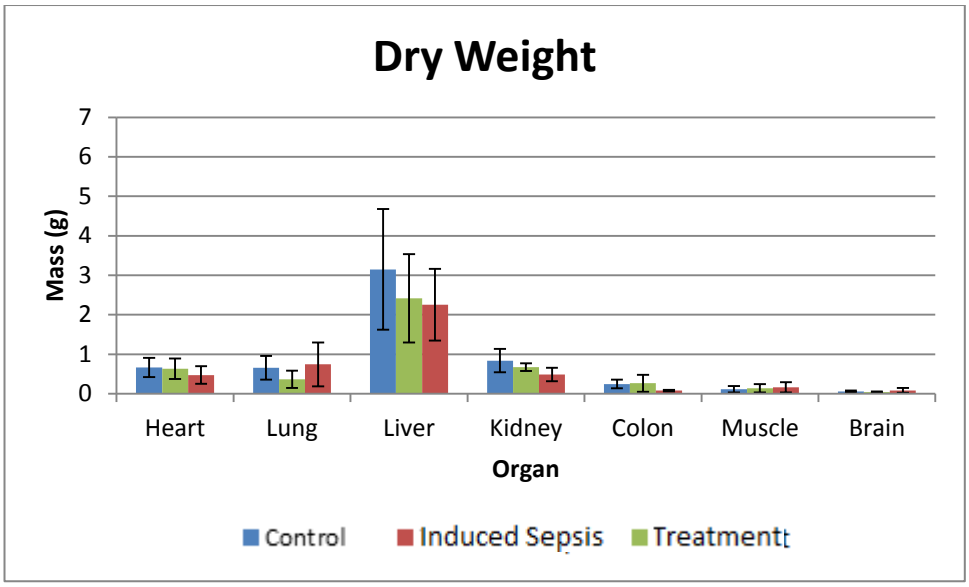

Figure 76: Phase 4: Sepsis, Control & Treatment. Harvested Organs Dry Weight

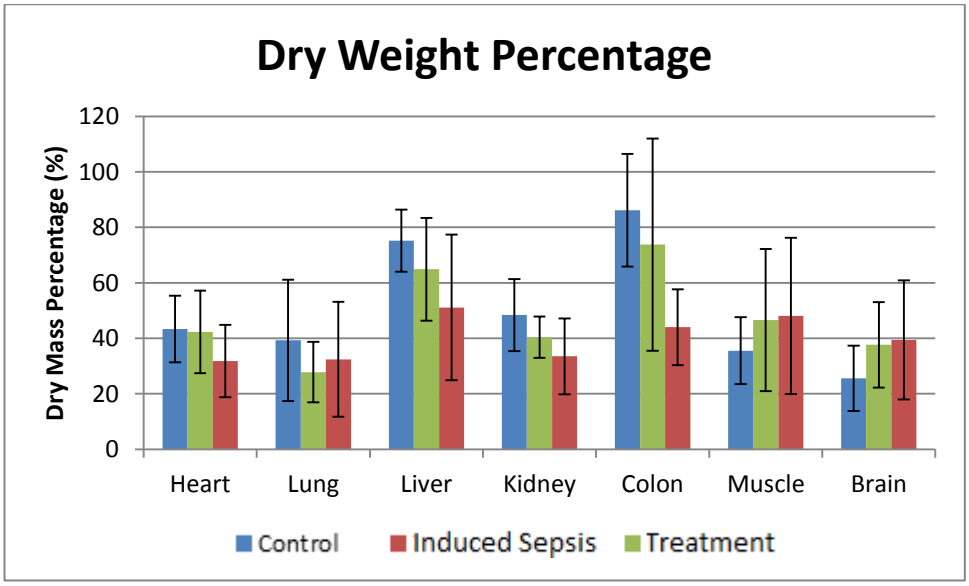

Figure 77: Phase 4: Sepsis, Control & Treatment. Harvested Organs Dry Percentage

5.2 Phase 4: Redesigned Extracorporeal Haemoperfusion Study Discussion

This section provides discussion on Phase 4, the redeveloped extracorporeal haemoperfusion study. Experimental aspects and procedures will be discussed in order to address the first thesis hypothesis identified in chapter 3.0, stating that a valid analogous model of early sepsis can be created and utilised for experimentation to evaluate a miniaturised extracorporeal circuit for testing treatment modalities. Results will be discussed in order to address the second hypothesis identified in chapter 3.0 that an extracorporeal haemoperfusion circuit will mitigate or halt the progression of the sepsis condition, extending the window for interventional therapy.

5.2.1 Discussion of Methods

Animals were anaesthetized, with inhalation of 2ml isoflurane (Abbott Laboratories Ltd Maidenhead) and subsequently injected subcutaneously with an anaesthetic mix containing 1ml Hypnorm (VetaPharma Ltd, Leeds), 1ml Hypnovel (Roche Products Ltd, Hertfordshire) and 2ml sterilised water for injection (W. & J. Dunlop Ltd, Dumfries) used as a dissolvent and diluents. An initial dose of 1.5ml of anaesthetic was used. Subsequent preparations of this 4ml anaesthetic mix were prepared should they be required, over the time course. Constant level of anaesthesia was maintained throughout the experiment by supplemental doses of 0.75 ml as needed (approximately once every hour, although this varied slightly animal to animal).

Following the volumetric challenges encountered in phase 3, it was recommended that greater care be taken in the fluid balance of the rats over the time course, which

had been undertaken in an ad hoc manner. In phase 4 this was carefully monitored, noting specific volumes drawn at any time point and balancing this between the time next blood draw with additional heparin doses and a fluid replacement cocktail composed of 50% saline and 50% gelofusine (gelofusine as a colloid is an effective fluid replacement), administered intravenously, a practice of ensuring 1:1 volume replacement was adopted. This practice was applied in all groups in order to ensure consistency and ensure valid comparison of results.

The new site of cannulation above the epigastric branch on the femoral artery did increase the difficulty of the surgical placement and the associated time required. However, this did not appear to present any significant effects. The cannulation of the femoral artery and vein provided stronger blood flow. In this phase it was possible to achieve a flow rate of 1ml/min above the minimal target flow rate, which equates to 3.125% of blood volume per minute which is sufficient. This would approximately equate to 171.875ml/min in a human placing it comfortably in the range of 120-240ml/min.

The same data logging equipment was utilised as in previous phases. The culmination of lessons learned, appropriate reflection and revision, from phases 1, 2 and 3 ensured that phase 4 was a considerably smoother series of experiments.

Blood samples were collected at hourly intervals. The plasma from these blood samples was then tested for the presence and levels of the cytokines by enzyme-linked immunosorbent assay (ELISA). Quantitative “plate-yourself” sandwich enzyme immunoassay technique kits were obtained for IL-6 (BD Biosciences,

Oxford) and TNFα (eBioscience, Hatfield). Both kits were suitable for reliable use with serum samples and the assays were carried out according to the protocols provided by the manufacturer. Each kit was prepared by coating with the supplied antigen and overnight incubation then washed. The samples were then added, washed again and lastly a colour reactive enzyme added which fluoresced when a stop solution was added. The intensity of this fluorescence was measured and could be used to quantify the levels of each cytokine present in each sample. Both assays were linear in calibration.

At the end of the time course, the animals were euthanized by intravenous administration of potassium chloride (JM Loveridge Ltd, Southampton), inducing cardiac arrest and death.

Following euthanasia of the animals, organs were harvested for subsequent delta dry weight analysis. The procedure for this was to open the chest cavity and remove the required organs and samples. They were then weighed prior to heating for 8 hours at 80°C to obtain the "wet weight" and weighed once more afterwards to obtain the "dry weight". The difference was calculated as a dry weight percentage of the initial weight. This allowed the investigator to observe what percentage of the weight in each group was composed of dry mass compared to fluid, the higher the dry weight percentage the lower the volume of fluid in each sample.

5.2.2 Discussion of Results

The results of phase 4 are presented in Figure 70, Figure 71, Figure 72, Figure 73, Figure 74, Figure 75, Figure 76 and Figure 77. These should be considered in

relation to the 3 experimental groups which were investigated; control, induced sepsis and treatment.

Beginning discussion with the observed mean arterial blood pressure readings, a clear overall trend can be seen. Very early in the time course, the induced sepsis group experiences a drop in BP which marks the beginning of a continual trend of predominantly low and falling BP. There is a marked deviation, even while considering the deviation, between this group and the control and treatment groups. This would appear to be in line with literature observations of the sepsis condition. By comparison, the treatment and control groups remain relatively similar in the results for this parameter. While there is a predominant dropping BP, it is a far less dramatic rate than the induced sepsis group, indicating that the treatment and control groups are experiencing a gradual decline over the 6 hours due to their blood being circulated extracorporeally. Most importantly, it would appear that the haemoperfusion circuit utilising the adsorbent is mitigating the effects of the induced sepsis.

Body temperature, a critical indicator of the animals condition, presents some interesting results. It should be noted that as in phase 2, no external heat source was provided. Although the animals were positioned on a heat mat, this was only to provide an insulated surface against the bench top, it was not turned on in any group. Accordingly, the observations in the results ensure valid comparison and appear to indicate a clear trend of progression into hypothermia for the induced sepsis group. The control and treatment groups too experience a decline. However similar to mean arterial blood pressure, they are closer to one another than to the induced sepsis

group, indicating a difference and again providing supporting evidence that the use of the adsorbent as a haemoperfusion device appears to be mitigating this effect. Control and treatment groups do however trend towards hypothermia themselves. However without any external heat source, this is to be expected. Having their blood circulated extracoporeally and receiving hourly blood sample collection will propagate this trend towards hypothermia. In the clinical setting, any procedures involving extracorporeal circulation would be supported by external temperature controls in the form of heat exchangers.

In the results regarding heart rate, the picture is not as clear. Results indicate that between the three groups there is little difference in the heart rate until the final hour where the induced sepsis group appears to be in serious decline, an observation supported by all sets of results for this group.

The ELISA results present some interesting information. Regarding TNF-α, a marked difference between all three groups is observed. The control group experiences an early spike, which gradually declines, although does appear to elevate gradually again towards the 6 hour mark. It would appear that the control group is experiencing upregulation of TNF-α due to blood being circulated extracoporeally, or perhaps a latent rise following completion of surgeries to place cannulas, before adjusting. The induced sepsis group however experiences a rise at a similar rate to the control group. However, where the control group drops again, in the induced sepsis group, this rise is sustained until the 4 hour mark where it begins to decline before reaching a similar level to control at completion of the 6 hour time course. This observation would appear to be in line with literature observations of early

sepsis. The treatment group by comparison experiences a clearly mitigated expression of TNF-α, mitigating even the early rise seen in the control group. This mitigation is sustained throughout the time course until very low levels are witnessed at the 6 hour time course completion. It is worth reiterating that the samples were collected from the draw side of the extracorporeal circuit before the adsorbent column to ensure that samples were indicative of the systemic circulation rather than being collected from the return side after the column. It would be a recommendation that for future work it would be a worthwhile investigation to observe blood samples from both the draw side and the return side of the circuit.

When observing IL-6, a known key indicator of sepsis and inflammation in general, little difference is observed in the results obtained until the end of the time course, where the control and induced sepsis groups levels remain elevated but the treatment group drops. IL-6 is a prolific cytokine key for many functions within the host and as such it is not surprising that in the context of an extracorporeal circuit its levels are elevated. It is considered that the effect of the haemoperfusion device is not being seen until the end of the time course due to the nature of IL-6. However, this cannot be conclusively stated. There are possible reasons for the sustained levels in all groups, one possibility would be in line with the "threshold level" theory discussed in section 2.7, that although the adsorbent is eliminating a proportion of circulating IL-6, it is being continually replaced by IL-6 drawn from the interstitial environment, and accordingly the effective circulating levels of IL-6 are not observable until later in the time course. Indeed, it has been reported that maximal expression of IL-6 is later in the time course of sepsis than that of TNF-α.

When considering the delta dry weight of the harvested organ and tissue samples the dry weight percentage, displayed in Figure 77 is of most significance It should be noted that while this is a useful measure for determining the amount of fluid present in a sample, it is not a completely conclusive indicator. It would be a recommendation for future work that histological studies be undertaken to assess the condition of the samples, not just in terms of fluid volume but also to assess other indicators of the condition. A trend is observable that in most samples, the control group has the highest percentage of dry mass in the samples followed by the treatment group and the induced sepsis group in the majority case appears to have less dry matter, indicating a high volume of fluid in the samples. However, it is not the case in all samples, notably the lung, muscle and brain samples. In the lung it is a well reported fact that pulmonary oedema is a key element of sepsis progression and an indicator of progression towards MODS. The control group lung does have the highest percentage of dry mass as would be expected and the induced sepsis group demonstrates more fluid content than the control group. However, the treatment group appears to possess the highest fluid content of the three groups, a consideration that would warrant further investigation in future work. In the brain and muscle samples, it would appear that induced sepsis and treatment groups have similar dry masses and the control group has the highest fluid content, which can be suggested to indicate misdistribution of fluid from these samples which would normally possess higher fluid content.

5.2.3 Key Outcomes of Phase 4

Phase 4 was developed following the unsuccessful phase 3. The purpose of the phase 4 (supposed by all previous phases) was to evaluate both the first and second thesis hypotheses Chapter 3.0. The first being that a valid analogous model of early sepsis can be created which will incorporate a miniaturised extracorporeal circuit for the testing of adsorbent (or other extracorporeal intervention) as a potential therapy for the condition. The second being that the deployment of adsorbent in an extracorporeal haemoperfusion circuit will mitigate or halt the progression of the condition, extending the window for interventional therapy. The key outcomes of phase 4 are summarised as follows:

- It is possible to successfully maintain a rat model, with minimal to no severity in a non-survival extracorporeal experimental circuit for a time course of 6 hours.

- It is possible to deploy an adsorbent in a designed haemoperfusion column device to evaluate its effects upon the progression of a condition within this extracorporeal circuit.

- It is possible to collect meaningful data dynamically and iteratively regarding a variety of parameters within this arrangement.

- The treatment group deviated from the induced sepsis group in a demonstrable manner.

- Treatment appeared to mitigate key mediators of the sepsis condition and additionally physiological data support the observation that survival, in this time course, is improved in comparison to induced sepsis.

- The results would appear to indicate an adsorptive action by the adsorbent of the sepsis mediators being examined.

- Data collected would appear to suggest a successful mitigation of the induced sepsis condition in the treatment group.

6.0 Conclusions

To reiterate the aim of this body of work:

To evaluate the use of an adsorbent as an interventional therapy for sepsis.

In order to achieve this, an extensive review of literature was conducted and reported upon. The profound demand for an effective therapeutic intervention for sepsis was established. Contemporary understanding of the sepsis condition and theories thereof were addressed. Key conclusions from this were that the progression of the condition is mediated by various host elements. However, their exact mechanisms and progression of doing so are intertwined, redundant and evade conclusive agreement or evidence at present. Accordingly as has been mentioned by many authors, a panacea or "magic bullet" for the treatment of sepsis may be unattainable based upon current understanding.

Upon that particular note, current therapies for sepsis were considered along with some experimental therapeutic options currently being investigated. Informed by this existing knowledge from research into the field at present a novel therapeutic option is proposed.

In order to provide clearer understanding and a method of evaluation for this complex condition, a stable and repeatable animal model has been developed, the results from which suggest a suitable analogue for the early sepsis condition. This model has also been developed to provide a means of experimental apparatus for

testing the proposed adsorbent. Its modular nature additionally ensures that it will provide a suitable apparatus for future testing and evaluation of any number of developmental therapies, which incorporate an extracorporeal method of deployment, particularly if they are haemoperfusion based.

This work has been presented at various conferences, both indexed (Coutts and Gourlay, 2013) and non-indexed. The results from 4 iterative phases of experimentation suggest that the deployment of the carbide-based mesoporous adsorbent in an extracorporeal haemoperfusion circuit could provide a suitable treatment modality that is worthy of further investigation and development. It has been demonstrated that survival can be improved and the progression of the induced sepsis condition in the experimental model mitigated. It is not stated that this is conclusively proven, instead that this treatment modality would merit further work and investigation for this application.

7.0 Limitations & Recommendations for Future Work

This section reflects upon the limitations of this work and makes recommendations for future work in the area. Perhaps the most significant limitation, already acknowledged in the course of discussion, is that the model, based upon an intraperitoneal injection of LPS, can only be considered relevant to gram negative sepsis. However in chapter 2.0, it was established sepsis arises from both gram-positive and gram-negative stimuli. The suitability of particular models is debated frequently in the literature on sepsis and frequently it is stated that an LPS based model is only indicative of early sepsis. While this was the main area of focus for this work, any future work should consider adapting the model by varying the method of sepsis inducement in order to evaluate the efficacy of the proposed treatment modality in the gram-positive sepsis setting and the longer term progression of the condition. Cecal ligation and puncture (CLP) is frequently cited as a suitable method for doing so.

A key limitation of the work is the sample size. Ideally, significantly larger sample sizes would be utilised. This was largely due to practical constraints; larger samples sizes would require significant financial, staff and resource commitments. It can be argued that this investigation could serve as justification for larger scale testing with a now refined protocol.

Lastly it should be noted that in order to make comparisons to clinical observations of sepsis in humans, experiments were reviewed according to pre-set criteria that

were extracted from clinically accepted literature. It is acknowledged that such criteria are the subject of judgement. Further to this, it is accepted that other criteria that were not considered could be tested. However for the purposes of the project, the readings and criteria were effective in the given context.

Throughout this thesis, it has been established that cytokines are a very valid area of investigation. However in further investigation, the suitability of an interventional sepsis therapy must not only focus on these markers. The actual impact to particular organs and their function must also be addressed. While organs and tissue samples were collected to establish dry mass percentage, future work could make use of harvested organs for histological analysis. In the future, perhaps through other methods of sepsis inducement, harvesting samples for analysis a deeper understanding of the effect of both the condition and therapy can be developed. Future work must also assess the suitability of a therapy in the adhesion and action of leukocytes and other interactions in the inflammatory cascade, at the cellular level. It is therefore another recommendation that experiments be developed to use these animal models but to assess their microvasculature in some way perhaps through means of micro vascular microscopy.

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8.0 References

ABRAHAM, E., LATERRE, P. F., GARG, R., LEVY, H., TALWAR, D., TRZASKOMA, B. L., FRANCOIS, B., GUY, J. S., BRUCKMANN, M., REA-NETO, A., ROSSAINT, R., PERROTIN, D., SABLOTZKI, A., ARKINS, N., UTTERBACK, B. G. & MACIAS, W. L. 2005. Drotrecogin alfa (activated) for adults with severe sepsis and a low risk of death. *N Engl J Med,* 353**,** 1332-41.

ADIB-CONQUY, M. & CAVAILLON, J. M. 2009. Compensatory anti-inflammatory response syndrome. *Thromb Haemost,* 101**,** 36-47.

ADRIE, C. & PINSKY, M. R. 2000. The inflammatory balance in human sepsis. *Intensive Care Medicine,* 26**,** 364-375.

AIRD, W. C. 2003. *The role of the endothelium in severe sepsis and multiple organ dysfunction syndrome*.

ANDERSEN, T. H., JENSEN, T. H. & ANDERSEN, L. W. 2009. Adjunctive therapy of severe sepsis and septic shock in adults. *Current Anaesthesia & Critical Care,* 20**,** 254-258.

ANGUS, D. C. 2011. Management of sepsis: a 47-year-old woman with an indwelling intravenous catheter and sepsis. *Jama,* 305**,** 1469-77.

ANGUS, D. C., BURGNER, D., WUNDERINK, R., MIRA, J. P., GERLACH, H., WIEDERMANN, C. J. & VINCENT, J. L. 2003. The PIRO concept: P is for predisposition. *Crit Care,* 7**,** 248-51.

ANGUS, D. C. M., MPH, FCCM, LINDE-ZWIRBLE, W. T., LIDICKER, J. M., CLERMONT, G. M., CARCILLO, J. M. & PINSKY, M. R. M., FCCM 2001. Epidemiology of severe sepsis in the United States: Analysis of incidence, outcome, and associated costs of care. *Critical Care Medicine,* 29**,** 1303-1310.

ANNANE, D., BELLISSANT, E. & CAVAILLON, J.-M. 2005. Septic shock. *The Lancet,* 365**,** 63-78.

ANNANE, D., SEBILLE, V., CHARPENTIER, C., BOLLAERT, P. E., FRANCOIS, B., KORACH, J. M., CAPELLIER, G., COHEN, Y., AZOULAY, E., TROCHE, G., CHAUMET-RIFFAUD, P. & BELLISSANT, E. 2002. Effect of treatment with low doses of hydrocortisone and fludrocortisone on mortality in patients with septic shock. *Jama,* 288**,** 862-71.

BALK, R. A. 2014. Systemic inflammatory response syndrome (SIRS). *Virulence,* 5**,** 20-6.

BAROCHIA, A., CUI, X. & EICHACKER, P. 2013. The Surviving Sepsis Campaign's Revised Sepsis Bundles. *Current Infectious Disease Reports,* 15**,** 385-393.

BATZOFIN, B. M., SPRUNG, C. L. & WEISS, Y. G. 2011. The use of steroids in the treatment of severe sepsis and septic shock. *Best Practice & Research Clinical Endocrinology & Metabolism,* 25**,** 735-743.

BAUE, A. E., DURHAM, R. & FAIST, E. 1998. Systemic Inflammatory Response Syndrome (SIRS), Multiple Organ Dysfunction Syndrome (MODS), Multiple Organ Failure (MOF): Are We Winning The Battle? *Shock,* 10**,** 79-89.

BELLINGAN, G. 1999. Inflammatory cell activation in sepsis. *British Medical Bulletin,* 55**,** 12-29.

BERNARD, G. R., VINCENT, J. L., LATERRE, P. F., LAROSA, S. P., DHAINAUT, J. F., LOPEZ-RODRIGUEZ, A., STEINGRUB, J. S., GARBER, G. E., HELTERBRAND, J. D., ELY, E. W. & FISHER, C. J., JR. 2001. Efficacy and safety of recombinant human activated protein C for severe sepsis. *N Engl J Med,* 344**,** 699-709.

BOCHUD, P. Y. & CALANDRA, T. 2003. Pathogenesis of sepsis: new concepts and implications for future treatment. *Bmj,* 326**,** 262-6.

BONE, R. C., BALK, R. A., CERRA, F. B., DELLINGER, R. P., FEIN, A. M., KNAUS, W. A., SCHEIN, R. M. & SIBBALD, W. J. 1992. Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis: American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference. *Chest,* 101**,** 1644-1655.

BOTERO, J. S. H. & PÉREZ, M. C. F. 2012. *The History of Sepsis from Ancient Egypt to the XIX Century,* INTECH Open Access Publisher.

BROUGHTON, G., 2ND, JANIS, J. E. & ATTINGER, C. E. 2006. A brief history of wound care. *Plast Reconstr Surg,* 117**,** 6s-11s.

BURAS, J. A., HOLZMANN, B. & SITKOVSKY, M. 2005. Animal Models of Sepsis: Setting the Stage. *Nature Reviews: Drug Discovery,* 4**,** 854-865.

CAI, B., DEITCH, E. A. & ULLOA, L. 2010. Novel Insights for Systemic Inflammation in Sepsis and Hemorrhage. *Mediators of Inflammation,* 2010.

CANNON, J. G., TOMPKINS, R. G., GELFAND, J. A., MICHIE, H. R., STANFORD, G. G., VAN DER MEER, J. W. M., ENDRES, S., LONNEMANN, G., CORSETTI, J., CHERNOW, B., WILMORE, D. W., WOLFF, S. M., BURKE, J. F. & DINARELLO, C. A. 1990. Circulating Interleukin-1 and Tumor Necrosis Factor in Septic Shock and Experimental Endotoxin Fever. *Journal of Infectious Diseases,* 161**,** 79-84.

CHARCHAFLIEH, J., WEI, J., LABAZE, G., HOU, Y. J., BABARSH, B., STUTZ, H., LEE, H., WORAH, S. & ZHANG, M. 2012. The role of complement system in septic shock. *Clin Dev Immunol,* 2012**,** 407324.

COHEN, J. 2002. The immunopathogenesis of sepsis. *Nature,* 420**,** 885-891.

COHEN, J. 2009. Sepsis. *Medicine,* 37**,** 562-565.

COURT, O., KUMAR, A., PARRILLO, J. E. & KUMAR, A. 2002. Clinical review: Myocardial depression in sepsis and septic shock. *Critical Care,* 6**,** 500-508.

COUSSENS, L. M. & WERB, Z. 2002. Inflammation and cancer. *Nature,* 420**,** 860-867.

COUTTS, E. & GOURLAY, T. 2013. Oral presentations and posters of the XL Annual ESAO Congress - 11-14 September 2013, Glasgow, Scotland (UK). *Int J Artif Organs,* 36**,** 541-86.

CRUZ, D. N., ANTONELLI, M., FUMAGALLI, R., FOLTRAN, F., BRIENZA, N., DONATI, A., MALCANGI, V., PETRINI, F., VOLTA, G., BOBBIO PALLAVICINI, F. M., ROTTOLI, F., GIUNTA, F. & RONCO, C. 2009. Early use of polymyxin B hemoperfusion in abdominal septic shock: the EUPHAS randomized controlled trial. *Jama,* 301**,** 2445-52.

CRUZ, D. N., PERAZELLA, M. A., BELLOMO, R., DE CAL, M., POLANCO, N., CORRADI, V., LENTINI, P., NALESSO, F., UENO, T., RANIERI, V. M. & RONCO, C. 2007. Effectiveness of polymyxin B-immobilized fiber column in sepsis: a systematic review. *Crit Care,* 11**,** R47.

DAHLBÄCK, B. 2000. Blood coagulation. *The Lancet,* 355**,** 1627-1632.

DANIEL G. REMICK, M. D. 2007. Pathophysiology of Sepsis. *The American Journal of Pathology,* 170**,** 1435-1444.

DANIELS, R. 2009. Defining the Spectrum of Disease. *In:* DANIELS, R. & NUTBEAM, T. (eds.) *ABC of Sepsis.* John Wiley & Sons.

DELLINGER, R. P. 2003. Inflammation and Coagulation: Implications for the Septic Patient. *Clinical Infectious Diseases,* 36**,** 1259-1265.

DELLINGER, R. P. 2014. The Surviving Sepsis Campaign 2014: An Update on the Management and Performance Improvement For Adults in Severe Sepsis. *Consultant,* 54**,** 767-771.

DELLINGER, R. P., CARLET, J. M., MASUR, H., GERLACH, H., CALANDRA, T., COHEN, J., GEA-BANACLOCHE, J., KEH, D., MARSHALL, J. C., PARKER, M. M., RAMSAY, G., ZIMMERMAN, J. L., VINCENT, J. L. & LEVY, M. M. 2004. Surviving Sepsis Campaign guidelines for management of severe sepsis and septic shock. *Intensive Care Med,* 30**,** 536-55.

DELLINGER, R. P., LEVY, M. M., CARLET, J., BION, J., PARKER, M., JAESCHKE, R., REINHART, K., ANGUS, D., BRUN-BUISSON, C., BEALE, R., CALANDRA, T., DHAINAUT, J.-F., GERLACH, H., HARVEY, M., MARINI, J. J., MARSHALL, J., RANIERI, M., RAMSAY, G., SEVRANSKY, J., THOMPSON, B. T., TOWNSEND, S., VENDER, J. S., ZIMMERMAN, J. & JEAN-LOUIS VINCENT, M. D., PH.D 2008. Surviving Sepsis Campaign: International guidelines for management of severe sepsis and septic shock: 2008. *Journal of Intensive Care Medicine,* 34**,** 17–60.

DELLINGER, R. P., LEVY, M. M., RHODES, A., ANNANE, D., GERLACH, H., OPAL, S. M., SEVRANSKY, J. E., SPRUNG, C. L., DOUGLAS, I. S., JAESCHKE, R., OSBORN, T. M., NUNNALLY, M. E., TOWNSEND, S. R., REINHART, K., KLEINPELL, R. M., ANGUS, D. C., DEUTSCHMAN, C. S., MACHADO, F. R., RUBENFELD, G. D., WEBB, S., BEALE, R. J., VINCENT, J. L., MORENO, R. & SURVIVING SEPSIS CAMPAIGN GUIDELINES COMMITTEE INCLUDING THE PEDIATRIC, S. 2013a. Surviving Sepsis Campaign: international guidelines for management of severe sepsis and septic shock, 2012. *Intensive Care Med,* 39**,** 165-228.

DELLINGER, R. P., LEVY, M. M., RHODES, A., ANNANE, D., GERLACH, H., OPAL, S. M., SEVRANSKY, J. E., SPRUNG, C. L., DOUGLAS, I. S., JAESCHKE, R., OSBORN, T. M., NUNNALLY, M. E., TOWNSEND, S. R., REINHART, K., KLEINPELL, R. M., ANGUS, D. C., DEUTSCHMAN, C. S., MACHADO, F. R., RUBENFELD, G. D., WEBB, S. A., BEALE, R. J., VINCENT, J. L. & MORENO, R. 2013b. Surviving sepsis campaign: international guidelines for management of severe sepsis and septic shock: 2012. *Crit Care Med,* 41**,** 580-637.

DI CARLO, J. V. & ALEXANDER, S. R. 2005. Hemofiltration for cytokine-driven illnesses: the mediator delivery hypothesis. *Int J Artif Organs,* 28**,** 777-86.

DINARELLO, C. A. 1991. The Proinflammatory Cytokines Interleukin-l and Tumor Necrosis Factor and Treatment of the Septic Shock Syndrome. *Journal of Infectious Diseases,* 163**,** 1177-1184.

DINARELLO, C. A. 2010. Anti-inflammatory Agents: Present and Future. *Cell,* 140**,** 935-950.

DOI, K., LEELAHAVANICHKUL, A., YUEN, P. S. T. & STAR, R. A. 2009. Animal models of sepsis and sepsis-induced kidney injury. *The Journal of Clinical Investigation,* 119**,** 2868-2878.

ESMON, C. T., FUKUDOME, K., MATHER, T., BODE, W., REGAN, L. M., STEARNS-KUROSAWA, D. J. & KUROSAWA, S. 1999. Inflammation, sepsis, and coagulation. *Haematologica,* 84**,** 254-9.

FORMICA, M., INGUAGGIATO, P., BAINOTTI, S. & WRATTEN, M. L. 2007. Coupled plasma filtration adsorption. *Contrib Nephrol,* 156**,** 405-10.

FORREST, R. D. 1982. Early history of wound treatment. *Journal of the Royal Society of Medicine,* 75**,** 198-205.

FORTENBERRY, J. D. & PADEN, M. L. 2006. Extracorporeal Therapies in the Treatment of Sepsis: Experience and Promise. *Seminars in Pediatric Infectious Diseases,* 17**,** 72-79.

FUNK, D., SEBAT, F. & KUMAR, A. 2009. A systems approach to the early recognition and rapid administration of best practice therapy in sepsis and septic shock. *Curr Opin Crit Care,* 15**,** 301-7.

GALLEY, H. & WEBSTER, N. 2008. Cytokines. *In:* FINK, M., HAYES, M. & SONI, N. (eds.) *Classic Papers in Critical Care.* Springer London.

GEROULANOS, S. & DOUKA, E. T. 2006. Historical perspective of the word "sepsis". *Intensive Care Med,* 32**,** 2077.

GIANNOUDIS, P. V. 2003. Current concepts of the inflammatory response after major trauma: an update. *Injury,* 34**,** 397-404.

GOURLAY, T., STEFANOU, D. & TAYLOR, K. M. 2002. The effect of methanol washing of plasticized polyvinyl chloride on biomaterial-contact-mediated CD11b (mac-1) expression in a rat recirculation model. *Artif Organs,* 26**,** 5-9.

GOURLAY, T., STEFANOU, D. C., ASIMAKOPOULOS, G. & TAYLOR, K. M. 2001. The effect of circuit surface area on CD11b(mac-1) expression in a rat recirculation model. *Artif Organs,* 25**,** 475-9.

GREEN, C., DINNES, J., TAKEDA, A., SHEPHERD, J., HARTWELL, D., CAVE, C., PAYNE, E. & CUTHBERTSON, B. H. 2005. Clinical effectiveness and cost-effectiveness of drotrecogin alfa (activated) (Xigris) for the treatment of severe sepsis in adults: a systematic review and economic evaluation. *Health Technol Assess,* 9**,** 1-126, iii-iv.

GREEN, R. S., DJOGOVIC, D. & HOWES, D. 2012. Sepsis update: management of severe sepsis and septic shock in the emergency department after the withdrawal of Xigris. *Cjem,* 14**,** 265-9.

GRIFFITHS, B. & ANDERSON, I. D. 2009. Sepsis, SIRS and MODS. *Surgery - Oxford International Edition,* 27**,** 446-449.

HACK, C. E., GROOT, E. R. D., J.F., R., FELT-BERSMA, NUIJENS, J. H., J.M., R., SCHIJNDE, S. V., EERENBERG-BELMER, A. J. M., THIJS, L. G. & AARDE, L. A. 1989. Increased Plasma Levels of Interleukin-6 in Sepsis. *Blood,* 74**,** 1704-1710.

HAENEY, M. R. 1998. The role of the complement cascade in sepsis. *Journal of Antimicrobial Chemotherapy,* 41**,** 41-46.

HIETBRINK, F., KOENDERMAN, L., RIJKERS, G. & LEENEN, L. 2006. Trauma: the role of the innate immune system. *World Journal of Emergency Surgery,* 1**,** 15.

HOLLENBERG, S. M., DUMASIUS, A., EASINGTON, C., COLILLA, S. A., NEUMANN, A. & PARRILLO, J. E. 2001. Characterization of a Hyperdynamic Murine Model of Resuscitated Sepsis Using Echocardiography. *American Journal of Respiratory and Critical Care Medicine,* 164**,** 891–895.

HONORE, P. M. & JOANNES-BOYAU, O. 2004. High volume hemofiltration (HVHF) in sepsis: a comprehensive review of rationale, clinical applicability, potential indications and recommendations for future research. *Int J Artif Organs,* 27**,** 1077-82.

HUNTER, P. 2006. Sepsis under siege. *European Molecular Biology Organization Reports,* 7**,** 667-669.

HUTTUNEN, R. & AITTONIEMI, J. 2011. New concepts in the pathogenesis, diagnosis and treatment of bacteremia and sepsis. *Journal of Infection,* 63**,** 407-419.

JAFFER, U., WADE, R. G. & GOURLAY, T. 2010. Cytokines in the systemic inflammatory response syndrome: a review. *HSR Proc Intensive Care Cardiovasc Anesth,* 2**,** 161-75.

JAWA, R. S., KULAYLAT, M. N., BAUMANN, H. & DAYTON, M. T. 2006. What is new in cytokine research related to trauma/critical care. *J Intensive Care Med,* 21**,** 63-85.

JEAN-LOUIS VINCENT, M. D., PH.D 2006. The last 100 years of sepsis. *American Journal of Respiratory and Critical Care Medicine,* 173**,** 256–263.

JOHARI, V. & LOKE, C. 2012. Brief Overview of the Coagulation Cascade. *Disease-a-Month,* 58**,** 421-423.

JOHN A. KELLUM, M., LAN KONG, P., MITCHELL P. FINK, M., LISA A. WEISSFELD, P., DONALD M. YEALY, M., MICHAEL R. PINSKY, M., JONATHAN FINE, M., ALEXANDER KRICHEVSKY, P., RUSSELL L. DELUDE, P. & DEREK C. ANGUS, M. 2007. Understanding the inflammatory cytokine response in pneumonia and sepsis: results of the Genetic and Inflammatory Markers of Sepsis (GenIMS) Study. *Archives of Internal Medicine,* 167**,** 1655-63.

JOSEPH E. PARRILLO, M. D. 1993. Pathogenetic Mechanisms of Septic Shock. *New England Journal of Medicine,* 328**,** 1471-1477.

KAWAI, T. & AKIRA, S. 2011. Toll-like Receptors and Their Crosstalk with Other Innate Receptors in Infection and Immunity. *Immunity,* 34**,** 637-650.

KELLUM, J. & DISHART, M. 2002. Effect of hemofiltration filter adsorption on circulating IL-6 levels in septic rats. *Critical Care,* 6**,** 429 - 433.

KELLY, M., HWANG, J. M. & KUBES, P. 2007. Modulating leukocyte recruitment in inflammation. *Journal of Allergy and Clinical Immunology,* 120**,** 3-10.

KIRSCHFINK, M. 2001. Targeting complement in therapy. *Immunol Rev,* 180**,** 177-89.

KRAMER, P., WIGGER, W., RIEGER, J., MATTHAEI, D. & SCHELER, F. 1977. Arteriovenous haemofiltration: A new and simple method for treatment of

over-hydrated patients resistant to diuretics. *Klinische Wochenschrift,* 55**,** 1121-1122.
KUCHAREWICZ, I., BODZENTA-LUKASZYK, A. & BUCZKO, W. 2008. Experimental asthma in rats. *Pharmacological Reports,* 60**,** 783–788.
KULABUKHOV, V. V. 2008. Use of an endotoxin adsorber in the treatment of severe abdominal sepsis. *Acta Anaesthesiol Scand,* 52**,** 1024-5.
KYLAT, R. I. & OHLSSON, A. 2012. Recombinant human activated protein C for severe sepsis in neonates. *Cochrane Database Syst Rev,* 4**,** Cd005385.
LAM, F. Y. & FERRELL, W. R. 1991. Neurogenic component of different models of acute inflammation in the rat knee joint. *Annals ofthe Rheumatic Diseases,* 50**,** 747-751.
LANGENBERG, C., BELLOMO, R., MAY, C., WAN, L., EGI, M. & MORGERA, S. 2005. Renal blood flow in sepsis. *Critical Care,* 9**,** R363-R374.
LATZ, E. 2010. The inflammasomes: mechanisms of activation and function. *Current Opinion in Immunology,* 22**,** 28-33.
LENZ, A., FRANKLIN, G. A. & CHEADLE, W. G. 2007. Systemic inflammation after trauma. *Injury,* 38**,** 1336-45.
LEVY, J. H., SNIECINSKI, R. M. & DEMMA, L. J. 2013. Blood and Coagulation. 615-627.
LEVY, M. M., FINK, M. P., MARSHALL, J. C., ABRAHAM, E., ANGUS, D., COOK, D., COHEN, J., OPAL, S. M., VINCENT, J.-L. & RAMSAY, G. 2003. 2001 SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference. *Intensive Care Med,* 29**,** 530–538.
LINDE-ZWIRBLE, W. T. & ANGUS, D. C. 2004. Severe sepsis epidemiology: sampling, selection, and society. *Critical Care,* 8**,** 222-226.
MAJNO, G. 1975. *The Healing Hand: Man and Wound in the Ancient World*.
MAJNO, G. 1991. The Ancient Riddle of σηψιζ (Sepsis). *Journal of Infectious Diseases,* 163**,** 937-945.
MARIK, P. E. 2011a. Surviving sepsis guidelines and scientific evidence? *J Intensive Care Med,* 26**,** 201-2.
MARIK, P. E. 2011b. Surviving sepsis: going beyond the guidelines. *Ann Intensive Care,* 1**,** 17.
MARIK, P. E. 2015. The demise of early goal-directed therapy for severe sepsis and septic shock. *Acta Anaesthesiologica Scandinavica***,** n/a-n/a.
MARSHALL, J. C., DEITCH, E., MOLDAWER, L. L., OPAL, S., REDL, H. & POLL, T. V. D. 2005. Preclinical Models of Shock and Sepsis: What Can They Tell Us? *Shock,* 24**,** 1-6.
MARTIN, G. S., MANNINO, D. M., EATON, S. & MOSS, M. 2003. The Epidemiology of Sepsis in the United States from 1979 through 2000. *New England Journal of Medicine,* 348**,** 1546-1554.
MATZINGER, P. 1994. Tolerance, danger, and the extended family. *Annu Rev Immunol,* 12**,** 991-1045.
MAYR, F. B., YENDE, S. & ANGUS, D. C. 2014. Epidemiology of severe sepsis. *Virulence,* 5**,** 4-11.
MIKHALOVSKY, S. V. 2003. Emerging technologies in extracorporeal treatment: focus on adsorption. *Perfusion,* 18**,** 47-54.
MORAN, T. A. & VIELE, C. S. 2005. Normal Clotting. *Seminars in Oncology Nursing,* 21**,** 1-11.

MORGERA, S., SLOWINSKI, T., MELZER, C., SOBOTTKE, V., VARGAS-HEIN, O., VOLK, T., ZUCKERMANN-BECKER, H., WEGNER, B., MÜLLER, J. M., BAUMANN, G., KOX, W. J., BELLOMO, R. & NEUMAYER, H.-H. 2004. Renal replacement therapy with high-cutoff hemofilters: impact of convection and diffusion on cytokine clearances and protein status. *American Journal of Kidney Diseases,* 43**,** 444-453.

NAMAS, R., GHUMA, A., HERMUS, L., ZAMORA, R., OKONKWO, D., BILLIAR, T. & VODOVOTZ, Y. 2009. The acute inflammatory response in trauma/hemorrhage and traumatic brain injury: current state and emerging prospects. *Libyan Journal of Medicine,* 4.

NAMAS, R., ZAMORA, R., NAMAS, R., AN, G., DOYLE, J., DICK, T. E., JACONO, F. J., ANDROULAKIS, I. P., NIEMAN, G. F., CHANG, S., BILLIAR, T. R., KELLUM, J. A., ANGUS, D. C. & VODOVOTZ, Y. 2012. Sepsis: Something old, something new, and a systems view. *Journal of Critical Care,* 27**,** 314.e1-314.e11.

NEMZEK, J. A., HUGUNIN, K. M. & OPP, M. R. 2008. Modeling Sepsis in the Laboratory: Merging Sound Science with Animal Well-Being. *Comparative Medicine,* 58**,** 120–128.

NILSSON, B., EKDAHL, K. N., MOLLNES, T. E. & LAMBRIS, J. D. 2007. The role of complement in biomaterial-induced inflammation. *Molecular Immunology,* 44**,** 82-94.

O'BRIEN, M. 2012. The Reciprocal Relationship Between Inflammation and Coagulation. *Topics in Companion Animal Medicine,* 27**,** 46-52.

OPAL, S. M. 2005. Concept of PIRO as a new conceptual framework to understand sepsis. *Pediatr Crit Care Med,* 6**,** S55-60.

OPAL, S. M. 2007. The host response to endotoxin, antilipopolysaccharide strategies, and the management of severe sepsis. *International Journal of Medical Microbiology,* 297**,** 365-377.

PANAGIOTOU, A., GAIAO, S. & CRUZ, D. N. 2013. Extracorporeal therapies in sepsis. *J Intensive Care Med,* 28**,** 281-95.

PAPE, H. C., TSUKAMOTO, T., KOBBE, P., TARKIN, I., KATSOULIS, S. & PEITZMAN, A. 2007. Assessment of the clinical course with inflammatory parameters. *Injury,* 38**,** 1358-64.

PENG, Z., SINGBARTL, K., SIMON, P., RIMMELE, T., BISHOP, J., CLERMONT, G. & KELLUM, J. A. 2010. Blood purification in sepsis: a new paradigm. *Contrib Nephrol,* 165**,** 322-8.

PHILIPPART, F., CAVAILLON, J.-M. & DINARELLO, C. 2009. Inflammatory Mediators. *Sepsis and Non-Infectious Systemic Inflammation.* Wiley-VCH Verlag GmbH & Co. KGaA.

PIERRE DAMAS, M. D., DIDIER LEDOUX, M. D., NYS, M., VRINDTS, Y., DONAT DE GROOTE, M. D., PAUL FRANCHIMONT, M. D. & MAURICE LAMY, M. D. 1992. Cytokine Serum Level During Severe Sepsis in Human IL-6 as a Marker of Severity. *Annals of Surgery,* 215**,** 356-362.

POULTON, B. 2006. Advances in the management of sepsis: the randomised controlled trials behind the Surviving Sepsis Campaign recommendations. *International Journal of Antimicrobial Agents,* 27**,** 97-101.

RAGHAVAN, M. & MARIK, P. E. 2006. Management of sepsis during the early "golden hours". *The Journal of Emergency Medicine,* 31**,** 185-199.

RATANARAT, R., BRENDOLAN, A., PICCINNI, P., DAN, M., SALVATORI, G., RICCI, Z. & RONCO, C. 2005. Pulse high-volume haemofiltration for treatment of severe sepsis: effects on hemodynamics and survival. *Critical Care,* 9**,** R294 - R302.

REDDY, R. C., CHEN, G. H., TEKCHANDANI, P. K. & STANDIFORD, T. J. 2001. Sepsis-induced immunosuppression: from bad to worse. *Immunologic Research,* 24**,** 273–287.

REMICK, D. G., BOLGOS, G., COPELAND, S. & SIDDIQUI, J. 2005. Role of Interleukin-6 in Mortality from and Physiologic Response to Sepsis. *Infection and Immunity,* 73**,** 2751–2757.

REMICK, D. G., NEWCOMB, D. E., BOLGOS, G. L. & CALL, D. R. 2000. Comparison of the Mortality and Inflammatory Response of Two Models of Sepsis: Lipopolysaccharide vs. Cecal Ligation and Puncture. *Shock,* 13**,** 110-116.

RICHARD S. HOTCHKISS, M. D. & IRENE E. KARL, P. D. 2003. The Pathophysiology and Treatment of Sepsis. *The New England Journal of Medicine,* 348**,** 138-150.

RIEDEMANN, N. C., GUO, R.-F. & WARD, P. A. 2003. The enigma of sepsis. *Journal of Clinical Investigation,* 112**,** 460–467.

RIMMELE, T. & KELLUM, J. A. 2011. Clinical review: blood purification for sepsis. *Crit Care,* 15**,** 205.

RITTIRSCH, D., HOESEL, L. M. & WARD, P. A. 2007. The disconnect between animal models of sepsis and human sepsis. *Journal of Leukocyte Biology Volume , January* 81**,** 137-143.

RITTIRSCH, D., HUBER-LANG, M. S., FLIERL, M. A. & AWARD, P. 2009. Immunodesign of experimental sepsis by cecal ligation and puncture. *Nature Protocols,* 4**,** 31-36.

RITTIRSCH, D., REDL, H. & HUBER-LANG, M. 2012. Role of complement in multiorgan failure. *Clin Dev Immunol,* 2012**,** 962927.

RIVERS, E., NGUYEN, B., HAVSTAD, S., RESSLER, J., MUZZIN, A., KNOBLICH, B., PETERSON, E. & TOMLANOVICH, M. 2001. Early Goal-Directed Therapy in the Treatment of Severe Sepsis and Septic Shock. *New England Journal of Medicine,* 345**,** 1368-1377.

ROGER C. BONE, M. 1991. The Pathogenesis of Sepsis. *Annals of Internal Medicine,* 115**,** 457-469.

ROGER C. BONE, M., PHD (HONORARY), MASTER FCCP, CHARLES J. GRODZIN, M. & ROBERT A. BALK, M., FCCP 1997. Sepsis: A New Hypothesis for Pathogenesis of the Disease Process. *Chest,* 112**,** 235-243.

RONCO, C., BELLOMO, R., HOMEL, P., BRENDOLAN, A., DAN, M., PICCINNI, P. & LA GRECA, G. 2000. Effects of different doses in continuous veno-venous haemofiltration on outcomes of acute renal failure: a prospective randomised trial. *Lancet,* 356**,** 26-30.

RONCO, C., BONELLO, M., BORDONI, V., RICCI, Z., D'INTINI, V., BELLOMO, R. & LEVIN, N. W. 2004. Extracorporeal therapies in non-renal disease: treatment of sepsis and the peak concentration hypothesis. *Blood Purif,* 22**,** 164-74.

RONCO, C., TETTA, C., MARIANO, F., WRATTEN, M. L., BONELLO, M., BORDONI, V., CARDONA, X., INGUAGGIATO, P., PILOTTO, L., D'INTINI, V. & BELLOMO, R. 2003. Interpreting the Mechanisms of Continuous Renal Replacement Therapy in Sepsis: The Peak Concentration Hypothesis. *Artificial Organs,* 27**,** 792-801.

ROSOLEM, M. D. M., RABELLO, L. S. C. F., LEAL, J. V., SOARES, M., LISBOA, T. & SALLUH, J. I. F. 2010. Entendendo o conceito PIRO: da teoria à prática clínica - parte 2. *Revista Brasileira de Terapia Intensiva,* 22**,** 64-68.

ROTELLI, A. E., GUARDIA, T., JUÁREZ, A. O., ROCHA, N. E. D. L. & PELZER, L. E. 2003. Comparative study of flavonoids in experimental models of inflammation. *Pharmacological Research,* 48**,** 601–606.

RUSSELL, J. A. 2006. Management of Sepsis. *New England Journal of Medicine,* 355**,** 1699-1713.

SADIK, C. D., KIM, N. D. & LUSTER, A. D. 2011. Neutrophils cascading their way to inflammation. *Trends in Immunology,* 32**,** 452-460.

SAGY, M., AL-QAQAA, Y. & KIM, P. 2013. Definitions and pathophysiology of sepsis. *Curr Probl Pediatr Adolesc Health Care,* 43**,** 260-3.

SAKATA, H., YONEKAWA, M. & KAWAMURA, A. 2006. Blood purification therapy for sepsis. *Transfusion and Apheresis Science,* 35**,** 245-251.

SCHEFOLD, J. & JÖRRES, A. 2010. Extracorporeal Therapies and Immunomodulation During Sepsis. *In:* JÖRRES, A., RONCO, C. & KELLUM, J. A. (eds.) *Management of Acute Kidney Problems.* Springer Berlin Heidelberg.

SCHETZ, M., FERDINANDE, R., BERGHE, G. V. D., VERWAEST, C. & LAUWERS, R. 1995. Removal of proinflammatory cytokines with renal replacement therapy: sense or nonsense? *Intensive Care Medicine,* 21**,** 169-176.

SCHORR, C. A., ZANOTTI, S. & DELLINGER, R. P. 2014. Severe sepsis and septic shock: management and performance improvement. *Virulence,* 5**,** 190-9.

SCHOUTEN, M., WIERSINGA, W. J., LEVI, M. & POL, T. V. D. 2008a. Inflammation, endothelium, and coagulation in sepsis. *Journal of Leukocyte Biology,* 83**,** 536-545.

SCHOUTEN, M., WIERSINGA, W. J., LEVI, M. & VAN DER POLL, T. 2008b. Inflammation, endothelium, and coagulation in sepsis. *Journal of Leukocyte Biology,* 83**,** 536-545.

SCHRODER, K. & TSCHOPP, J. 2010. The Inflammasomes. *Cell,* 140**,** 821-832.

SCHULTE, W., BERNHAGEN, J. & BUCALA, R. 2013. Cytokines in sepsis: potent immunoregulators and potential therapeutic targets--an updated view. *Mediators Inflamm,* 2013**,** 165974.

SEMERARO, N., AMMOLLO, C. T., SEMERARO, F. & COLUCCI, M. 2012. Sepsis, thrombosis and organ dysfunction. *Thrombosis Research,* 129**,** 290-295.

SHAHEEN, I., HARVEY, B. & WATSON, A. R. 2009. Haemofiltration therapy. *Paediatrics and Child Health,* 19**,** 121-126.

SHERWOOD, E. R. & TOLIVER-KINSKY, T. 2004. Mechanisms of the inflammatory response. *Best Practice & Research Clinical Anaesthesiology,* 18**,** 385-405.

SHERWOOD, E. R. & TRABER, D. L. 2012. Chapter 21 - The systemic inflammatory response syndrome. *In:* HERNDON, D. N. (ed.) *Total Burn Care (Fourth Edition).* London: W.B. Saunders.

SHIGA, H., HIRASAWA, H., NISHIDA, O., ODA, S., NAKAMURA, M., MASHIKO, K., MATSUDA, K., KITAMURA, N., KIKUCHI, Y. & FUKE, N. 2014. Continuous Hemodiafiltration with a Cytokine-Adsorbing Hemofilter in Patients with Septic Shock: A Preliminary Report. *Blood Purification,* 38**,** 211-218.

SHIMAOKA, M. & PARK, E. J. 2008. Advances in understanding sepsis. *European Journal of Anaesthesiology,* 42**,** 146–153.

SHUM, H. P., LEUNG, Y. W., LAM, S. M., CHAN, K. C. & YAN, W. W. 2014. Alteco endotoxin hemoadsorption in Gram-negative septic shock patients. *Indian Journal of Critical Care Medicine : Peer-reviewed, Official Publication of Indian Society of Critical Care Medicine,* 18**,** 783-788.

SILVA, E., PASSOS, R. D. H., FERRI, M. B., FRANCISCO, L. & FIGUEIREDO, P. D. 2008. Sepsis: From Bench to Bedside. *Clinics,* 63**,** 109-20.

SINGER, M. 1998. Management of multiple organ failure: guidelines but no hard-and-fast rules. *Journal of Antimicrobial Chemotherapy,* 41**,** 103–112.

SPRUNG, C. L., ANNANE, D., KEH, D., MORENO, R., SINGER, M., FREIVOGEL, K., WEISS, Y. G., BENBENISHTY, J., KALENKA, A., FORST, H., LATERRE, P. F., REINHART, K., CUTHBERTSON, B. H., PAYEN, D. & BRIEGEL, J. 2008. Hydrocortisone therapy for patients with septic shock. *N Engl J Med,* 358**,** 111-24.

STEGMAYR, B. G. 2005. A survey of blood purification techniques. *Transfusion and Apheresis Science,* 32**,** 209-220.

SUCKOW, M. A., WEISBROTH, S. H. & FRANKLIN, C. L. 2006. *The laboratory rat,* London, Elsevier Academic Press.

TAKEUCHI, O. & AKIRA, S. 2010. Pattern Recognition Receptors and Inflammation. *Cell,* 140**,** 805-820.

THIJS, L. G., SCHNEIDER, A. J. & GROENEVELD, A. B. J. 1990. The haemodynamics of septic shock. *Intensive Care Medicine,* 16**,** 182-186.

THURSTON, A. J. 2000. Of blood, inflammation and gunshot wounds: the history of the control of sepsis. *Australian and New Zealand Journal of Surgery,* 70**,** 855-861.

TREACHER, D. F. & ALUN BROWN, K. 2009. The basic science of sepsis. *Surgery (Oxford),* 27**,** 465-469.

TSIOTOU, A. G., SAKORAFAS, G. H., ANAGNOSTOPOULOS, G. & BRAMIS, J. 2005. Septic shock; current pathogenetic concepts from a clinical perspective. *Med Sci Monit,* 11**,** Ra76-85.

TSOKOS, M. 2007. Postmortem diagnosis of sepsis. *Forensic Science International,* 165**,** 155-164.

TSUKAMOTO, T., CHANTHAPHAVONG, R. S. & PAPE, H.-C. 2010. Current theories on the pathophysiology of multiple organ failure after trauma. *Injury,* 41**,** 21-26.

TUFFERY, A. A. 1987. *Labratory Animals: An Introduction For New Experimentors*, Chilchester, John Wiley & Sons Ltd.

VALE, J. A., REES, A. J., WIDDOP, B. & GOULDING, R. 1975. Use of charcoal haemoperfusion in the management of severely poisoned patients. *British Medical Journal,* 1**,** 5-9.

VAN DER POLL, T. & OPAL, S. M. 2008. Host–pathogen interactions in sepsis. *The Lancet Infectious Diseases,* 8**,** 32-43.

VANZANT, A. M. & SCHMELZER, M. 2011. Detecting and treating sepsis in the emergency department. *J Emerg Nurs,* 37**,** 47-54.

VENKATARAMAN, R., SUBRAMANIAN, S. & KELLUM, J. 2003. Clinical review: Extracorporeal blood purification in severe sepsis. *Critical Care,* 7**,** 139 - 145.

VILLA, P., SARTOR, G., ANGELINI, M., SIRONI, M., CONNI, M., GNOCCHI, P., ISETTA, A. M., GRAU, G., BUURMAN, W., TITS, L. J. H. V. & GHEZZI, P. 1995. Pattern of Cytokines and Pharmacomodulation in Sepsis Induced by Cecal Ligation and Puncture Compared with That Induced by Endotoxin. *Clinical and Diagnostic Labratory Immunology,* 2**,** 549–553.

VINCENT, J.-L. & ABRAHAM, E. 2006. The Last 100 Years of Sepsis. *American Journal of Respiratory and Critical Care Medicine,* 173**,** 256-263.

VINCENT, J. L. 2008. Clinical sepsis and septic shock--definition, diagnosis and management principles. *Langenbecks Arch Surg,* 393**,** 817-24.

VINCENT, J. L. & MARSHALL, J. C. 2008. Surviving sepsis: a guide to the guidelines. *Crit Care,* 12**,** 162.

VRIESE, A. S. D., COLARDYN, F. A., PHILIPPE, J. J., VANHOLDER, R. C., SUTTER, J. H. D. & LAMEIRE, N. H. 1999. Cytokine Removal during Continuous Hemofiltration in Septic Patients. *Journal of the American Society of Nephrology,* 10**,** 846–853.

WARD, P. A. 2008. Sepsis, apoptosis and complement. *Biochemical Pharmacology,* 76**,** 1383-1388.

WENZEL, R. P. & EDMOND, M. B. 2001. Severe sepsis-national estimates. *Crit Care Med,* 29**,** 1472-4.

WHEELER, A. P. & BERNARD, G. R. 1999. Treating patients with severe sepsis. *N Engl J Med,* 340**,** 207-14.

WIERSINGA, W. J., LEOPOLD, S. J., CRANENDONK, D. R. & VAN DER POLL, T. 2014. Host innate immune responses to sepsis. *Virulence,* 5**,** 36-44.

WILLIAMS, M. R., AZCUTIA, V., NEWTON, G., ALCAIDE, P. & LUSCINSKAS, F. W. 2011. Emerging mechanisms of neutrophil recruitment across endothelium. *Trends in Immunology,* 32**,** 461-469.

WINCHESTER, J. F., KELLUM, J. A., RONCO, C., BRADY, J. A., QUARTARARO, P. J., SALSBERG, J. A. & LEVIN, N. W. 2003. Sorbents in acute renal failure and the systemic inflammatory response syndrome. *Blood Purif,* 21**,** 79-84.

XIAO, H., SIDDIQUI, J. & REMICK, D. G. 2006. Mechanisms of Mortality in Early and Late Sepsis. *Infection and Immunity,* 74**,** 5227–5235.

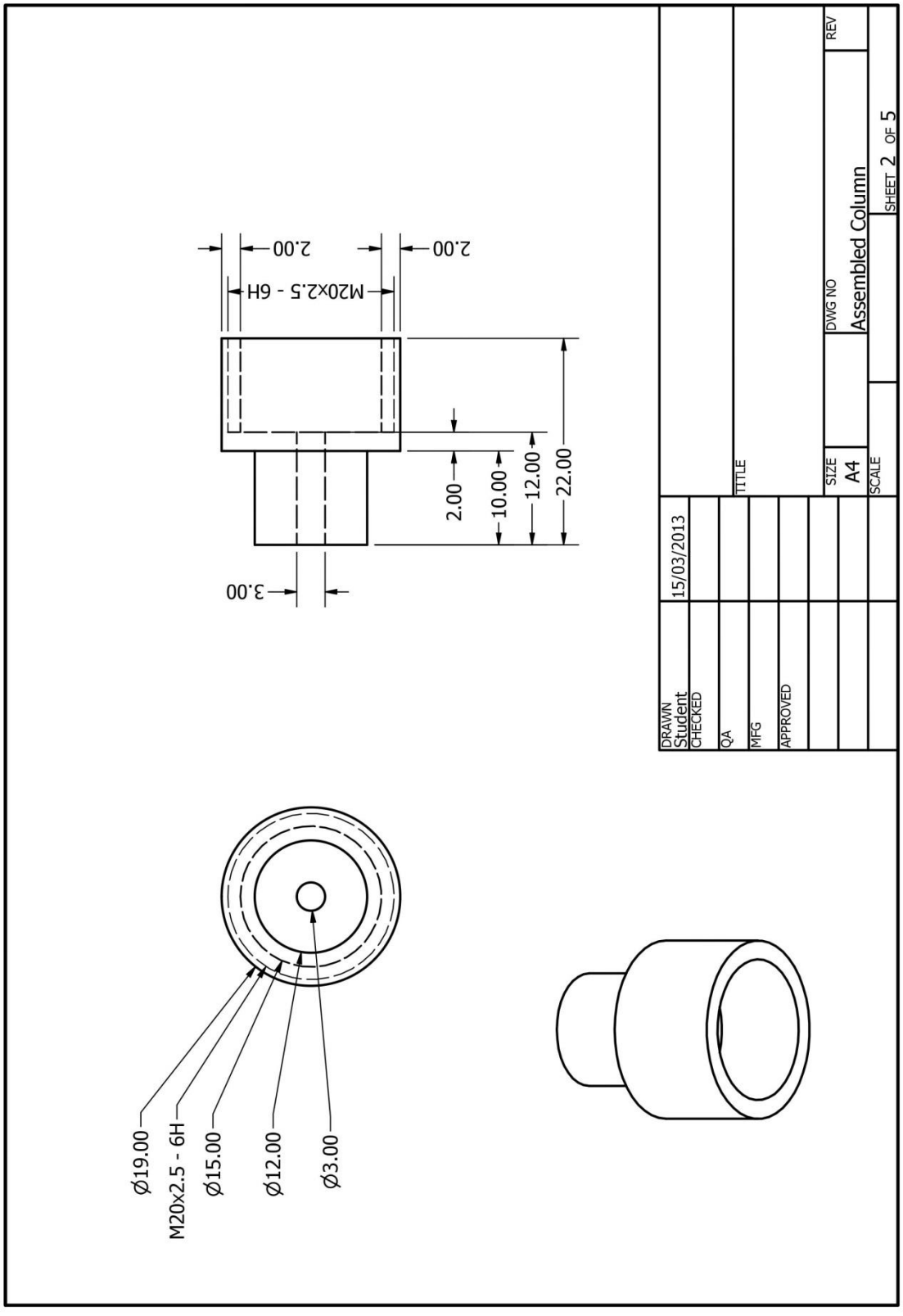
YACHAMANENI, S., YUSHIN, G., YEON, S.-H., GOGOTSI, Y., HOWELL, C., SANDEMAN, S., PHILLIPS, G. & MIKHALOVSKY, S. 2010. Mesoporous carbide-derived carbon for cytokine removal from blood plasma. *Biomaterials,* 31**,** 4789-4794.

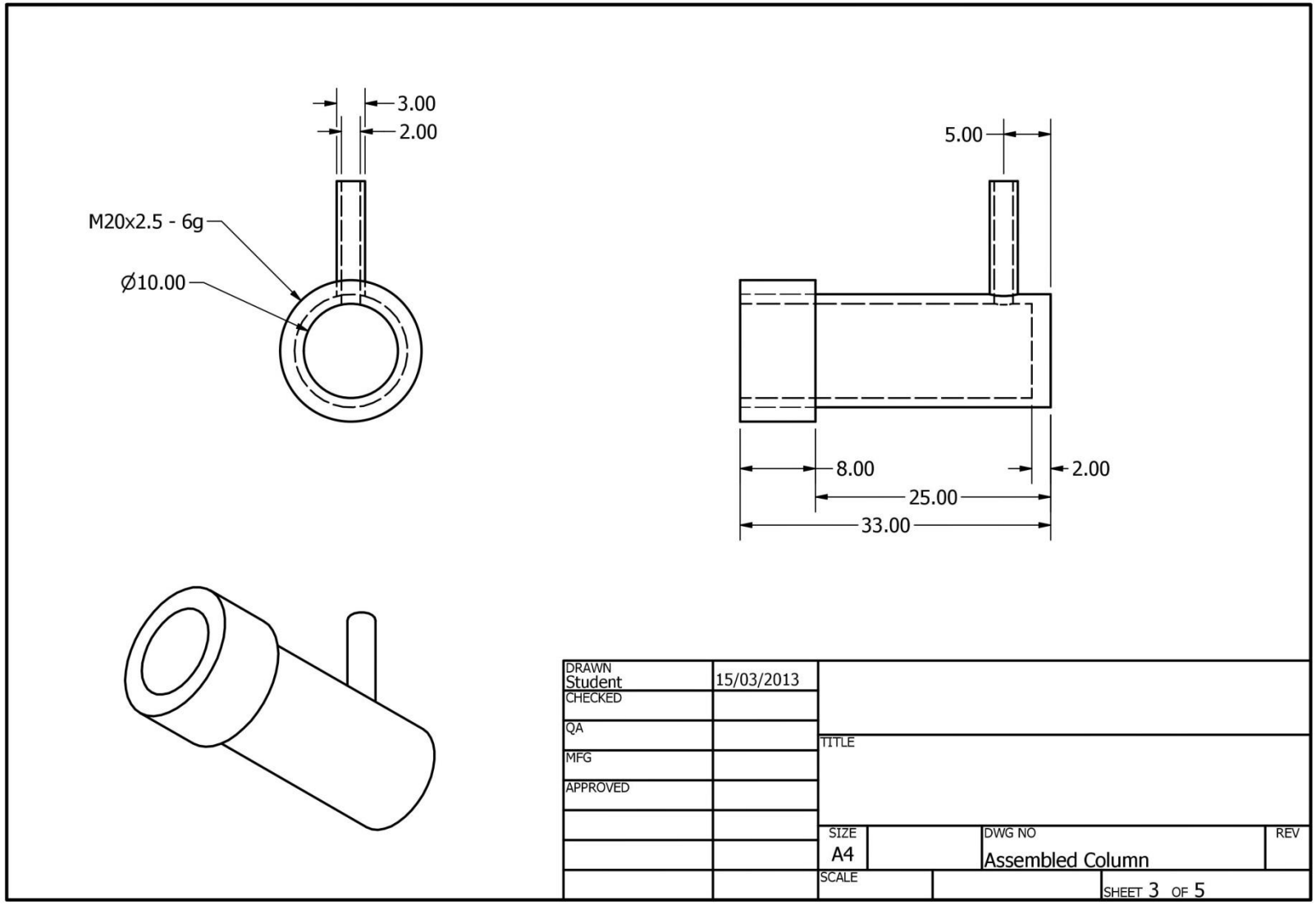
YOSHIHIKO KANNO, M., PHD, HIRONORI NEMOTO, M., PHD, HIDETOMO NAKAMOTO, M., PHD, HIROKAZU OKADA, M., PHD, SOICHI SUGAHARA, M., PHD, KOICHIRO NISHIDOME, N., YOSHIHISA YAMASHITA, N. & HIROMICHI SUZUKI, M., PHD 2003. Selection of hemoperfusion therapy for patients with septic shock on the basis of the primary disease. *The International Journal of Artificial Organs,* 6**,** 205–210.

YOU, S., YUAN-XU, J., SAN-PENG, X., YAN, W., ZHOU-YANG, W., SHI-YING, Y. & SHANG-LONG, Y. 2009. Reduction of pulmonary inflammatory response by erythropoietin in a rat model of endotoxaemia. *Chinese Medical Journal,* 122**,** 834-838.

ZIMBLER, N. & CAMPBELL, A. 2004. Sepsis, SIRS and MODS. *Surgery (Oxford),* 22**,** 73-76.

Appendix I: Layout Drawings for Adsorbent Device





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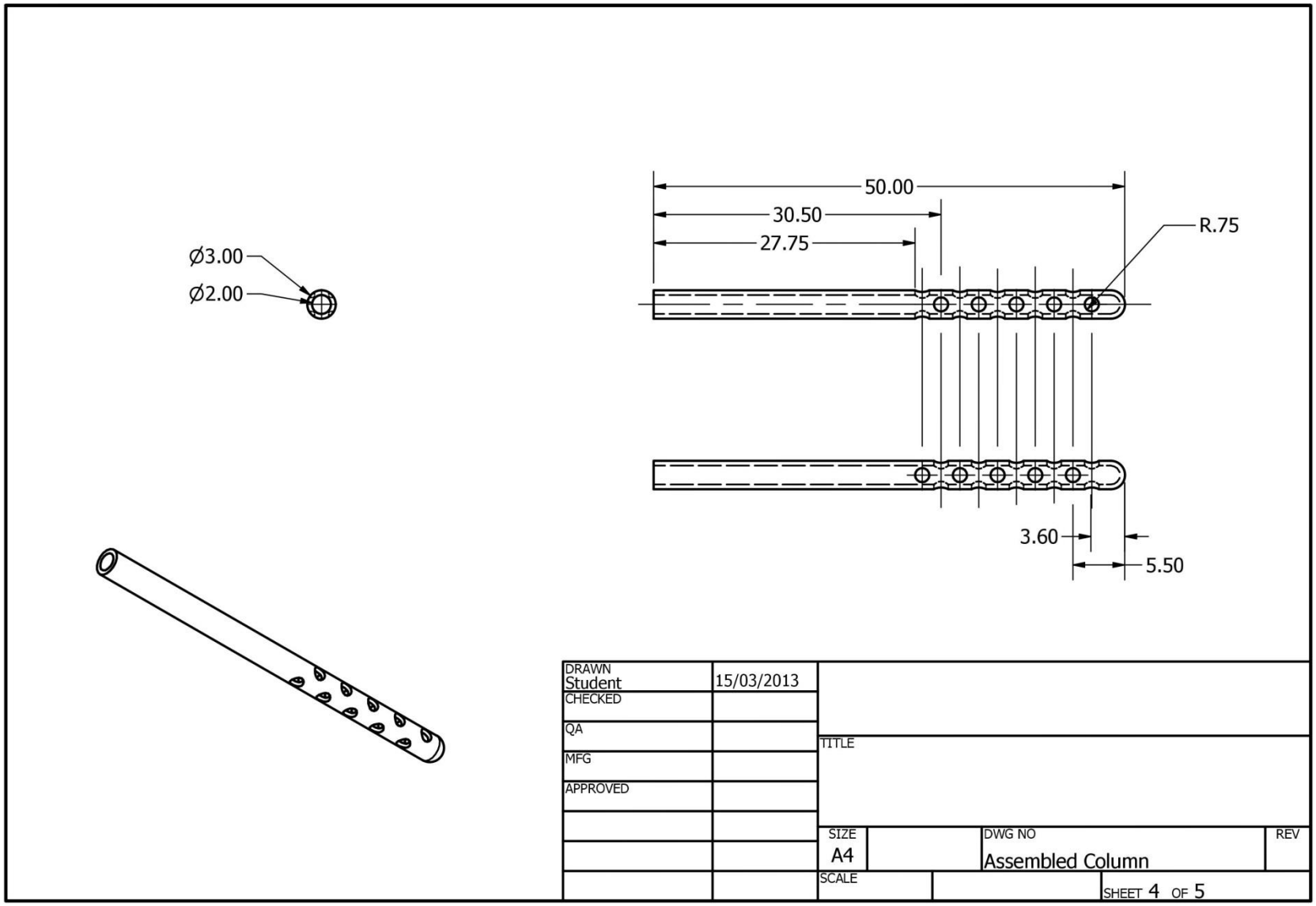

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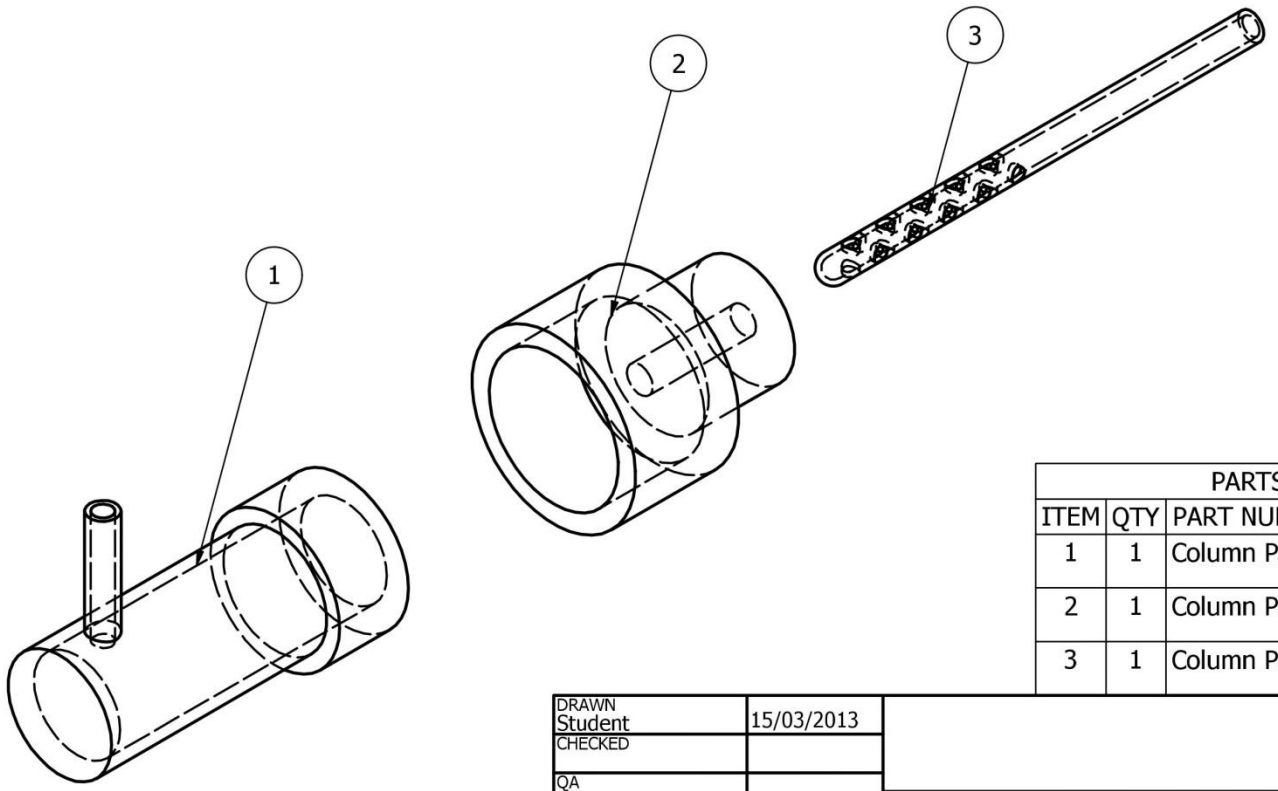

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3	1	Column Part C	

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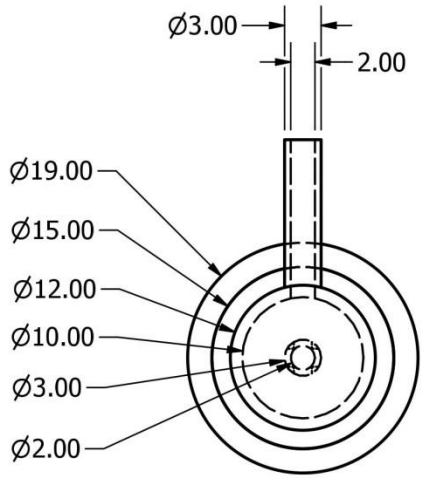

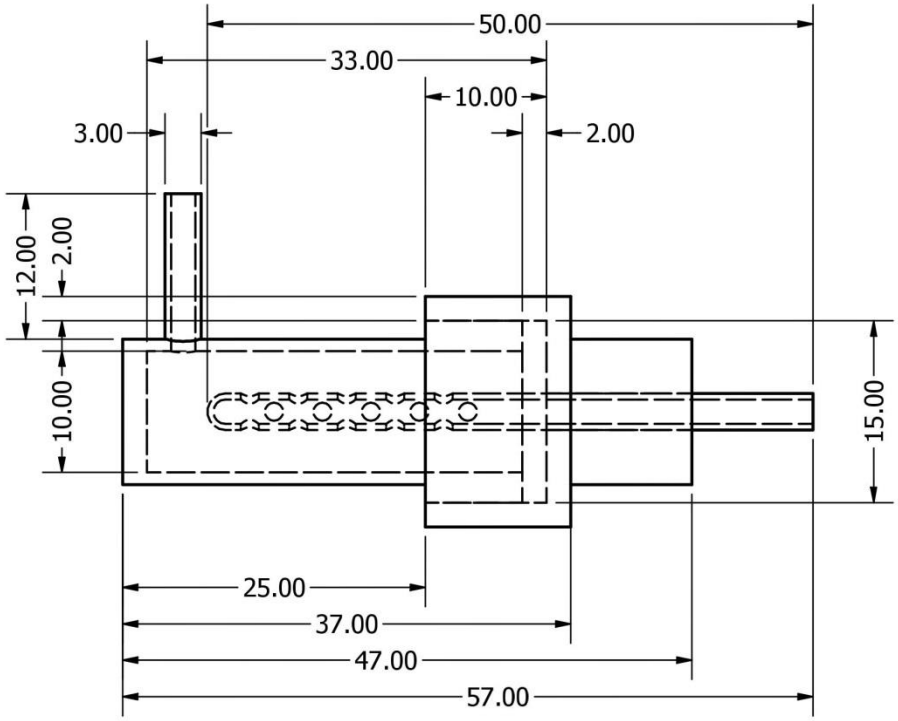

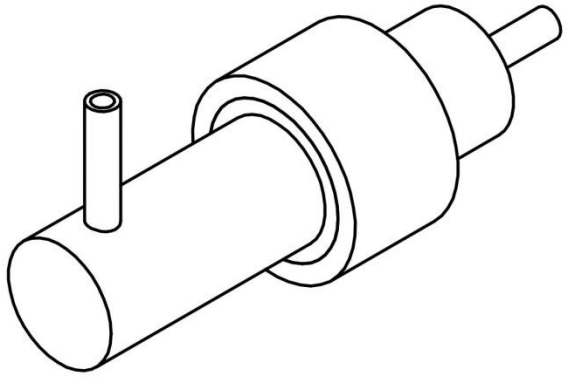

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Appendix II Home Office Personal Licence

Home Office

No. PIL 60/12989

ANIMALS (SCIENTIFIC PROCEDURES) ACT 1986

PERSONAL LICENCE

to

carry out regulated procedures on living animals.

In pursuance of the powers vested in him by the above Act, the Secretary of State hereby licenses

Mr E Coutts
Wolfson Centre
University of Strathclyde
106 Rottenrow East
Glasgow
G1 0NW

to apply the techniques specified in column a of paragraph 15 of the attached Schedule to the kinds of animals in column b of the same paragraph at the place or places specified in paragraph 14 of this Schedule, subject to the restrictions and provisions contained in the Act, and subject also to the limitations and conditions contained in this licence and to such other conditions as the Secretary of State may from time to time prescribe.

This licence shall be in force until revoked by the Secretary of State and shall be periodically reviewed by him.

Home Office
2 Marsham Street
London SW1P 4DF

For the Secretary of State

13 April 2011

NB. This licence does not authorise the licensee to perform any of the procedures specified in it unless they are carried out in the course of a project for which there is a project licence in force under the Act.

WORKING TOGETHER TO PROTECT THE PUBLIC

Home Office

No. PIL 60/12989
13 April 2011

Personal Licence - Additional Conditions

This licence is subject to the following additional conditions -

The performance of all techniques in the attached schedule shall be given the appropriate level of supervision by the project licence holder or an experienced personal licensee deputed by him/her for such time as may be needed to achieve competence.

Home Office

PO Box 6779
DUNDEE DD1 9WN
Tel 01382 223189 Fax 01382 221571
Web Site
http://www.homeoffice.gov.uk/

Mr E Coutts
C/O Ms Horan
S.I.P.B.S.
BPU
161 Cathedral Street
Glasgow
G4 0RE

Your Reference

Our Reference PIL 60/12989

Date 13 April 2011

Dear Mr Coutts

ANIMALS (SCIENTIFIC PROCEDURES) ACT 1986

I am pleased to inform you that the Secretary of State has granted your application for a personal licence.

You should check through your licence carefully for any endorsements on this licence in relation to animal types, procedures, establishments, and the conditions attached to it. A personal licence on its own does not authorise you to perform regulated procedures on protected animals. You may only perform the procedures specified on it in the course of a project for which a project licence has been issued under the Act. The use of unauthorised procedures is a breach of the Act and may result in a prosecution and/or revocation of your licence.

You are required to keep a record of all procedures performed under the authority of your licence. This information must be made readily available to the Inspector or Secretary of State when required. If you cease to carry out work requiring a licence (for example leaving the UK to work abroad) you must return your licence to the Home Office.

As soon as you cease to work at the establishment given as the primary availability on your licence, or it ceases to be the place where you wish your licence to be primarily available, you must notify the Home Office, as this change will affect the fees charged. Under the Act you may not delegate the authorities granted to you under this licence to any other person. No other person may perform, either in whole or in part, any procedure authorised by your licence. The only exceptions are certain specific tasks of a non-technical nature. If you have been granted permission to delegate any such tasks, this will be recorded in additional conditions attached to your licence. No other delegation is permitted.

Should you wish any part of this licence to be amended, you must apply to the Home Office giving details of the changes requested and using the Application for change(s) to a Personal Licence form located at: http://www.homeoffice.gov.uk/science-research/animal-research/

If a condition of supervision is attached to your licence it is your responsibility to ensure that your supervisor is aware of the authority granted to you under this licence.

Yours sincerely

Miss L Doggart

Version Date:	25-02-2011

Applicant to complete version date

PERSONAL LICENCE APPLICATION FORM

		FOR OFFICE ONLY
13 Is this application submitted with a project licence application?	YES ☐ NO ☑ (tick appropriate box)	25 FEB 2011
a. If YES please state the TITLE of the project	NA	PPL No
b. If NO for which projects (identify if possible) or area of work do you require this licence? (First-time applicants must specify the project(s) on which they intend to work)	Investigation of the mechanisms of cardiopulmonary bypass pathophysiology. Professor T. Gourlay, Bioengineering PPL 6003767	PPL No

14 **a.** Title and address (including department) of the designated establishment at which you wish your licence to be primarily available to carry out procedures on living animals subject to the Act.	TITLE University of Strathclyde	PRIMARY AVAILABILITY
	ADDRESS BPU & Registered area within the Wolfson centre 106 Rottenrow East, Glasgow	
	SUPERVISOR Professor Terence Gourlay	
b. If you intend to carry out procedures at more than one establishment please give the names and addresses of the additional establishments and supervisors' names if applicable (Please attach an additional sheet if necessary)	TITLE NA	CODE
	ADDRESS NA	DEPT
	SUPERVISOR NA	
	TITLE NA	CODE
	ADDRESS NA	DEPT
	SUPERVISOR NA	
c. If you intend to carry out procedures at a place other than a designated establishment, please specify the location	NA	CODE

Version Date:	25-02-2011

Applicant to complete version date

15 The techniques and animals for which you seek authority
(Please continue on additional sheets if necessary)

FOR OFFICE USE ONLY

25 FEB 2011

Number	a. Technique Please use codes S and NS respectively to indicate surgical and non-surgical techniques	b. Animal(s)	c. Anaesthesia Please use codes given in note
1	Induction and maintenance of general/local/regional anaesthesia by use of agents and by routes suitable for the species, nature and duration of the procedure (NS)	Rats/Mice	AA/AB/AC
2	Administration of substances, infectious agents or cellular material by the following routes: a) Intravenous injection (NS) b) Subcutaneous injection (NS) c) Intradermal injection (NS) d) Intramuscular injection (NS) e) Intraperitoneal injection (NS) f) Inhalation (NS) g) Topically to the skin (NS)	Rats/Mice	AC
3	Insertion of catheter into jugular, femoral or tail vein. (S)	Rats/Mice	AC
4	Administration of substances, or withdrawal of fluids via previously implanted cannula, catheter, or delivery system. (NS)	Rats/Mice	AC
5	Administration of substances by injection into, or application onto, tissue or organs previously exposed under separate authority. (NS)	Rats/Mice	AC
6	Withdrawal of blood, including by: a) Superficial venepuncture/venesection (NS) b) Cardiac puncture (including exsanguination) (NS) c) Removal of a tail tip or scab (NS)	Rats/Mice	AC
7	Non-surgical placement or removal of electrodes, probes or other devices that may cause pain, suffering, distress or lasting harm. (NS)	Rats/Mice	AC
8	Blood pressure monitoring. (NS)	Rats/Mice	AC
9	Electrocardiograph monitoring. (NS)	Rats/Mice	AC

ANNEX B

Conditions in personal licences

1. In exercising his or her responsibilities, the licence holder shall act at all times in a manner that is consistent with the principles of replacement, reduction and refinement.

2. The licence holder is entrusted with primary responsibility for the welfare of the animals on which he or she has performed regulated procedures; the licence holder must ensure that animals are properly monitored and cared for.

3. The licence holder must not apply a regulated procedure to an animal if the procedure may cause the animal severe pain, suffering or distress that is likely to be long-lasting and cannot be ameliorated.

4. The licence holder must not apply a regulated procedure to an animal unless the holder has taken precautions to prevent or reduce to the minimum consistent with the purposes of the procedure any pain, suffering, distress or discomfort that may be caused to the animal.

5. Where the licence holder is applying a regulated procedure to an animal the holder must ensure that any unnecessary pain, suffering, distress or lasting harm that is being caused to the animal is stopped.

6. Where the licence holder is applying or has applied a regulated procedure which is causing the animal severe pain, suffering or distress the holder must take steps to ameliorate that pain, suffering or distress.

7. The licence holder shall ensure that where the holder applies a regulated procedure death as the end-point of the procedure is avoided as far as possible and is replaced by an early and humane end-point.

8. In all circumstances where an animal which is being, or has been, subjected to a regulated procedure is in severe pain, suffering or distress which is likely to be long-lasting and cannot be ameliorated, the licence holder must ensure that the animal is immediately killed in accordance with section 15A.

9. The licence holder may apply a regulated procedure without the use of general or local anaesthesia only if the holder is satisfied that—

(a) the procedure will not inflict serious injuries capable of causing severe pain; and

(b) the use of general or local anaesthesia would be more traumatic to the animal than the procedure itself or would frustrate the purposes of the procedure.

10. When anaesthesia (whether general or local) is used, it shall be of sufficient depth to prevent the animal from being aware of pain arising during the procedure.

11. If the licence holder applies a regulated procedure to an animal with the use of general or local anaesthesia the holder must, unless it would frustrate the purpose of the procedure, use such analgesics or other pain-relieving methods as may be necessary to reduce any pain that the animal may experience once the anaesthesia wears off.

12. The licence holder must use analgesia or another appropriate method to ensure that the pain, suffering and distress caused by regulated procedures are kept to a minimum.

13. It is the responsibility of the personal licence holder to notify the project licence holder as soon as possible when it appears either that the severity limit of any procedure listed in the project licence or that the constraints upon adverse effects described in the project licence have been or are likely to be exceeded.

14. The licence holder shall ensure that suitable arrangements exist for the care and welfare of animals during any period when the personal licence holder is not in attendance.

15. The licence holder shall ensure that, whenever necessary, veterinary advice and treatment are obtained for the animals in his or her care.

16. The licence holder shall ensure that all cages, pens or other enclosures are clearly labelled. The labelling must be such as to enable Inspectors, named veterinary surgeons and named animal care and welfare officers to identify the number of the project licence authorising the procedures, the project licence protocol in which the animals are being used, the date the protocol was started, and the responsible personal licence holder.

17. In order to ensure that regulated procedures are performed competently, the licence holder shall not apply regulated procedures unless given the appropriate level of supervision by the project licence holder or an experienced personal licence holder deputed by him/her for such time as may be needed to achieve competence.

18. The licence holder is authorised to delegate to assistants, who do not themselves possess the requisite personal licence authority but are under his or her control, the delegable tasks which form an integral part of the regulated procedures the licence holder is authorised to perform by this licence. The tasks must not require technical knowledge or skill, and delegation shall be in accordance with any relevant guidance published by the Secretary of State under section 21.

19. The licence holder must take all reasonable steps to ensure appropriate personal and project licence authorities exist before performing regulated procedures. The licence holder must be aware of the nature of the authorities given by this licence and the project licence, and of the conditions of issue attached to the licences.

20. The licence holder shall maintain a record of all animals on which procedures have been carried out, including details of supervision and declarations of competence by the project licence holder as appropriate. This record shall be retained for at least five years and shall, on request, be submitted to the Secretary of State or made available to an Inspector.

21. The licence holder must give any necessary assistance to inspectors carrying out visits by virtue of section 18(2A)(b) ; and to experts of the European Commission carrying out duties under Article 35 of the Animals Directive.

22. The licence remains the property of the Secretary of State, and shall be surrendered to him on request.

Home Office

Appendix E

STANDARD CONDITIONS: PERSONAL LICENCES

The authority conferred by this licence is subject to the following conditions. Licences may be revoked or varied for a breach of conditions.

In addition, breaches of conditions 1-9 and failure to comply with a requirement under condition 10 may be criminal offences under the Act.

For the purpose of these conditions, "Inspector" means a person appointed under the terms of section 18 of the Animals (Scientific Procedures) Act 1986.

1. No personal licensee shall carry out a regulated procedure for which authority has not been granted in his or her personal licence.
2. No personal licensee shall use in any regulated procedure any type of protected animal not authorised by his or her personal licence.
3. No personal licensee shall carry out any regulated procedure unless authorised by a project licence.
4. No personal licensee shall carry out any regulated procedure as an exhibition to the general public or carry out any such procedure which is shown live on television for general reception.
5. No personal licensee carrying out any regulated procedure shall use any neuromuscular blocking agent in place of an anaesthetic.
6. No personal licensee shall use any neuromuscular blocking agent without express authority from the Secretary of State which must be contained in both the project and personal licences.
7. Unless otherwise authorised by the Secretary of State in both project and personal licences, personal licensees shall perform the procedures for which they have authority only at the place or places specified in their personal licences, and only in suitable areas specified in the relevant certificate of designation.
8. The personal licence holder shall arrange for any animal which, at the conclusion of a series of procedures for a particular purpose, is suffering or is likely to suffer adverse effects to be promptly and humanely killed:
   (i) by a competent person using an appropriate method under Schedule 1 to the Act or another method authorised in the certificate of designation; or
   (ii) by another method authorised by the personal licence of the person by whom the animal is killed.
9. No animal which has completed a series of regulated procedures for a particular purpose may be re-used without express authority in the project licence.
10. If an Inspector requires that an animal must be killed because that Inspector believes that it is undergoing excessive suffering, it must be promptly and humanely killed in accordance with 8(i) or 8(ii) above.
11. It is the responsibility of a personal licensee to ensure that all cages, pens or other enclosures are clearly labelled. The labelling must be such as to enable Inspectors, Named Veterinary Surgeons and Named

Animal Care and Welfare Officers to identify the project in which the animals are being used, the regulated procedures which have been performed, and the responsible personal licensee.

12. The personal licensee is entrusted with primary responsibility for the welfare of the animals on which he or she has performed regulated procedures; the personal licensee must ensure that animals are properly monitored and cared for, and must take effective precautions, including the appropriate use of sedatives, tranquillisers, analgesics or anaesthetics, to prevent or reduce to the minimum level consistent with the aims of the procedure any pain, suffering, distress or discomfort caused to the animals used.

13. It is the responsibility of the personal licensee to notify the project licence holder as soon as possible when it appears either that the severity limit of any procedure listed in the project licence (section 19a) or that the constraints upon adverse effects described in the protocol sheets (section 19b) have been or are likely to be significantly exceeded.

14. In all circumstances where an animal which is being, or has been, subjected to a regulated procedure is in severe pain or severe distress which cannot be alleviated, the personal licensee must ensure that the animal is promptly and humanely killed in accordance with 8(i) or 8(ii) above.

15. It is the responsibility of the personal licensee to ensure that suitable arrangements exist for the care and welfare of animals during any period when the personal licensee is not in attendance.

16. It is the responsibility of the personal licensee to ensure that, whenever necessary, veterinary advice and treatment are obtained for the animals in his or her care.

17. The personal licensee is subject to such supervision requirements as may be stated on the licence or which the project licence holder may deem necessary in order to ensure that regulated procedures are performed competently.

18. Before any animal, or group of animals, that has been subject to procedures is released into the wild, to a farm, or for use as a pet, the personal licensee must ensure that appropriate authority exists in the project licence for the animal or animals to be released.

19. When anaesthesia (whether general, regional or local) is used, it shall be of sufficient depth to prevent the animal from being aware of pain arising during the procedure.

19A. All authorised procedures shall, in accordance with the relevant project licence authorities, be carried out under general, regional or local anaesthesia unless:

(i) anaesthesia would be incompatible with the purposes of the procedures; or

(ii) anaesthesia would be more traumatic to the animal concerned than the procedures themselves.

19B. Except where incompatible with the purposes of the procedures, when an anaesthetised animal suffers considerable pain once the anaesthesia has worn off, the personal licensee shall ensure that, wherever possible, the animal is given pain-relieving treatment in good time or that the animal is promptly and humanely killed in accordance with 8(i) or 8(ii) above.

19C. Where, in accordance with 19A above, anaesthesia is not used, analgesics or other appropriate methods must be used in order to ensure as far as possible that pain, suffering, distress and harm are limited and that in any event the animal is not subject to severe pain, distress or suffering.

20. Personal licensees must take all reasonable steps to ensure appropriate personal and project licence authorities exist before performing regulated procedures, and must be aware of the nature of the current authorities, and the conditions of issue attached to the licences.

21. The personal licensee shall maintain a record of all animals on which procedures have been carried out, including details of supervision and declarations of competence by the project licence holder as appropriate. This record shall be retained for at least five years and shall, on request, be submitted to the Secretary of State or made available to an Inspector.

22. The licence remains the property of the Secretary of State, and shall be surrendered to him on request.

Appendix III: Statement of Compliance Regarding Animal Work

All experimental procedures used in this study were approved by the United Kingdom Home Office under the project licensee, and all procedures were carried out by appropriately trained and licensed personnel.

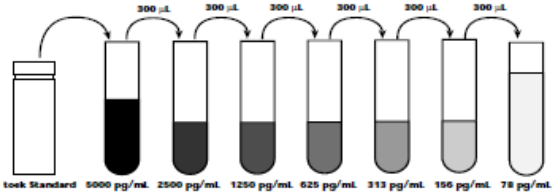
Adult male Sprague-Dawley albino rats (480g-590g) were utilised. All animals were supplied by the Strathclyde Institute of Pharmacy & Biomedical Sciences animal facility at the University of Strathclyde. Prior to the day of experiment all animals were caged in a standard fashion, overseen by University Animal Care, and given food and water ad libitum. The animals were kept in a pathogen free environment in the institutional animal care facility with room temperature maintained at 25ºC and an automated 12-hour light, 12 hour dark cycle.

All experiments conducted throughout this body of work were non-survival. During a procedure and throughout experiments animals were heavily anaesthetised and considered with minimal severity, all experiments were non-survival concluding with euthanasia of each animal.

Appendix IV ELISA Procedure

Standards Preparation and Handling

1. **Reconstitution:** After warming lyophilized standard to room temperature, carefully open vial to avoid loss of material. Reconstitute lyophilized standard with 1.0 mL of deionized water to yield a stock standard. Allow the standard to equilibrate for at least 15 minutes before making dilutions. Vortex gently to mix.
2. **Storage/ handling of reconstituted standard:** After reconstitution, **immediately** aliquot standard stock in polypropylene vials at 50 µl per vial and freeze at -80°C for up to 6 months. If necessary, store at 2-8° C for up to 8 hours prior to aliquotting/freezing. Do not leave reconstituted standard at room temperature.
3. **Standards Preparation for Assay:**
   a. Prepare a 5000 pg/mL standard from the stock standard. Vortex to mix. (See dilution instructions on Instruction/Analysis Certificate.)
   b. Add 300 µL Assay Diluent to 6 tubes. Label as 2500 pg/mL, 1250 pg/mL, 625 pg/mL, 313 pg/mL, 156 pg/mL, and 78 pg/mL.
   c. Perform serial dilutions by adding 300 µL of each standard to the next tube and vortexing between each transfer. Assay Diluent serves as the zero standard (0 pg/mL).



Serial dilutions within the plate may also be performed by pipetting 100 µL of Assay Diluent into each standard well except the highest (5000 pg/mL), then adding 100 µL of the 5000 pg/mL standard to both that well and the 2500 pg/mL well, mixing the well contents by rinsing the pipette tip, and adding 100 µL of the 2500 pg/mL standard to the 1250 pg/mL well. Continue these dilutions to the 78 pg/mL standard well, out of which the extra 100 µL should be discarded.

Warnings and Precautions

1. Reagents which contain preservatives may be toxic if ingested, inhaled, or in contact with skin.
2. Handle all serum and plasma specimens in accordance with NCCLS guidelines for preventing transmission of blood-borne infections.
3. Capture Antibody contains < 0.1% sodium azide. Sodium azide yields highly toxic hydrazoic acid under acidic conditions. Dilute azide compounds in running water before discarding to avoid accumulation of potentially explosive deposits in plumbing.
4. Detection Antibody contains 1% BSA and ProClin™-150 as a preservative.
5. Enzyme Reagent contains 1% BSA and ProClin™-300 as preservative.
6. *Source of all serum proteins is from USDA inspected abattoirs located in the United States.*

Recommended Assay Procedure

1. Coat microwells with 100 µL per well of Capture Antibody diluted in Coating Buffer. For recommended antibody coating dilution, see lot-specific Instruction/Analysis Certificate. Seal plate and incubate overnight at 4° C.
2. Aspirate wells and wash 5 times with ≥ 300 µL/well Wash Buffer. After last wash, invert plate and blot on absorbent paper to remove any residual buffer.
3. Block plates with ≥ 200 µL/well Assay Diluent. Incubate at RT for 1 hour.
4. Aspirate/wash as in step 2.
5. Prepare standard and sample dilutions in Assay Diluent. See "Standards Preparation and Handling".
6. Pipette 100 µL of each standard, sample, and control into appropriate wells. Seal plate and incubate for 2 hours at RT.
7. Aspirate/ wash as in step 2, but with 5 total washes.
8. Add 100 µL of diluted Detection Antibody to each well. Seal plate and incubate for 1 hour at RT.
9. Aspirate/ wash as in step 2, but with 5 total washes.
10. Add 100 µL of diluted Enzyme Reagent (SAv-HRP) to each well. Seal plate and incubate for 30 minutes at room temperature.
11. Aspirate/ wash as in step 2, but with 7 total washes. NOTE: In this final wash step, soak wells in wash buffer for 30 seconds to 1 minute for each wash.
12. Add 100 µL of TMB Substrate Solution to each well. Incubate plate (without plate sealer) for 30 minutes at room temperature in the dark.
13. Add 50 µL of Stop Solution to each well.
14. Read absorbance at 450 nm within 30 minutes of stopping reaction. If wavelength correction is available, subtract absorbance at 570 nm from absorbance 450 nm.

Assay Procedure Summary

1. Add 100 µL diluted Capture Ab to each well. Incubate overnight at 4° C
2. Aspirate and wash 5 times.
3. Block plates: 200 µL Assay Diluent to each well. Incubate 1 hr RT
4. Aspirate and wash 5 times.
5. Add 100 µL standard or sample to each well. Incubate 2 hr RT.
6. Aspirate and wash 5 times.
7. Add 100 µL diluted Detection Ab to each well. Incubate 1 hr RT
8. Aspirate and wash 5 times
9. Add 100 µL diluted SAv-HRP to each well. Incubate 30 min RT.
10. Aspirate and wash 7 times (with 30 sec. to 1 min soaks)
11. Add 100 µL TMB Substrate Solution to each well. Incubate 30 min RT in dark
12. Add 50 µL Stop Solution to each well. Read at 450 nm within 30 min with λ correction 570 nm.