The Receptor for Advanced Glycation End-Products (RAGE) and its Ligands in Systemic Inflammation following Surgery Necessitating Cardiopulmonary Bypass

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Declaration

I hereby declare that this submission is my own work, or if not it is clearly stated and fully acknowledged. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person.
Abstract

Surgery necessitating cardiopulmonary bypass (snCPB) is associated with systemic inflammation which can be severe. Systemic inflammation is common in the critically ill, is associated with adverse outcome and currently has no specific therapy. Insight into the pathogenesis of systemic inflammation may lead to therapies. The receptor for advanced glycation end-products (RAGE) may represent a novel target for intervention.

RAGE is a ubiquitous multi-ligand receptor that is up-regulated in the presence of its ligands. Initially characterised as a receptor for glycated proteins, it is also binds the S100 proteins and high mobility group box 1 (HMGB1); causing pro-inflammatory responses via NF-κB and the MAP kinases. RAGE inhibition has been associated with improved outcomes in animal models of infectious and sterile systemic inflammation.

Of the snCPB patients assessed (n=2440) for relationships between age (associated with RAGE up-regulation) with systemic inflammation and clinical outcome, the oldest patients met more SIRS criteria in the first 1h and 24h following snCPB than those aged 40-80 y. This was accompanied by higher scores of organ dysfunction. Also, plasma levels of RAGE ligands and soluble RAGE increased (n=18-120) around surgery with pre-operative levels correlating with duration of intensive care. Leukocyte cell-surface and intracellular levels of RAGE were assessed and cell surface levels on neutrophils decreased following surgery, possibly contributing to the sRAGE levels in plasma. Cytokine release from whole blood increased following incubation with RAGE ligands, with a diminished effect on whole blood obtained after snCPB, suggesting leukocyte hypo-responsiveness. Finally, genotyping 8 single nucleotide polymorphisms in the RAGE, HMGB1 and S100A8 genes in 187 snCBP patients indicated statistically significant relationships to clinical outcomes such as impaired oxygenation and incidence of acute kidney injury.

The findings from these investigations, inform understanding of the involvement of the RAGE axis in systemic inflammation.

293 words (<300)
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Chapter 1, the image for Figure 3 (SIRS) is reproduced from [1]. The image for Figure 4 (DAMPS) is reproduced from [2]. The image for Figure 12 (S100) is reproduced from [3]. The image for Figure 15 (old age) is reproduced from [4]. The image for Figure 16 (sepsis incidence) is reproduced from [5]. The image for Figure 17 (sepsis incidence in diabetes) is reproduced from [6].

Chapters 6-9, materials and methods: The majority of the methods and materials are common to other members of the group and the descriptions and some images have been adapted from their descriptions.

Chapter 10, SIRS after snCPB and age: The database of clinical characteristics and scores of 2440 patients were compiled by Dr Niall McCallum with the assistance of Dr Simon Finney. All other aspects including the analysis, interpretation and discussion are original.

Chapter 11, release of ligands and soluble RAGE: Plasma was analysed from patients in cohorts B and C. These patients were recruited for clinical research studies by Lauren Hector (LH) and Dr Anna Lagan (AL). Clinical data was collected by me and others. All other aspects including the assays, analysis, interpretation and discussion are original. Advice on statistical analysis was obtained from Winston Banya (WB), Imperial College.

Chapter 12, leukocyte RAGE expression around snCPB: All aspects of sample collection and processing, assay development and use, analysis, interpretation and discussion are original.

Chapter 13, ex vivo assessment of pro-inflammatory effects of RAGE ligands: Collection of patient samples, experimental protocols and assays were collected by me or Myuran Kaneshamoorthy (MK, BSc student). Analysis, interpretation and discussion are original.

Chapter 14, genetic polymorphisms in the genes for RAGE and its ligands: patient samples were analysed from patients in cohorts B; previously collected by AL and LH. Study design and choice of SNPs was original. Primer design and testing was performed by Nekisa Zakeri (NZ, BSc student) with assistance from Dr Panos Pantelidis (PP) and LH. Genotyping was conducted by NZ and me. ‘Phototyping’ was performed by NZ and checked by LH. Clinical data collection was by AL, LH, NZ, Richard Hewitt, Dr Dan Melley and me. Data mining and statistical analysis was performed by NZ and me, with assistance from WB and PP. Interpretation and discussion are original.
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Abstracts


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1. CARDIAC SURGERY AND THE INFLAMMATORY RESPONSE

1.1. Cardiac surgery

1.1.1. Historical perspective

In the modern era, cardiac surgery is a safe and accepted treatment for a range of conditions, with more than 30,000 patients having cardiac surgery per year in the UK alone [7, 8]. However, the practice of surgery on the heart is a relatively recent development. Prior to the development of effective cardiopulmonary bypass (CPB), cardiac surgery was largely confined to a few enthusiasts performing a limited range of procedures with highly variable success rates [9, 10]. The limiting factors were movement of the heart and overwhelming bleeding if the chambers were opened. The heart-lung machine or cardiopulmonary bypass (CPB) was conceived and built by Dr John Gibbon; to provide the surgeon with a motionless and bloodless operating field whilst maintaining the supply of oxygenated blood to the patient. Gibbon’s machine was first used successfully in an 18 year-old with an atrial septal defect in 1953 [11]. Unfortunately, Gibbon’s next four patients died and he abandoned surgery using CPB. During this hiatus, innovators developed cross-circulation techniques to treat congenital heart defects in children by connecting them to the circulation of their parents, blood group compatibility permitting; which met with considerable success [12]. Cross-circulation was superseded by the development of a modification of Gibbon’s machine and by the end of 1956 open-heart surgery was being offered around the world [13]. The safety of CPB greatly improved over the following few decades. Concurrent with this, the range of potential applications of ‘open-heart’ surgery increased to include valve repairs and replacement, coronary artery bypass grafting (CABG), surgery on the aorta, and correction of congenital defects [10].

1.1.2. Modern CPB

Cardiopulmonary bypass describes the diversion of the patient’s circulation around their heart and lungs, into a circuit in which gas exchange occurs and via which newly arterialised blood is returned to the patient; a diagrammatic illustration is shown (Figure 1).
Prior to the initiation of CPB, the patient is anticoagulated using heparin to prevent thrombus formation [14]. The systemic blood is drained from the inferior vena cava via a plastic cannula into the venous reservoir. Additional blood enters the CPB circuit from vents and suction; systems which collect shed mediastinal blood to reduce blood transfusion requirements. Blood is drawn from the reservoir through an oxygenator (which also removes carbon dioxide) by a pump, and the temperature is adjusted. Patients are routinely cooled during surgery to reduce the metabolic requirements of tissues, including the brain and myocardium, that will temporarily be receiving diminished perfusion [15, 16]; they are rewarmed prior to cessation of CPB. The newly oxygenated blood is passed through a particle filter and bubble trap before returning to the patient via a cannula inserted into the aorta at an appropriate pressure and flow. The aortic cannula is sited above the level of a clamp placed across the aorta which restricts the flow of blood to the aortic root and therefore stops blood entering the coronary arteries or flowing back through the aortic valve into the left ventricle. During CPB, cardioplegia solution is instilled into the coronary circulation in order to minimise myocardial oxygen consumption and reduce the accumulation of toxic metabolites.

Important developments in CPB technology have included the use of circuits and oxygenator membranes than are heparin-bonded and bio-compatible, the inclusion of filters, improved efficiency of the oxygenators and monitoring such as real-time blood gas analysis and near-
patient coagulation assessment, controlled hypothermia and re-warming, and advances in the performance of the pumps and anticoagulation (and its reversal).

1.1.3. Complications of snCPB
Modern surgery necessitating CPB (snCPB) is safe; in the UK those having had a CABG have a 30-day survival of 98.4% [8]. However, there are many possible complications including cerebral or myocardial ischaemia, or bleeding requiring blood transfusion or further surgery. There are also complications that develop following progression of the common physiological derangements seen in the immediate post-operative period. In the vast majority of cases such derangements resolve without consequence and recovery is rapid. In a minority, organ failures develop that may lead to death or prolonged illness; which is associated with poor functional outcome, low quality of life and increased mortality [17-19]. CPB also causes systemic inflammation that is a major contributor to the development of acute organ failures [20].

1.1.4. Systemic inflammation and CPB
Systemic inflammation due to CPB develops through a variety of mechanisms. Firstly, during bypass the heart and lungs are ischaemic. Upon reinstitution of circulation the phenomenon of ischaemia-reperfusion injury occurs, resulting in the release of reactive oxygen species (ROS) and pro-inflammatory mediators (e.g. cytokines). Secondly, activation of leukocytes, platelets, the complement and clotting cascades, and release of other inflammatory mediators may occur following the direct contact of blood with the extracorporeal bypass circuitry. Thirdly, relative hypoperfusion of the splanchnic bed, both intra- and post-operatively, may lead to gut wall ischaemia, an increase in villous capillary permeability and translocation of enteral bacteria and bacterial antigens into the systemic circulation (Section 2.3). Finally, it is recognised that haemolysis occurring in the CPB circuit and following the routine suction and transfusion of mediastinal shed blood may contribute to inflammatory responses [20, 21]. These mechanisms are illustrated in Figure 2.
In recent years, cardiac operative procedures have been developed that can take place without CPB. However, off-pump coronary artery bypass (OPCAB) has not yet seen its theoretical advantages reflected in improved outcomes [22]. A large number of, mostly small and uncontrolled, studies have compared different aspects of the inflammatory response between traditional CABG and OPCAB techniques. The off-pump technique is associated with less elevation of some pro-inflammatory cytokines and acute phase proteins in the immediate post-operative period; however, after this time the differences disappear and thus are of uncertain significance [23, 24]. By-products of the development of CPB technology include ventricular assist devices (VADs) used for severe myocardial failure, and extracorporeal membrane oxygenation (ECMO) used for severe respiratory failure. VADs now represent possible destination therapy for selected patients with end-stage cardiac failure [25] and ECMO can be used as temporary support to facilitate oxygenation during reversible severe acute pulmonary failure, as seen during the 2009 H1N1A/Influenza pandemic [26, 27].

1.2. Sepsis, SIRS and systemic inflammation

The concept of sepsis is ancient, the word being derived from the ancient Greek word sepo (σηπω), which means “I rot” [28]. Hippocrates (460-370BC) recognised fever as a major
symptom but it was Celsus (25BC-50AD) who described the four cardinal signs of inflammation as: rubor (redness), tumor (swelling), calor (heat) and dolor (pain) [29]. In 2011, sepsis remains the single most important cause of morbidity and mortality in the intensive care unit (ICU).

Modern use of the term sepsis varied prior to the development of consensus definitions for sepsis and its related conditions at a conference of the American College of Chest Physicians and the Society of Critical Care Medicine (ACCP/SCCM) in 1991 [1, 30]. The aims of standardizing and defining these terms were to enable clinicians and researchers to be more consistent in diagnosis, monitoring and treatment and to facilitate research in the investigation and management of sepsis and its related conditions. The term ‘systemic inflammatory response syndrome’ (SIRS) was introduced to describe the inflammatory process common to both sepsis and the similar clinical condition of inflammatory response following non-infectious insults such as pancreatitis, CPB, burns injury or trauma.

SIRS is defined by acute alterations from baseline in two or more physiological parameters in the absence of any known cause for the abnormalities. These are temperature, heart rate, respiratory rate and white cell count. Sepsis is defined as SIRS due to confirmed infection and is clinically distinct from SIRS alone. Progressively more compromising sepsis is known as ‘severe sepsis’ when organ dysfunction is present, and ‘septic shock’ when there is hypotension resistant to fluid resuscitation (Table 1). Together these conditions are termed the sepsis syndromes and the relationships between infection, bacteraemia, sepsis and SIRS (and some causes) are demonstrated in Figure 3.
<table>
<thead>
<tr>
<th>Condition</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colonisation</td>
<td>Presence of micro-organisms with no host response</td>
</tr>
<tr>
<td>Infection</td>
<td>Microbial phenomenon characterised by an inflammatory response to the presence of microorganisms or the invasion of normally sterile host tissue by those organisms.</td>
</tr>
<tr>
<td>Bacteraemia</td>
<td>The presence of viable bacteria in the blood</td>
</tr>
<tr>
<td>Systemic Inflammatory</td>
<td>The response is manifested by two or more of the following:</td>
</tr>
<tr>
<td>Response Syndrome (SIRS)</td>
<td>• Temperature &gt; 38°C or &lt; 36°C</td>
</tr>
<tr>
<td></td>
<td>• Heart rate &gt; 90 beats per minute (min)</td>
</tr>
<tr>
<td></td>
<td>• Respiratory rate &gt;20 breaths/min or PaCO$_2$ &lt; 32mm Hg (4.3kPa)</td>
</tr>
<tr>
<td></td>
<td>• White blood cell count &gt; 12,000 cells/mm$^3$, &lt; 4,000 cells/mm$^3$ or &gt;10 % immature (band) forms.</td>
</tr>
<tr>
<td>Sepsis</td>
<td>The systemic response to infection. SIRS with documented infection</td>
</tr>
<tr>
<td>Severe sepsis</td>
<td>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.</td>
</tr>
<tr>
<td>Septic shock</td>
<td>Sepsis with arterial hypotension despite adequate fluid resuscitation along with the presence of perfusion abnormalities. Patients who are on inotropic or vasopressor support may not be hypotensive at the time that perfusion abnormalities are measured.</td>
</tr>
<tr>
<td>Sepsis induced hypotension</td>
<td>A systolic blood pressure &lt;90mmHg or a reduction of &gt;40 mmHg from baseline in the absence of other causes of hypotension.</td>
</tr>
<tr>
<td>Multiple organ dysfunction syndrome (MODS)</td>
<td>Altered organ function in an acutely ill patient such that homeostasis cannot be maintained without intervention.</td>
</tr>
</tbody>
</table>

Table 1: Definitions of the sepsis syndromes [1]
Sepsis is a common problem, affecting more than 750,000 patients in United States each year [31]. In the UK, an estimated 31,000 patients were admitted to intensive care units (ICUs) with sepsis in 2004 [32].

A European survey of the prevalence and outcomes of sepsis in the ICU (the SOAP study) revealed it to be present on ICU admission in 66% of patients [33]. The commonest sites for infection during the ICU stay were lung (68%), abdomen (22%), blood (20%) and urinary tract (14%). The overall European ICU and hospital mortality rates were 27% and 36% respectively; rates for the UK were 32% and 41%, respectively. Organ failure occurred in 71% and there was a direct relationship between the number of organs failing and ICU mortality rate [33].

A further analysis of the SOAP study examined specifically the relationship between SIRS criteria and outcome in those with and without infection. In the latter, 82.2% of ICU patients had SIRS (≥2 criteria met), with increasing ICU mortality with the number of criteria met: 2 criteria (10.0%), 3 criteria (19.0%), all criteria (25.0%); there was also an overall increase in organ system failure as the number of SIRS criteria increased from two to three but not to
four. In those ICU patients with infection on ICU, SIRS was universally present and increasing number of SIRS criteria was associated with increased organ failure, prolonged length of stay and increased mortality [34].

Key findings of an influential review of published cohort studies examining the epidemiology of the sepsis syndromes were the very high incidence of SIRS in ICU patients (>50%) and the increasing 28-day mortality from SIRS (10%) to sepsis (20-25%) to severe sepsis (20-40%) to septic shock (40-70%) [30]. The prognosis of the sepsis syndromes, is also related to the nature of underlying disease and the severity of the inflammatory response and its sequelae, reflected clinically as shock and organ system failure [30].

The ACCP/SCCM definitions have been accepted within the critical care community and despite robust criticism and a further consensus conference (2001) have not been revised [35, 36]. Criticisms include being overly sensitive and therefore unsuitable as a trial entry criterion, not reflecting disease severity, not helping understand pathophysiology, and failing to define a distinct pattern of host response to injury [30, 36].

1.2.1. Modern management of the sepsis syndromes

Consensus guidelines on the management of severe sepsis and septic shock were first published in 2004 and revised in 2008 [37, 38]. The guidelines are comprehensive and cover all aspects of therapy. The least controversial recommendations for initial treatment are for the early administration of broad spectrum antibiotics (following drawing blood for culture) and intravenous fluids, prompt confirmation of the source of the infection and source control measures if appropriate, and use of vasoactive drugs to increase persistently low blood pressure following adequate fluid resuscitation. Together these therapies represent the key components of optimal ‘supportive’ therapy. Other recommendations include the use of early goal-directed therapy, measurement of serum lactate concentration, specific haemodynamic targets for resuscitation and time targets for administration of antibiotics (<1h from clinical presentation). The revised guideline recommends administration of activated protein C to patients with severe sepsis and a high risk of death (in the absence of recent surgery) and intravenous hydrocortisone for septic shock when hypotension responds poorly to adequate fluid resuscitation and vasopressors.
The clinical manifestations of SIRS in the absence of infection may be identical to SIRS of infectious origin, and the level of organ support required may be similar. In the absence of clinical trial data specifically obtained from non-infected patients, clinical management is identical to the ‘supportive’ care described above, with the exception of use of anti-microbial drugs.

Despite initially promising pre-clinical studies, no immunomodulatory therapies for the treatment of the sepsis syndromes have entered routine clinical practice [39].

1.2.2. Complications of the sepsis syndromes

All organ systems are susceptible to dysfunction or failure due to the onset and progression of the sepsis syndromes, but only the commonest requiring specific therapy on the ICU will be reviewed. These relate to the pulmonary, renal and cardiovascular systems.

1.2.2.1. Acute lung injury

Systemic inflammation, whether caused by infection or injury, commonly results in acute lung injury (ALI) and its extreme manifestation, the acute respiratory distress syndrome (ARDS). A recent prospective study examining risk factors found 6% of patients with sepsis, 8% with pneumonia and 17.8% with undifferentiated ‘shock’ developed ALI [40].

ALI is defined by refractory hypoxaemia due to non-cardiogenic pulmonary oedema, in the presence of a known predisposing factor. A joint American–European Consensus Conference on ARDS formally defined ALI and ARDS as acute severe respiratory distress of sudden onset; bilateral infiltrates on frontal chest radiograph, in the absence of left atrial hypertension (a pulmonary capillary wedge pressure less than 18mmHg or no clinical signs of left ventricular failure); but with severe hypoxemia (assessed by the PaO$_2$/FiO$_2$ ratio, PFR)[41]. ALI exists when the PFR is <300mmHg (40kPa) and ARDS when it is <200mmHg (26kPa). The definitions have been subject to much debate but have yet to be revised [42].

Pathologically, ALI is characterised by alveolar inflammation and injury leading to increased pulmonary capillary permeability. The syndrome is known to evolve through exudative, inflammatory, and fibroproliferative phases, usually over two to three weeks. The clinical consequences are impaired gas exchange with refractory hypoxaemia attributable to
ventilation-perfusion mismatch; physiological shunting, atelectasis of lung units, and reduced compliance [43].

The treatment of ALI includes management of the precipitating condition and maintenance of acceptable gas exchange without adversely contributing through the application of excessive ventilation (ventilator-induced lung injury, VALI). Therefore ‘lung-protective’ ventilation with small tidal volumes and appropriate levels of positive end-expiratory pressure (PEEP) is the cornerstone of what remains supportive management [44]. Conservative fluid replacement is associated with significantly shortened duration of mechanical ventilation and intensive care unit stay without increasing non-pulmonary organ failures [45]. Early neuromuscular blockade has recently been associated with significantly improved mortality, possibly by indirectly minimizing various manifestations of VALI [46]. Techniques generally reserved for refractory cases (‘rescue’ therapies) include inhaled nitric oxide (NO), placing the patient prone, high frequency oscillatory ventilation (HFOV), and ECMO [47]. There is no effective pharmacological intervention for ALI [48]; corticosteroid administration remaining controversial [49].

The associated mortality has been reported to lie between 15-72% and analyses of change in mortality over time are discrepant [50, 51]. Mortality is partly dependent upon the nature of the underlying precipitant.

1.2.2.2. Acute kidney injury

Acute kidney injury (AKI) is common (36-73%) amongst critically ill patients, measures of incidence varying according to which definitions are used and the specific population [52]. Sepsis, and particularly septic shock, is the commonest cause of AKI in ICU patients [53]. Mortality attributable to AKI, as opposed to the precipitating condition, increases in proportion to its severity. Severe AKI necessitates the use of renal replacement therapy (RRT) and mortality may be as high as 70-90%. Even small increases in serum creatinine are associated with increased mortality [54]. The mechanisms responsible for the increased mortality and morbidity risks are elusive [52].

1.2.2.3. Cardiovascular failure

The cardiovascular dysfunction that frequently accompanies the sepsis syndromes is vasodilatory shock with relative and absolute hypovolaemia, myocardial depression and
altered distribution of blood flow. Vasodilatation is due to decreased ability of vascular smooth muscle to contract, causing hypotension that may be refractory to standard catecholamine therapy. This may be due to endogenous overproduction of NO and adrenoceptor desensitization, potentially attributable to high circulating levels of catecholamines [55]. There is relative and absolute hypovolaemia such that even after the restoration of circulating volume, there remains maldistribution of cardiac output with perturbations in microvascular flow [56, 57]. Up to 50% of those with prolonged septic shock have myocardial depression, as defined by depressed ejection fraction with ventricular dilatation on echocardiography [58].

Management of cardiovascular failure relies upon optimisation of intravascular volume status, may require use of vasoconstrictor drugs to increase vascular tone and thus systemic blood pressure, or the use of positive inotropes to increase cardiac output [57, 59]. The optimal means of assessing the circulation, cardiac output and adequacy of resuscitative efforts are beyond the scope of this thesis.

1.2.3. Post snCPB SIRS and sequelae

Systemic inflammation is common following snCPB; the incidence of SIRS having been reported to be between 28% and 63% [60-63]. Complications of post-snCPB SIRS are common. In a recent prospective cohort study the incidence of ALI following snCPB was 10.2% [40], with earlier estimates of the incidence of post-snCPB ARDS of 0.5-2.5% [64-67]. The incidence of AKI following snCPB varies by use of definitions, however a recent large retrospective study from RBH identified AKI in 25.9% of all patients within the first 7d of surgery (AKIN stage 1 or more) [68]; similar studies have demonstrated associations between severity of AKI and adverse outcome [69].

A common complication following snCPB is cardiovascular failure which is often multifactorial in aetiology. Pathological vasodilatation (vasoplegia) is common, with an incidence reported to be 5-44%, and may require high doses of vasoconstrictors [70, 71]. Post-operative myocardial dysfunction may be due to operative factors, the effects of systemic inflammation and the contribution of pre-operative impairment, with an incidence reported to be 3.9-9.1%. Operative factors include myocardial ischaemia due to prolonged cross-clamp time and inadequate myocardial protection, incomplete revascularisation or
graft failure, reperfusion injury, and hypothermia [72-74]. Management of post-snCPB myocardial failure may include intra-aortic balloon counter-pulsation, selective pulmonary vasodilators, and ventricular assist devices in refractory cases.

1.2.3.1. **Therapeutic approaches**

There are currently no pharmacological interventions that have unequivocally been shown to improve clinical outcomes following snCPB. However, this remains a highly sought-after goal, reflected in the multitude of current/recent trials registered with clinicaltrials.gov. A selection are summarised in Table 2.
<table>
<thead>
<tr>
<th>Redox/nutrition</th>
<th>Inflammation/clotting</th>
<th>Process/technique</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Acetylcysteine</td>
<td>AP214 (α-melanocyte-stimulating hormone analogue)</td>
<td>Perioperative haemodynamic optimization</td>
</tr>
<tr>
<td>20% human albumin solution</td>
<td>TP10, complement inhibitor</td>
<td>Hypertonic saline with hetastarch</td>
</tr>
<tr>
<td>Fish oil emulsion</td>
<td>Pexelizumab (monoclonal antibody targeted against component 5 of the complement system)</td>
<td>Use of cell-saver vs. non-cell saver suction</td>
</tr>
<tr>
<td>Selenium peri-op</td>
<td>Lactobacillus probiotic administration pre-operatively</td>
<td>sevoflurane vs. propofol</td>
</tr>
<tr>
<td>n-3 enriched nutrition therapy peri-op</td>
<td>Ulinastatin (protease inhibitor) and Tranexamic Acid during CPB</td>
<td></td>
</tr>
<tr>
<td>Ascorbic acid pre and post-op</td>
<td>Atorvastatin pre-op</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Peri-op ACE inhibitor or ARB</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bovine Intestinal Alkaline Phosphatase (Anti-LPS) peri-op infusion</td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Summary of registered trials (clinicaltrials.gov on 29/11/10) of interventions for snCPB with markers of systemic inflammation as a primary or secondary endpoint. Grouped by putative mechanism of action.

Many anti-inflammatory therapies have been evaluated as potentially useful adjuncts to standard care. Firstly, corticosteroids are used in many acute and chronic inflammatory conditions and have multiple actions including suppression of leukocyte-mediated inflammation. In the 1960s these were introduced into clinical practice as an anti-inflammatory prophylactic therapy for post-snCPB SIRS, and there followed a series of investigations showing seemingly beneficial effects on levels of circulating mediators and organ function but with mixed results on clinical outcomes. To date there remains no consensus on their use in this setting [75, 76].

Secondly, a non-specific protease inhibitor named aprotinin (trade name Trasylol) was incorporated into routine clinical use in cardiac surgery during the 1990s. Its actions were mediated by the inhibition of complement activation and fibrinolysis (the break-down of
products of coagulation) and it therefore promoted haemostasis and reduced peri-operative bleeding. Additionally it possesses broad anti-inflammatory properties and in vitro data suggested it would limit the development of SIRS responses post-operatively [77-81]. However, over the following decade reports of increased complications culminated in two large multi-centre RCT that found use of aprotinin was associated with significantly increased short and long-term mortality [82, 83] and despite a meta-analysis showing contrary findings [84], led to withdrawal of the drug.

Pharmacological strategies that have yet to provide evidence of clinical benefit but remain under investigation include anti-oxidants, statins, anti-arrhythmics, complement inhibitors, and phosphodiesterase inhibitors. Non-pharmacological treatments that currently have insufficient clinical trial data to support their use include leukocyte filtration and endotoxin absorption [85-88].

1.3. Assessing the severity of systemic inflammation
There are several methods of measuring the severity of systemic inflammatory response, all with specific advantages and limitations. An ideal biomarker would vary in proportion to the severity of inflammation and any therapy, be highly specific and sensitive, add independent prognostic information, have biological plausibility and potentially identify patient subgroups that would benefit from specific interventions. Further desirable characteristics would include low cost, rapid results and high reproducibility.

In the specific context of measuring systemic inflammation following snCPB, an International expert committee met and reported in 2008 [89]. In this report the limitations of applying standard SIRS criteria were discussed and a division drawn between markers likely to be causally involved in the systemic inflammatory process (e.g. levels of circulating pro-inflammatory cytokines), and clinical end-points (e.g. ICU length of stay, LOS) or markers of organ dysfunction. A recommendation was made that both types are reported with a strong emphasis on the clinical end-points, including ICU LOS [89].

1.3.1. Clinical assessment
The parameters assessed when determining SIRS criteria are purposely readily observable clinically (temperature, heart rate and respiratory rate) and incorporate one laboratory result (white blood cell count). The SIRS criteria were designed to be highly sensitive but not
to reflect severity. Therefore, the individual parameters cannot be expected to vary in proportion to extent of systemic inflammation, nor should the number of SIRS criteria met be considered proportionate to its extent. Furthermore, each of the defining parameters can be altered by factors unrelated to systemic inflammation. For example, tachycardia can be masked by the use of beta blockers or induced by the use of a cardiac pacemaker, tachypnea altered by the use of invasive mechanical ventilation, and the febrile response is frequently impaired or obfuscated by concurrent heat losses, deliberate cooling or antipyretic drugs.

These limitations may restrict the validity of conclusions drawn regarding the extent of systemic inflammation based on these parameters in an individual patient. Although when considering a large population of patients this is not the case; as previously discussed, in a large observational study increased numbers of SIRS criteria met was related to increased organ failure and mortality, irrespective of the presence of infection [34].

1.3.2. Scoring systems

Scoring systems have been developed to quantify the severity of illness and extent of organ failure. The most widely used is the Acute Physiology and Chronic Health Evaluation (APACHE) II score. Simpler systems include Multiple Organ Dysfunction Score (MODS [90]), the New Simplified Acute Physiology Score (SAPS II [91]), and Sequential Organ Failure Assessment (SOFA [92]) scores. All have been validated in cardiac surgical patients [93-97]. The Euroscore has been designed specifically for cardiac surgery; it uses clinical and operative variables to predict early mortality and allows risk-adjusted comparison between surgical populations [98].

Scoring systems are useful for predicting mortality and in some cases, duration of ICU LOS and mechanical ventilation, as an indicator of quality of care, by comparing observed with predicted outcome, and for benchmarking to aid comparison between ICUs. They are not intended to guide individual patient management as calculated probabilities of mortality are valid only for populations, not individuals [99, 100].

1.3.3. Plasma assays

CRP, an acute-phase protein, is an exquisitely sensitive systemic marker of inflammation and tissue damage. The 99th centile of plasma CRP in healthy adults is 10mg/l. It is produced by
hepatocytes, predominantly under transcriptional regulation by IL-6. Synthesis starts rapidly following a stimulus, reaching a peak around 48h. The biological role of CRP is to bind a range of ligands, autologous and extrinsic, and promote phagocytosis and initiation of the complement cascade [101]. The major shortcoming in use of CRP is the delay between the onset of inflammatory stimulus and peak plasma levels.

An elevated white blood cell (WBC) count, or more specifically, neutrophil count, is an accepted, simple and established marker of inflammation or infection, but which has major limitations to its use as a marker of extent of systemic inflammation. Concurrent bone marrow failure or a ‘toxic left shift’ phenomenon (bone marrow stimulation resulting in release of immature neutrophil precursors) will reduce the numbers of circulating neutrophils in spite of infection. The extent of elevated neutrophil count (neutrophilia) may fluctuate during the course of illness or be elevated in the absence of inflammation, by procedures such as splenectomy, medications such as corticosteroids or conditions such as malignancy. Moreover, neutrophilia is insensitive for discriminating systemic response to infection from localised infections [102, 103].

A more recently identified marker of inflammation is procalcitonin (PCT); a pro-hormone peptide secreted by non-neuroendocrine cells in response to infection or inflammation. Levels rise within 2-4h of any stimulus, peaking at 8-24h and remaining elevated as long as the inflammation continues. Despite the tendency for PCT to rise massively above minimum detectable levels, there are multiple reports of patients with severe inflammatory illnesses with serum levels <2ng/ml or even <0.5ng/ml (the indeterminate level of the common LUMItest assay). Thus, a lack of sensitivity and specificity limit its clinical use [104].

Levels of systemic pro- and anti-inflammatory cytokines may be assayed and in the context of snCPB have been demonstrated to be raised in numerous studies [105-109]. Circulating cytokine levels in patients with sepsis relate to the development of organ dysfunction and death [110] and may augment traditional scoring systems [111, 112]. However, no such relation to organ failure and outcome has been demonstrated in patients following snCPB [113]. Cytokine assays have not entered routine clinical use, probably due to the expense and lack of provision of sufficient additional clinically useful information.
1.3.4. Cellular assays

The measurement of cell surface markers on circulating leukocytes through flow cytometry can provide detailed information about specific populations of leukocytes and their cellular activation and function, and are of established use in the laboratory investigation of haematological malignancies and immunodeficiencies [114, 115]. It is also possible to measure biomolecules including phosphorylated cell signalling proteins within cells through rendering the plasma membrane permeable (permeabilisation) to the antibodies used in the preparation for flow cytometry, which can provide mechanistic insight [116].

Measures of leukocyte membrane expression are being developed for investigation of the sepsis syndromes and associated perturbations in immune status. The extent of immune suppression that can follow acute severe systemic inflammation may be assessed by measurement of monocyte Human Leukocyte Antigen (HLA) DR expression [117]; levels have been associated with development of nosocomial sepsis and death [118] and have been used to guide immuno-stimulatory therapy [119]. The analysis of expression of CD64 (an Fc receptor) and CD35 (a complement receptor) on circulating neutrophils from patients can distinguish between bacterial infection, viral infection and sterile inflammation [120]. Similarly, quantification of expression of CD69 (human transmembrane C-Type lectin protein) on lymphocytes has shown potential to discriminate infectious from injurious systemic inflammation [121]. A study of the potential advantages of leukocyte monitoring over routine clinical plasma assays of systemic inflammation is currently recruiting, enrolling patients following surgical procedures (clinicaltrials.gov NCT01250574).

A distinct form of cellular monitoring is the measurement of the RNA expression profile of leukocytes; this has been described as the ‘riboleukogram’. Characterisation of the transcriptional profile of circulating leukocytes in clinical inflammatory conditions has the potential to improve diagnostics, particularly differentiation between infectious and non-infectious SIRS [122-125]. The characterisation could involve examination of expression of specific genes of interest, or use gene array chips that analyse the expression of thousands of genes. Although only studied in a small number of patients, there are indications that onset of infection-specific transcriptional changes may precede the development of clinical features, thus permitting earlier diagnosis and treatment [122].
1.3.5. Proxy measures

The length of ICU stay (LOS) in patient populations relates primarily to the need for advanced supportive therapies and monitoring. As such, it represents a composite measure of organ failures and extent of any inflammatory response.

1.4. Post-operative SIRS as a model of human systemic inflammation

To study the onset of systemic inflammation it is necessary to identify patients who can be studied prior to its development. Therefore elective surgery is an appropriate model and snCPB particularly useful as it is associated with frequent and significant systemic inflammation. Since patients are identified when they are well, there are fewer difficulties obtaining consent for research participation. Patients undergoing snCPB are routinely monitored using arterial and central venous cannulae that facilitate painless acquisition of blood samples.

Patients undergoing snCPB have a range of underlying pathologies, treated with different procedures (e.g. CABG versus aortic valve replacement). However, there are many similarities including the route of surgical access and the techniques of CPB. The procedures are sufficiently similar to induce comparable systemic inflammation, assessed with a range of assays [126].

2. MECHANISMS OF SYSTEMIC INFLAMMATION

2.1. Mediators and cytokines

Cytokines are low molecular weight proteins or glycoproteins that function as intercellular messengers. They include interleukins (ILs), interferons (IFNs), tumour necrosis factors (TNFs), growth factors, transforming growth factors, colony stimulating factors and chemokines. Their synthesis and secretion are highly regulated; they are highly potent and have short-lived effects. Their roles are varied and include control of cell proliferation, differentiation and metabolism; immune modulation of cytotoxic cells, phagocytic cells, and immunoregulatory cells; regulation of acute phase response and fever; and wound healing and remodelling.
2.1.1. Chemokines

Chemokines are a subgroup of cytokines that direct the movement of circulating leukocytes to sites of inflammation or injury i.e. they are chemotactic. Chemokines and their receptors have a standardized nomenclature based on their cysteine-related structures. CC chemokines have two adjacent cysteine residues and the group includes monocyte chemoattractant protein 1 (MCP-1). The archetypal chemokine IL-8 is in the CXC group, characterised by a single amino acid residue between the first two cysteines and is also known as CXCL8, paired with its receptor CXCR1. The third group (CX3C) has only one member, fractalkine, similarly the fourth only has lymphotactin.

IL-8 is produced by both neutrophils and monocytes and it has well defined and important functions including chemoattraction and neutrophil activation. In patients with septic shock, changes in plasma levels of IL-8 are associated with those of other inflammatory mediators and to clinical outcome [127, 128]. Elevated circulating levels of IL-8 have been similarly demonstrated in other systemic inflammatory conditions including snCPB [129], pancreatitis [130] and polytrauma [131].

2.1.2. IL-1β

Classical pro-inflammatory cytokines include IL-1β, IL-6 and TNF-α. Interleukin-1β is a 15kDa protein secreted by monocytes and macrophages and has the capacity to induce a range of genes including those for more cytokines, adhesion molecules and chemokines. IL-1β is thought to be partially responsible for some of the classical features of infection or inflammation including fever (acting on the hypothalamus) and raised numbers of circulating neutrophils (acting on the bone marrow to induce neutrophil production, maturation and release)[132]. Recombinant analogues of the naturally-occurring IL-1 receptor antagonist (e.g. Anakinra) have been shown to be beneficial in patients with systemic inflammatory conditions such as rheumatoid arthritis, but not in those with severe sepsis [133, 134].

2.1.3. IL-6

IL-6 is a 22-27kDa protein secreted predominantly from immune cells but also by endothelial cells and adipocytes. It binds the IL-6 receptor (IL-6R) and through interaction with a complex of glycoproteins (gp130), activates pro-inflammatory responses via the
MAPK pathways. The IL-6 receptor can disassociate from the cell membrane and continue to function as a soluble receptor (sIL-6R), binding IL-6 and then interacting with gp130. The importance of this is that IL-6 mediated cellular responses are possible in the wider range of cells that possess gp130, but do not endogenously express IL-6R [135].

Similarly to IL-1β, IL-6 stimulates the febrile response via actions in the hypothalamus. IL-6 stimulates synthesis of acute phase proteins, including CRP by hepatocytes. In addition to its pro-inflammatory actions, IL-6 has anti-inflammatory attributes, suppressing TNF-α and IL-1β and promoting synthesis of IL-1ra and soluble TNF receptor p55. Relative to IL-1β and TNF-α, IL-6 levels remain elevated in the circulation for longer during systemic inflammation. IL-6 has been described as a cytokine mediating the resolution of acute inflammation by balancing pro-inflammatory effects and orchestrating the transition from innate to acquired immunity [136]. Antagonism of IL-6 with humanized monoclonal antibodies (Tocilizumab) is an effective therapy for rheumatoid arthritis but has not been studied in the sepsis syndromes [137].

2.1.4. TNF-α

TNF-α is a 17kDa protein produced mostly by monocytes and macrophages. It has pleiotropic actions on cellular activity and includes the release of soluble mediators, the induction of gene expression, growth inhibitory and cytotoxic effects, and the enhancement of proliferation [138]. There are other tumour necrosis factors including TNF-β (also known as lymphotoxin-α), lymphotoxin-β and Fas ligand. There is also a family of TNF receptors. TNF-α is initially formed as a transmembrane protein cleaved by TNF-α converting enzyme (TACE) to release soluble TNF-α, which forms a homo-trimer. It binds the TNFR1 (p55 or CD120a) and TNFR2 (p75 or CD120b) receptors, each initiating different intra-cellular signalling events. TNFR1/2 are constitutively cleaved by TACE, causing release of soluble sTNFR1/2. Levels of the soluble receptors reflect the activation state of the TNF-α/TNF receptor system and can be used to monitor the progression of inflammation [139].

Soluble TNFR1/2 may compete for plasma TNF-α and reduce cellular TNFR1/2 signalling, thereby attenuating inflammatory responses in a negative feedback loop. However, in vivo the pathway is far more complex and administration of exogenous sTNFR1/2 has adverse effects, possibly by preventing elimination and prolonging the effects of TNF-α [140, 141].
By contrast, TNF-α antibodies have been found to be clinically efficacious in rheumatoid arthritis, inflammatory bowel disease and some inflammatory dermatoses [142, 143]. Antagonism of TNF-α in the sepsis syndromes has not been successful and this is likely to be due to several reasons. Thus, levels peak very early after the onset of acute illness and are already declining when patients present for immuno-modulatory intervention, the selection of patients may have been suboptimal, or the early elevations of TNF-α may be a necessary and advantageous response promoting appropriate immune cell recruitment and activation.

2.1.5. SIRS, CARS or both?
Excessively elevated levels of circulating pro-inflammatory cytokines can be measured in patients with SIRS and are implicated in the development of organ failure. Elevated levels of anti-inflammatory mediators including IL-10, IL-1ra, and sTNFR1/2 are also detectable concurrently. This response has been described as the compensatory anti-inflammatory response syndrome (CARS) and was initially thought to succeed SIRS; it is now believed to overlap with pro-inflammatory responses. CARS is implicated in the development of a hypo-responsive immune system (immunoparesis) with the consequent development of nosocomial infections. It has been argued that the CARS response is of greater clinical significance than SIRS, but in general the pro- and anti-inflammatory responses are equally proportionate to the severity of illness [144-147].

2.2. Leukocyte responses

2.2.1. Neutrophils
Neutrophils are formed in the bone marrow in response to the growth factor granulocyte colony-stimulating factor (G-CSF). Following 14-16 days of maturation they are released into the circulation and after 4-6h they transmigrate into tissues. After 2-6 days they undergo spontaneous programmed cell death, termed apoptosis. During inflammation, large increases in the numbers of effective cells are achieved rapidly by the delay of tissue apoptosis, the increased release of mature neutrophils from the marrow and the mobilization of neutrophils from a marginated pool within the bone marrow sinusoids. Apoptosis may be an evolutionarily conserved mechanism for limiting excessive inflammation and is delayed in patients with the sepsis syndromes [148, 149]. Conversely, augmentation of neutrophil apoptosis is necessary for the resolution of inflammation [150,
Following neutrophil apoptosis the shrunken cell remnant is phagocytosed by macrophages (efferocytosis), resulting in release of G-CSF.

Neutrophils are the principal circulating inflammatory cells and in addition to phagocytosis they have other important functions. They migrate to inflammatory foci by margination, rolling, then adherence and transmigration (via a process termed diapedesis) across the endothelium into the tissue. Neutrophils move towards a focus of infection by following concentration gradients of chemoattractants, a process termed chemotaxis. Neutrophil activation is a two-step process. Priming occurs through interaction with cytokines or activated endothelial surfaces. Neutrophil degranulation follows further exposure to inflammatory stimulus and aims to destroy invading pathogens through the release of preformed granular enzymes and proteins, and production of reactive oxygen species (ROS). An unfortunate side effect of neutrophil degranulation is tissue injury mediated via the proteases and ROS which damage cells and the extracellular matrix. Tissue injury acts as a further stimulus for recruitment of inflammatory cells and a cascade effect results.

In patients with the sepsis syndromes there are elevated levels of circulating cytokines that have been implicated in impairing the ability of circulating neutrophils to migrate to the site of infection by reducing neutrophil-endothelium adhesion. The combination of neutrophil activation and lifespan extension with failure to appropriately migrate has been implicated in the development of organ dysfunction, particularly acute lung injury [152-155].

Neutrophils can synthesize and secrete a number of chemokines, including IL-8, growth-related oncogene-α (GROα), Macrophage Inflammatory Proteins-1α/β (MIP1-α/β), Interferon gamma-induced protein 10 kDa (IP-10) and monokine induced by gamma interferon (MIG). These cytokines are chemotactic for monocytes, immature dendritic cells, T lymphocytes and more neutrophils [156].

Microbes or microbial particles that have been opsonised bind to specific receptors on neutrophils initiating phagocytosis. Once the opsonised particles have been engulfed within a phagosome, intracellular killing takes place. The respiratory burst is the formation of toxic ROS and hypochlorous acid within the phagosome. A second mechanism of intracellular killing is the fusion of granules rich in hydrolytic and digestive enzymes.
Neutrophil extracellular traps (NETs) are extracellular fibres of granule proteins and chromatin that bind bacteria and fungi, preventing them from spreading; ensuring a high local concentration of antimicrobial agents to degrade virulence factors and kill bacteria [157].

2.2.1.1. Assessing neutrophil inflammatory status

Neutrophil function may be assessed by a variety of means. A functional assay involves an assessment of alteration in leukocyte function in response to a stimulus. Alterations in cytokine secretion represent a simple and accessible functional endpoint. The measurement may be the secreted cytokines, or may include the quantity from lysed cells. Common methods of quantitation of cytokine protein levels include ELISA or Western blot. To assay relative quantity of cytokine mRNA expression, qRT-PCR can be used.

Proteins that are components of up-stream signalling pathways that progress to changes in inflammatory cell function may also be assayed. The mitogen-activated protein kinases (MAPK) are commonly studied. These cell signalling pathways link activating stimuli to phosphorylation of numerous proteins including transcription factors, cytoskeletal proteins and other enzymes. There is an exhaustive array of interconnected and tightly controlled intracellular signalling pathways that are activated or deactivated through protein phosphorylation. Commonly, investigators compare the expression of phosphorylated to total forms of ERK1/2, JNK or p38 in whole cell lysates using Western blotting.

Dynamic features of leukocyte function may be examined using in vitro assays. Chemotaxis may be studied through use of a cell-staining fluorescent dye (such as CFSE), and a potent chemo-attractant (such as IL-8) deployed in wells with permeable cell-counting inserts. Phagocytosis may be studied through the use of pH-sensitive fluorescent probes. Intracellular killing may be studied through the use of fluorescent probes that become activated upon oxidation (such as carboxy-H2DCFDA). Neutrophil degranulation may be studied with fluorogenic substrates of myeloperoxidase (MPO), a major constituent of the azurophilic granules that are released extracellularly during degranulation.

2.2.2. Monocytes

Mononuclear phagocytes share many properties with neutrophils and have distinct functional properties. Their major functions include phagocytosis, antigen presentation, and
immunomodulation. They are formed in the bone marrow from a common myeloid progenitor that is shared with neutrophils before they enter the circulation while still immature and circulate for several days before entering tissues and differentiating into either macrophages or dendritic cells (DCs). They account for 5-10% of circulating leukocytes [158].

Within the circulating population of monocytes there is heterogeneity, and initially they were divided into subsets on the basis of their morphology. Their classical appearance is of an irregular cell shape with a large kidney-shaped nucleus and cytoplasmic vesicles [159]. Differences in chemotaxis, cytokine secretion, respiratory burst, and antigen presentation were described. More recently, distinct populations of circulating monocytes have been described based upon differences in cluster differentiation (CD) markers.

Of the many subsets of monocytes, expression of CD14 (a co-receptor with TLR-4 for bacterial endotoxin) and CD16 (the immunoglobulin receptor, FcγRIII) permits the categorization of monocytes. The largest group (80-90%) are CD14+CD16- ‘classical’ monocytes; with higher phagocytic activity but lower cytokine expression. By contrast, CD14+CD16+ monocytes (~10% of the total) have been characterised as 'pro-inflammatory'. They are increased during infections and produce more pro- than anti-inflammatory cytokines in response to stimulation [160, 161]. There are many other subgroups defined by CD markers and postulated to have divergent functions, with minor subsets having common features to macrophages and dendritic cells [162].

In common with other leukocytes, monocytes are recruited to sites of inflammation by margination, rolling, adherence and transmigration. They accumulate more gradually at sites of inflammation and persist for longer. Consistent with this, their respiratory burst is less extreme. Their microbial killing relies on the formation of ROS and reactive nitrogen species (RNS) as well as upon the production of phagolysosomal enzymes. Monocytes have the capacity to kill a wider range of microbes than neutrophils.

During severe sepsis monocytes may become hypo-responsive to inflammatory stimuli and turn to producing anti-inflammatory cytokines such as IL-10 and IL-1ra (receptor antagonist). In parallel with this, their surface expression of HLA-DR decreases [163].
2.2.2.1. **Dendritic cells (DCs)**

DCs have a primary role as antigen-presenting cells interacting with T cells and modulating their responses. They are present in the blood, the lymphoid tissues, epithelia such as mucosa and epidermis, and in the interstitium of organs. Exogenous antigens are internalised and then processed for MHC (Major Histocompatibility Complex) presentation. The major subsets are myeloid and plasmacytoid DCs, the latter being originally considered to arise from a lymphoid lineage [164]. Infections and microbial components accelerate differentiation of monocytes into DCs and cause phenotypic changes; migration from peripheral sites to lymphoid tissues, up-regulation of antigen presenting molecules, up-regulation of molecules that interact with T lymphocytes and production of cytokines (IL-12, TNFα, IL-10, IL-6, IFN-α/β)[165]. DCs regulate which class of effector T cells are induced. Naïve CD4+ T cells can differentiate either into T helper 1 cells (Th1) or T helper 2 cells (Th2) cells [166].

2.2.2.2. **Macrophages**

Following their exposure to the environment of the circulation, monocytes selectively ‘home’ to different tissues to reside. Their functions in the tissue parenchyma alter in response to their microenvironment, particularly the presence of inflammatory stimuli or microbes. Resident macrophages fulfil a wide variety of roles and are known by different names according to tissue: Kupffer cells in the liver, Langerhans cells in the skin, microglial cells in the brain, osteoclasts in the bone and histiocytes in connective tissues.

Macrophages may become polarised in response to their microenvironment. A simplified explanation of the types includes a ‘classical’ macrophage type (M1) that is an efficient producer of ROS and inflammatory cytokines and participates in Th1 responses. The M2, non-classical, macrophage participate in Th2 reactions, promote encapsulation and killing of parasites, and promote tissue repair and remodelling [167].

2.2.3. **Lymphocytes**

There are T (thymus) and B (bone) lymphocytes, and natural killer (NK) cells. T lymphocytes can be divided into CD4+ cells, known as helper T cells and CD8+ cells, killer T cells. Helper T cells are prolific cytokine producers and are further divided by the type of cytokines they produce. Th-1 cytokines promote inflammation and anti-microbial responses, particularly
against intra-cellular organisms. Th-2 cytokines include IL-4, -5 and -13, associated with promotion of IgE and eosinophilic responses, and also the anti-inflammatory cytokine IL-10. A balanced Th-1 and Th-2 response is desirable. B lymphocytes are responsible for the production of antibodies, formation of plasma cells and memory B cells - the humoral immune response.

In patients with sepsis, lymphocyte populations are universally and significantly decreased and remain so for at least the first 48h [168]. Their function is also impaired with increased expression of inhibitory receptors, diminished ex vivo responsiveness, increased Th-2 to Th-1 ratio, induction of regulatory T cells and increased apoptosis [169]. Inhibition of lymphocyte apoptosis is associated with improved outcome in murine models of sepsis [170, 171] and has thus been seen as a modifiable response to severe infection with a potential therapeutic role [172].

T-cell activation affects neutrophils. Experimental data suggest that inadequate CD4+ activity produces dysfunctional neutrophils, facilitating bacterial dissemination; and that excessive CD4+ activity promotes bacterial clearance but with increased tissue damage [173].

Regulatory T cells, characterised by the cell surface marker CD25 and intracellular expression of the transcription factor Foxp3, mediate effector T cell responsiveness, as well as having the capacity to induce apoptosis of monocytes and neutrophils [174, 175]. Furthermore, their numbers increase during sepsis and are implicated in the development of T cell hypo-responsiveness [176].

### 2.2.4. DAMPS and PAMPS

Microbial pathogens and their derived products are potent stimulators of the innate immune system. These pathogen-associated molecular patterns (PAMPs) include surface molecules such as endotoxin (e.g. lipopolysaccharide, LPS; lipoproteins; outer-membrane proteins; flagellin; fimbriae; peptidoglycan, PGN; peptidoglycan-associated lipoprotein; and lipoteichoic acid, LTA), and internal motifs released following bacterial lysis (e.g. heat-shock proteins, HSP; DNA fragments)[177]. In the host, Toll-like receptors (TLR) and cytoplasmic pattern recognition receptors (PRRs) such as NOD1 and NOD2 act as sensors which recognize the exogenous noxious stimuli.
Tissue damage and cellular necrosis causes an inflammatory response through the release of damage-associated molecular patterns (DAMPs) and their detection by pattern recognition receptors. DAMPs are usually components of cells that fulfil a distinct role in health but when released into the extracellular space due to disruption of the plasma membrane, mediate inflammation. DAMPs include HMGB1, DNA, uric acid, IL-1α, IL-18, heat shock proteins, the S100 proteins and components of the extracellular matrix such as hyaluronan and heparan sulfate (Figure 4).

Together DAMPS and PAMPs have been described as danger signals, or Alarmins.
Figure 4: Pathways of Development of Systemic Inflammation. Overwhelming and dysregulated systemic inflammation results from the release of endogenous cellular contents from damaged tissue or dying cells in the form of damage-associated molecular patterns (DAMPs) or in response to exogenous microbial pathogen-associated molecular patterns (PAMPs). Endogenous DAMPs and exogenous PAMPs are recognized by pattern-recognition receptors, such as extracellular toll-like receptors or intracellular nucleotide-binding oligomerization domain–like receptors or retinoic acid–inducible gene-1–like receptors on cells of the innate immune system, including polymorphonuclear leukocytes and macrophages. The abbreviation dsRNA denotes double-stranded RNA, HMGB1 high mobility group box 1, HSP heat-shock protein, LPS lipopolysaccharide, ssRNA single-stranded RNA, and TNF tumor necrosis factor. Diagram taken from NEJM [2].
2.3. Endotoxin and bacterial translocation

Endotoxin is a historical term for lipopolysaccharide (LPS), a major constituent of Gram-negative bacterial cell walls that contributes substantially to bacterial toxicity. Gram-negative bacteria are those that do not retain crystal violet dye due to the characteristics of their cell walls. LPS comprises of a polysaccharide chain that varies between bacterial species and a lipid moiety (Lipid A) that shows less variability and is predominantly responsible for the pathological effects associated with infection by Gram-negative bacteria.

LPS is commonly used in vitro as a positive control for activation of inflammatory pathways in cells and tissues in culture, and has also been used extensively in studies performed in animals and humans in vivo. Intravenous administration of high-dose LPS reproduces the clinical features of septic shock in animals. At lower doses in humans, flu-like symptoms emerge, accompanied by fever, tachycardia, and mild hypotension [178]. Inhaled LPS has been used as a model of direct acute lung injury in humans [179].

The phenomenon of bacterial translocation explains how LPS is important in conditions other than Gram-negative sepsis. A vast number of live bacteria from >300 species live within the lumen of the normal human gut. The passage of these through the epithelial mucosa is called bacterial translocation and may have a physiological role in immune surveillance [180]. In both infectious and non-infectious systemic inflammatory processes there may be both global and regional hypoperfusion leading to increased enteric bacterial translocation [181, 182]. Translocated bacteria usually belong to Gram-negative aerobic genera such as escherichia, proteus, klebsiella; and thus express endotoxin. The presence of bacterial components in the circulation has a potent immunostimulatory effect, exacerbates the systemic inflammatory response, and has been implicated in the development of organ failure [183].

In part this mechanism provides justification for the consideration of infectious and non-infectious systemic inflammatory states as a continuum rather than distinct pathological processes. For example, in non-infectious (or “sterile”) systemic inflammation such as complicates haemorrhagic shock, there may be substantial bacterial translocation. The immune response to this may be similar to that seen in classical infectious (“non-sterile”) systemic inflammation such as that of urinary tract infection. Further evidence in support of
this hypothesis is the finding that increased levels of antibodies to endotoxin are associated with a decreased incidence of SIRS following non-infectious injury [184, 185].

2.3.1. Endotoxin tolerance
Early experiments demonstrated that animals receiving a low dose of bacterial endotoxin had a markedly reduced mortality when re-challenged with a lethal dose; this was termed endotoxin tolerance or hypo-responsiveness. Similar decreased responsiveness has been demonstrated in terms of cytokine release from LPS-stimulated leukocytes in vitro [178]. Endotoxin tolerance is not specific to the action of LPS, and cross-reactivity with other exogenous stimuli occurs [186].

Early endotoxin tolerance has several potential mechanisms: including the induction of the anti-inflammatory cytokine IL-10; altered cell signalling pathways, including several of the MAP kinases, and NF-κB; decreased expression of cell-surface receptors such as TLR4; and finally via gene silencing of pro-inflammatory pathways [187], an area in which the RAGE ligand HMGB1 may have a role [188]. By contrast, late tolerance is mediated through antibodies directed against moieties of endotoxin [189].

Endotoxin tolerance is generally considered to be beneficial rather than a deleterious or maladaptive response, protecting the host from excessive inflammation and beneficially altering immune responses [189]. Hypo-responsiveness may be a less accurate description than tolerance, as not all responses are suppressed; indeed some are enhanced. The exact effect of subsequent responses depends upon factors that include the degree of LPS exposure [190].

2.3.2. Clinical relevance to snCPB
Levels of endotoxin in the circulation of patients who have undergone snCPB are elevated [191-196]. A significant contributor to this observed endotoxaemia is thought to be diminished intestinal perfusion and increased mucosal permeability [197, 198]. Therefore, leukocytes taken from patients prior to snCPB can be considered LPS-naïve and those taken from patients following snCPB can be considered LPS-exposed. In this context, snCPB induced an early LPS hypo-responsiveness of whole blood [199-202], and was associated with a reduction in LPS-induced IL-8 release from neutrophils sourced from patients following snCPB versus healthy volunteers [203].
Given the postulated role of intestinal bacteria in the development of infectious complications and organ dysfunction in critical illness and specifically following snCPB, sterilisation of the bowel has been considered. Theoretically, the goal would be to selectively remove potential pathogenic aerobic bacteria without that of benign anaerobic intestinal micro-organisms through the enteral administration of non-absorbable antibiotics prior to surgery, termed ‘selective digestive decontamination’, SDD. A small randomised controlled trial (RCT) of SDD in snCPB patients did not demonstrate any difference in endotoxaemia, cytokine response or clinical complications [195]. One possible contributory reason for these findings could be that the bacterial components remained within the intestinal lumen and although their viability may have been reduced, their immunogenicity was not. SDD has been studied in many different populations of critical ill patients and remains a highly contentious area with widely differing degrees of implementation between countries and institutions [204-207].
2.4. Genetic variability and outcome from systemic inflammation

The role of genetic factors influencing the susceptibility to, and progression of, acute and chronic systemic inflammatory conditions can be investigated using genetic association. This may shed light on the pathways involved in complex disease and identify new targets for therapeutic intervention and is achieved by comparison of the distribution of genetic markers, such as single nucleotide polymorphisms (SNPs), in genes of interest amongst cases and controls.

SNPs are the common (>1%) single base pair ‘variants’ in the DNA sequence at a particular point compared with the ‘common’ sequence. Some SNPs are within the exons, parts of the gene that are translated into protein. Among these, non-synonymous SNPs lead to a change in amino acid sequence of the resultant protein, whereas synonymous SNPs do not result in amino acid change. Other SNPs are in introns and do not directly code for protein but may still influence cell function through other means, including so-called intron-mediated enhancement [208]. Counter-intuitively, analysis of genetic association studies show that non-synonymous SNPs (change in coding) are no more likely to be involved in disease mechanisms than synonymous SNPs. Intronic SNPs are less likely to be involved in disease mechanisms than extronic SNPs [209]. Synonymous SNPs may affect function through alterations in messenger RNA splicing, stability, and structure as well as protein folding [210].

Haplotypes are combinations of alleles at different loci on the chromosome that are transmitted together. They are constructed using knowledge of linkage disequilibrium (LD) between markers and offer information about ancestral chromosomes. LD is a mark of association between alleles at different loci such that if two SNPs are in LD, they are present together more frequently than would be expected by chance (i.e. they are co-inherited). Conjoining SNPs into multi-SNP haplotypes may improve the power to assigning a phenotype to a genetic region in association studies. A SNP may be associated to a clinical phenotype through another SNP that is in LD with it.

Complex diseases or syndromes are the result of the interplay between genetic and environmental factors. The progression of infection to sepsis, to severe sepsis, and to septic shock only occurs in a minority of cases; similarly the progression of post-operative SIRS to
severe systemic inflammation, to the development of multiple organ dysfunction is rare. Although environmental considerations are important, the genetic susceptibility to progression of inflammation is also an important determinant. The landmark study that initiated investigations in this area determined the causes of death in those who were adopted in relation to the causes of death of their biological parents. They found that death before the age of 50 in the biological parents resulted in a relative risk of death in the adoptees of 5.81 (2.47-13.7, 95% confidence intervals, p<0.001) for infectious causes [211]. More recently, a genetic variant in a component of the clotting cascade (factor V Leiden, affecting ~5% of the population) that is associated with an increased risk of venous thrombosis, has been associated with improved outcome from severe sepsis [212].

When the gene of interest is known studies can focus on a ‘candidate’ gene. By contrast, non-hypothesis driven studies can study a large number of SNPs on a wide range of genes and look for previously unidentified associations that may lead to new investigative approaches. Thus a SNP within the RAGE gene (G82S) was recently associated with impaired pulmonary function in a genome-wide association study [213].

2.4.1. The limitations of genetic association studies

Genetic association studies are frequently underpowered as sample size calculations may not have been performed, or the numbers of recruited patients was less than planned. The power of a statistical test is its ability to reject a false null hypothesis. A study that is underpowered is at risk of both a type II error, when a null hypothesis is not rejected despite being false, and a type I error, when a null hypothesis is rejected when it should not be, a ‘false positive’.

Moreover, when performing multiple statistical tests, it becomes inevitable that some will appear to show statistical significance, even in the absence of a true effect and failure to compensate for this eventuality increases the risk of false positives. Use of post-hoc subgroup analyses increases the chances of detecting a false positive result.

Publication bias and consequent misleading systematic reviews is a further limitation. Publication bias describes the phenomenon of negative results being less likely to be submitted for publication, let alone accepted. Thereafter any systematic reviews will only have positive papers to analyse leading to false conclusions.
Some would argue that complex outcomes, such as duration of ICU stay following snCPB, have a vast number of gene-gene and gene-environment interactions and to suppose that variation in one gene, however significant it is, will affect this may be overly simplistic and reductionist.

Finally, conducting genetic association studies in different populations may lead to multiple reports of contradictory findings, or failure to replicate positive results. This may be attributable to the lack of genuine effect or variation in the extent of effect between different populations [214].

2.4.2. Genes of the immune response

The influence of genetic variability in key components of the immune system (such as cytokines, cytokines receptors and intracellular signalling pathway proteins) on outcome in patients with the sepsis syndromes has been extensively studied. Unfortunately, a large proportion of trials in this area have had methodological shortcomings, and their results must be interpreted with caution [215]. Predisposition to sepsis and to the progression of sepsis to severe sepsis or septic shock have been the most widely studied aspects of the sepsis syndromes but inflammatory responses to snCPB have also been investigated (Table 3).

TNF-α is a pivotal pro-inflammatory cytokine with several SNPs affecting gene function, and has been studied extensively in relation to critical illness, with inconsistent results [216]. A recent study included 854 patients and matched controls and identified an association between a SNP in TNF-β (+252) and predisposition to severe sepsis but none between several SNPs (in TNF-α and β, IL-1β, IL-6, IL-10 and CD14) and severity of sepsis [217]. A systematic review of studies of a promoter region SNP in TNF-α (−308A>G) demonstrated an association to predisposition to, but not to severity of, sepsis [218].
<table>
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<tr>
<td>IL-8, IL-6, TNF-α</td>
<td>-174G&gt;C, -251A&gt;T, -308G&gt;A respectively</td>
<td>154</td>
<td>IL-6 (-174GG) and IL-8 (-251AA) associated with higher levels of post-op IL-6 and -8 respectively. IL-8 -251AA associated with prolonged ventilation and hospital stay.</td>
<td>[224]</td>
</tr>
<tr>
<td>IL-6, IL-10, TNF-α</td>
<td>-572, -1082, -308 respectively</td>
<td>100 patients, 112 controls</td>
<td>IL-6 -572 polymorphism was associated with acute lung injury after cardiac surgery with cardiopulmonary bypass</td>
<td>[225]</td>
</tr>
<tr>
<td>IL-6</td>
<td>-174G&gt;C, -572G&gt;C, and -597G&gt;A</td>
<td>127</td>
<td>Peak IL-6 levels were significantly higher in carriers of the -572C allele than in those of the -572GG genotype and in those with genotype -174CC compared with -174G allele carriers</td>
<td>[226]</td>
</tr>
<tr>
<td>IL-6</td>
<td>-174 G&gt;C</td>
<td>111</td>
<td>-174 G associated with greater IL-6 plasma levels and longer ICU and hospital stays</td>
<td>[227]</td>
</tr>
<tr>
<td>IL-6</td>
<td>-174 G&gt;C</td>
<td>111</td>
<td>G homozygotes had significantly higher IL-6 levels postoperatively, worse renal function and prolonged mechanical ventilation</td>
<td>[228]</td>
</tr>
<tr>
<td>IL-10 and TLR-4</td>
<td>Many</td>
<td>156</td>
<td>Haplotype effects on post-operative plasma IL-10 levels</td>
<td>[229]</td>
</tr>
<tr>
<td>IL-10</td>
<td>-1082</td>
<td>150</td>
<td>Minor allele (GG) associated with lower IL-10 plasma levels 3h post-CPB</td>
<td>[230]</td>
</tr>
<tr>
<td>TNF-α, IL-1β, IL-6, IL-10, IL-1ra, ICAM-1, P-selectin and E-selectin.</td>
<td>17 polymorphisms</td>
<td>759</td>
<td>Carriage of minor allele (T) of the E-selectin SNP at +98 was associated with coagulopathy and increased post-operative bleeding. ICU LOS not reported.</td>
<td>[231]</td>
</tr>
<tr>
<td>IL-18</td>
<td>-607 C/A, -137 G/C, 8148 C/T and 9545 T/G</td>
<td>658</td>
<td>Homozygosity for the major (T) allele at 9545 T/G was associated with prolonged ICU stay, more severe vasodilatation, increased IL-18 and TNF-α levels, with decreased IL-10 levels.</td>
<td>[232]</td>
</tr>
</tbody>
</table>

Table 3: Studies relating SNPs in genes of the innate immune response and clinical outcomes related to systemic inflammation in adult patients who have had snCPB, negative studies not shown.
3. THE RECEPTOR FOR ADVANCED GLYcation END-PRODUCTS (RAGE)

3.1. Introduction

3.1.1. History
Originally characterised in 1992 as a receptor for advanced glycation end-products [233], RAGE has since become an area of active research in diverse fields including those relating to inflammation, immunity, development, and oncogenesis. Advanced glycation end-products (AGEs) are modifications of proteins or lipids resulting from non-enzymatic glycation or oxidation and are a diverse range of compounds, as are their receptors, each being known by several different names.

3.1.2. Receptors for AGE
OST-48 (oligosaccharyltransferase-48) is a membrane protein originally considered to have a primary role as a glycosyltransferase, but later found to be present on the plasma membrane and to bind and internalise AGE [234]; it was originally termed p60 and later renamed AGE-R1. 80K-H is an intracellular substrate for protein kinase C but was also later found to bind and internalise AGE [234]; it was originally termed p90 and later renamed AGE-R2. Galectin-3 is a carbohydrate-binding protein (lectin) of complex composition and diverse roles including binding and degradation of AGE [235, 236]; subsequently renamed AGE-R3. AGE receptors 1, 2 and 3 may work together as the AGE-receptor complex [237].

Macrophage scavenger receptor class A (SRA) is a transmembrane glycoprotein that binds oxidized low density lipoprotein (Ox-LDL), but also endocytoses and degrades AGE [238]. The defining member of the class B macrophage scavenger receptors is CD36. This glycoprotein, in addition to various other ligands, binds and degrades AGE [239]. The protein lysozyme also binds AGE and this may adversely affect its antimicrobial properties [240]. Finally, the receptors “fasciclin EGF-like, laminin-type EGF-like, and link domain-containing scavenger receptor” -1 and -2 (FEEL-1 and -2) may recognize AGEs [241] and are expressed in vascular tissues. Further discussion of non-RAGE receptors for AGE is beyond the scope of this thesis. In contrast to other AGE receptors, RAGE is a member of the immunoglobulin superfamily, and upon ligand inflammatory signalling events are initiated.
3.1.3. Structure

The receptor structure consists of three extracellular domains that participate in ligand binding, one V-type (variable) immunoglobulin like domain and two C-type (constant) immunoglobulin like domains; a transmembrane domain and a cytoplasmic tail essential for intracellular signalling (Figure 5). Different ligands preferentially interact with these individual domains [242] and result in different intra-cellular events [243]. It has been reported that the V and C1 domains form an integrated structural unit for ligand recognition [244].

![Diagram of RAGE structure](image)

**Figure 5: Diagrammatic representation of RAGE**

Other immunoglobulin-like receptors include the Fc-γ immunoglobulin receptors, the families of receptors named ‘leukocyte immunoglobulin-like receptors’, and the ‘killer cell immunoglobulin-like receptor’ [245]; all are involved in mediating the immune response.

3.1.4. Genesis

The gene for RAGE (named AGER by the human genome nomenclature committee) is located within the gene-dense major histocompatibility class III region on chromosome 6. Polymorphisms within the gene and their functional consequences are discussed within Section 3.1.6.

Genetic modified RAGE deleted mice appear phenotypically normal, and are no more susceptible to experimental development of diabetes than control mice and breed normally. They do however, develop spontaneous pulmonary fibrosis [246] and have abnormal osteoclast development causing increased bone mass [247].
3.1.5. RAGE variants

An exhaustive study of splice variants has attempted to harmonise divergent naming conventions and reports of splice variants. Full-length membrane-bound RAGE has several splice variants and these are likely to affect ligand-binding and signal transduction [248]. However, the finding of novel variants has not been replicated in other laboratories [249, 250].

In addition to splice variants there may be post-translational modifications of RAGE that affect ligand binding. The V-type immunoglobulin domain of RAGE has two potential N-glycosylation sites. N-glycosylation is the enzymatic addition of an oligo- or poly-saccharide to a nitrogen molecule within a peptide. Such modifications increased RAGE affinity for S100A8/9, S100A12 and HMGB1 [251-253].

Genetic variation in the RAGE gene has been related to altered gene function and associations have been reported in relation to increase risk of development of diseases and in those with diseases, of complications.

3.1.6. Genetic variation in the RAGE axis

The human genome nomenclature committee (HGNC) use the name AGER for RAGE. This gene has 1492 base pairs and is on chromosome 6p21 in the major histocompatibility (MHC) complex locus in the class III region, and is given the Entrez Gene [254] ID 177. The genomic context of the RAGE gene is shown in Figure 6.

![Figure 6: RAGE gene (termed AGER) on chromosome 6 and the genomic context](image)

Once transcribed the resultant messenger RNA (mRNA) includes 11 exons and is illustrated in Figure 7. Following splicing and translation the RAGE protein is 404 amino acids in length.
There are no published investigations of SNPs in the RAGE gene relating to acute inflammatory conditions. SNPs affecting the RAGE gene have been studied in association with the development of chronic diseases and particularly associated complications thereof. The largest literature exists for studies of patients with diabetes (Table 4), with some investigations in haemodialysis-dependent patients and those with rheumatoid arthritis (Table 5), also in healthy subjects (Table 6).
<table>
<thead>
<tr>
<th>Gene</th>
<th>SNP</th>
<th>Populations</th>
<th>Findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAGE</td>
<td>−429 T/C</td>
<td>Patients with type 2 DM: 106 with and 109 without retinopathy</td>
<td>−429 C allele associated with retinopathy and with increased in vitro transcriptional activity</td>
<td>[255]</td>
</tr>
<tr>
<td>RAGE</td>
<td>G82S, 1704G/T, 2184A/G, and 2245G/A</td>
<td>202 Caucasian patients with type 2 DM and 169 controls</td>
<td>G82S and 2245G/A were associated with T2 DM. Complex relationships to antioxidant status in those with diabetes</td>
<td>[256]</td>
</tr>
<tr>
<td>RAGE</td>
<td>G82S</td>
<td>200 Asian Indians, 100 with retinopathy and 100 without, 50 unrelated controls.</td>
<td>G82S was associated with a decreased incidence of retinopathy</td>
<td>[257]</td>
</tr>
<tr>
<td>RAGE</td>
<td>−429 T/C, −374 T/A, and G82S</td>
<td>996 Finnish patients with T1 DM</td>
<td>Less coronary heart disease, acute myocardial infarction and peripheral vascular disease in patients with the AA genotype of the −374 T/A polymorphism</td>
<td>[258]</td>
</tr>
<tr>
<td>RAGE</td>
<td>−374A</td>
<td>703 Brazilians with type 2 diabetes (520 Caucasian- and 183 African-Brazilians)</td>
<td>−374A allele was associated with a decreased risk of having ischemic heart disease in African-Brazilian type 2 diabetic patients</td>
<td>[259]</td>
</tr>
<tr>
<td>RAGE</td>
<td>G82S</td>
<td>487 type 1 diabetic patients with proliferative retinopathy subdivided into four groups according to their level of renal involvement and in 351 control subjects</td>
<td>G82S associated with advanced nephropathy</td>
<td>[260]</td>
</tr>
<tr>
<td>RAGE</td>
<td>−374 T/A</td>
<td>Scandinavians: 867 T1 DM, 2467 T2DM and 205 non-diabetic controls</td>
<td>374 T/A polymorphism was associated with development of diabetic nephropathy and was found to be in LD with HLA-DQB1</td>
<td>[261]</td>
</tr>
<tr>
<td>RAGE</td>
<td>Nine SNPs</td>
<td>Dutch subjects with normal glucose metabolism (301), impaired glucose metabolism (127), and T2DM (146)</td>
<td>The minor allele of G82S was strongly associated with lower sRAGE levels</td>
<td>[262]</td>
</tr>
<tr>
<td>RAGE</td>
<td>−374 T/A</td>
<td>1291 Dutch individuals, with normal glucose metabolism (44%), impaired glucose metabolism (23%) or T2 DM (33%)</td>
<td>In those with normal glucose metabolism, the -374A allele of the RAGE gene is protectively associated with blood pressure and arterial stiffness, whereas in individuals with impaired glucose metabolism or T2 DM, it is adversely associated with these variables.</td>
<td>[263]</td>
</tr>
</tbody>
</table>

Table 4: RAGE polymorphisms and incidence of onset and complications of diabetes
<table>
<thead>
<tr>
<th>Gene</th>
<th>SNP</th>
<th>Populations</th>
<th>Findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAGE</td>
<td>G82S</td>
<td>205 Caucasian patients with RA and 169 matched controls</td>
<td>G82S associated with increased risk of RA but possibly due to LD with HLA-DRB1*0401. Ex vivo monocyte stimulation with S100A12 demonstrated increased inflammatory signalling in those heterozygous for G82S than wildtype.</td>
<td>[265]</td>
</tr>
<tr>
<td>RAGE</td>
<td>−429 CC and 2184 GG</td>
<td>261 unrelated Caucasian patients on chronic HD, 100 healthy controls</td>
<td>Plasma sRAGE levels were higher in those with −429 CC and 2184 GG, and higher for all patients than controls.</td>
<td>[266]</td>
</tr>
</tbody>
</table>

Table 5: RAGE polymorphisms and incidence of rheumatoid arthritis, and effect on plasma sRAGE levels in haemodialysis-dependent patients

<table>
<thead>
<tr>
<th>Gene</th>
<th>SNP</th>
<th>Populations</th>
<th>Findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAGE</td>
<td>G82S</td>
<td>262 infants aged 3-16m and 122 mothers of recruited children</td>
<td>Minor allele of the RAGE gene polymorphism G82S was associated with reduced plasma sRAGE (30-40%) in all age groups</td>
<td>[267]</td>
</tr>
<tr>
<td>RAGE</td>
<td>G82S</td>
<td>1676 healthy Koreans</td>
<td>Minor allele of the RAGE gene polymorphism G82S was associated with reduced plasma sRAGE. Heterozygotes were intermediate. Additionally plasma levels of TNFα and hsCRP were higher in the SS group.</td>
<td>[268]</td>
</tr>
</tbody>
</table>

Table 6: RAGE polymorphisms and effect on plasma sRAGE levels and inflammatory markers in healthy humans
Of the many SNPs within the RAGE gene the most frequently studied are -374 T/A, -429 C/T, G82S, 1704 G/T And 2184 A/G. These descriptions of the SNPs are either the position relative to the gene and the nucleotide change (i.e. -374 T/A means position 374 before the start codon and change of T (thymine) for A (adenine), or the change in amino acid and the position within the protein (G82S). The standardised method of described these SNPs is the refSNP (rs) number and together with their functional significance are listed in Table 7.

<table>
<thead>
<tr>
<th>refSNP</th>
<th>SNP name</th>
<th>Location</th>
<th>Change in nucleotide (common, variant)</th>
<th>Change in amino acid</th>
<th>Functional significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs1800624</td>
<td>-374 T/A</td>
<td>Promoter region</td>
<td>T to A</td>
<td>N/A</td>
<td>Increased transcriptional activity of RAGE[255]</td>
</tr>
<tr>
<td>rs1800625</td>
<td>-429 T/C</td>
<td>Promoter region</td>
<td>T to C</td>
<td>N/A</td>
<td>Increased transcriptional activity of RAGE[255]</td>
</tr>
<tr>
<td>rs2070600</td>
<td>Gly82Ser G/A</td>
<td>Ligand binding domain – exon3</td>
<td>G to A</td>
<td>Glycine to Serine</td>
<td>Missense, results in damage to structure and function of RAGE[269]</td>
</tr>
<tr>
<td>rs184003</td>
<td>1704 G/T</td>
<td>Intron 7</td>
<td>G to T</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>rs3134940</td>
<td>2184 A/G</td>
<td>Intron 8</td>
<td>A to G</td>
<td>N/A</td>
<td></td>
</tr>
</tbody>
</table>

Table 7: SNPs in the RAGE gene that we chose to genotype for. Adenine (A), Cytosine (C), Thymine (T), Guanine (G)

There have been few genetic association studies of the S100 proteins. Indeed, only S100A8 has been investigated in the context of the inflammatory dental condition, periodontitis [270]. In common with other S100A genes, its gene is on chromosome 1 and is given the Entrez Gene [254] ID 6279. The genomic context of the S100A8 gene is shown in Figure 8.

Figure 8: S100A8 gene on chromosome 1 and the genomic context

Once transcribed the resultant messenger RNA (mRNA) includes 3 exons and is illustrated in Figure 9. Following splicing and translation the RAGE protein is 93 amino acids in length.
SNPs of the HMGB1 gene have been studied and although their functional significance at the molecular levels remains undetermined, the -1377delA promoter polymorphism in HMGB1 was significantly associated with increased risk of delayed mortality in patients with SIRS and the 982C>T polymorphism in HMGB1 was associated with significantly higher probability of early death due to infection [271]. The gene is on chromosome 13 and is given the Entrez Gene [254] ID 3146. The genomic context of the HMGB1 gene is shown in Figure 10.

Once transcribed the resultant messenger RNA (mRNA) includes 5 exons and is illustrated in Figure 11. Following splicing and translation the HMGB1 protein is 215 amino acids in length.

3.1.7. Signalling

3.1.7.1. Transduction

The cytoplasmic tail of RAGE is essential for its role in signal transduction, with its deletion exerting a dominant-negative effect [243]. However, the lack of either endogenous kinase activity or any known motifs involved in receptor signalling left its mechanism of signal transduction unknown until very recently when several mechanisms for RAGE signal transduction were proposed (Figure 12).
The RAGE cytoplasmic domain was found to interact with Diaphanous-1, a member of the formin protein family, which in turn, interacts with mediators of the actin cytoskeleton and various signal transduction pathway molecules including the members of the Rho family of GTPases, Rac-1 and Cdc42 [272].

RAGE forms oligomers on the cell surface and this may be necessary for ligand binding [273]; similarly sRAGE [274]. sRAGE and RAGE V-domain can interfere with this process inhibiting RAGE dimerization and downstream signalling events [275]. Receptor homodimerization is important for signal transduction in other receptor classes such as the toll-like receptors [276]. Alternatively, internalisation of the RAGE-ligand complex may be important in signal transduction [277].

RAGE activation results in the generation of intra-cellular ROS and this is a potent factor initiating signal transduction, including the redox-sensitive NF-κB and MAPK pathways [278]. In support of this, antioxidants such as N-acetylcysteine and vitamin E, have demonstrated inhibition of RAGE-mediated effects [279].
The extracellular signal-regulated kinase-1 and -2 (ERK-1/2) proteins are important members of the mitogen-activated protein kinase (MAPK) pathway and have been found to interact with the cytosolic tail of RAGE[280].

3.1.7.2. Signalling pathways

An array of intra-cellular signalling pathways has been implicated in the transduction of ligand binding to cellular response. The majority of findings from these studies have been identified in cells other than leukocytes, from animals other than humans and often in transformed cells. The extent to which these findings are generalisable is also challenged as different ligand-RAGE interactions result in alternative downstream events. These limitations restrict confidence in knowing which pathways are important in human primary leukocyte RAGE signal transduction. However, there is sufficient overlap to be able to draw some conclusions.

The most commonly implicated signal pathway is that of nuclear factor κB (NF-κB), a complex and highly regulated network of intra-cellular proteins. NF-κB consists of two polypeptides (p50 and p65, also known as RelA) and when inactive, resides in the cytoplasm in physical association with its inhibitor proteins (the inhibitor-κB or IκB proteins). In response to diverse stimuli, including redox changes, the IκB proteins are phosphorylated by a complex of protein kinases (The IKKs). Once IκB has been degraded, NF-κB translocates to the nucleus where it regulates the expression of hundreds of immune and inflammatory genes [281].

Secondly, the mitogen-activated protein kinase (MAPK) pathways, c-Jun-NH₂-terminal kinase/stress-activated protein kinase (JNK/SAPK), p38, and extracellular signal-regulated kinases (ERK) have been implicated. These pathways comprise of a complex sequence of kinases that rapidly connect single signals to multiple cellular events. They are interconnected and incorporate negative feedback loops and redundancy [282]. The MAPK pathways lead to activation of transcription factors including NF-κB, specificity protein 1 (Sp-1), and activating protein-1 (AP1).

Formation of intracellular ROS may link RAGE activation through initiating the NF-κB pathway directly or through the phosphatidylinositol 3'-kinase (PI3K) system to ERK1/2 [283].
In addition to altered cellular function through activation of transcription factors, protein kinases and cytoskeletal changes, there are two important positive feedback loops. Firstly, NF-κB promotes further RAGE gene expression [284] and secondly, RAGE activation results in increased levels of \textit{de novo} synthesized NF-κBp65 overriding endogenous negative feedback mechanisms and perpetuating the response [285]. Finally, through a less direct feedback mechanism, the inflammatory mediator C-reactive protein, increases expression of RAGE [286].

\textbf{3.1.7.3. In primary human leukocytes}

There are a paucity of investigations into RAGE signalling that utilise primary human leukocytes, partly due to their limited longevity in culture, their reluctance to tolerance experimental conditions (such as siRNA techniques) and the necessarily limited quantity of material for assays. However, the integral nature of ERK1/2 and NF-κB to RAGE signal transduction has been demonstrated in neutrophils in response to HMGB1, AGE and S100B [287-289] and the role of ROS has been demonstrated in monocytes in response to AGE [290].

\textbf{3.1.8. Soluble RAGE isoforms}

The full-length membrane-bound RAGE protein has been variously described in the literature as mRAGE, FL-RAGE, or simply RAGE to distinguish it from the soluble forms of the protein that are present in human plasma (Figure 13). The major soluble forms are sRAGE (soluble RAGE) and esRAGE (endogenous secretory RAGE). Despite efforts to standardize terminology, these terms are used inconsistently in the literature.

Several studies that have exhaustively studied soluble RAGE isoforms have identified other forms in human tissues; potentially the RAGE isoform expressed may depend upon the cell type and its environment [248, 291, 292].
3.1.8.1. sRAGE

sRAGE is the predominant soluble form in human plasma and is produced by intramembrane proteolytic cleavage of full-length RAGE by the membrane metalloprotease (MMP) known as ADAM10 (A Disintegrin And Metalloprotease 10)\cite{250, 293}. sRAGE retains the extracellular domains but lacks both the transmembrane domain and the cytoplasmic tail.

The full-length RAGE protein is ubiquitous and as the soluble form lacks distinct epitopes, it is difficult to distinguish using immunoassays. It is present in the plasma of healthy humans, with levels that vary over time and between individuals \cite{294}. By contrast, it is not present in the murine circulation \cite{295}.

Although primary human neutrophils and monocytes have been demonstrated to possess mRNA for RAGE and the RAGE protein on their cell surfaces \cite{296-298}, there are few published data suggesting that leukocytes are a major source of circulating sRAGE \cite{250, 299}. Circulating sRAGE is largely presumed to originate in the lung which has high basal RAGE expression \cite{300, 301}; but another important potential source is the endothelium, RAGE being expressed on healthy and diseased human endothelial cells \cite{302}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure13.png}
\caption{Diagrammatic illustration of full-length RAGE on the left and the two soluble forms: sRAGE and esRAGE.}
\end{figure}
3.1.8.2. esRAGE

Endogenous secretory RAGE (esRAGE) is a product of alternative splicing of mRNA resulting in a novel stretch of amino acids in the C2-Ig domain, creating an epitope to which specific antibodies may be raised. Like sRAGE, it retains ligand-binding but does not have signal transduction capacity [303].

3.1.9. Differential regulation and expression

By contrast to the hypothesis of differential regulation and expression, an immunohistochemical study of human expression of the two forms of RAGE, using antibodies directed against the V-type domain of RAGE, the cytosolic domain of RAGE and the C-terminal end of esRAGE, found very similar distributions across a wide range of tissues [304]. Similarly, in a large cohort of patients with chronic kidney disease, levels of sRAGE correlated very closely with levels of esRAGE [266].

Levels of circulating RAGE forms have been variably demonstrated to increase in vivo in response to medications including statins (e.g. atorvastatin), thiazolidinediones (e.g. pioglitazone), and angiotensin-converting enzyme inhibitors (e.g. perindopril). Supportive in vitro work has shown similar results with angiotensin receptor blockers (e.g. candesartan) [299, 305-307]. However, there was no convincing evidence of differential regulation in these studies. By contrast, stimulation of human endothelial cells in vitro with distinct AGEs differentially enhances expression of RAGE isoforms [308].

3.2. RAGE Ligands

With the exception of advanced glycation end-products, RAGE ligands are all proteins that have distinct intra- and extra-cellular properties, and lacking defined secretory signal sequences, crossing the plasma membrane through non-classical means [309]. For several of these ligands, RAGE is not their exclusive receptor. In a murine model of delayed-type hypersensitivity, sRAGE significantly attenuated the inflammatory response both in wild-type and RAGE-deleted animals [310]. Similarly, sRAGE maintained beneficial anti-inflammatory effects in a murine endotoxaemia model both in wild-type and RAGE-deleted animals [311].
3.2.1. S100 proteins

These are low-molecular-weight (9-13kDa) calcium-binding proteins with intracellular regulatory functions and extracellular effects on inflammatory cell activity. They possess a defining N-terminal EF-hand (Figure 14).

![Schematic representation of the secondary structure of an S100 protein.](image)

When intracellular, they mostly exist as anti-parallel homodimers held together by non-covalent bonds. They regulate the activity of effector proteins across diverse pathways including cell growth and differentiation, cytoskeletal maintenance and transduction of calcium signalling [3, 312, 313]. They have similarities to the archetypal calcium sensing protein calmodulin. The S100 proteins A8/9 and A12 are also known as the calgranulins, reflecting their calcium-binding properties, and are highly expressed in granulocytes.

There are at least 25 identified S100 proteins which are expressed exclusively in vertebrates. The majority are of the S100A subfamily and their genes are located on chromosome 1q21. S100 genes from other chromosomal regions carry the stem symbol S100 followed by a single letter (e.g. S100B).

S100 proteins are secreted by alternative, rather than classical pathways as they lack the relevant signal peptides for the classical endoplasmic reticulum-golgi network. Non-classical secretion is typical of DAMPs. The mechanisms by which they are secreted are poorly understood. Once in the extracellular space they undergo conformation change and acquire oligomeric structures in response to the increased calcium ion concentration. These oligomeric structures are important in signalling [314]. Zinc and copper concentrations are also important in regulating conformation and therefore signalling [315].
Of the S100 proteins, those that have been most studied in relation to acute inflammation in humans are S100A8/9, S100A12 and S100B.

### 3.2.1.1. S100A8 and S100A9

S100A8 and S100A9 associate to form the heterodimer known as calprotectin[316]. Other names include: A8 as Calgranulin A, or myeloid-related protein-8 (MRP-8), and A9 as Calgranulin B or MRP-14. When not in association, they form homodimers and their activity is greatly reduced [317]. Calgranulin also exists as heterotetramers and as such sequesters Zinc ions [318].

S100A8/9 is the most abundant cytoplasmic protein of neutrophils and monocytes (~49% of total cytosolic protein) and is released during their activation [319]. Plasma levels in healthy humans have been reported across a wide range of values from 58ng/ml to 1000ng/ml [320].

When extracellular the major role of S100A8/9 is as a potent chemoattractant for neutrophils and monocytes and its blockade significantly attenuates inflammatory cell migration [321, 322]. In addition, it can cause neutrophil degranulation [323, 324] and has been proposed to have inherent anti-microbial properties, possibly as a consequence of its zinc sequestering [325-329]. RAGE is an important receptor for S100A8/9, but there is also evidence of activity mediated by TLR4-MD2 complex [330-332].

Post-translational modifications of these proteins affect their function. S100A8 may be modified by S-nitrosylation (addition of -NO to cysteine) or oxidation; S100A9 by S-glutathionylation (addition of glutathione to a cysteine residue). These modifications generate novel anti-inflammatory activities and may function as a negative feedback loop [333-335]. By binding ROS they may restrict tissue damage and promote resolution of inflammation.

S100A9 genetically deleted mice live and breed normally. As well as lacking S100A9 protein, they also lack S100A8 and therefore the A8/9 heterodimer [336].

### 3.2.1.2. S100A12

S100A12 has also been known as calgranulin C and extracellular newly identified ligand of RAGE (EN-RAGE). It has a high degree of structural homology with S100A9 and is absent in
the rodent genome [337]. Like calprotectin, it is constitutively expressed in phagocytes, but to a lesser extent (~5% total cytosolic protein) [338]. It binds RAGE on monocytes, neutrophils, endothelial cells, macrophages, and lymphocytes [339, 340], provoking pro-inflammatory responses including chemoattraction and adhesion. S100A12 is susceptible to conformational rearrangement in response to altered metal ion concentration; in particular the increased extracellular calcium concentration causes formation of S100A12 hexamers, greatly increasing their affinity to RAGE [274]. In small cohorts of healthy humans, S100A12 plasma levels have been found to be between 8 and 75ng/ml [341-344].

3.2.1.3. S100B

The first of the identified S100 proteins was the heterodimer of S100B with S100A1 purified from bovine brain [345]. S100B exists within cellular cytoplasm as a homodimer in which the two subunits are held together by non-covalent bonds and are arranged in an anti-parallel fashion [346]. S100B also forms a variety of oligomeric structures including dimeric, tetrameric, hexameric and octameric forms, depending upon calcium ion concentration. The oligomeric structure of S100B strongly influences its affinity for RAGE and this is reflected in subsequent RAGE activity, with tetrameric forms having greater activity than dimeric forms in experimental models [347]. A practical consideration when using S100B experimentally is that repeat freeze-thaw cycles may decrease the relative abundance of the more complex associations [347].

The human gene encoding S100B maps to chromosome 21q22.3 and several SNPs have been described [348, 349] but without published studies relating to inflammatory diseases.

S100B is present in the cytoplasm in a wide variety of cells within the nervous, renal, and endocrine systems as well as muscle, skin and adipose tissue. Within cells S100B acts as a calcium ion sensor protein and upon binding undergoes conformational change, exposing a hydrophobic cleft through which dimeric S100B binds to intracellular target proteins. Functional effects are diverse and include the regulation of cell proliferation, differentiation and shape, calcium homeostasis, protein phosphorylation, transcription, enzyme activity and metabolism [346].

In monocytes, S100B binds to RAGE and causes pro-inflammatory responses including TNF-α and prostaglandin secretion, ROS generation, and promotion of adherence to endothelium
In neutrophil-like HL60 cells it promotes ROS generation and in neutrophils, enhances chemoattraction and induces expression of pro-inflammatory cytokines [287, 353]. These functional effects are mediated through changes in the MAPK and NF-κB cell signalling pathways. Radioligand studies confirm binding of S100B to RAGE that is blocked by the application of sRAGE [339].

Extracellular S100B may originate either from secretion or leakage from damaged cells. Secretion has been most comprehensively studied in astrocytic multifunctional glial cells, within the central nervous system. They release S100B in response to various stimuli including pro-inflammatory cytokines and metabolic stress [354, 355]. S100B is secreted under normal physiological conditions and plasma levels are high during development, decreasing in adults [356-359]. Plasma levels in healthy humans are affected by variation within the gene and by adiposity [358, 360, 361]. In a cohort of 200 healthy humans, the median level of S100B was 50pg/ml [358].

3.2.2. HMGB1

High mobility group box 1 (HMGB1, previously named HMG1 or amphoterin) is a 25kDa nuclear DNA-binding protein that is highly conserved across species and widely distributed in all mammalian tissues. Structurally, it is composed of three domains: two homologous DNA binding motifs named A box and B box, and a negatively charged C-terminus [362].

HMGB1 may exist in its native form or after a variety of post-translational modifications. Nuclear HMGB1 may be acetylated in response to inflammatory cell activation resulting in HMGB1 passing into the cytosol, prior to packaging into lysozymes and being secreted [363]. HMGB1 can exist in oxidised or reduced states (via an intra-molecular disulphide bond) which may affect extracellular activity [364]. Indeed, all HMG proteins are subjected to extensive post-translational modifications [365] and the extent to which this affects extracellular activity remains to be fully elucidated.

In the nucleus, HMGB1 binds DNA in a non-specific manner and acts primarily by manipulating nucleoprotein complexes to regulate gene transcription and to fluidize chromatin by loosening the wrapped DNA-core histone complex [365]. HMGB1 genetically deleted mice die shortly after birth from hypoglycaemia, supporting the functional
significance of the role of HMGB1 in the transcriptional enhancement of the glucocorticoid receptor [366].

In a large cohort of healthy humans the mean level of circulating HMGB1 was 1.69±0.04 ng/ml in males and 1.62±0.04 ng/ml in females; sRAGE levels being independently inversely related, and CRP and WBC being (independently) positively related [367]. Anti-HMGB1 antibodies have been found in the plasma of patients with auto-immune disease and in more than one third of patients with septic shock [368, 369]; it is uncertain how this affects the accuracy of immunoassays.

The role of extracellular HMGB1 is contentious; a large body of work suggesting it fulfils a cytokine-like role, eliciting pro-inflammatory responses. By contrast, there are data suggesting that HMGB1 may have only weak or absent inflammatory activity per se and is more likely to function as a molecular chaperone, augmenting the inflammatory responses to other mediators.

### 3.2.2.1. The case for HMGB1 being a pro-inflammatory mediator

#### 3.2.2.1.1. Interventional models

Initially, murine experiments revealed that HMGB1 levels were elevated during sepsis and administration of HMGB1 was lethal, and that antibodies to HMGB1 were protective against lethal endotoxaemia. Furthermore, they demonstrated that endotoxin caused release of HMGB1 from macrophages and levels of HMGB1 were elevated in patients with sepsis [370]. These elevations occurred late and persisted far beyond those of the classical cytokines; thus HMGB1 was considered to have a larger potential ‘therapeutic-window’ for intervention and was described as a late-mediator of sepsis. An inhibitor of HMGB1 release, ethyl pyruvate (EP) was shown to have efficacy in reducing mortality in murine endotoxaemia and polymicrobial sepsis, even when administered 24h after the onset of injury[371]. Later, other inhibitors (anti-HMGB1 antibodies or DNA-binding A box) were also shown to be similarly effective in murine models of sepsis even when given late [372]. Indeed, a large number of HMGB1-inhibiting reagents have been evaluated in murine models of sepsis, (reviewed by Wang et al [373]). In addition to models of sepsis, inhibition of HMGB1 was found to be efficacious in experimental acute lung injury, pancreatitis, haemorrhagic shock and polytrauma [374-378]. HMGB1-inhibition is particularly attractive
as a potential treatment in humans as the therapeutic window is wide and the experimental interventions were effective when started after the onset of systemic inflammation; a situation akin to the use of antibiotics in patients presenting with sepsis.

3.2.2.1.2. In vitro data
Inflammatory stimuli cause monocytes and macrophages to secrete HMGB1 in a non-classical, leaderless manner [379], an effect that can be attenuated by immunomodulatory drugs such as corticosteroids [380]. In response to extra-cellular HMGB1, monocytes show altered chemotaxis (spreading), transendothelial migration [381] and release of IL-6, IL-1β and TNF-α [382, 383]. HMGB1 also mediates neutrophil adhesion and transmigration across endothelium as well as inducing pro-inflammatory cytokine expression [374, 384, 385]. The actions of HMGB1 have been shown to be mediated through interaction with RAGE, TLR2, TLR4, and TLR9 [386-389].

3.2.2.1.3. Patients
Levels of HMGB1 have been found to be elevated, and in some cases related to clinical outcome, in the plasma of patients with acute inflammatory conditions including pneumonia, sepsis and septic shock, haemorrhagic shock, disseminated intravascular coagulation, falciparum malaria, burns, trauma and severe acute pancreatitis [390-401]. Soon after surgery, monocytes exhibit increased HMGB1 expression. Later this is diminished, concurrent with elevation in plasma HMGB1 levels, thereby implicating monocytes as a source of plasma HMGB1 [383].

3.2.2.2. The case against HMGB1 being a pro-inflammatory mediator
The form of HMGB1 used in early experiments was recombinant and sourced from E. Coli. In 2007, a pivotal advance suggested that when eukaryotic HMGB1 was appropriately purified, it lacked the observed cytokine-like activities [402, 403]. In fact, lesser inflammatory effects of native, compared to recombinant, HMGB1 had previously been reported [404]. Affinity chromatography revealed that bacterially-derived material, including lipids, were tightly bound to HMGB1 and contributed to the observed activity [403]. Thus, in addition to the effect of many possible post-translational modifications to HMGB1, there remains the important effect of smaller molecules bound to HMGB1 including LPS, IL-1β, IFN-γ, TNF-α and fragments of DNA [388, 405, 406]. These findings have altered the view of the scientific
community of HMGB1 which is now generally considered to act as a chaperone protein, in molecular collaboration with other immuno-stimulatory factors [407, 408].

A compelling reason to doubt that inhibition of HMGB1 will offer therapeutic benefit in patients is that in a large cohort of patients with severe community acquired pneumonia, HMGB1 levels were persistently elevated at hospital discharge [409, 410]. It remains possible that anti-HMGB1 therapies will retain efficacy through diminishing the adjuvant role of HMGB1 in potentiating inflammatory responses.

### 3.2.3. Advanced glycation end-products

Advanced glycation end-products (AGEs) result from post-translational non-enzymatic glycation and oxidation of proteins and lipids and accumulate in diverse biological settings. The biochemical reactions leading to the formation of AGEs (Maillard reaction and Amadori rearrangement) occur in all tissues and body fluids. The amount of AGE modifications to a protein is dependent upon the inherent reactivity of specific amino groups, their microenvironment, the glucose concentration, and the half-life of the protein [411].

More than a dozen AGE have been found in tissue proteins such as collagen. Adducts include Nε carboxyethyl-lysine (CEL) and Nε carboxymethyl-lysine (CML), and crosslinks like glyoxal-lysine dimer (GOLD), methlyglyoxal-lysine (MOLD), pentosidine and verperlysines. The commonest AGE modification present in human tissue is CML [412, 413]. Albumin is the predominant plasma protein, with approximately 30-50g in every litre of plasma and a half-life of 20 days; it is also a major target of oxidant stress [414]. CML-modified albumin is elevated in those with diabetes and is implicated in the development of vascular complications [415].

Clearance of plasma AGEs is achieved predominantly through glomerular filtration and therefore chronic kidney disease is associated with raised plasma AGE, including CML-albumin. Levels correlate to plasma creatinine, and diminish with dialysis and following renal transplantation [416-418].

AGE accumulation is believed to be a consequence rather than a cause of aging. In humans, age-related increases in AGE have been demonstrated in tissues of high extra-cellular matrix content with slow, little, or no turnover, including human dura mater, skin and cartilage.
Similarly, in nucleated cells with intact metabolic machinery (peripheral T lymphocytes) there is a curvilinear increase in the AGE pentosidine with age. This increase is potentiated by the presence of end-stage renal disease [420]. The formation of AGEs is predominantly endogenous, but they can also be derived from exogenous sources such as food and tobacco smoke [421]. Studies of human female twins suggest that the degree of plasma CML is largely determined by genetic factors [422]. AGE formation can also occur acutely and is facilitated by oxidative stress, acute hyperglycaemia and the actions of neutrophil myeloperoxidase [423, 424].

AGE-modified proteins, including plasma proteins such as albumin, have been shown to induce inflammatory responses in leukocytes, mediated via RAGE. CML-albumin has been shown to induce an inflammatory response in neutrophils and monocytes, mediated by RAGE [287, 297, 425]. AGE both increases surface RAGE expression on monocytes experimentally and has been observed to be an independent determinant of the extent of monocyte RAGE expression [296, 426]. By contrast, in a cell-free model, AGE was not found to bind RAGE [427] and AGEs were not found to induce inflammatory responses in peripheral blood mononuclear cells [352]. These discrepant results can be partly explained by consideration of the extent of AGE modification. It is simple to generate AGE-modified proteins such as albumin experimentally but the degree of glycation may be excessive and lack physiological relevance; in vivo glycation modifications are minimal, perhaps one modified amino acid per protein [428].

In support of the pathogenic role of AGE and the pivotal role of RAGE, a recent study demonstrated that commercially-available albumin used in the resuscitation fluid ‘human albumin solution’, has extensive AGE modifications; furthermore, its use in mice with experimental sepsis worsened mortality. Importantly, RAGE-deletion protected mice from this effect [429].

### 3.2.4. Amyloid proteins

Amyloid proteins are insoluble fibrous proteins that are implicated in the pathogenesis of diverse conditions including the systemic amyloidoses, Alzheimer’s disease and rheumatoid arthritis. Of the varied amyloid proteins, there is evidence of interaction with RAGE for serum amyloid A (SAA), transthyretin (TTR) and amyloid β protein [430-433]. The SAA family
comprises a number of differentially expressed lipid-binding proteins [434]. Amyloid β protein is a short peptide formed by cleavage of the transmembrane glycoprotein named amyloid precursor protein (APP) and has a propensity to coalesce to forms fibrils. TTR is a 55-kDa homotetramer that is physiologically present in the plasma and cerebrospinal fluid and binds substances including thyroxine and vitamin A, but also accumulates in systemic amyloidoses. SAA has been demonstrated to cause pro-inflammatory responses via RAGE on human monocytes and fibroblasts [432, 435].

RAGE-mediated inflammation due to amyloid β protein neuronal deposition has been implicated as a fundamental contributor to the pathogenesis of Alzheimer’s disease [436]. Indeed, an early-phase clinical trial of the RAGE inhibitor TTP-488 in patients with Alzheimer’s reported a favourable safety profile [437].

3.2.5. Integrins

Integrins are a large family of receptors with functions that include mediation of cellular adhesion, either between cells or between the cell and its extracellular matrix. Leukocytes express distinct patterns of integrins dependent upon their ontogeny and environment. Macrophage-1 antigen (Mac-1) is a heterodimeric integrin consisting of alpha-M and beta-2 (αMβ2) components, is known as a β-2 integrin and is present on neutrophils, monocytes and macrophages and NK cells. The αM component is also known as CD11b or integrin alpha M; the β-2 component is also known as CD18 [438]; Mac-1 is also known as complement receptor 3 (CR3).

RAGE has been identified as a counter-receptor for the integrin Mac-1. In an experimental model of murine peritonitis, sRAGE diminished neutrophil recruitment. The effect was more pronounced when RAGE had been up-regulated. RAGE-deleted mice exhibited diminished neutrophil recruitment that was reversed by restoring the expression of RAGE on endothelial cells. The RAGE-deleted mice also exhibited decreased macrophage recruitment. Further work revealed that Mac-1 bound directly to RAGE, an effect augmented by the presence of S100B [439]. Ligation with Mac-1 results in inflammatory responses mediated by NF-κB [440, 441]. Furthermore, the chemoattractant properties of HMGB1 may be partly attributable to the interaction of Mac-1 and RAGE on neutrophils [442].
3.3. RAGE axis in systemic inflammation

The role of the RAGE axis has been extensively studied in animal models of acute systemic inflammatory conditions, particularly sepsis. More recently, clinical observational studies have begun to measure aspects of the RAGE axis in humans with acute systemic inflammatory conditions. Interventional trials in this area are yet to be performed.

3.3.1. Animal models

3.3.1.1. Sepsis

Strategies aimed at reducing RAGE-mediated inflammation, explored specifically via the use of genetically modified mice or the administration of inhibitors, have shown lethality to be reduced in various models of sepsis (Table 8). That most studied has been caecal ligation and puncture (CLP), in which significant survival benefit was demonstrated following administration of anti-RAGE and anti-HMGB1 antibodies, or after administration of pharmacological HMGB1-inhibitors. Similar effects were seen in RAGE-deleted mice [310, 371, 372, 443, 444]. Importantly, the survival benefits associated with anti-RAGE antibodies, anti-HMGB1 antibodies and ethyl pyruvate remain even when they are administered up to 24h after the CLP procedure [371, 372, 443]. The administration of sRAGE was associated with a trend towards improved survival [310]. The efficacy of anti-RAGE strategies is not limited to models of intra-abdominal sepsis, but also those employing systemic Listeria monocytogenes, intra-nasal Streptococcus pneumoniae or influenza A [443, 445, 446].

Targeting relevant ligands to limit RAGE-mediated effects is also associated with substantially improved survival and diminished early bacterial dissemination in mice genetically deprived of S100A8/9 compared to wild-type, both in systemic endotoxaemia and Escherichia coli–induced abdominal sepsis [331, 447]. As discussed in Section 3.2.2, inhibition of HMGB1 also showed beneficial effects in murine models of septic and non-septic systemic inflammation. In addition to anti-HMGB1 antibodies, agents that inhibit the release of HMGB1, such as ethyl pyruvate (EP), stearoyl lysophosphatidylcholine, intravenous immunoglobulin (IVIG), vasoactive intestinal peptide, ghrelin, and nicotine; or those that bind fragments of the HMGB-1 protein (DNA-binding A box), or thrombomodulin have all ameliorated adverse inflammatory responses [373]. The substantially reduced lethality in RAGE-deleted mice, exposed to AGE-rich human albumin solution as
resuscitation fluid in septic shock provides compelling evidence for the centrality of RAGE and its ligands [429].

Further evidence of the capacity of sRAGE to reduce inflammation and its adverse sequelae in acute systemic inflammation was seen when administration of sRAGE reduced end-organ damage and levels of pro-inflammatory cytokines and improved mortality in a murine systemic endotoxaemia model; effects also seen in RAGE-deleted mice [311]. The investigators proposed that LPS binding to RAGE mediated the effect of sRAGE. Alternatively sRAGE bound canonical RAGE ligands released in response to endotoxaemia and abrogated receptor-mediated inflammatory responses – RAGE-mediated in the wildtype mice and TLR-mediated in the RAGE-deleted mice.

3.3.1.2. Non-infective SIRS

Studies of RAGE inhibition in non-septic systemic inflammation published to date are summarised in Table 9. In models of haemorrhagic shock and resuscitation, administration of sRAGE attenuated the associated systemic inflammation and RAGE-deleted mice exhibited a protected phenotype [448]. Anti-HMGB1 antibodies prevented death and gut barrier dysfunction [378]. Use of a resuscitation fluid containing the HMGB1-inhibitor EP was associated with complete protection from lethal haemorrhage compared to control fluid [449].

Anti-HMGB1 antibodies reduced systemic inflammation and end-organ damage following bilateral femoral fracture and severe acute pancreatitis (SAP) [376, 377]. EP also limited systemic inflammation and improved survival in rat models of pancreatitis, even when given 12h after onset of SAP [450]. As EP is a small molecule effective at clinically achievable concentrations it seems particularly attractive as a pharmacological inhibitor of HMGB1 release.
<table>
<thead>
<tr>
<th>Author and year</th>
<th>Model</th>
<th>Mode of RAGE inhibition and control</th>
<th>Indication of altered bacterial dissemination</th>
<th>Organ failure</th>
<th>Survival difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yamamoto 2011[311]</td>
<td>Intraperitoneal LPS</td>
<td>RAGE-deleted, sRAGE</td>
<td>Not reported</td>
<td>Less lung and liver damage with sRAGE</td>
<td>Improved survival in RAGE KO vs. WT Improved survival with sRAGE in both RAGE KO and WT</td>
</tr>
<tr>
<td>Van Zoelen 2010[451]</td>
<td>Intraperitoneal E.Coli</td>
<td>RAGE-deleted vs. wildtype</td>
<td>Increased CFU in peritoneal fluid, blood, liver and lungs at 20h</td>
<td>Worse hepatocellular damage</td>
<td>Not assessed</td>
</tr>
<tr>
<td>Anti-RAGE IgG vs. control IgG</td>
<td></td>
<td>Increased CFU in peritoneal fluid and distant organs at 20h</td>
<td>No change</td>
<td>Not assessed</td>
<td></td>
</tr>
<tr>
<td>Van Zoelen 2009[445]</td>
<td>S. pneumoniae intranasally</td>
<td>RAGE-deleted vs. wildtype</td>
<td>Decreased CFU in lung, blood and spleen</td>
<td>Less lung inflammation</td>
<td>Improved survival</td>
</tr>
<tr>
<td>Van Zoelen 2009[446]</td>
<td>Influenza A intranasally</td>
<td>RAGE-deleted vs. wildtype</td>
<td>Increased clearance of influenza A</td>
<td>Not reported</td>
<td>Improved survival</td>
</tr>
<tr>
<td>Zhu 2009[444]</td>
<td>CLP</td>
<td>Spermine (inhibitor of HMGB1 release) vs. control</td>
<td>Not reported</td>
<td>Not reported</td>
<td>Improved survival when given early and when given late. Excessive doses given late worsened survival.</td>
</tr>
</tbody>
</table>

Table 8 (part I): Summary of major papers assessing effects of RAGE inhibition in murine models of sepsis. sRAGE soluble RAGE, HMGB1 high mobility group box 1, CFU colony forming units, CLP caecal ligation and puncture, LPS lipopolysaccharide, IV intravenous.
<table>
<thead>
<tr>
<th>Author and year</th>
<th>Model</th>
<th>Mode of RAGE inhibition and control</th>
<th>Indication of altered bacterial dissemination</th>
<th>Organ failure</th>
<th>Survival difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Su 2008[452]</td>
<td>Intrapertoneal LPS</td>
<td>Ethyl pyruvate vs. placebo</td>
<td>Not reported</td>
<td>No change</td>
<td>Worsened survival</td>
</tr>
<tr>
<td>Lutterloh 2007[443]</td>
<td>CLP</td>
<td>RAGE-deleted vs. wildtype</td>
<td>No change to CFU in liver, spleen and peritoneal lavage fluid</td>
<td>Not reported</td>
<td>Improved survival</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anti-RAGE IgG vs. control IgG</td>
<td>No change to CFU in liver, spleen and peritoneal lavage fluid</td>
<td>Less lung and gut injury</td>
<td>Improved survival, even when given as a single dose 24 hours after CLP</td>
</tr>
<tr>
<td>Vogl 2007[331]</td>
<td>Intrapertoneal LPS</td>
<td>S100A9-deleted vs. wildtype</td>
<td>Not reported</td>
<td>Not reported</td>
<td>Improved survival, returned to normal with IV S100A9.</td>
</tr>
<tr>
<td>Liliensiek 2004[310]</td>
<td>CLP</td>
<td>RAGE-deleted vs. wildtype (and RAGE over-expressing mutants)</td>
<td>Not reported</td>
<td>Less hypotension</td>
<td>Improved survival in RAGE-deleted and no change in over-expressing mutants.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>sRAGE intra-peritoneal repeat doses vs. vehicle</td>
<td>Not reported</td>
<td>Not reported</td>
<td>Trend towards improved survival.</td>
</tr>
<tr>
<td>Yang 2004[372]</td>
<td>Intrapertoneal LPS</td>
<td>Anti-HMGB1 vs. control Ig, given 24h after onset</td>
<td>No change to CFU in spleen</td>
<td>Not reported</td>
<td>Improved survival</td>
</tr>
<tr>
<td>Ulloa 2002[371]</td>
<td>CLP</td>
<td>Ethyl pyruvate (40mg/kg) vs. placebo, given before LPS</td>
<td>No change to CFU in spleen</td>
<td>Not reported</td>
<td>Improved survival</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ethyl pyruvate (40mg/kg) vs. placebo, given repeatedly from 24h after CLP</td>
<td>Not reported</td>
<td>Not reported</td>
<td>Improved survival</td>
</tr>
<tr>
<td>Wang 1999[370]</td>
<td>Intrapertoneal LPS</td>
<td>Anti-HMGB1 IgG intra-peritoneal repeat doses vs. control IgG</td>
<td>Not reported</td>
<td>Not reported</td>
<td>Improved survival, even when delayed</td>
</tr>
</tbody>
</table>

Table 8 (part II): Summary of major papers assessing effects of RAGE inhibition in murine models of sepsis. sRAGE soluble RAGE, HMGB1 high mobility group box 1, CFU colony forming units, CLP caecal ligation and puncture, LPS lipopolysaccharide, IV intravenous.
<table>
<thead>
<tr>
<th>Author and year</th>
<th>Model</th>
<th>Mode of RAGE inhibition and control</th>
<th>Indication of altered bacterial dissemination</th>
<th>Organ failure</th>
<th>Survival difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cai 2009[449]</td>
<td>Haemorrhagic shock and resuscitation</td>
<td>Ethyl pyruvate vs. none</td>
<td>Not reported</td>
<td>Not reported</td>
<td>Dose-dependent, survival benefit</td>
</tr>
<tr>
<td>Yang 2006[378]</td>
<td></td>
<td>HMGB1 AB vs. control</td>
<td>Decreased bacterial translocation to mesenteric lymph nodes</td>
<td>Attenuated ileal mucosal hyperpermeability</td>
<td>Survival benefit (90% vs. 46%)</td>
</tr>
<tr>
<td>Levy 2007[376]</td>
<td>Bony fractures</td>
<td>HMGB1 AB vs. control</td>
<td>Not reported</td>
<td>Attenuated hepatic and systemic inflammation</td>
<td>Not assessed</td>
</tr>
<tr>
<td>Sawa 2006[377]</td>
<td>Severe acute pancreatitis (SAP)</td>
<td>HMGB1 AB vs. control</td>
<td>Increased bacterial translocation to pancreas</td>
<td>Attenuated pancreatitis and pulmonary and renal injury</td>
<td>Not assessed</td>
</tr>
<tr>
<td>Yang 2008[450]</td>
<td></td>
<td>Delayed ethyl pyruvate vs. control</td>
<td>Not reported</td>
<td>Attenuated hepatic, pulmonary and renal injury</td>
<td>Prolonged survival</td>
</tr>
<tr>
<td>Raman 2006[448]</td>
<td>Haemorrhagic shock and resuscitation</td>
<td>RAGE-deleted vs. wildtype sRAGE vs. sham</td>
<td>Decreased bacterial translocation to mesenteric lymph nodes</td>
<td>Not reported</td>
<td>Not assessed</td>
</tr>
</tbody>
</table>

Table 9: Summary of major papers assessing effects of RAGE inhibition in murine models of non-septic systemic inflammation. AB antibodies, LPS lipopolysaccharide, KO knockout, WT wildtype, LD50 median lethal dose.
<table>
<thead>
<tr>
<th>Condition</th>
<th>Measurement</th>
<th>Association</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nakamura 2011[453]</td>
<td>ARDS and sepsis</td>
<td>sRAGE and HMGB1 levels in blood</td>
</tr>
<tr>
<td></td>
<td>sRAGE was independently associated with death</td>
<td></td>
</tr>
<tr>
<td>Jaboudon 2011[454]</td>
<td>ARDS ± severe sepsis</td>
<td>sRAGE levels in blood</td>
</tr>
<tr>
<td></td>
<td>sRAGE levels correlated with severity of ARDS, independent of presence of severe sepsis</td>
<td></td>
</tr>
<tr>
<td>Cohen 2010[455]</td>
<td>Trauma</td>
<td>sRAGE levels in blood</td>
</tr>
<tr>
<td></td>
<td>sRAGE levels were increased early after severe trauma and correlated with the severity of injury, early posttraumatic coagulopathy and hyperfibrinolysis, and endothelial cell activation</td>
<td></td>
</tr>
<tr>
<td>Manganelli 2010[383]</td>
<td>Major elective surgery</td>
<td>HMGB1 levels in blood and on monocytes</td>
</tr>
<tr>
<td></td>
<td>HMGB1 is increased on monocytes early post-operatively, and then concurrent with increases in plasma HMGB1, decreases.</td>
<td></td>
</tr>
<tr>
<td>Kikkawa 2010[456]</td>
<td>Sepsis, major elective surgery ± ALI</td>
<td>sRAGE and S100A12 plasma levels</td>
</tr>
<tr>
<td></td>
<td>sRAGE increases in those who do not develop ALI; S100A12 increases more in those that develop ALI.</td>
<td></td>
</tr>
<tr>
<td>Agonstoni 2010[457]</td>
<td>snCPB</td>
<td>sRAGE levels in blood</td>
</tr>
<tr>
<td></td>
<td>sRAGE increases following snCPB</td>
<td></td>
</tr>
<tr>
<td>Kohno 2010[458]</td>
<td>Aortic snCPB</td>
<td>HMGB1 levels in blood</td>
</tr>
<tr>
<td></td>
<td>Higher HMGB1 was associated with more severe SIRS and a higher incidence of impaired oxygenation</td>
<td></td>
</tr>
<tr>
<td>Calfee 2008[459]</td>
<td>ALI/ARDS</td>
<td>sRAGE levels in blood</td>
</tr>
<tr>
<td></td>
<td>Levels associated with severity of illness in all. In subset (high tidal volume arm) correlated with mortality</td>
<td></td>
</tr>
</tbody>
</table>

Table 10 (Part I): Human studies of the sepsis syndromes relating to measurements of the RAGE axis. BAL broncho-alveolar fluid
<table>
<thead>
<tr>
<th>Condition</th>
<th>Measurement</th>
<th>Association</th>
</tr>
</thead>
<tbody>
<tr>
<td>Payen 2008[460]</td>
<td>Recovery from septic shock</td>
<td>Leukocyte gene expression array</td>
</tr>
<tr>
<td>Bopp 2008[461]</td>
<td>Sepsis (and ARDS)</td>
<td>sRAGE levels in blood</td>
</tr>
<tr>
<td>Kocsis 2009[462]</td>
<td>Severe acute pancreatitis; sepsis</td>
<td>sRAGE and HMGB1 levels in blood</td>
</tr>
<tr>
<td>Makam 2009[463]</td>
<td>Cystic fibrosis</td>
<td>RAGE on neutrophils from airways and blood</td>
</tr>
<tr>
<td>Determann 2008[464]</td>
<td>Major elective surgery</td>
<td>sRAGE levels in blood and BAL</td>
</tr>
<tr>
<td>Wang 1999[370], Sunden-Cullberg 2005[392]</td>
<td>Sepsis</td>
<td></td>
</tr>
<tr>
<td>Angus 2007[409]</td>
<td>Community acquired pneumonia</td>
<td>HMGB1 levels in blood</td>
</tr>
<tr>
<td>Peltz 2009[400]</td>
<td>Trauma</td>
<td></td>
</tr>
<tr>
<td>Alleva 2005[396]</td>
<td>Infection with Falciparum malariae</td>
<td></td>
</tr>
<tr>
<td>Lantos 2010[397]</td>
<td>Burn injury &gt;10% body surface area</td>
<td></td>
</tr>
<tr>
<td>van Zoelen 2009[447]</td>
<td>Severe sepsis</td>
<td>S100A8/9 levels in blood</td>
</tr>
<tr>
<td>Piazza 2007[465]</td>
<td>Severe sepsis</td>
<td>S100B levels in blood</td>
</tr>
<tr>
<td>Korfias 2006 [466]</td>
<td>Trauma without head injuries</td>
<td>S100B levels in blood</td>
</tr>
</tbody>
</table>

Table 10 (Part II): Human studies of the sepsis syndromes relating to measurements of the RAGE axis. BAL broncho-alveolar fluid
3.3.2. Human studies

3.3.2.1. Sepsis

Levels of plasma sRAGE have been quantified in observational studies of patients with sepsis, in the presence or absence of co-existent ALI/ARDS (Table 10). The first description of raised sRAGE in patients with severe sepsis or septic shock was made in a cohort of surgical patients; levels were higher than those in controls, and highest in non-survivors. Although the presence of ALI/ARDS was not expressly described, both survivors and non-survivors had oxygenation indices within the defining range for ARDS [461]. A far larger cohort of patients with ALI/ARDS, of whom 26% had sepsis as the primary cause, was found to demonstrate a correlation between severity of illness (APACHE III score) and plasma sRAGE levels [459]. In 20 patients with sepsis (mean APACHE II score of 18.5) sRAGE was elevated (2,214±252pg/ml) and correlated positively with plasma HMGB1 levels [462]. No description of any relationship to oxygenation was reported. Experimental endotoxaemia has been shown to increase levels of plasma sRAGE in healthy volunteers [467].

Several recent studies have investigated the relationship between sRAGE and clinical parameters in patients with ALI/ARDS and severe sepsis. In patients with both ARDS and severe sepsis, sRAGE and HMGB1 levels correlated and were higher in non-survivors, sRAGE being an independent predictor of death. No relationship was found between lung injury score and sRAGE levels [453]. In another study, levels of plasma sRAGE were compared between patients with severe sepsis, severe sepsis with ARDS, ARDS alone and controls. sRAGE was found to be elevated in proportion to several markers of ALI/ARDS and without difference in levels between those with severe sepsis (without ARDS) and controls [454]. Finally, in patients who underwent surgery for abdominal sepsis, some of whom developed ALI, plasma sRAGE was higher in those who did not develop ALI, S100A12 higher in those who did [456].

It is difficult to reconcile these contradictory findings. What is clear is that plasma sRAGE levels increase in systemic inflammatory conditions, and ALI/ARDS rarely if ever occurs in its absence. Additionally, plasma sRAGE levels undoubtedly bear an important relationship to alveolar injury, as is discussed in more detail in Section 3.4.
Measurement of plasma sRAGE represents only one aspect of the RAGE axis and may not be the best gauge of RAGE-axis activity – to date no studies have quantified the extent of RAGE leukocyte expression in acute systemic inflammation. However, airway neutrophil RAGE expression in patients with chronic airway inflammation was found to be greater than in circulating neutrophils [463]. The only other published study of cellular RAGE expression was outside the context of acute illness, and demonstrated a positive correlation between monocyte RAGE expression and levels of serum AGE [296].

Circulating levels of the RAGE ligands (S100A8/9, S100A12, S100B, HMGB1) have been shown to be elevated in the plasma of patients with sepsis [341, 370, 392, 447, 460, 465, 468, 469]. Changes in the expression profile of leukocytes in those recovering from septic shock revealed that of the 340 genes studied, some of the greatest changes were in the genes for S100A8 and S100A12 and that non-survivors had significantly higher plasma levels of S100A8/9 [460].

### 3.3.2.2. Non-infective SIRS

Levels of plasma sRAGE and RAGE ligands have been quantified in observational studies of patients with pancreatitis, burns, trauma, and following major elective surgery. Firstly, elective surgical patients undergoing cardiac, thoracic or major abdominal surgery demonstrated elevations in plasma sRAGE post-operatively [457, 464, 470]. Those undergoing thoracic aneurysm repairs (snCPB) demonstrated elevated levels of HMGB1 [458]. Secondly, those with pancreatitis also showed elevated plasma sRAGE levels compared to controls, but with inverse correlations to disease severity and plasma HMGB1 levels [462]. Finally, burns and trauma were both associated with increased levels of circulating HMGB1 [400]. Levels of HMGB1 were positively correlated with the extent of the burn injury and were higher in non-survivors [397]. Levels of sRAGE are elevated very early following trauma and positively correlated with the extent of injury, though did not relate to mortality [455].

Together, these findings indicate that sRAGE levels increase as a consequence of tissue damage. To what extent alveolar injury, either attributable to mechanical ventilation (in the elective surgery patients), or in response to circulating mediators (in the other patients) was relevant is uncertain.
3.3.3. Potential role of RAGE inhibition

Inhibition of RAGE mediated signalling can be achieved through several means. Firstly, reduction of the amount of available ligand, either through reduced release or enhanced elimination. Agents that inhibitors release of HMGB1 include spermine and ethyl pyruvate. Secondly, blockade of RAGE-RAGE ligand interaction has been demonstrated through the use of anti-RAGE antibodies, anti-HMGB1 antibodies or excessive quantities of ligand-binding sRAGE or derivates thereof. Derivates include agents such as TTP-4000, which is a fusion protein consisting of the variable region and N-terminal constant domain of RAGE mated to the Fc region of human immunoglobulin G (Section 9.8.5).

With few exceptions, inhibition of RAGE in murine models of systemic inflammation has been associated with beneficial effects (Section 3.3.1). The question of therapeutic potential in various patient groups has been made, but several considerations require discussion.

Firstly, there is a disconnect between results from murine studies and clinical trials in humans. Historically, interventions designed to modulate the immune response in systemic inflammation (usually sepsis) have shown promise in animal models but have largely failed to replicate this in humans [39, 471, 472]. The reason that RAGE inhibition may have a greater chance of demonstrating efficacy compared to previously investigated pathways is based on the observation that RAGE inhibitors remain efficacious even when given hours after the experimental injury. This is in contrast to the supporting animal studies of the largest failed trials of immunomodulation in human sepsis (TNF-α, IL-1ra, PAF antagonists [473-475]), all of which involved pre-treatment.

Secondly, there are specific differences in the RAGE axis between mice and men: unlike in humans, sRAGE is not found in mouse plasma [295], and S100A12 is absent from the mouse genome [337].

Thirdly, and perhaps most importantly, an intact RAGE axis may be necessary for antibacterial defence. In a model of bacterial peritonitis, RAGE-deleted mice had enhanced bacterial growth, increased bacterial dissemination, and more severe inflammation [451]; the effect on mortality or surrogate thereof was not reported. Contradictory findings have been reported with RAGE-deleted mice exhibiting diminished bacterial dissemination [445,
In non-infectious systemic inflammation treated with anti-HMGB1 antibodies, bacterial dissemination has been found to be both reduced [378] and increased [377]. Genetic deletion of key components of the innate immune response may result in improved outcomes from both endotoxaemia and live bacteria (CD14-deficiency [476]) or worsened outcomes from both endotoxin and live bacteria (iNOS-deficiency [477]). Importantly, genetic deletion may also result in improved outcomes from endotoxaemia and worsened outcomes from live bacteria (MyD88-deficiency [478, 479]). The innate immune response is often described as a “double-edged sword”, meaning that it has capacity to help or harm; RAGE-mediated inflammation may be necessary to prevent bacterial dissemination but should not be excessive and contribute towards mortality. When considering genetic deletion models, compensatory changes in the immune system are likely to play a significant role but are challenging to evaluate.

Finally, sRAGE may inherently possess pro-inflammatory capacity. The interaction between RAGE and the Mac-1 integrin resulted in inflammatory activity in murine splenocytes [480]. Similarly, when sRAGE was given intra-peritoneally in a murine model of arthritis, the joint improved at the cost of increased peritoneal inflammation [480]. Perhaps of greater clinical relevance is the recent finding that sRAGE modifies human monocyte behaviour: causing recruitment, inducing pro-inflammatory cytokine activation, and promoting survival and differentiation [481]. The recombinant source of the sRAGE may be relevant. Indeed it is possible that the structure of sRAGE may be responsible for adverse effects rather than the inhibition of RAGE per se. In support of this, the small molecule RAGE inhibitor TTP-488 (also known as PF-04494700) has shown good initial safety data in early stage trials [437].

3.4. RAGE axis in acute lung injury (ALI)

Systemic inflammation, whether caused by infection or injury, is frequently complicated by the development of ALI, and its extreme manifestation, ARDS. The clinical and pathological characteristics, defining criteria, and reported mortality rates are discussed in Section 1.2.2.1.

3.4.1. RAGE-mediated inflammation is implicated in the pathogenesis of ALI

RAGE is implicated in ALI both as a marker of alveolar injury and as an important contributor to alveolar inflammation. Thus, in rodents with experimental lung injury induced either by
intra-tracheal LPS or acid, increased levels of sRAGE in serum and broncho-alveolar lavage (BAL) and serum correlate with the severity of insult [482]. In experimental models, BAL RAGE levels could distinguish between direct (precipitating factor involves the lung) and indirect (precipitating factor does not involve the lung) types of ALI, with raised RAGE levels only detectable in the direct lung injury group [483]. However, this finding was not replicated in patients with ALI [454].

Intra-tracheal administration of HMGB1 induced ALI in mice, thereby implicating RAGE more directly in the genesis of ALI. Moreover, the pathological effects of intra-tracheal LPS were partially ameliorated by systemic administration of sRAGE or anti-HMGB1 antibodies [374, 484]. Experimental endotoxaemia in rats induced ALI and increased tissue and circulating levels of HMGB1, effects that were aggravated by concurrently induced hyperglycaemia; and abrogated when combined with insulin therapy [485]. Experimental murine pulmonary ischaemia followed by reperfusion also caused ALI that was ameliorated by pre-treatment with sRAGE, and reduced when performed in RAGE deleted mice [486]. In rabbits, injuriously high tidal volume ventilation caused ALI and increased HMGB1 content in BAL when compared with lower tidal volumes, an effect attenuated by antibodies to HMGB1 [487]. Together, these data suggest strongly that RAGE plays a fundamental role in modulating pulmonary inflammation.

### 3.4.2. sRAGE as a biomarker for ALI

Patients with ALI have increased levels of sRAGE in pulmonary oedema fluid compared to those with cardiogenic pulmonary oedema. Moreover, sRAGE levels in oedema fluid were 100 times higher than plasma levels, supporting the hypothesis that sRAGE is generated in the lung [482]. Plasma sRAGE levels from patients enrolled in the influential ARMA trial (comparing high versus low tidal volume ventilation for ALI) correlated with the severity of illness and of ALI. Once corrected for other dependent variables (including age and severity of illness scores), baseline levels of sRAGE in patients randomised to receive high tidal volume ventilation correlated with mortality [459]. In a more recent study, extent of elevation of sRAGE levels in plasma from patients with ALI, with or without concurrent severe sepsis, correlated with markers of lung injury severity (quantified by PFR and Murray lung injury score) and radiographic appearance [454]. Another recent study demonstrated serum levels of HMGB1 and sRAGE were significantly higher in non-survivors of ARDS.
compared with survivors; with plasma sRAGE levels being independently predictive of mortality [453]. Further observational trials are currently recruiting patients with ALI in order to measure RAGE ligands and soluble forms and gain further insight into their relationship with clinical parameters (clinicaltrials.gov, NCT01270295).

Primary graft dysfunction (an ALI-like syndrome) is a common and serious complication of lung transplantation. Plasma sRAGE measured shortly after reperfusion correlated with adverse short-term outcomes [488]. These results may be partially explained by the observation that in isolated perfused human lungs, elevated levels of sRAGE in the airspace were associated with impaired alveolar fluid clearance, a measure of epithelial integrity [489]. Patients with ARDS have increased levels of S100A12 and A8/9 in their BAL and increased S100A12 expression in lung biopsies [343, 490]. Furthermore, inhalation of LPS by healthy volunteers increased S100A12 content in BAL [343] and clinical ALI is associated with elevated levels of serum S100A12 [456].

The utility of measuring aspects of the RAGE axis to monitor the contribution of ventilation to lung injury has been assessed in several studies. Firstly, patients receiving mechanical ventilation for major elective surgery were either randomized to receive lung protective (low tidal volume, positive end expiratory pressure, PEEP) or conventional (high tidal volume, no PEEP) ventilation. sRAGE was found to be elevated post-operatively irrespective of ventilatory strategy [464]. Secondly, in critically ill patients without ALI, BAL levels of HMGB1 were greater in those who had undergone long-term (7.6 days mean) mechanical ventilation (MV) in comparison to short-term (5 hours mean) MV [491]. Finally, in patients undergoing lung resection surgery requiring one-lung ventilation (a major risk for post-operative ALI) levels of sRAGE did not relate to ventilatory parameters or clinical outcomes [470].

Despite extensive study of potential biomarkers of ALI none have entered routine clinical use [492, 493]; predominantly because they have not demonstrated any clear advantage over clinical predictors. An ideal biomarker for ALI would be highly specific and sensitive, vary in proportion to the severity of injury and reflect the effect of therapy. It would add independent prognostic information, have biological plausibility and potentially identify patient subgroups that would benefit from specific therapy. Finally, the assay should be
inexpensive, rapid and highly reproducible. Systemic inflammation typically either precipitates or complicates ALI, and the utility of plasma sRAGE as a specific biomarker of alveolar damage is therefore likely to be limited.
4. INFLUENCES ON THE RAGE-AXIS AND OUTCOME FROM ACUTE SYSTEMIC INFLAMMATION

A range of conditions and acquired characteristics affect the RAGE-axis and the acute inflammatory response. The same conditions may be associated with adverse outcome from acute systemic inflammation. Of particular relevance are conditions associated with increased RAGE ligands: old age, diabetes, chronic kidney disease and rheumatoid arthritis, the commonest chronic inflammatory arthritis.

4.1. Old age

4.1.1. Demographics

The population of the UK is ageing. Over the last 25 years the percentage of the population aged 65 and over increased from 15 per cent in 1984 to 16 per cent in 2009, an increase of 1.7 million people. The fastest increase has been in the number of those aged 85 and over, the “oldest old” (Figure 15). In 1984, there were around 660,000 people in the UK aged 85 and over, since then the numbers have more than doubled to 1.4 million in 2009. By 2034 the number of people aged 85 and over is predicted to reach 3.5 million, accounting for 5% of the total population [4].

![Figure 15: Population by age in the UK, 1984, 2009 and predicted for 2034](image_url)
4.1.2. Changes in immune status with ageing

4.1.2.1. Immuno-senescence

A state of dysregulated immune function is found in the elderly and is termed immuno-senescence [494, 495]. Table 11 summarises changes in leukocyte function.

<table>
<thead>
<tr>
<th>Neutrophils</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell number</strong></td>
</tr>
<tr>
<td><strong>Migration</strong></td>
</tr>
<tr>
<td><strong>Tissue penetration</strong></td>
</tr>
<tr>
<td><strong>Phagocytosis</strong></td>
</tr>
<tr>
<td><strong>Production of ROS</strong></td>
</tr>
<tr>
<td><strong>NK cells</strong></td>
</tr>
<tr>
<td><strong>Cell number</strong></td>
</tr>
<tr>
<td><strong>Per cell killing</strong></td>
</tr>
<tr>
<td><strong>Macrophages</strong></td>
</tr>
<tr>
<td><strong>Phagocytosis</strong></td>
</tr>
<tr>
<td><strong>Killing</strong></td>
</tr>
<tr>
<td><strong>Cytokines</strong></td>
</tr>
<tr>
<td><strong>Wound healing</strong></td>
</tr>
<tr>
<td><strong>T lymphocytes</strong></td>
</tr>
<tr>
<td><strong>Repertoire</strong></td>
</tr>
<tr>
<td><strong>Cytokine response</strong></td>
</tr>
<tr>
<td><strong>Antigen presentation</strong></td>
</tr>
<tr>
<td><strong>B lymphocytes</strong></td>
</tr>
<tr>
<td><strong>Repertoire</strong></td>
</tr>
<tr>
<td><strong>Antibody response</strong></td>
</tr>
<tr>
<td><strong>Dendritic cells</strong></td>
</tr>
<tr>
<td><strong>Migration</strong></td>
</tr>
<tr>
<td><strong>Cytokine response</strong></td>
</tr>
<tr>
<td><strong>Phagocytosis</strong></td>
</tr>
</tbody>
</table>

Table 11: Summary of age-related changes to immune cells, adapted from Castle et al. (2000)

Immuno-senescence contributes to increased susceptibility to infection, decreased response to vaccination, and predisposition to development of malignancy [495-498].

4.1.2.2. Inflamm-aging

In addition to a general diminution in the immune response there is a concomitant progressive increase in pro-inflammatory status. A state of low-grade chronic inflammation,
termed inflamm-aging exists, and may be provoked by a continuous antigenic load and stress. It is postulated that this is partly due to incomplete eradication of pathogens and continued inflammatory cell signalling [499]. The age-associated low-grade inflammatory state is reflected in increased levels of circulating cytokines TNF-α, IL-6, IL-1Ra, sTNFR; and acute phase proteins such as CRP and Serum Amyloid A (SAA)[500].

4.1.2.3. Immuno-responsiveness

Similar to studies of endotoxin tolerance (Section 2.3.1) there have been ex vivo studies of leukocyte response to inflammatory stimuli investigating the effect of the age of leukocyte donor on responsiveness. Although data are conflicting [501] the prevailing consensus is that there is relative hypo-responsiveness with increased age [502-504].

To minimise conflicting immunological studies of older patients the strict SENIEUR protocol was developed which classifies only 10–15% of aged subjects (and 75% of young subjects) as perfectly healthy and fit [505]. The danger of this approach is that a rigidly defined group of “healthy elderly” becomes increasingly irrelevant to a “normal” elderly population with advancing age.

The effect of age on cytokine responses and outcome in animal models of sepsis is rarely considered; young animals tend to be chosen for logistical and economic reasons. In a murine CLP model, aged animals displayed decreased survival and increased plasma cytokines compared to younger counterparts [506]; similar results have been demonstrated in endotoxaemia models [507, 508].

Of greater clinical relevance are in vivo observational studies of inflammatory activation and cytokine responses. Compared to younger patients, older subjects with pneumonia have similar increases in circulating cytokines (IL-1β, IL-6, IL-1ra, sTNFR-I, and IL-10) but a greater elevation of TNF-α on the day of initial assessment. One week later the older group had persistent elevation of pro- and anti-inflammatory cytokines in comparison to the younger group [509]. Similarly, at enrolment to an interventional study of septic shock, those over 85y had greater levels of TNF-α than younger subjects [510]. In an experimental endotoxaemia model, older animals exhibited greater increases in inflammatory cytokines, that were also relatively prolonged [511, 512]. Conversely, levels of cytokines during the
acute phase of sepsis have also been found to be lower in the elderly group [504] possibly explaining a lower incidence of fever [513] and CRP response [514].

4.1.3. Age and the RAGE-axis
Non-enzymatic glycation reactions occur slowly and relentlessly, leading to gradual accumulation of AGEs; episodes of acute systemic inflammation may result in abrupt increases in AGE. As discussed in Section 3.2.3, AGE levels in plasma and tissues have been related to aging [515]. In the paradigm under consideration within this thesis, such accumulated AGEs up-regulate the RAGE-axis and predispose to excessive and perpetuated inflammatory responses. However, there are currently few data to suggest that tissue RAGE levels increase with age. Thus, levels of RAGE protein (but not mRNA) increase with age in human myocardium [516], but not in endothelial cells obtained from peripheral veins [517].

4.1.4. Age and outcome from critical illness
Increased age has been repeatedly associated with adverse outcome from most, if not all causes of critical illness; for this reason patient age is a key variable in the majority of scoring systems that relate patient characteristics to adverse outcome [91, 98, 518-523]. The question is whether this association relates to the changes of aging in themselves, or due to other considerations.

Studies designed to answer this question variably suffer from particular limitations. Firstly, failure to consider or precisely correct for all the contributory factors (confounders). For example a study of patient outcome following snCPB showed that the incidence of myocardial dysfunction (left ventricular systolic dysfunction) increased with age and related to increased mortality [524]. Thus, studies that did not assess myocardial function would tend to over-estimate the effect of age on mortality. More generally, with advanced age there are important confounders that may not be routinely assessed, such as the accumulation of co-morbidities, increased use of prescription medications [525] and decreased strength and dexterity that may result in increased dependence, associated with adverse outcome [526].

Comparing patients of different ages and different degrees of severity of illness requires consideration of severity of illness, typically through the use of a scoring system. However, these are imperfect tools which vary in their sensitivity and specificity [527]. A related pitfall
would be to over-correct for age by using an unmodified severity of illness score that includes age within it. A simple example of this is the CURB-65 score for community acquired pneumonia [522].

Secondly, when assessing the effect of age on outcomes from an elective intervention such as surgery, as opposed to an emergent condition such as sepsis, an important consideration is selection bias. Only elderly patients that are deemed likely to benefit are chosen to undergo surgical interventions. Further, only a subset of elderly patients who might technically benefit will choose to have the surgery – this potentially selects fitter and more motivated patients. These selection biases are arguably impossible to correct for and would tend to hide any association between age and adverse outcome and may go some way towards explaining divergent findings.

Thirdly, the most commonly used methods of multivariable analysis such as linear or logistic regression incompletely eliminate selection factors, or to provide an accurate assessment of the effect of the variable of interest being compared after properly adjusting for patient differences. The technique of propensity score matching attempts to eliminate these limitations [528, 529].

Finally, considerations that are difficult to measure. Logically, humans have a limited lifespan and those that are elderly have fewer years remaining, therefore the 5y survival rate of patients over 85y for anything under consideration will always be less than patients who are 55y. There are relevant non-medical factors that differ with age, including access to health care, delivery of healthcare and patient care preferences [5]. Related to this, older patients are more likely to have considered limitations to their therapy and possess advanced directives [530]; and are more likely to have care withdrawn, a relatively common practice internationally amongst the critically ill [531].

It is hypothesised that critical illness triggers a pathophysiological process that persists long after discharge when patients have seemingly recovered from acute illness. In older adults, there may be an inability to appropriately down-regulate inflammation after resolution of initiating injury [532]. Similarly, hospitalization of older persons often results in functional decline, despite cure or repair of the condition for which they were admitted [533].
4.1.4.1. **Sepsis**

A longitudinal observational study using national discharge data of 10,422,301 adult sepsis patients hospitalized between 1979 and 2002 in the United States showed an increased incidence of sepsis with age and increased case-fatality (Figure 16).

![Figure 16: Incidence (filled circles, left abscissa) and case-fatality (open boxes, right abscissa) for sepsis, adjusted and stratified by age deciles. Points represent mean and error bars SEM. Taken from [5].](image)

To assess if the observed relationship between age and case fatality was related to age or confounding variables, multivariate logistic regression modelling considered age, gender, race, source of infection and chronic co-morbid medical conditions (cancer, chronic renal failure, congestive heart failure, COPD, coronary artery disease, diabetes, hepatic cirrhosis, HIV and hypertension). Age > 65y was independently associated with a 2.3 times higher risk of death among patients with sepsis (adjusted OR, 2.26; 95% CI 2.17–2.36). Survivors >65y were less likely to be discharged home (54% vs. 76%, p <0.001) [5]. These results mirror other observations made in the United States [31] and Singapore [534].

As sepsis is not a homogeneous condition, there may be benefit in considering a better circumscribed infection, such as pneumonia. A prospective population-based cohort study of 3415 patients in Canada examined the association between age and 30-day (short-term)
and 1-year (long-term) mortality using Cox proportional hazards regression. The association corrected for potential confounders: pneumonia severity, mechanical ventilation, sex, functional status, nursing home residence, and having a living will. There was a strong statistically significant positive relationship between increased age and increased mortality (Table 12) [535]. With each decade increase there was a 24% increase in mortality at 30 days and 39% increase at 1 yr. This may contrast to previous contradictory reports which over-corrected for age when using severity of illness scores that include age.

<table>
<thead>
<tr>
<th>Variable</th>
<th>30 day</th>
<th></th>
<th>1 year</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>aHR (95% CI)</td>
<td>P</td>
<td>aHR (95% CI)</td>
<td>P</td>
</tr>
<tr>
<td>Age per decade, yrs</td>
<td>1.24 (1.03-1.49)</td>
<td>0.03</td>
<td>1.39 (1.21-1.60)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Age categories, yrs</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;60</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>60-69</td>
<td>0.78 (0.30-2.00)</td>
<td>.6</td>
<td>1.14 (0.60-2.18)</td>
<td>.7</td>
</tr>
<tr>
<td>70-79</td>
<td>1.79 (0.91-3.51)</td>
<td>0.09</td>
<td>2.62 (1.59-4.31)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>&gt;80</td>
<td>2.54 (1.21-5.36)</td>
<td>0.01</td>
<td>3.47 (1.99-2.81)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Male sex</td>
<td>1.36 (0.78-2.37)</td>
<td>.3</td>
<td>1.32 (0.89-1.96)</td>
<td>.2</td>
</tr>
<tr>
<td>Functional limitation</td>
<td>2.02 (0.83-4.89)</td>
<td>.1</td>
<td>1.60 (0.92-2.81)</td>
<td>.1</td>
</tr>
<tr>
<td>Living will</td>
<td>3.08 (1.61-5.90)</td>
<td>&lt;0.001</td>
<td>2.00 (1.21-3.32)</td>
<td>0.007</td>
</tr>
<tr>
<td>Nursing home residence</td>
<td>0.71 (0.36-1.42)</td>
<td>.3</td>
<td>1.13 (0.71-1.81)</td>
<td>.6</td>
</tr>
<tr>
<td>Mechanical ventilation</td>
<td>2.09 (0.87-4.99)</td>
<td>.1</td>
<td>1.55 (0.90-2.69)</td>
<td>.1</td>
</tr>
<tr>
<td>Modified PSI score (pre 10 points)</td>
<td>1.11 (1.03-1.21)</td>
<td>.01</td>
<td>1.12 (1.05-1.19)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Table 12: Results of multivariable regression examining increasing age as a correlate of 30 day and 1-yr mortality in ICU patients with pneumonia. aHR, adjusted hazard ratio; CI, confidence interval; PSI, Pneumonia Severity Index. Reproduced from [535]. aHR emboldened when p<0.05

4.1.4.2. SIRS

4.1.4.2.1. snCPB

This is increasing demand for cardiac surgical procedures in the elderly due to an ageing population and the fact that a principle cause of death is cardiovascular pathology, including coronary artery disease (CAD). The most common (~70%) snCPB is coronary artery bypass grafting (CABG). Although the use of percutaneous coronary intervention (PCI) for obstructive CAD has increased, CABG remains the treatment of choice for many types of CAD [536]. The second commonest procedure is aortic valve surgery for stenosis, another degenerative disease process that increases in incidence with age. One in five patients
having CABG are now aged over 75y, and the average age of aortic valve replacement patients increased from 61 in 1994 to 68 in 2008 [8].

The majority of studies investigating the relationship between age of patient and mortality or morbidity following cardiac surgery have found statistically significant, independent relationships (Table 13).

<table>
<thead>
<tr>
<th>Year</th>
<th>Subjects</th>
<th>Surgery and condition</th>
<th>Statistical analysis</th>
<th>Age associated with mortality</th>
<th>Age associated with morbidity</th>
<th>Reference</th>
</tr>
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<td>1991</td>
<td>13,625</td>
<td>CABG</td>
<td>LR</td>
<td>Yes</td>
<td>Yes, Stroke</td>
<td>[537]</td>
</tr>
<tr>
<td>2002</td>
<td>31,688</td>
<td>mitral valve replacement (MVR) ± other</td>
<td>LR</td>
<td>Yes</td>
<td>Yes</td>
<td>[538]</td>
</tr>
<tr>
<td>2005</td>
<td>7726</td>
<td>snCPB</td>
<td>LR</td>
<td>Yes</td>
<td>Yes, stroke and re-operation</td>
<td>[539]</td>
</tr>
<tr>
<td>2005</td>
<td>1746</td>
<td>CABG</td>
<td>LR</td>
<td>Yes, &gt;80</td>
<td>Yes, LOS and cost</td>
<td>[540]</td>
</tr>
<tr>
<td>2008</td>
<td>122 in each group</td>
<td>CABG</td>
<td>PS</td>
<td>No</td>
<td>Yes, AF and LOS</td>
<td>[541]</td>
</tr>
<tr>
<td>2009</td>
<td>3094</td>
<td>snCPB</td>
<td>LR</td>
<td>Yes in those &gt;70y</td>
<td>Higher incidence of many complications in those &gt;70y</td>
<td>[524]</td>
</tr>
<tr>
<td>2009</td>
<td>3683</td>
<td>CABG</td>
<td>LR</td>
<td>Yes, &gt;80 for early mortality not late</td>
<td>N/A</td>
<td>[542]</td>
</tr>
<tr>
<td>2010</td>
<td>10141 &gt;85y</td>
<td>CABG or PCI for ACS</td>
<td>LR</td>
<td>Yes at 60d and 3y</td>
<td>N/A</td>
<td>[543]</td>
</tr>
</tbody>
</table>

Table 13: Summary of selection of retrospective observational studies of the relationship between age and outcome in cardiac surgery. ACS acute coronary syndrome, RRT renal replacement therapy, LR logistic regression, PS propensity score.
4.1.4.2.2. Non-cardiac surgery

Studies investigating the relationship between age of patient and mortality or morbidity following non-cardiac surgery have found statistically significant independent relationships. The largest was a multivariate regression database analysis of 594,911 patients having non-cardiac surgery, in which age was independently associated with mortality [544]. More recently, a prospective study of 4158 non-cardiac surgical patients aged 70 years or more assessed the relationship between patient factors and mortality at 30 days. Patients in the 80-89 cohort had an odds ratio of mortality of 2.1 (95% CI 1.6-2.8), p < 0.001; those in the 90+ cohort OR 4.0 (95% CI 2.6-6.2), p < 0.001) [545]. In two smaller series from California of 544 patients >70y and 367 patients >80y having non-cardiac surgery, no association between age and mortality or morbidity was found [546, 547].

4.1.4.2.3. Trauma

In a retrospective review of trauma registry data collected prospectively on 26,237 blunt trauma victims (age ≥65y, n=7,117) the elderly had significantly higher mortality rates after stratification by Injury Severity Score (ISS), Revised Trauma Score, and for other pre-existing co-morbidities. Age ≥ 65 years was associated with a two- to threefold increased mortality risk in mild (ISS < 15, 3.2% vs. 0.4%; < 0.001), moderate (ISS 15-29, 19.7% vs. 5.4%; < 0.001), and severe traumatic injury (ISS > or = 30, 47.8% vs. 21.7%; < 0.001) compared with patients aged < 65 years. Logistic regression analysis confirmed that elderly patients had a nearly twofold increased mortality risk (odds ratio, 1.87; confidence interval, 1.60-2.18; < 0.001). Elderly patients also had significantly longer ICU and hospital LOS after stratifying for severity of injury [548].

Similarly, patients >50y (n = 1,251) had a longer LOS (12.5 versus 8.4 days; p < 0.001) and increased mortality (22.1% versus 8.0%; p < 0.001) than those <50y. Age remained an independent predictor of mortality when controlling for ISS [549].

4.1.5. Conclusions

Putting aside the considerable, and sometimes unavoidable, limitations, it seems that there is a significant relationship between advanced age and adverse outcome. This may be due to altered immunity and the inevitable decline in physiological reserve that occurs with age.
4.2. Diabetes

4.2.1. Demographics
Diabetes in this context refers to both type 1 diabetes, characterised by young onset and autoimmune destruction of the \( \beta \)-cells in the pancreas; and type 2 diabetes, characterised by later onset, insulin resistance and progressive insulin deficiency due to pancreatic \( \beta \)-cell dysfunction. The incidence of both is increasing internationally [550-553]. Prevalence of diabetes was 4.3% in the UK and 7% in the US in 2005 [6, 554].

Hyperglycaemia is the *sine qua non* of diabetes and poorly controlled diabetes is typified by chronic hyperglycaemia. Acute critical illness is almost invariably associated with hyperglycaemia and insulin resistance, even in those without pre-existing diabetes [555, 556]. As discussed in Section 3.2.3, hyperglycaemia predisposes to formation of AGE modifications, particularly when coupled with redox stress.

4.2.2. Changes in immune status with diabetes
Alterations in immune status in patients with diabetes has been studied extensively [557] and although perturbations in innate and adaptive responses have been demonstrated *in vitro*, to what extent these are clinically relevant remains unresolved [558]. Contrary to expectations, attenuated immune function may even be beneficial under some circumstances: diabetes having been associated with decreased risk of ALI in those with sepsis, compared to those without diabetes [6].

Hyperglycaemia is conducive to microbial growth and those with diabetes are generally considered to be predisposed to the acquisition of infection. Despite some controversy, this relationship has been shown to be robust [6, 559, 560] (Figure 17).
4.2.2.1. **Innate**

Neutrophil functions including chemotaxis, phagocytosis and intracellular killing are significantly impaired during hyperglycaemia, and possibly in associated with its severity [561-563]. Indeed, improved glycaemic control may lead to improvements in neutrophil function [564]. The *ex vivo* cytokine response to endotoxin stimulation has been found to be impaired in those with type 2 diabetes [565].

4.2.2.2. **Adaptive**

Defects in the adaptive immune response in those with diabetes are less well described, with no consistent defects in cell mediated immunity and normal antibody responses to vaccinations [558]. Circulating antibodies are however, subject to AGE-modifications, particularly the antigen-binding fragment (Fab) which may affect antigen affinity [566].

4.2.3. **Diabetes and the RAGE-axis**

RAGE ligands are increased in patients with diabetes. Circulating levels of S100A12 are elevated in the plasma of patients with diabetes and relate to Hba1c levels and white blood cell count [344]. Plasma CML-albumin is elevated in diabetes and implicated in the development of vascular complications [415]. Indeed, a wide range of AGE modifications are found in the vasculature and other tissues in those with diabetes [567]. sRAGE plasma levels have been shown to be higher in those with diabetes than matched controls [568].
Hyperglycaemia affects circulating leukocytes, with evidence of altered RAGE signalling in neutrophils and up-regulation of monocyte membranous RAGE expression [287, 296]. Hyperglycaemia up-regulates RAGE, S100A8, S100A12, and HMGB1 expression on human endothelial cell expression in vitro [569].

The RAGE-axis has a well-established role in the development of diabetic complications including retinopathy, nephropathy, neuropathy and atherosclerosis, particularly through effects on the vascular endothelium [570-574]. Substantial work has been conducted on the role of RAGE in the development of atherosclerosis in diabetes and euglycaemia, but it is beyond the scope of this thesis and readers are referred to recent reviews [575-577].

### 4.2.4. Diabetes and outcome from critical illness

Associations between diabetes and increased morbidity and mortality following critical illness could be due to the underlying causes for the diabetes, including obesity [578] (type 2) or autoimmunity (type 1), the effects of hyperglycaemia and altered immune function; or the complications of chronic diabetes including chronic kidney disease and atherosclerosis resulting in coronary artery disease [579], peripheral vascular disease and cerebro-vascular disease. Lastly, therapies used in the treatment of diabetes, such as insulin, statins and glitazones may influence the host response to infection [580].

#### 4.2.4.1. Sepsis

Diabetes traditionally has been considered as a risk factor for the progression of infection and subsequent adverse outcome. Recently, an investigation of the influence of diabetes on the clinical course of, and outcome from, community acquired pneumonia (CAP) in 3534 patients was reported [581]. Diabetes was associated with increased severity of illness (APACHE III) and severity of pneumonia (PSI) scores, increased incidence of acute kidney injury (AKI), and increased mortality at one year. An absence of difference in plasma biomarkers of inflammation or coagulation, or cytometric measures of monocyte immune status casts doubt on the significance of altered immune function. Deaths due to cardiovascular disease and chronic kidney disease were higher among those with diabetes (34.4% vs 26.8% and 10.4% vs 4.5% for cardiovascular and kidney disease, respectively).
The second commonest infection causing hospitalisation following CAP is urosepsis. Patients with diabetes have increased incidence, increased rates of complications and higher mortality rates from urinary tract infection than those without diabetes [582, 583].

A recent analysis of National Hospital Discharge Survey data from 12.5 million hospital admissions with sepsis in the United States confirmed increased rates of infection in those with diabetes (for respiratory, urinary, skin and soft tissue and bone infections) but had the unexpected finding of decreased organ failure and improved outcome in those with diabetes [6]. The authors suggest the lower incidence of respiratory failure observed in those with diabetes may be due to protection from ALI/ARDS, consistent with their previous findings [584]. Further, they suggest that a blunted immune response may be responsible for the decreased incidence of ALI. Alternative explanations include patients with diabetes seeking medical attention earlier or an unidentified protective effect of their medications. A major limitation of this study is its reliance on coding data and the absence of detailed clinical data.

4.2.4.2. SIRS

4.2.4.2.1. snCPB

There have been many studies of the influence of diabetes on outcome from cardiac surgery, mostly in patients undergoing CABG. A meta-analysis and systematic review of >100,000 patients has recently reported associations between diabetes and increased risk of mortality, stroke, renal failure, sternal infection, and blood transfusion [585].

4.2.4.2.2. Non-cardiac surgery

Diabetes is widely considered to be a significant risk factor for adverse outcome following non-cardiac surgery, but this not appear to be independent, in that its associated complications account for the attributable risk; in particular, coronary artery disease and consequent peri-operative myocardial infarction [586, 587].

4.2.4.2.3. Trauma

Early studies found significant associations between diabetes and mortality [588] but more recent analyses identified only increased rates of prolonged ICU stay and infectious complications [589, 590].
4.2.5. Conclusions
The increased incidence of infection in those with diabetes may be solely due to hyperglycaemia. The extent to which impaired immune responses contribute is uncertain. Once systemic inflammation has developed, any progression to death seems to relate more to any pre-existing complications of diabetes, rather than immune dysfunction.

How RAGE-mediated effects of diabetes on the innate immune response contribute to the development and progression of systemic inflammation remains uncertain but may be less important than the well-established role of the RAGE-axis in the development of diabetic complications [574].

4.3. Chronic kidney disease (CKD)
CKD is defined as a structural or functional deficit in the kidneys, manifest by either kidney damage detected as excessive urinary protein (proteinuria) with or without a decreased glomerular filtration rate (GFR, <60 mL/min/1.73 m², a measure of kidney function) [591]. CKD is divided into stages: 1, normal GFR but intrinsic disease; 2, mild impairment; 3, moderate impairment; 4, severe impairment; 5, minimal or absent function, requiring renal replacement therapy (RRT), previously known as ‘end-stage renal failure’.

4.3.1. Demographics
The prevalence of CKD varies according to which definitions are used, how the renal function is measured (or calculated) and whether proteinuria is included. However, CKD (stages 3-5) is estimated to affect 5-10% of the adult population in the Western world [592] and is growing [593].

The commonest causes of CKD are diabetes and hypertension, followed by glomerulonephritis. CKD is associated with an increased risk of death, particularly from cardiovascular (CV) causes and although this partly relates to the underlying conditions, there is an appreciable excess mortality proportional to the degree of renal impairment [594]. The majority of people with mild-to-moderate CKD die from CV disease before needing RRT [595].
4.3.2. Changes in immune status with CKD

Biomarkers of inflammation, including classical pro-inflammatory mediators, markers of endothelial activation, oxidative stress and coagulation, have been demonstrated repeatedly to be elevated in proportion to stage of CKD, when compared to matched controls [596-598].

Patients with CKD have been shown to have defective leukocyte priming and diminished respiratory burst [599, 600], but the effects of CKD on other aspects of the innate and acquired immune systems have not been examined in detail.

4.3.3. CKD and the RAGE-axis

The RAGE-axis may be important in the development of various types of CKD, not just diabetic nephropathy [601]. Of greater relevance to these investigations are the findings of increased circulating RAGE ligands, soluble forms, and up-regulated leukocyte RAGE expression in CKD.

Soluble RAGE levels have been noted to be elevated in those with CKD, in proportion to the extent of renal impairment, possibly through reduced renal elimination [602, 603]. Levels positively correlate with plasma AGE in CKD patients [603]. Similarly, HMGB1 levels in the plasma positively correlate with extent of renal impairment [604]. In patients with stage 5 CKD, S100A12 levels were elevated and associated with all-cause and cardiovascular mortality [605].

RAGE expression on monocytes is increased in those with CKD, in proportion to the abundance of the AGE pentosidine; furthermore, monocyte cytokine response to AGE was proportionate to the extent of RAGE expression [606]

4.3.4. CKD and outcome from critical illness

The predisposition of patients with CKD to cardiovascular death is highly relevant as critical illness may precipitate CV death. CKD is an important risk factor for CV mortality for many reasons: there is a high prevalence of co-morbidities; there is abnormal vascular biology with endothelial dysfunction; accumulation of phosphate and increased levels of parathyroid hormone contributing to vascular calcification, anaemia and left ventricular hypertrophy; and increased circulating levels of homocysteine, fibrinogen, and uric acid.
Mediations with prognostic benefit are underused and there may be therapeutic nihilism associated with these patients [594, 607, 608].

Consideration of the relationship between CKD status and adverse outcome from critical illness is hampered by the difficulty of correcting for all relevant confounding factors. Additionally, the high risk of CV mortality independent of the onset of critical illness will lead to bias in any longer-term outcome studies.

4.3.4.1. Sepsis

Patients with CKD may be pre-disposed to the development of infection due to a combination of factors which include: malnutrition, chronic inflammation, retention of uraemic solutes, trace element deficiencies, metabolic abnormalities, relatively higher prevalence of chronic urinary sepsis; and in some of those receiving RRT the presence of indwelling catheters or grafts [609]. Indeed, infection is the second commonest cause of death after cardiovascular disease in those with stage 5 CKD [609]. Indeed, CKD of all stages is associated with increased mortality from infections [610]. Bloodstream infection (BSI) is especially common in those with CKD, even in the absence of in-dwelling vascular access devices. The risk of acquisition of a BSI relates to degree of renal impairment, as does the risk of death [611]. Similarly, the risk of hospitalisation with pneumonia and subsequent death relate to severity of CKD [612].

4.3.4.2. SIRS

The most-studied non-infectious cause of SIRS is snCPB. The presence of CKD in such patients is invariably associated with higher mortality [607, 613, 614].

4.3.5. Conclusion

CKD is associated with accelerated atherosclerosis contributing to cardiovascular mortality. This is the predominant factor determining outcome from acute systemic inflammation. However, even non-cardiac all-cause mortality is higher in those with CKD than without [610], reflecting the diverse pathological consequences of impaired kidney function.

The effects of increased activity of the RAGE-axis in CKD on the development and progression of systemic inflammation remain uncertain. However, the RAGE-axis is
fundamentally important in the development of the accelerated atherosclerosis of CKD that accounts for much of the excess adverse outcome.

4.4. Rheumatoid arthritis

4.4.1. Demographics
Rheumatoid arthritis (RA) is a common chronic inflammatory disease with increased prevalence with advanced age [615, 616]. Overall, the prevalence of RA in the UK may be in decline for women (1.16%) but has remained constant for men (0.44%) since the 1950s [617]. Patients with RA suffer from increased cardiovascular morbidity and mortality due to accelerated atherosclerosis and premature coronary artery disease [618, 619] believed to relate to chronic vascular inflammation.

4.4.2. Changes in immune status with RA
Rheumatoid arthritis is a complex, systemic, auto-immune inflammatory disease resulting in synovial inflammation and associated damage to articular cartilage and bone [615]. It is beyond the scope of this thesis to describe the immunopathology in detail.

4.4.3. RA and the RAGE-axis
RAGE ligands are implicated in the development of arthritis, with high level expression of S100A12 and S100A8/9 in synovial tissue and elevated plasma levels correlated with disease activity and response to therapy [620-624]. sRAGE levels have been found to be suppressed in one cohort of patients with RA [625], and unchanged in another [626], both in comparison to healthy controls.

4.4.4. RA and outcome from critical illness
Patients with rheumatoid arthritis have significantly increased mortality compared to the general population, with particularly striking increases in cardiovascular (CV) and infective deaths [627]. The increased incidence of CV events in RA patients is independent of traditional CV risk factors. A close relationship between markers of systemic inflammation and future CV events implicates an inflammatory aetiology in accelerated atherosclerosis [619, 628].

117
4.4.4.1. **Sepsis**

Individuals with RA are at increased risk of infection [629]. The increasing use of immune-suppressive therapy, such as biological disease modifying drugs and oral corticosteroids, further increases the risk [630]. The association between RA and susceptibility to infection was observed prior to use of immune-suppressive therapy [631]. Sites of infection with the highest risk ratios were bone, joints, skin, soft tissues, and the respiratory tract [629].

Retrospective observational studies from the 1990s describe high short-term mortality in patients with systemic rheumatic diseases admitted to ICU [632], but possibly not significantly different from non-selected patients [633]. Another study noted mortality that was higher than would be expected from predictive models [634]. Most recently, a matched case-control study revealed similar mortality despite lower severity of illness scores [635]. Although none of these studies are without significant shortcomings, their conclusions are mostly consistent.

4.4.4.2. **SIRS**

Given that patients with RA have a high incidence of macrovascular and microvascular atherosclerosis and myocardial infarction is their leading cause of death they might be expected to have an increased mortality following CABG surgery. Paradoxically, a large retrospective multivariate analysis of hospital in-patient data from the US demonstrated improved mortality in patients with RA [636]. There are few studies examining outcome of patients with RA and concomitant acute non-infectious critical illness.
5. SCOPE OF THIS THESIS

The work described in this thesis was designed to explore the hypothesis that the RAGE axis is an important determinant of systemic inflammation following surgery necessitating cardiopulmonary bypass.

Figure 18: Overview of RAGE axis in snCPB with numerals 1 – 5 demonstrating where the specific aims of the thesis lie.

The specific aims of the work were therefore:

1: To conduct an analysis of the relationship between the age of patients undergoing snCPB and the severity of their systemic inflammatory response, the relationship between these variables and adverse clinical outcomes (Section 10).

2: To characterise levels of relevant RAGE ligands and levels of soluble RAGE forms in plasma obtained around snCPB and establish their relationship to relevant clinical variables (Section 11).

3: To develop flow cytometric and quantitative reverse transcriptase real-time polymerase chain reaction (qRT-PCR) methods to facilitate subsequent measurement of leukocyte RAGE

---

First hit: chronic influences on the RAGE axis

<table>
<thead>
<tr>
<th>1</th>
<th>Age</th>
<th>Chronic kidney disease</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diabetes</td>
<td>Diet</td>
</tr>
<tr>
<td></td>
<td>Chronic inflammatory conditions</td>
<td></td>
</tr>
</tbody>
</table>

Second hit: acute influences on the RAGE axis

<table>
<thead>
<tr>
<th>2</th>
<th>Hyperglycaemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Oxidative stress</td>
</tr>
<tr>
<td>4</td>
<td>Inflammation</td>
</tr>
<tr>
<td>5</td>
<td>Tissue injury</td>
</tr>
</tbody>
</table>

Exaggerated and perpetuated inflammation

- Systemic inflammation
  - SIRS criteria, CRP, WBC
- Organ failure
  - SOFA score, P1, Cr
- Adverse outcomes
  - Prolonged ICU LOS
  - Death

---
expression at the protein and transcription levels in biological samples from patients pre- and post- snCPB (Section 12).

4: To conduct ex vivo investigations on the inflammatory activity of RAGE ligands on leukocytes, the inhibition thereof, and the effect of snCPB on ligand responsiveness (Section 13).

5: To investigate genetic variation in select genes relevant to the activity of the RAGE axis and their possible influence on outcome from snCPB (Section 14).

These specific aims are summarized within Figure 18.
Methods
6. CLINICAL METHODS

6.1. Patient cohorts

**Cohort A:** snCPB patients retrospectively identified between 1/1/2002 and 31/5/2005 at the Royal Brompton Hospital (RBH). Patients had routine clinical data collected. The study was examined by the Royal Brompton and Harefield NHS Foundation Trust Research Ethics Committee and deemed to fall within the remit of ‘service evaluation’ and thereby did not require patient consent. Patients within this cohort numbered 2440. Patients were included within this cohort if they cardiac surgery following by admission to ICU. Patients were excluded if their admission was a re-admission or if records were incomplete or unobtainable.

**Cohort B:** Patients enrolled in a clinical observational trial studying influence of genetic polymorphisms and inflammatory mediators on outcome from snCPB, particularly with respect to development of acute lung injury. RBHT ethics committee code 00-033 v1 (amended 2006); patient consent form and information sheet in the appendix. For the majority of the plasma assays the patients had been recruited previously and banked specimens were used (n=20). The 20 patients chosen from the larger cohort were chosen at random. Banked DNA was used for genotyping. Patients were also prospectively recruited for some assays (flow cytometry, qRT-PCR and ex vivo stimulation, n=29). Patients were included in this cohort if they were having cardiac surgery involving CPB with patients preferentially recruited who were having complex surgery i.e. valve replacement surgery and coronary artery bypass grafting or more than one heart valve replaced or repaired. The only exclusion was absence of informed consent.

**Cohort C:** snCPB patients enrolled in a clinical interventional trial comparing two alternative fluids in the cardiopulmonary bypass circuit prior to initiation of CPB. RBHT ethics committee code 07/H0712/137); patient consent form and information sheet is in the appendix. Patients had been recruited previously and banked specimens of plasma were assayed (n=19). The first 19 patients recruited into the study were chosen for these analyses. Patients were included in this cohort if they were having cardiac surgery involving CPB. Patients with a Euroscore of ≥ 7 were excluded.
6.2. Electronic databases
Each patient admitted to the ICU had clinical, physiological and laboratory data automatically entered into an electronic database. In 2009 the clinical information system changed from CareVue to IntelliVue Clinical Information Portfolio (ICIP), both provided by Philips Healthcare Ltd (Guildford, UK).

6.3. Measured and recorded variables
The results of routinely measured laboratory assays were incorporated into the clinical information systems and then used in analyses including the calculation of severity of illness scores. Such assays included: full blood count (white blood cell count, neutrophil count, haemoglobin concentration, platelet count); clotting profiles (international normalised ratio, prothrombin time); biochemical profiles (urea, creatinine, sodium, CRP, bilirubin, alanine transaminase); and arterial blood gas values, ABG (partial pressure of oxygen, partial pressure of carbon dioxide).

The results of measured physiological variables were also incorporated into the clinical information systems and then used similarly. Such measures included: temperature, arterial oxygenation saturations, respiratory rate, heart rate, blood pressure, right atrial pressure.

Details relating to the therapy the patients received were similarly gathered, including inspired oxygen concentration, details of mechanical ventilation and vasoactive drug therapies; details relating to the operations including type of procedure, CPB time, ischaemic time, and volume of blood transfused; details relating to the post-operative recovery: duration of ICU stay, duration of hospital stay, vital status (dead/alive).

Demographic details that were used similarly included age, height, weight, body mass index (BMI, kg/m²), ethnicity, and gender.

In some cases, pre-operative clinical characteristics were gathered from the clinical written notes: Presence of diabetes, ischaemic heart disease (IHD), treatment with statin, presence of pulmonary hypertension, pre-op creatinine and chronic kidney disease (CKD) stage.

6.4. Derived variables
PaO₂/FiO₂ ratio (PFR) is the result of dividing the partial pressure of oxygen (PaO₂) by the fraction of inspired oxygen (FiO₂). The values used in the original definitions of ALI/ARDS
were in mmHg but where kPa are to be used, 1kPa = 7.6 mmHg. The PFR values used in the analyses of cohort B were taken from the clinical information system and for every 24h period (for example, post-operative day 2, POD2) the PFR used was the lowest (most severely impaired) recorded in that period; a PFR was only used if a PaO$_2$ and FiO$_2$ were both recorded within an hour of each other.

The alveolar-oxygen difference (sometimes erroneously termed alveolar-oxygen gradient), or D(A-a) is calculated from a simplified formula derived from the alveolar gas equation. D(A-a) = Partial pressure of oxygen in the Alveolus (PAO$_2$) – Partial pressure of oxygen in the arteries (PaO$_2$). The PaO$_2$ is taken from the ABG and the PAO$_2$ is calculated, assuming a respiratory quotient of 0.8. Thus, PAO$_2$ = 100 x FiO2 – PaCO2*1.25. For example, a patient breathing 50% oxygen (FiO2 0.5) with a PaCO$_2$ of 5.0 and a PaO$_2$ of 10.0 has a D(A-a) of: (100 x 0.5 – (5.0*1.25)) – 10.0 = 33.75kPa. A normal value, (100 x 0.21 – (5.0*1.25)) – 12.5 = 2.25kPa.

**6.5. Definitions and scores**

Acute Lung Injury (ALI) and Acute Respiratory Distress Syndrome (ARDS) represent diagnoses along the spectrum of a disease process characterised by pathologically increased pulmonary alveolar-capillary membrane permeability and the consequent leakage of high oncotic pressure fluid into the alveolar space causing refractory hypoxaemia. The criteria were defined at a consensus conference [41], all four criteria must be met:

1. Acute onset of respiratory failure in presence of recognised cause
2. Diffuse bilateral infiltrates on chest radiograph
3. Absence of left atrial hypertension (pulmonary artery occlusive pressure [PAOP] ≤ 18 mm Hg) or no clinical evidence of left atrial hypertension
4. Hypoxaemia, defined as PaO$_2$:FiO$_2$ ratio ≤ 300mmHg (ALI) or ≤ 200mmHg (ARDS). In SI units: ≤ 40kPa (ALI) or ≤ 27kPa (ARDS).

Acute physiology and chronic health evaluation (APACHE) II score uses a point score based on previous health status, age and 12 routine measures of physiology. The values are recorded from the first 24h of assessment. The APACHE II system is most commonly used critical illness scoring system in the UK and was initially described in 1985 [518].
The Sequential Organ Failure Assessment (SOFA) score is an alternative scoring system that can be used to serially quantify extent of organ dysfunction [92]. The score is based on six different scores, one each for the respiratory, cardiovascular, hepatic, coagulation, renal and neurological systems. For each organ system a score from 0 to 4 is given, with 4 representing the most severe organ dysfunction. Thus the total SOFA score ranges from 0 to 24. The SOFA score was originally designed to predict morbidity in patients with sepsis but has been evaluated in many other settings, including assessment of risk of adverse outcome following elective cardiac surgery. Initially a single-centre study of 218 patients related increased SOFA scores with increased morbidity [97]. In a larger cohort of 857 patients, the SOFA score was associated with mortality and morbidity even when assessed in the early postoperative period after adult cardiac surgery [96]. Most recently, the discriminatory value of four scoring systems was compared in a large cohort and the SOFA score found to be inferior to another scoring system, but superior to two others (SAPSII and APACHE II) [637].

CPB time is the duration that blood is passing from the patient through the cardiopulmonary bypass circuit. During this time there is a period when no blood is flowing through the pulmonary or coronary circulation and this is the ischaemic time.

Euroscore is scoring system for the prediction of early mortality in cardiac surgical patients on the basis of objective risk factors [98]. Patient-related factors include: age over 60, gender, chronic pulmonary disease, extracardiac arteriopathy, neurological dysfunction, previous cardiac surgery, serum creatinine >200µmol/l, active endocarditis and critical preoperative state. Cardiac factors are unstable angina on intravenous nitrates, reduced left ventricular ejection fraction, recent (<90 days) myocardial infarction and pulmonary systolic pressure >60 mmHg. Operation-related factors were emergency, other than isolated coronary surgery, thoracic aorta surgery and surgery for postinfarct septal rupture.

Acute Kidney injury (AKI) is the current preferred term for acute renal failure and there are two scoring systems for AKI. Acute Dialysis Quality Initiative Group published a consensus definition/classification system termed the RIFLE criteria. The Acute Kidney Injury Network (AKIN) group modified this system [638]. The analysis of incidence of post-operative AKI and its relation to genotypic variation (Section 14.4.2) uses a modification of the AKIN criteria as
urine output data was not used in our analyses. Therefore, the following scoring system was used (Table 14).

<table>
<thead>
<tr>
<th>AKI stage</th>
<th>Change in serum creatinine</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Increase in serum creatinine; ≥ 26.4 µmol/l or ≥ 1.5- to 2-fold from baseline</td>
</tr>
<tr>
<td>2</td>
<td>Increase in serum creatinine; &gt; 1.5- to 2-fold from baseline</td>
</tr>
<tr>
<td>3</td>
<td>Increase in serum creatinine &gt; 3-fold from baseline or serum creatinine of ≥ 354µmol/l (with an acute increase of at least 44µmol/l); or use of renal replacement therapy</td>
</tr>
</tbody>
</table>

Table 14: AKI scoring system

6.5.1. Duration of intensive care therapy and ‘level 3 care’

ICU length of stay (ICU LOS) is an important outcome variable for two reasons: firstly, the time a patient spends in ICU primarily relates to the degree to which they require advanced therapies and monitoring, which is largely proportionate to their severity of illness; secondly, a prolonged ICU stay is not benign and is associated with serious adverse outcomes including decreased quality of life and increased mortality after hospital discharge [639, 640].

Hospital administrative database will usually collect ICU LOS and therefore it can be a convenient measure to use. However, non-clinical considerations such as availability of ‘step-down’ bed may be important and compromise the relationship between severity of illness and ICU LOS. To overcome this limitation it is possible to analyse electronic databases of patient physiological parameters and calculate the duration that they require advanced therapies and monitoring. The use of such outcome variables has been encouraged [89].

“Level 3 care” duration is such a calculated variable and is derived from the UK Critical Care Minimum Data Set (CCMDS), a system used for billing purposes; available online:


For the purposes of the study, the ‘level 3 care duration’ was the time in hours that a patient required advanced therapies or monitoring at a level that would be usually thought of as “intensive care”. Level 3 care is defined as either advanced respiratory, cardiovascular or renal support alone, or basic respiratory support in combination with support for at least
two other organ systems. In this study we did not consider gastrointestinal or liver support parameters. The definitions are shown in Figure 19.
Advanced respiratory support

- Invasive mechanical ventilatory support (excluding mask CPAP or noninvasive methods e.g. mask ventilation but including BiPAP or CPAP applied via a tracheal tube).
- Extracorporeal respiratory support

Basic respiratory support

- More than 50% oxygen delivered by face mask.
- Close observation due to the potential for acute deterioration to the point of needing advanced respiratory support. (e.g. severely compromised airway or deteriorating respiratory muscle function).
- Physiotherapy or suction to clear secretions at least two hourly, whether via tracheostomy, minitracheostomy, or in the absence of an artificial airway.
- Patients recently extubated after a prolonged period of intubation and mechanical ventilation, (e.g. more than 24 hours of tracheal intubation)
- Mask CPAP or non invasive ventilation.
- Patients who are intubated to protect the airway but needing no ventilator support and who are otherwise stable

Advanced cardiovascular support

- Multiple intravenous vasoactive and/or rhythm controlling drugs used to support arterial pressure, cardiac output or organ perfusion, (e.g. inotropes, amiodarone, nitrates).
- Patients resuscitated after cardiac arrest where intensive therapy is considered clinically appropriate.
- Observation of cardiac output and derived indices (e.g. pulmonary artery catheter, lithium dilution, pulse contour analyses, oesophageal doppler).
- Intra aortic balloon pumping

Basic cardiovascular support

- Treatment of circulatory instability due to hypovolaemia from any cause
- Use of a CVP line for basic monitoring or central venous access to deliver therapeutic agents.
- Use of an arterial line for basic monitoring of arterial pressure or sampling of arterial blood.
- Single intravenous vasoactive drug used to support arterial pressure, cardiac output or organ perfusion
- Intravenous drugs to control cardiac arrhythmias
- Non-invasive measurement of cardiac output (e.g. echocardiography, thoracic impedance)

Renal support

- Acute renal replacement therapy (e.g. haemodialysis, haemofiltration etc.)

Neurological support

- Central nervous system depression sufficient to prejudice the airway and protective reflexes, excepting that caused by sedation prescribed to facilitate mechanical ventilation.
- Invasive neurological monitoring e.g. ICP, jugular bulb sampling.
- Severely agitated or epileptic patients requiring constant nursing attention and/or heavy sedation

Dermatological support

- Patients with major skin rashes, exfoliation or burns. (e.g. greater than 30% body surface area affected).
- Use of multiple, large trauma dressings, (e.g. multiple limb or limb and head dressings).
- Use of complex dressings (e.g. open abdomen or large skin area greater than 30% of body surface area)

Figure 19: Level 3 care definitions from UK CCMDS
6.6. Operative details

6.6.1. Induction and maintenance of anaesthesia

Patients within all cohorts received snCPB at the Royal Brompton Hospital with specific management of anaesthesia and cardiopulmonary bypass at the discretion of the attending anaesthetist, perfusionist and surgeon.

6.6.2. CPB

The extra-corporeal circuit used during CPB consisted of a roller pump, a reservoir, and a membrane oxygenator. The circuit was primed with a gelatine solution or human albumin solution. Heparin was administered before starting CPB. The pump flow rate was ~4L/min/m² and hypothermia (28-32°C) was maintained during CPB. In each patient, the myocardium was protected with repeated cardioplegia. Before aortic unclamping, the patient was re-warmed. The injected heparin was neutralized by administration of protamine sulphate.

6.7. Patient sample acquisition and preparation

Healthy volunteers: informed consent was obtained and 30-40ml of venous blood was drawn from the non-dominant antecubital fossa with a 20G ‘butterfly’ cannula. The blood was mixed with sodium citrate in a 9:1 ratio, and gently mixed to prevent coagulation.

In experiments using whole blood (WB) the blood was then either used without further dilution, or mixed 1:1 with DMEM medium. In experiments using neutrophil suspension the blood was further processed as described in Section 7.2.3.

Patients: following induction of anaesthesia and placement of a radial arterial line, 30-40ml of arterial blood was drawn from the arterial line. There was usually no more than 10mins between induction of anaesthesia and acquisition of blood. Depending on the specific assay, blood was anti-coagulated by addition of sodium citrate, heparin or EDTA. Post-operative samples were either obtained as soon after the end of CPB as possible or 2h following the end of CPB.
7. LABORATORY METHODS

7.1. General methods

7.1.1. Centrifugation
For volumes less than 2ml a fixed angle bench top centrifuge was used either Hawk 15/05 centrifuge (Sanyo GallenKemp, Etten Leur, Netherlands) or Boeco M-240 R (Hettich, Germany) centrifuge. For larger volumes a Hettich Rotina 46R centrifuge was used with swing-out buckets (Hettich AG). Centrifugation speeds are quoted as relative centrifugal force (RCF or g) or rotor speed in revolutions per minute (RPM) followed by time, temperature and brake.

7.1.2. General cell counting
Cells were counted under either an inverted phase contrast microscope (CK40, Olympus UK, Southend, UK) or a conventional upright microscope (Olympus, CH30, x100-x400 objective) using a haematocytometer (improved Neubauer model). The total numbers of cells in each of the four (1mm$^3$) larger corner squares were counted. Cells were included if they were entirely within the boundaries of the squares or if they partially overlapped the upper or left borders. Appropriate dilutions and repeat counts were undertaken to ensure that the total number of cells counted was between 100 and 400. The total number of cells in suspension was estimated using the following formula.

Total cell count (cells/ml) = \( \text{Cell count for four squares} \times 10^4 \times \text{dilution factor} \)

7.2. Cells

7.2.1. Cell culture of THP-1 cells
The THP-1 cell line is derived from immortalised blood monocytes from human monocytic leukaemia. The cells resemble human monocytes in their morphology, expression of membrane antigens and secretory proteins. The cells were purchased from the European Collection of Cell Cultures and grown in RPMI 1640 media containing 10% heat inactivated fetal calf serum, L-Glutamine, penicillin/streptomycin and incubated at 37°C in 95% air, 5% CO$_2$. Cells were maintained at 0.5 - 1 x 10$^6$ cells/ml.
7.2.2. *Ex vivo* leukocyte models

Whole blood (WB) contains erythrocytes (red blood cells), leukocytes (white blood cells), thrombocytes (platelets), plasma, and plasma proteins. The leukocyte population includes neutrophils, monocytes, lymphocytes, eosinophils, and basophils. Use of whole blood for *ex vivo* experimentation requires absolutely minimal processing, only the addition of an anticoagulant and optional the addition of culture medium. More importantly than the ease of use, WB assays most authentically mimic the *in vivo* state and thus may be the most appropriate milieu in which to study cytokine production *in vitro* [641] and offer superior reproducibility to alternative methods [642].

Isolation of leukocyte subsets is cumbersome as it involves multiple stages and importantly, it can activate cells and this may cause their *ex vivo* responses to differ from their *in vivo* responses [642]. Moreover, removal of the possibility of interactions between the components of whole blood, further removes the model from physiological relevance.

7.2.3. Neutrophil isolation

Following dextran sedimentation to generate a leukocyte rich suspension, granulocytes were purified over a discontinuous Percoll plasma gradient [643]. Residual erythrocytes were removed by hypotonic lysis. The detailed protocol is outlined in Figures 20 and 21. Cells were re-suspended, at 1-2X10⁶ cells/ml, in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% v/v foetal calf serum (containing 100IU/mL penicillin, 100μg/mL streptomycin and 2mM L-glutamine).
1. Freshly prepare dextran and percoll, place in waterbath with DMEM, and 0.25% and 1.6% saline.
2. Obtain blood into sodium citrate, to final concentration of 10% v/v.
3. Spin whole blood 300g, 20mins at 20˚C, Acceleration 9, Deceleration 4
4. Remove all plasma into PRP (platelet rich plasma) falcon and spin 2000g for 20min at 20˚C
5. Add 6ml of 6% dextran through 0.45µm filter to blood tube
6. Top up to 50ml with 0.9% saline
7. Invert once and leave on bench for 30mins
8. Take PRP out of centrifuge and put supernatant into new PPP (platelet poor plasma) falcon
9. After dextran sedimentation there will be two layers: aspirate WBC into 50ml falcon and spin 300g 7min
10. Prepare percoll gradient (90% percoll)
11. 42%: 2.1ml 90% percoll (3 x 700µl), with 2.9ml PPP (3 x 966 µl)
12. 51%: 2.55ml 90% percoll (3 x 850µl), with 2.45ml PPP (3 x 816.66µl)
13. 15ml falcon with white needle and 2ml syringe; 3ml 51% percoll then 3ml 42% percoll gently layered on top
14. Take WBC out of centrifuge, white cell pellet and red stain
15. Remove supernatant (waste), flick to dislodge pellet, add 1ml PPP and pipette up/down (wide tip), add 1ml PPP
16. Add this to gradient, further 200µl of PPP into syringe
17. Spin gradient 260rcf, 12min, Acc 4, Dec 0
18. Remove gradient from centrifuge (Figure 21)
19. Collect neutrophils into 50ml falcon and add remaining PPP
20. Spin neutrophils 300rcf, 7min
21. Remove neutrophils from centrifuge
22. Discard supernatant, flick tube to dislodge cells
23. Add 1ml 0.2% saline and pipette up/down x7 (wide tip)
24. Add 24ml 0.2% saline and invert x10
25. Top up to 50ml with 1.6% saline, invert once
26. Spin 300rcf, 7min
27. Pour off supernatant, flick to dislodge cells and resuspend in medium (2ml FCS, 18ml DMEM)
28. Count cells (10µl sample and 90µl TURKS stain)
29. Re-spin and resuspend in medium and desired concentration for experiment

Figure 20: Neutrophil isolation protocol
Figure 21: Photographs of Percoll density gradient centrifugation. On the left: A) concentrated leukocyte suspension, lying on top of B) two layers of percoll, which are indistinguishable in appearance. On the right, following gentle centrifugation, C) monocytes at the interface of two density gradients, and D) neutrophils present at this density interface (hard to visualise).

7.2.4. Neutrophil staining for counting

At the completion of experimental conditions, the cell suspension was spun at 400g for 10mins and supernatant aspirated. The remaining cell pellet was re-suspended in PBS prior to use of a cytopsin to centrifuge the cells onto glass slides. The slides were fixed by air drying until staining with May-Grünwald-Giemsa stain prior to mounting with DPX (Figure 22). Each slide has 2-4 fields photographed and following transfer of the digital images to computer the cells are counted.

Contamination with non-neutrophil cells including eosinophils was consistent with levels reported by others (<10%) [643]. Neutrophils spontaneously apoptose in the circulation and the isolation technique can precipitate this. Immediately following neutrophil preparation there was negligible neutrophil apoptosis, however, after 4h of co-incubation with experimental conditions up to 40% of cells had undergone apoptosis.
Figure 22: Representative photomicrograph (x65 magnification) of slide stained with May-Grünwald-Giemsa. Arrow indicates viable neutrophil, star indicates contaminating eosinophil and arrowhead indicates an apoptotic neutrophil.

7.3. Enzyme immunoassays (ELISA)

7.3.1. Basic principles

Enzyme linked immunosorbent assays (ELISA) accurately quantify the presence of soluble analytes in a biological sample using specific antibodies directed against antigens of interest. In a sandwich ELISA the capture antibody is immobilised on a plate and binds the target antigen in solution. ELISA may be purchased in kits that contain all the necessary reagents or assay-specific kits can be purchased with the generic parts of the assay purchased separately. For the later type of ELISA a description follows and the general cytokine sandwich ELISA protocol is described in Figure 23.

A ‘capture’ antibody is attached to a solid-phase such as the plastic of a 96 well microtitre plate by passive adsorption. Surplus antibody is washed away and a blocking buffer containing a high concentration of albumin is used to fill excess non specific binding sites. Samples are then added to the wells and any antigen present is bound specifically by the immobilised primary ‘capture’ antibody. After washing away any unbound substances, a second ‘detection’ antibody labelled with biotin and directed at a different epitope on the
antigen of interest is added which binds to the now immobilised analyte. After further washing, streptavidin horseradish peroxidase (HRP) is added. Streptavidin binds to biotin on the detection antibody. Thus, the amount of HRP present is proportionate to the amount of antigen in the original sample. Where the secondary antibody is conjugated directly to HRP the streptavidin step can be omitted. HRP is quantified by the colorimetric reaction following the addition of HRP substrate 3, 3’, 5, 5’ tetramethylbenzidine (Reagent B, BD OptEIA) in the presence of hydrogen peroxide (Reagent A, BD OptEIA). The colour develops in proportion to the amount of antigen present in the initial step. The reaction is terminated at 20-30mins by acidification. The developed colour is read by a dedicated fluorometer.

All ELISA reactions used to assay concentration of cytokine within a biological specimen were performed in duplicate in neighbouring wells of the same 96 well plate and the assay result averaged. Each plate included eight conditions (in duplicate) representing serial dilutions of a known concentration of analyte (provided by the manufacturer) and the optical density of these wells is used to create a standard curve. ELISA plates were read using a Biotek EL800 plate reader and Gen5 (Biotek) software. Data were exported to excel and then analysed using GraphPad Prism (v 4.0). The concentration of the analyte was calculated by comparing the optimal densities against those from the standard curve using appropriate mathematical functions within GraphPad (sigmoidal dose-response curve).
General cytokine sandwich ELISA protocol

**Reagents**
Maxisorp 96 well microtitre plates
Appropriate antibodies (capture and detection), standards and streptavidin-HPR from kit.

**Wash buffer**
PBS (5 tablet / 1000mls H₂O)  
0.05% Tween 20 (0.5ml/1000mls)  
pH 7.4. Store 2-8°C, use within 30 days.

**Blocking buffer**
PBS (1 tablet/200mls H₂O)  
1% BSA (2g/200mls)  
0.05% NaN₃ (0.1g/200mls)  
Store 2-8°C, use within 7 days

**Reagent diluent**
Tris buffered saline (20mM Trizma base- 1.211g /500ml, 150mM NaCl- 4.38g /500ml)  
0.1% BSA-0.5g/500ml  
0.05% Tween 20 0.25ml/500ml  
pH 7.3. Store 2-8°C, use within 7 days

**Substrate solution**
50% Reagent A (H₂O₂)  
50% Reagent B (Tetramethylbenzidine)  
Use immediately

**Stop solution**
1M H₂SO₄

**Protocol**
1. Coat wells of Maxisorp 96 well microtitre plate with 100µl capture antibody. Seal and incubate overnight at room temperature.
2. Wash each well 3 times with 400µl wash buffer. Invert plate and tap dry on paper towel.
3. Add 300µl/well blocking buffer and incubate for a minimum of 1h at room temperature. During this stage prepare standards and sample dilutions using reagent diluent.
4. Wash 3 times and tap dry.
5. Add 100µl of control /standard / sample to appropriate wells. Seal and incubate for 2h at room temperature.
6. Wash 3 times and tap dry.
7. Add 100µl/well of detection antibody, seal and incubate for 2hrs at room temperature.
8. Wash 3 times and tap dry.
9. Add 100µl/well Streptavidin HRP, seal and incubate for 20 mins at room temperature avoiding direct light.
10. Wash 3 times and tap dry.
11. Add 100µl/well substrate solution, seal and incubate for 20mins at room temperature avoiding direct light.
12. Add 50µl/well stop solution.
13. Read O.D. at 450nm (correction of 570nm) within 30mins

Figure 23: Details of generic ELISA protocol
7.4. Multiplex assays

7.4.1. Principle of electrochemiluminescence (MSD)

Meso-Scale Discovery (MSD) plates allows the detection of multiple proteins from a single biological sample across a wide range of concentrations. Each well of a microplate features several micro-spots, each corresponding to a specific analyte (Figure 24). The samples are added to the wells and processed very similarly to a standard ELISA. The detection antibody is conjugated to a Sulpho-Tag™ group. When the protocol has been completed the plate is placed within a plate-reader (MSD Sector) a small electric current is run across each micro-spot within each well. By electrochemiluminescence, a luminous intensity is generated in proportion to the extent the Sulpho-tagged antibody is present and this varies in proportion to the quantity of analyte present within each well, Figure 24. A series of dilutions of known concentrations of analytes provided a reference and the on-computer software (MSD Discovery workbench) calculates the levels of each analyte in each well and exports the data to a spreadsheet.

In addition to measuring multiple analytes simultaneously, the advantage of the MSD system is that wide range of concentrations that can be measured without sample dilution – a traditional ELISA can measure over 2 to 3 log ranges, the MSD system can measure over 4 to 5. The main disadvantage of the system is expense.

Figure 24: Cytokine capture antibody is pre-coated on specific spots of a 4-Spot MSD MULTI-Spot plate. Calibrator solutions or samples are incubated in the MULTI-Spot plate, and each cytokine binds to its corresponding capture antibody spot. Cytokine levels are quantitated using a cytokine-specific detection antibody labelled with MSD SULFO-TAG reagent.
7.4.2. Protocol

Samples were collected and processed as per the manufacturers’ protocol, Figure 25.

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Addition of Diluent: Dispense 25 µL of Diluent 2 into each well. Pipette to the bottom of the plate so as to allow the fluid to cover the entire bottom of the well. A slight tap may be necessary to allow the fluid to settle to the bottom.</td>
</tr>
<tr>
<td>2</td>
<td>Seal the plate with an adhesive plate seal and incubate for 30 minutes with vigorous shaking (300-1000 rpm) at room temperature.</td>
</tr>
<tr>
<td>3</td>
<td>Addition of Sample or Calibrator: Dispense 25 µL of each Calibrator or Sample Solution into a separate well of the MSD plate.</td>
</tr>
<tr>
<td>4</td>
<td>Seal the plate with an adhesive plate seal and incubate for 2 hours with vigorous shaking (300-1000 rpm) at room temperature.</td>
</tr>
<tr>
<td>5</td>
<td>Wash and Addition of Detection Antibody Solution: Wash the plate 3X with PBS + 0.05% Tween-20.</td>
</tr>
<tr>
<td>6</td>
<td>Dispense 25 µL of the 1X Detection Antibody Solution into each well of the MSD plate.</td>
</tr>
<tr>
<td>7</td>
<td>Seal the plate and incubate for 2 hours with vigorous shaking (300-1000 rpm) at room temperature.</td>
</tr>
<tr>
<td>8</td>
<td>Wash and Read: Wash the plate 3X with PBS + 0.05% Tween-20. Add 150µL of 2X Read Buffer T to each well of the MSD plate.</td>
</tr>
<tr>
<td>9</td>
<td>Analyze the plate on the SECTOR reader.</td>
</tr>
</tbody>
</table>

Figure 25: Protocol for MSD use
7.5. Flow cytometry (FC)

Flow cytometry is a technique that can be used for counting, characterizing, and sorting cells suspended in a stream of fluid either by their physical characteristics, or by the extent of presence of bound, labelled antibodies or dyes.

7.5.1. Basic principles of FC

Cells are processed to allow binding of fluorescently-labelled monoclonal antibodies to the cells. Following removal of excess antibodies, the cell suspension is processed such that cells pass individually through a series of lasers and paired detectors. The lasers excite the fluorescent molecules, which are excited at one range of wavelengths and emit at a second range. Filters in front of each of a series of detectors restrict the light that reaches the detector to only a small range of wavelengths. Each detector provides a value for the degree of fluorescence in that wavelength for each cell. There may be more than ten detectors and tens of thousands of cells are typically processed for each experimental condition. The flow cytometer machine (LSR II, Becton Dickinson, Oxford, UK) passes the acquired information to a microcomputer (Standard PC running FACSDiva software v6.0) that allows the user to manipulate how the data is presented. Data may be further analysed ‘off-line’ using specialised software such as FlowJo v9.1 (Treestar, Oregon, USA).

Identification of specific populations of cells is possible through comparison of the fluorescence in several channels and the use of ‘gating’ strategies. Extent of fluorescence data is usually represented as a histogram with number of events on the y axis and the fluorescence along the x axis as a logarithmic scale. If the distribution of fluorescence is parametric, then the mean fluorescence intensity (MFI) may be used as a summary statistic to represent degree of fluorescence.

7.5.2. Staining whole blood for FC

Details of the development of this method are to be found within Section 12.4.1. The final protocol that was used is described in Figure 26.
STAINING

- Obtain 3ml blood into citrate tube (BD Vacutainer™ purple), mix gently
- Take 1.0ml and add to 10ml of cold PBS/EDTA in 15 ml Falcon tube
- Spin 400g (10mins 4°C), Prepare bead controls
- Aspirate supernatant
- Add 10ml PBS/EDTA
- Spin 400g
- Aspirate supernatant
- Top up to 1.0ml volume with PBS/EDTA, gentle mix (resuspended WB)
- 50µl into each of the duplicate labelled 2ml eppendorf tubes:
  - RAGE
  - FMO
  - Intracellular RAGE
  - Intracellular FMO
- Make controls for the live/dead assay by heating 50µl of the resuspended WB in 450µl of PBS to 90°C for 5m. Cool, then mix 50µl with 50µl unheated resuspended WB.
- Staining:
  - 0.5µl of undiluted AB to each tube, 0.5µl of undiluted AB to each control for CD14, CD16 and Live/dead
  - 0.5µl of undiluted Anti-RAGE AB to the RAGE tube and to bead control
- Give all tubes a very brief vortex then leave on ice in dark for 20mins
- Add 2ml PBS/EDTA to all tubes
- Spin 400g
- Aspirate supernatant, leave about 100ul
- Add 200ul of PBS to the beads and put in fridge
- Resuspend all with up to 10x U/D with small wide tip
- Add 2ml lyse/fix, wait 30mins+ on ice
- Leave some tubes in the fridge – L/D controls, RAGE, and FMO. With the Intracellular RAGE and Intracellular FMO:
  - Spin 600g
  - Aspirate supernatant
  - Add 2ml of perm/wash, gentle u/d, wait 20mins on ice
  - Spin 1500g 5m
  - Aspirate supernatant
  - Add Anti-RAGE AB to RAGE tube
  - Give all tubes a very brief vortex then leave on ice in dark for 20m
- Add 2ml EDTA-PBS, Spin 1500g, 5m
- Aspirate most off, add 2ml lyse/fix, in fridge overnight
- Next day: wash cells in PBS, Spin, resuspend in 300µl PBS

ACQUISITION

- Live cells were distinguished with the cell viability stain
- Monocytes were characterized on the basis of their CD14 expression and neutrophils on the basis of their CD16 expression.
- At least 10,000 neutrophil events were recorded.
- Results are expressed as means of fluorescence intensities (MFI) related to the specified population, reflecting the RAGE density per cell.

Figure 26: Final flow cytometry staining protocol for whole blood
7.6. DNA extraction

7.6.1. Acquisition

10mls of peripheral blood was collected from each patient via an indwelling arterial catheter into an anticoagulant vacutainer containing sodium citrate. DNA was extracted from the blood samples within 24h according to the following protocol.

Blood was transferred into a 15ml polypropylene tube (Becton Dickinson, UK). Centrifugation of the tube at 2,500 rpm for 20 mins at room temperature separated red and white blood cells (WBC) from the serum. The buffy coat of WBCs lying between the plasma and erythrocyte fractions was aspirated into a new 15ml tube. 10mls of red cell lysis buffer (144mM NH₄Cl, 1 mM NaHCO₃) was added to eliminate contaminating erythrocytes and the tubes were inverted several times to ensure complete haemolysis. Each sample was centrifuged for 20 minutes at 2,500 rpm, the supernatant was disposed of and red cell lysis buffer used to wash the resultant WBC pellet.

7.6.2. DNA extraction from WBC pellet

The WBC pellet was immersed in 3 mls of nuclei lysis buffer (10 mM Tris-HCl pH 8.2, 400 mM, NaCl, 2 mM EDTA pH 8.0). The addition of 1ml of 6M NaCl precipitated proteins and 2mls of 24:1 Chloroform: Isoamyl alcohol separated the WBC pellet from the DNA. A milky solution was formed by agitation of the sample and this was centrifuged for 30 minutes at 3,000 rpm.

Three phases were visible after centrifugation; the upper phase containing DNA was transferred into a new tube. 10mls of absolute ethanol was added and the tube was inverted several times to precipitate the DNA. The DNA was placed in a sterile 1.5ml microfuge, suspended in sterile water (Baxter, UK) in aliquots and frozen at -20°C until required for SSP-PCR.
7.7. Polymerase chain reaction (PCR)

7.7.1. Basic principle

The polymerase chain reaction (PCR) enables a selected region of the genome with a known DNA sequence to be amplified. Two primers (synthetic oligonucleotides) are designed that are complementary to each strand of double stranded DNA that surrounds the region of interest. Each strand of DNA is replicated, catalysed by the heat stable enzyme Taq polymerase. As the process continues through repeated cycles each newly generated strand is used as a template for further replication thus exponentially amplifying the initial DNA sequence of interest.

Each cycle consists of 3 steps:

1. Denaturation: Samples are heated briefly to 94°C causing disruption of the hydrogen bonds between the two strands of DNA resulting in their separation.

2. Annealing: Subsequent cooling to 55-65°C in the presence of an excess of the two primers allows the primers to bind to the complementary DNA sequences through hydrogen bonds.

3. Extension: Reaction temperature is increased to 72°C, the optimal temperature for Taq polymerase to attach to the primer/cDNA segment and extend from the primer by adding dNTPs that are complementary to the template in a 5’to 3’ direction. Provided there are surplus reagents the amount of target DNA is doubled. Extension of the original template produces strands longer than the target sequence in a linear manner. By contrast, amplification of the DNA PCR products only generates DNA of the required length. Since these are generated in an exponential manner, after several cycles they greatly outnumber the longer sequences.

4. A final elongation step ensures that any remaining single stranded DNA is fully extended.
7.8. Sequence specific primer PCR (SSP-PCR) genotyping

7.8.1. Principle

Sequence specific primer PCR (SSP-PCR) uses primers designed with their 3’ end complementary to a particular SNP variant and identifies the presence of this variant by PCR amplification [644]. Thus, for a single locus, two different PCR reactions detect the two allelic variants: one containing a specific primer complementary to one variant, and the other containing a specific primer complementary to the other variant in conjunction with a consensus primer. The presence of a PCR product of the expected size indicates the presence of the allele in the specimen (Figures 27 and 28).

Figure 27: Diagram illustrating SSP-PCR. This example of a SNP has one variant with a complementary primer with an adenine (A) at the locus and the other variant with a guanine (G) – top left of diagram. The consensus primer is the same for both. For each patient’s DNA two PCR reactions take place, one with the A primer and one with the G primer. In the case of DNA1, the A primer is shown to match and the G primer is shown to mismatch – therefore on the diagram of the gel photograph there is a specific band for A but none for G.
7.8.2. Primer design

All primers were designed to work under the same amplification procedures. For this reason, a set of primer design rules were established. Primers were designed optimally with a length of 19 to 21 bases, a salt adjusted melting temperature (Tm) of 58 to 61 °C, a GC content of 45 to 55 % and with no secondary structures (e.g. hairpin loops). The online program oligonucleotide properties calculator was used to determine these conditions:

http://www.basic.northwestern.edu/biotools/oligocalc.html

Primers that could potentially form dimers with themselves or with other primers in the reaction mix were avoided, having been identified using the program FastPCR:

http://primerdigital.com/fastpcr.html

Once designed, primers were checked for specificity using the basic local alignment search tool (BLAST):


BLAST is a search tool which looks for matches between the sequence you submit and a comprehensive database of all known DNA sequences called GenBank. This step avoids non-specific priming ensuring as far as possible that primers will only anneal to an exact site match.

The oligonucleotides used as primers for each SNP are listed in Table 15.

Figure 28: Photograph of representative gel showing two heterozygotes, two homozygotes for the first allele and two for the second allele.
Table 15: Oligonucleotides used as primers for each SNP

<table>
<thead>
<tr>
<th>Reference</th>
<th>Variant</th>
<th>Specific primer complementary to SNP</th>
<th>Consensus primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs1800624</td>
<td>WT</td>
<td>CTG TTG TCT GCA AGG GTG CAT</td>
<td>AGG TTG GAA GTG TGA TGG GT</td>
</tr>
<tr>
<td></td>
<td>variant</td>
<td>CTG TTG TCT GCA AGG GTG CAA</td>
<td></td>
</tr>
<tr>
<td>rs1800625</td>
<td>WT</td>
<td>C AGG AGA GAA ACC TGT TTG GAA</td>
<td>TAA AGA TCC GGG CAG GAC TG</td>
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<tr>
<td></td>
<td>variant</td>
<td>AGG AGA GAA ACC TGT TTG GAG</td>
<td></td>
</tr>
<tr>
<td>rs2070600</td>
<td>WT</td>
<td>GCT CGT GTC CTT CCC AAC A</td>
<td>GCC CTC ATG GGC CAA GGC TG</td>
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<tr>
<td></td>
<td>variant</td>
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<td></td>
</tr>
<tr>
<td>rs184003</td>
<td>WT</td>
<td>AGG TAG GGT GAA CCA TAA CTA T</td>
<td>TGA CAG CAC GGC TTT CCT G</td>
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<td>CCA CCC ATT CCA GCC ACG</td>
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<td></td>
<td>variant</td>
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</tr>
<tr>
<td>rs3795391</td>
<td>WT</td>
<td>GGG CAG TAC GCA GAG GAC</td>
<td>GAG TGA GGC AGG GAT AAG GA</td>
</tr>
<tr>
<td></td>
<td>variant</td>
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<td></td>
</tr>
<tr>
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<td>WT</td>
<td>CGA TAC TCA GAG CAG AAG AGG</td>
<td>TGT CCA ATG CAT CAC AGA AA</td>
</tr>
<tr>
<td></td>
<td>variant</td>
<td>CGA TAC TCA GAG CAG AAG AGA</td>
<td></td>
</tr>
</tbody>
</table>

Primers were manufactured by Invitrogen in nanomolar quantities. Stock solutions of primer were prepared by adding DEPC-treated water to give a resultant concentration of 1nmol.μl⁻¹ (1mM). Stock solutions were stored at -20°C.

7.8.3. SSP-PCR amplification

10μl of oil was added to each well in a 96 well plate and 5 μl of primer mix was dispensed into the oil. The primer mix contained two pairs of primers; one pair for a sequence specific reaction and one pair by an internal positive control reaction to validate the results. Each well received 8 μl of DNA/PCR reaction mix containing TDMH (1X NH4 buffer, 300 μM of each dNTP, 2 mM MgCl2) 0.08 μg DNA and 0.32 units Taq DNA polymerase (Bioline UK). The plate was sealed with a Thermowell sealer (costar) and secured in an MJ Research PTC-200V Thermal Cycler machine. SSP-PCR was performed with the following cycling parameters:
1 minute at 96°C; 4 cycles of 20 seconds at 96°C; 45 seconds at 75°C; 25 seconds at 72°C; 20 cycles of 25 seconds at 96°C, 50 seconds at 65°C, 30 seconds at 72°C, 3 cycles of 30 seconds at 96°C, 60 seconds at 55°C, 90 seconds at 72°C, 10 minutes at 5°C.

Control primers were multiplexed (mixed in the same reaction well and amplified simultaneously) into each PCR in order to confirm that amplification was successful.

7.8.4. Gel electrophoresis

1.5% agarose gel was prepared using 0.5 X Tris Borate EDTA (TBE) buffer, stained with 20µl of SYBRSafe (Invitrogen). On completion of SSP-PCR, 10µl of orange G loading Buffer (glycerol, 0.5x TBE, orange G) was added to the PCR product in each well of the 96-well plate. 25 µl of PCR product and orange G was then loaded into each well of the gel. Electrophoresis was carried out at 200V for 20 minutes. The gel was visualised under ultraviolet light (UV, 320nm) light; resultant amplicons were photographed and digitally recorded using the alpha digidoc system. Any equivocal results were repeated.

The stringent conditions outlined in this section ensured amplification using the SSPs occurred only with an exact primer match. Therefore, the presence of a specific allele was scored by observing an amplicon of the expected size in association with a control PCR product. The absence of a specific allele was scored by observing only a control PCR product. Through careful design, all primers worked optimally at the same conditions. It was therefore possible to ‘phototype’ several SNPs simultaneously in one DNA sample.

7.8.5. Reading the gels

The images of the gels were read by two independent observers and any discordance or uncertainty was resolved by re-typing. For each SNP, for each patient their genotype was entered into anonymised excel spreadsheet for later analysis.
7.9. Quantitative reverse transcription real-time polymerase chain reaction (qRT-PCR)

Reverse transcription PCR describes the use of PCR following the conversion of coding information from RNA to DNA, reverse transcription (RT). The RT-PCR described within this thesis is a two-stage process, although an integrated process is possible. Prior to RT, the mRNA must be isolated from the DNA, protein and other cellular components.

7.9.1. mRNA isolation from leukocytes

Total ribose nucleic acid (RNA) was extracted from leukocytes using the phenol-chloroform extraction method [645]. Cell suspension (either whole blood or isolated neutrophils) was thoroughly mixed 1:3 with TRI Reagent™ (Invitrogen). TRI reagent combines phenol and guanidine thiocyanate in solution. The later denatures proteins including RNAse that might otherwise cause RNA degradation. The samples can be stored in TRI reagent at -80°C until processing. The RNA extraction relies upon phase separation and upon addition of chloroform (200µl for every 1ml) and following centrifugation (120g for 15mins at 4°C), the RNA becomes dissolved in the upper aqueous phase (Figure 29). Addition of isopropanol results in precipitation of the RNA. Following centrifugation and aspiration of the supernatant, the RNA pellet is washed in 75% ethanol. The supernatant is discarded and the RNA pellet air-dried prior to addition of RNase-free water to dissolve the RNA prior to RT.
7.9.2. Reverse transcription

7.9.2.1. Basic principle
The isolated RNA isolated must be non-specifically transcribed into complementary DNA (cDNA) prior to PCR. Mature mRNA has a chain of adenosine nucleotides on the 3’ end – this is known as the Poly-A tail. The RT primer used in this thesis is an oligo-(dT) that, by complementarity, hybridizes with the poly-A end of mRNA. Polymerization starts from this primer, and proceeds until a complete cDNA transcription of the mRNA has formed.

7.9.2.2. Protocol

Reagents:

<table>
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<tr>
<th>Reagent</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT buffer (x5)</td>
<td>250nM Tris HCl pH 8.3, 375nM KCl, 15mM MgCl₂</td>
</tr>
<tr>
<td>DTT</td>
<td>0.1M</td>
</tr>
<tr>
<td>dNTP</td>
<td>10mM dATP, dCTP, dGTP and dTTP (For 200μl: 20μl of each, each at 100mM + 120μl DEPC H₂O)</td>
</tr>
<tr>
<td>OligoDT</td>
<td>12-18</td>
</tr>
<tr>
<td>RNasine</td>
<td>0.5U/μl</td>
</tr>
<tr>
<td>RTase</td>
<td>200U/μl</td>
</tr>
<tr>
<td>DEPC H₂O</td>
<td>Dissolve DEPC in 200mls distilled H₂O, leave on agitator in water bath at 37°C overnight, autoclave once DEPC is dissolved completely.</td>
</tr>
<tr>
<td>Ice</td>
<td></td>
</tr>
</tbody>
</table>

Reverse Transcription mixture buffer (for 1 sample):

<table>
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<tr>
<th>Reagent</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT buffer (x5)</td>
<td>2.8μl, DTT 2μl, dNTP 10mM 2μl, DEPC H₂O 1.7μl, RNasine 0.5μl, RTase 1μl</td>
</tr>
</tbody>
</table>

Protocol

1. Turn heat blocks on to 37°C and 65°C in advance.
2. For each sample dilute 0.6μg mRNA in 7μl DEPC H₂O
3. Add 1μl Oligo DT to each sample
4. Incubate at 65°C for 10 mins followed by 5 minutes on ice
5. Add 10μl of reverse transcription mixture buffer to each sample
6. Incubate at 37°C for 1 hour
7. Incubate at 65°C for 5 mins
8. Store at -20°C

Figure 30: Protocol for cDNA preparation by Reverse Transcription
7.9.3. Principles of real-time PCR

Real time PCR enables quantification of PCR product as the amplification process proceeds. Based on conventional PCR it has the advantage that it allows more accurate, quantitative measurements of PCR product concentrations after each cycle using the incorporation of a fluorescent dye or oligonucleotide probe. The simplest and most cost-effective method is to add a fluorescent DNA binding dye such as SYBR Green into the reaction mixture. SYBR Green fluoresces when it binds to the minor groove of the double stranded DNA. SYBR Green emits a low signal when in solution but a much higher signal when bound to DNA. Thus, as the reaction proceeds there is a corresponding increase in signal intensity (Figure 31).

Individual reactions are characterised by the PCR cycle at which fluorescence first rises above background levels. It is at this point that PCR amplification becomes exponential, termed the threshold cycle (Ct). The more target present in the starting material the lower the Ct. The correlation between fluorescence and the amount of amplified product enables quantification of target molecules over a wide range. Figure 32 shows an example graph of fluorescence plotted against time/cycle.
Figure 32: The progress of the reaction is monitored by tracking the increase in fluorescence over time. Each coloured line represents one condition/reaction/tube within the machine. The x axis is cycle number and the y axis is fluorescence intensity. The red horizontal line represents the threshold. It can be seen that the first black line crosses the threshold earlier than the other lines, this indicates it had a greater quantity of cDNA specific for the primer in that tube than the other samples had of their respective primers.

7.9.4. qRT-PCR protocol

20μl PCR reactions were set up in duplicate in 0.1 ml Thermo-PCR tubes, containing SYBR Green PCR Master Mix, forward primer, reverse primer, template cDNA and RNAse free water and loaded into the PCR machine (Rotor-Gene 6000, Corbett). The loading volume of reagents and samples were based on duplicate measurements for each sample. Preparation of reagents and materials are shown in and details of cycling conditions (Figures 33 and 34).

Reagents:
cDNAs (15μl cDNA from RT of 1.2μg RNA + 345μl H₂O)
SYBR Green
Primer mix (20μM)
DEPC H₂O
Keep all reagents on ice

Protocol
1. For new primers reconstitute with TE [10 mM Tris-HCl (pH 8.0), 1 mM EDTA] and vortex
2. Calculate total volume of SYBR green and primer mixture to make a master mix according to sample number (N)
3. Add SYBR green (10xNx1.1) to primer mix (2xNx1.1) and vortex for each gene.
4. Aliquot 12μl of Sybr/Primer and 8μl of cDNA into each PCR tube. Performed in duplicate.
5. Load the tubes into the rotor of the PCR machine (Rotor-Gene 6000). Set up the running program and start amplification procedure

Figure 33: Details of qRT-PCR protocol
7.9.5. Quantification of gene expression

Under identical cycling conditions, all primer sets worked with similar efficiencies to obtain simultaneous amplification in the same run. The choice of reference genes is discussed within Section 12.5.1. HPRT1, GUSB and RPL13 were chosen; the gene of interest was RAGE. Analysis of relative expression and CT values was with rotor-gene software (v 1.7), followed by use of Excel software to implement the delta delta CT method [646] to compare the difference between post-snCPB and pre-snCPB expression of RAGE relative to the mean change in reference genes. Relative transcript abundance is expressed as ΔCt value (ΔCt = Ct reference – Ct target), with higher ΔCt values indicate higher transcript abundances, and negative ΔCt values represent genes that are less expressed compared with the reference gene. The fold-change of the transcript levels in post versus control can be estimated by $2^{\Delta\Delta Ct}$, where ΔΔCt values are calculated as ΔCtpost – ΔCtpre. Positive $2^{\Delta\Delta Ct}$ values indicate up-regulation, negative values indicate down-regulation of a target gene.

7.9.6. Melt Curve

The melt curve is a post PCR analysis tool that is used to ensure the specificity of amplified products. End products are incubated through a range of increasing temperatures. The amplicon (specific PCR product resulting from amplification of the region of sequence targeted by the PCR primers) is melted from a double stranded to single stranded state. At low temperatures the DNA is double stranded and a high fluorescence is recorded; at high temperature the strands dissociate and low fluorescence is detected due to release of the dye. Melt curves are presented as a derivative plot showing rate of change of fluorescent signal. The x axis represents temperature and the y axis the negative derivative (rate of

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturing</td>
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<td>15 mins</td>
</tr>
<tr>
<td>Denaturing</td>
<td>94°C</td>
<td>20 sec</td>
</tr>
<tr>
<td>Annealing</td>
<td>60°C</td>
<td>20 sec</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>20 sec</td>
</tr>
<tr>
<td>Final Extension</td>
<td>72°C</td>
<td>15 sec</td>
</tr>
</tbody>
</table>

Figure 34: Details of PCR cycling conditions
change) of fluorescence (F) with respect to temperature (T), shown as $dF/dT$ (Figure 35).

The melting temperature depends on the length of DNA, sequence and G:C content. The peak of the plot represents the melting point (Tm) at which 50% of the DNA is single stranded. More than one peak indicates more than one amplified product. Primer dimers denature into single strands at high temperatures.

Figure 35: A representative melt curve, temperature (x axis) versus the rate of change of fluorescence (F) with respect to temperature (T), shown as $dF/dT$ (y axis).
8. STATISTICAL METHODS

8.1. Data processing

Data were entered in Excel spreadsheets prior to statistical analysis using PRISM v4.0 (GraphPad software, La Jolla, CA, USA) or SPSS v17.01 (IBM, Somers, NY, USA).

8.2. Data presentation and analysis

Data are presented as mean and standard error of the mean (SEM), or median (interquartile range, IQR) for non-parametric data. For each dataset the distribution was determined using the Kolmogorov-Smirnov test. The statistical tested used for individual experiments are outlined as the results are presented. Statistical significance was defined at the 95% level. Statistical tests used are presented in Table 16.

<table>
<thead>
<tr>
<th>Distribution</th>
<th>Groups</th>
<th>Paired</th>
<th>Test</th>
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<tbody>
<tr>
<td>Parametric</td>
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<td>N</td>
<td>t-test</td>
</tr>
<tr>
<td>Non-parametric</td>
<td>2</td>
<td>N</td>
<td>Mann Whitney U (MWU)</td>
</tr>
<tr>
<td>Parametric</td>
<td>&gt;2</td>
<td>N</td>
<td>ANOVA with Bonferroni correction</td>
</tr>
<tr>
<td>Non-parametric</td>
<td>&gt;2</td>
<td>N</td>
<td>Kruskal-Wallis test (KWT) with Dunn’s multiple comparisons test (DMCT) correction</td>
</tr>
</tbody>
</table>

| Parametric   | 2      | Y      | Paired t-test                             |
| Non-parametric | 2 | Y      | Wilcoxon matched pairs test (WMPT)        |
| Parametric   | >2     | Y      | Repeated measures ANOVA with Bonferroni correction |

Table 16: Statistical tests used according to dataset distribution and number of groups

Contrary to standard methodology but to more clearly demonstrate statistical relationships, the mean and SEM were charted in preference to the median and IQR for some non-parametric variables in Section 10; appropriate non-parametric statistical analyses were performed.
8.3. Correlations

If data were parametric in distribution, Pearson’s correlation was used; if non-parametric, Spearman’s rank correlation. In both cases, a value is stated for $r$ (degree of correlation) and $p$ (statistical significance), $r$ values of $>0.3$ or $<-0.3$ are considered significant when associated with a $p$ value of $<0.05$.

8.4. Binary logistic regression

To assess relationship between continuous variables and binary outcomes binary logistic regression was used. Continuous variables were necessarily parametric in distribution and were this not the case, a $\log_{10}$ transform was used. Statistical relationship of variables to outcome was initially assessed individually and if $p<0.10$ then these variables were used in the multivariate analysis. For each analysis, odds ratio, 95% confidence intervals and $p$ values were presented.

In the presentation of the data, for each outcome, 0 indicates absence of the outcome and the mean and standard deviation for the relevant clinical variable are shown, 1 indicates presence of outcome and similarly the mean and standard deviation are shown. A variable that, in multivariate analysis, has 95% confidence intervals that do not span 1.0 will have a $p$ value of less than 0.05 is considered to be have an independent relationship with the outcome under consideration. An odds ratio of less than 1.0 equates to reduced risk and greater than 1.0, increased risk.
9. MATERIALS

9.1. General reagents

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<thead>
<tr>
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<td>BD</td>
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<td>Sigma Aldrich</td>
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<td>Sigma Aldrich</td>
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<td>Methanol</td>
<td>-</td>
<td>Merk</td>
</tr>
<tr>
<td>Microcentrifuge tubes</td>
<td>-</td>
<td>Fisher</td>
</tr>
<tr>
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<td>-</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Percoll</td>
<td>17-0891-02</td>
<td>GE Healthcare</td>
</tr>
<tr>
<td>Perm/wash buffer</td>
<td>554723</td>
<td>BD</td>
</tr>
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<td>BD</td>
</tr>
<tr>
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<tr>
<td>Tween 20</td>
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<td>Roche</td>
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</table>
### 9.2. Equipment

- EL800 plate reader with Gen 5 (Biotek) software: Biotek
- LSRII: BD
- RG-6000 RT-PCR machine: Corbett

### 9.3. Antibodies

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<thead>
<tr>
<th>Target</th>
<th>Host species</th>
<th>Monoclonal/Polyclonal and conjugate</th>
<th>Code</th>
<th>Producer</th>
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<td>Rabbit</td>
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<td>Ab37647</td>
<td>Abcam</td>
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<td>Goat</td>
<td>Polyclonal, FITC</td>
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<td>Rabbit</td>
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<td>Isotype</td>
<td>Rabbit</td>
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<tr>
<td>RAGE</td>
<td>Mouse</td>
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<td>Millipore</td>
</tr>
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<td>Goat</td>
<td>Polyclonal, FITC</td>
<td>DAM1487570</td>
<td>Millipore</td>
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<tr>
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<td>Millipore</td>
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<td>Polyclonal, FITC</td>
<td>sc-2010</td>
<td>Santa Cruz</td>
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<td>CD16</td>
<td>Mouse</td>
<td>302030</td>
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<td>Mouse</td>
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<td>BioLegends</td>
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</tr>
<tr>
<td>CD14</td>
<td>Mouse</td>
<td>Monoclonal, PE-Cy7</td>
<td>557742</td>
<td>BD</td>
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<td>Mouse</td>
<td>Monoclonal, PE</td>
<td>FAB14781P</td>
<td>R&amp;D</td>
</tr>
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<td>TLR-9</td>
<td>Mouse</td>
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<td>IMG-305D</td>
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<td>Abcam</td>
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<td>ab18447</td>
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<tr>
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<td>Mouse</td>
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### 9.4. Antibody related

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</tr>
<tr>
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<td></td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Lightning link™ – APC kit conjugation kit</td>
<td>705-0005</td>
<td>Innova Bioscience, Cambridge, UK</td>
</tr>
<tr>
<td>Live/Dead FITC kit</td>
<td>V13242</td>
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### 9.5. Cells and cell culture

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<tr>
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<tr>
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### 9.6. qRT-PCR

<table>
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<td>DEPC</td>
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<td>Gibco</td>
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<td>Y00147</td>
<td>Invitrogen</td>
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<td>Invitrogen</td>
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<td>M531a</td>
<td>Invitrogen/Promega</td>
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<td>Invitrogen</td>
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<td>58862</td>
<td>Invitrogen</td>
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Quantitect Primer assay: RAGE  
Quantitect Primer assay: TLR-4  
RNasin, 0.5\(\text{U}/\mu\text{l}\)  
RNeasy Kit  
RT buffer  
SYBR green PCR kit  

9.7. ELISA and related

Calprotectin (S100A8/9) ELISA  
CML-albumin ELISA  
esRAGE  
HMGB1 ELISA  
Human IL-8  
Human proinflammatory-7 Ultra-sensitive plate  
IL-8 duoset  
Quantikine sRAGE ELISA  
Rapid Endotest  
S100A12  
S100B  

9.8. Recombinant proteins

9.8.1. S100B

A recombinant human S100 \(\beta\)-chain (RC790, Randox) comprises a 91 amino acid fragment (2-92) corresponding to processed S100 \(\beta\)-chain and is expressed in E. coli with an amino terminal hexahistidine tag, necessary for affinity chromatography purification. It was not certified endotoxin-free.
9.8.2. S100A12
A partial recombinant human protein, produced using the in vitro Wheat Germ expression system for Novus Biologicals (Littleton, CO 80160) under license from CellFree Sciences Co., Ltd of Japan. Supplied by Abnova. H00006283-Q01

9.8.3. LPS
Lipopolysaccharide (LPS), was provided by my colleague Dr Susanna Leaver, following extensive purification and validation. The LPS was purchased from a commercial manufacturer (Sigma, L2880) prior to the availability of highly purified LPS. The LPS was derived by hot phenolic extraction from Escherichia coli (serotype 055:B5) and this may leave residual non-LPS contaminants. It was therefore further purified using a phenol extraction method and quantified using Limulus amoebocyte lysate (LAL) assay performed commercially by BioWhitaker, Verviers, Belgium.

9.8.4. HMGB1
A recombinant human protein synthesised in murine myeloma cell line (NS0-derived), purchased from R&D (Catalogue number 1690-HM-025). Certified endotoxin free <1.0 EU per 1µg of the protein. The method of purification was not specified.

9.8.5. sRAGE
A recombinant human protein (Prospec, Israel) produced in HEK (Human Embryonic Kidney) cells as a single, glycosylated, polypeptide chain containing 331 amino acids and having a molecular mass of 35.2 kDa. It is fused to a Flag tag at C-Terminus and purified by proprietary chromatographic techniques. It was not certified endotoxin-free.
9.8.6. TTP-4000

A protein provided as a research donation from Transtech Pharma; it is a fusion protein containing the variable region and N-terminal constant domains of RAGE mated to the Fc region derived from human IgG (Figure 36). It was not certified endotoxin-free.

Figure 36: Diagrammatic representation of how TTP-4000 relates to the IgG, RAGE, and sRAGE biomolecules
Results
10. THE RELATIONSHIP BETWEEN SIRS AFTER SNCPB AND AGE

10.1. Introduction

Advanced age is associated with adverse outcomes from a variety of clinical conditions associated with SIRS, including infection and following snCPB (as described in Section 4.1). However, the relationship between age and the severity of systemic inflammation consequent upon snCPB has not been characterised. Thus, significant changes in immune function occur with age, and heightened inflammatory responses have been demonstrated, exemplified by positive associations between age and levels of circulating inflammatory cytokines during sepsis [509, 510]. Secondly, oxidative modifications to long-lived biomolecules occur with age, and these advanced glycation end-products are ligands for RAGE. There is therefore justification for exploring the hypothesis that accumulation of RAGE ligands may contribute to age-related changes in immune function, predisposing to greater inflammatory responsiveness which is reflected in adverse outcomes for elderly patients displaying an acute systemic inflammatory response to the stimulus of snCPB.

Unfortunately, optimal clinical and other parameters that might quantify the extent of systemic inflammation are undefined; patients displaying such a reaction to snCPB typically fulfilling several SIRS-defining criteria. However, these were not designed to quantify systemic inflammation, but rather as defining criteria. Thus, fulfilling three defining criteria for SIRS does not infer that the extent of a systemic inflammatory response is more severe than if only two were met. Whilst retrospective observational studies have shown that meeting more SIRS criteria is associated with worse outcome from both infectious and non-infectious acute systemic inflammatory conditions (Section 1.2) [34, 647], the relationship between the number of SIRS criteria fulfilled and outcome in patients undergoing snCPB has not been reported. Partly this may be because of significant limitations to using standard SIRS criteria to assess systemic inflammation in these patients. Indeed, body temperature is influenced by cooling for cardiopulmonary bypass, heart rate by pacing, respiratory rate by mandatory invasive ventilation, and white blood cell count by haemodilution. By contrast, meeting two or more SIRS defining criteria after snCPB is common (estimated to be 28-63%); the variables under consideration are therefore almost universally present in a given population. Moreover, proposals have been made concerning the best way to report
systemic inflammation following snCPB in the form of a combination of causal inflammatory markers known to be associated with adverse sequelae, such as circulating inflammatory cytokines; and evidence of at least one clinical end-point of organ injury, such as organ failure scores or ICU length of stay (LOS) [89].

Increased numbers of circulating white blood cells (leukocytosis) and more specifically neutrophils (neutrophilia) are non-specific markers of inflammation and incorporated into the some severity of illness scores (including APACHE II [518]). Both excessively high (>12 X10^9/l) and low (<4X10^9/l, leucopenia) leukocyte counts are defining criteria for SIRS although leucopenia is often attributable to immunosuppressant therapies or bone marrow failure; both uncommon following snCPB. CRP is an acute-phase protein under transcriptional control by IL-6 and rises rapidly following a stimulus, peaking around 36-48h. It is routinely measured in the critically ill as an inflammatory marker but has limited prognostic value, particularly as an isolated measurement [648].

The SOFA and APACHE II scores are derived from routinely collected clinical variables and quantify different aspects of severity of illness, and have been validated in relation to adverse outcome in cardiac surgical patients [94, 96]. SOFA score reflects the extent of organ dysfunction and does not include age as a variable. APACHE II includes age and information regarding chronic health status.

The duration of time that a patient spends on an ICU or the length of stay (LOS), relates primarily to the need for advanced supportive therapies and monitoring. As such, it represents a composite measure of organ failures and extent of inflammatory response. Prolonged ICU has serious implications as it is associated with poor functional outcome, low quality of life and increased mortality [17-19].
10.2. **Aims and objectives**

The analyses reported within this chapter were aimed at determining the relationship between the age of patients undergoing snCPB and the severity of their systemic inflammatory response, and whether such an association has influence upon clinical outcome. The specific objectives were:

i. To demonstrate the relationship between patient age and SIRS criteria, as a measure of extent of systemic inflammation; and patient age and SOFA score, as a measure of organ dysfunction.

ii. To determine the relationship between patient age and leukocytosis and levels of CRP, as measures of systemic inflammation.

iii. To establish the relationship between patient age and duration of ICU stay (ICU LOS).

iv. To assess predictors of fatal outcome or prolonged ICU LOS.

10.3. **Specific methods**

Patients admitted to the Adult Intensive Care Unit following elective cardiac surgery between 1st Jan 2002 until 31st May 2005 at the Royal Brompton Hospital were retrospectively identified as part of a related study (termed ‘Cohort A’). The study was examined by the Royal Brompton and Harefield NHS Foundation Trust Research Ethics Committee and deemed to fall within the remit of ‘service evaluation’ and thereby did not require patient consent.

The characteristics of the patients, their operations and subsequently clinical course including physiological and laboratory data were extracted from electronic databases (Section 6.2). Admissions with conditions unrelated to cardiac surgery, re-admissions and those in whom electronic data was incomplete were excluded. Patients had undergone coronary artery bypass-graft (CABG), valvular surgery (Valve), CABG with valve surgery (CABG & Valve), or other. The influence of age on systemic inflammation was considered within the surgical categories to avoid confounding by surgery type. Within the surgical category ‘CABG and Valve’ there were very few patients aged less than 40 years and the group was merged with that of the 40-60 year cohort for analysis.

To determine the number of SIRS criteria fulfilled physiological data were assessed in one hour epochs. Standard SIRS criteria were used with the following modifications: immature
forms of leukocyte were not considered, as this information was not available; axillary temperatures were increased by 0.5°C and core temperatures were not adjusted as recommended[649]. SOFA and APACHE scores were calculated as described in Section 6.5. ICU LOS was defined as the duration (in hours) between admission and discharge from ICU.

10.3.1. Data analysis

Data were entered in Excel spreadsheets prior to statistical analysis using PRISM v4.0 (GraphPad software, La Jolla, CA, USA) and SPSS v17.01 (IBM, Somers, NY, USA).

Patients were allocated to one of 4 groups: <40 (young), 40-60 (middle aged), 60-80 (old), >80 (oldest) years. When divided by quartile the relative abundance of patients between 60 and 80 years reduced the age difference between the groups; an approach employed by others in studies attempting to relate age to inflammatory response [510]. The distribution of scores was compared between groups using ANOVA with Bonferroni correction for multiple comparisons when data were parametric; and with the Kruskal Wallis Test with Dunn’s Multiple Comparison Test when non-parametric. The Kolmogorov-Smirnov test was used to determine the distribution of each variable. For some non-parametric variables, the mean and standard error of the mean were charted in preference to the median and interquartile range, in order to more clearly demonstrate statistical relationships. Binary logistic regression is explained in Section 8.4. To assess possible relationships between potential predictors of adverse outcome and survival, binary logistic regression analysis was conducted.
10.4. Results

10.4.1.1. Age of patients

![Histogram of ages of patients in the cohort with a parametric curve superimposed for reference. N=2440.](image_url)

Data from 2440 patients of the total cohort of A were analysed. The median age was 67 years and the mean 65.3 years. There were 103, 532, 1603 and 202 patients in the young, middle aged, old and oldest groups respectively. The distribution of age was therefore non-parametric in distribution (Kolmogorov-Smirnov test \( p<0.0001 \)) (Figure 37).
10.4.2. Description of patients

The distribution of ages within each of the surgical categories differed significantly (Table 17), p<0.001 for all comparisons. There was no difference between the groups in distribution of white blood cell count measured within the first 24h of surgery. There were significant differences in CRP within the first 24h of surgery between the groups; the Valve & CABG group having the highest CRP, followed by the CABG group, and the valve group (p<0.001 for all comparisons). APACHE II was highest in the Valve and CABG group and similar in the two remaining (p<0.001 for valve and CABG vs. either CABG or valve) groups. These differences between the surgical categories justify consideration of the relationships between age and other variables within categories, as opposed to consideration of the entire cohort together.

Consistent with expectations, the ICU LOS and mortality were highest in the valve & CABG group, followed by the valve group, and were lowest in the CABG group. Overall, LOS was short (median one day), although many patients had prolonged ICU LOS with 456 patients (18.7%) staying more than 3 days.

<table>
<thead>
<tr>
<th>Population</th>
<th>CABG n = 1425</th>
<th>Valve n=763</th>
<th>CABG and Valve n=252</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years (median, IQR)</td>
<td>67, 60-73</td>
<td>65, 53-74</td>
<td>72, 65-78</td>
</tr>
<tr>
<td>WBC, x10^9/L (median, IQR)</td>
<td>11.8, 9.4-15</td>
<td>12.2, 9.5-16</td>
<td>12.2, 9.3-15</td>
</tr>
<tr>
<td>CRP, mg/L (median, IQR)</td>
<td>72, 55-91</td>
<td>67, 50-88</td>
<td>74, 65-78</td>
</tr>
<tr>
<td>APACHE II (median, IQR)</td>
<td>15, 12-18</td>
<td>15, 12-18</td>
<td>17, 14-20</td>
</tr>
<tr>
<td>ICU Length of stay (days, IQR)</td>
<td>1, 0.8-1.9</td>
<td>1.6, 0.9-2.9</td>
<td>1.8, 0.9-3.8</td>
</tr>
<tr>
<td>ICU mortality (number, %)</td>
<td>22, 1.54%</td>
<td>22, 2.88%</td>
<td>12, 4.76%</td>
</tr>
</tbody>
</table>

Table 17: Demographics, markers of systemic inflammation, severity of illness and outcome. Groups were compared using the Kruskal Wallis test with Dunn’s Multiple Comparison Test correction, *** p<0.001 for comparisons between each group with the others. CABG (n=1425) Valve (n=763) Valve & CABG (n=252).
As seen in Figure 38, the SOFA score was highest in the Valve & CABG group, followed by the Valve group, and was lowest in the CABG group (p<0.001 for all comparisons).

10.5. Incidence and severity of SIRS

10.5.1. For all patients

<table>
<thead>
<tr>
<th>SIRS criteria met</th>
<th>Proportion of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Within 1h</td>
</tr>
<tr>
<td>0</td>
<td>7.13%</td>
</tr>
<tr>
<td>1</td>
<td>35.31%</td>
</tr>
<tr>
<td>2</td>
<td>42.97%</td>
</tr>
<tr>
<td>3</td>
<td>13.64%</td>
</tr>
<tr>
<td>4</td>
<td>1.05%</td>
</tr>
<tr>
<td>≥2</td>
<td>57.66%</td>
</tr>
</tbody>
</table>

Table 18: Proportion of patients meeting SIRS criteria within 1h and 24h of admission to ICU.

As seen in Table 18, on the basis of meeting 2 or more defining criteria, 57.66% of patients in the total cohort had SIRS within the first hour following snCPB and 96.17% within the first 24h.
10.5.2. SIRS criteria in relation to age of patient and type of surgery

![CABG - SIRS criteria](Mean SEM)

![Valve - SIRS criteria](Mean SEM)

![Valve & CABG - SIRS criteria](Mean SEM)

**Figure 39:** SIRS criteria in the first hour (left) and in the first 24h (right) for patients in the three operative categories, split into age groups. Data are presented as mean values with bars indicating SEM, although non-parametric in distribution (Section 8.2). Kruskal Wallis test with Dunn’s Multiple Comparison Test correction, * p<0.05, ** p<0.01, *** p<0.001. CABG (n=1425) Valve (n=763) Valve & CABG (n=252).
Statistically significant differences in the distribution of the maximum number of SIRS criteria met within the first 1h and 24h were seen (Figure 39). A common pattern was seen across the 3 surgical groups in terms of the maximum number of SIRS criteria met in the first hour. Higher mean numbers of criteria were met with increased age, a finding most apparent in the CABG group (p<0.001 for all comparisons). By contrast, a different pattern was seen in the distribution of SIRS criteria met within the first 24h, a non-linear, ‘U-shaped’ relationship emerging with the oldest (>80y) and youngest(<40y) cohorts having a higher number, which reached statistical significance in the CABG (p<0.001) but not the valve group. The numerically smallest group, Valve & CABG, showed no significant differences between age groups.
10.5.3. SOFA score in relation to age of patient and type of surgery

Figure 40: SOFA scores in the first 24h for patients in the three operative categories, split into age groups. Data are presented as median values with bars indicating IQR. Kruskal Wallis test with Dunn’s Multiple Comparison Test correction, *** p<0.001. CABG (n=1425) Valve (n=763) Valve & CABG (n=252).
Statistically significant differences in the distribution the SOFA score was seen across surgical categories, with an association between higher SOFA scores and increased age (Figure 40). In the CABG and Valve groups this was significant (p<0.001 for all comparisons). The smallest group, Valve & CABG, showed no significant differences between age groups.
10.5.4. CRP and WBC in relation to age of patient and type of surgery

Figure 41: Markers of systemic inflammation, CRP (left) and WBC (right) for patients in the three operative categories, split into age groups. Data are presented as median values with bars indicating IQR. Kruskal Wallis test with Dunn’s Multiple Comparison Test correction, * p<0.05, ** p<0.01, *** p<0.001. CABG (n=1425) Valve (n=763) Valve & CABG (n=252).
As seen in Figure 41, statistically significant differences in the distribution of CRP and white blood cell count (WBC) were seen. In both the CABG and Valve groups, increasing age was associated with decreasing WBC and CRP, which was significant in the CABG patients (p<0.001 for all WBC, p<0.05 for >80 vs. 40-60 CRP).

In the Valve group CRP was significantly lower in the >80 group than the <40 (p<0.05) or 40-60 groups (p<0.001). Similarly, the CRP was significantly lower 60-80 group than the 40-60 group (p<0.01). The WBC was significantly lower in the >80 than <40 groups (p<0.05). The numerically smallest group, Valve & CABG, showed no significant differences between age groups.
10.5.5. ICU LOS in relation to age of patient and type of surgery

Figure 42: Duration of intensive care stay (ICU LOS) for patients in the three operative categories, split into age groups. Data are presented as median values with bars indicating IQR. Kruskal Wallis test with Dunn’s Multiple Comparison Test correction, * p<0.05, *** p<0.001. CABG (n=1425) Valve (n=763) Valve & CABG (n=252).
ICU LOS was statistically significantly different by age, particularly in the CABG group, $p<0.001$ for comparisons between all groups (Figure 42). ICU LOS increased with age, with the exception of the <40y group who also had prolonged LOS. The same trend was seen in the valve group, and reached statistical significance for ICU LOS in those >80 vs. 40-60 ($p<0.05$). The numerically smallest group, Valve & CABG showed no significant differences between age groups.

### 10.5.6. Determinants of outcome in mixed cohort of snCPB patients

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Survived Mean(std)</th>
<th>Died Mean(std)</th>
<th>Univariate analysis Odds Ratio, 95% CI</th>
<th>P</th>
<th>Multivariate analysis Odds Ratio, 95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>65.1(12.3)</td>
<td>71.8(9.8)</td>
<td>1.064 (1.03-1.10)</td>
<td>0.000</td>
<td>1.042 (1.01-1.07)</td>
<td>0.008</td>
</tr>
<tr>
<td>CRP</td>
<td>74.7(34.6)</td>
<td>70.1(38.6)</td>
<td>0.996 (0.99-1.01)</td>
<td>0.358</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WBC</td>
<td>12.8(5.9)</td>
<td>15.8(6.6)</td>
<td>1.035 (1.01-1.06)</td>
<td>0.004</td>
<td>0.997 (0.96-1.03)</td>
<td>0.856</td>
</tr>
<tr>
<td>Max SIRS in 1h</td>
<td>1.65(0.8)</td>
<td>2.01(0.8)</td>
<td>1.677 (1.22-2.31)</td>
<td>0.001</td>
<td>0.971 (0.67-1.40)</td>
<td>0.877</td>
</tr>
<tr>
<td>Max SIRS in 24h</td>
<td>2.64(0.7)</td>
<td>3.40(0.7)</td>
<td>4.588 (3.03-6.96)</td>
<td>0.000</td>
<td>2.950 (1.75-4.98)</td>
<td>0.000</td>
</tr>
<tr>
<td>SOFA score</td>
<td>5.9(2.1)</td>
<td>10.0(2.6)</td>
<td>2.068 (1.82-2.35)</td>
<td>0.000</td>
<td>1.894 (1.64-2.19)</td>
<td>0.000</td>
</tr>
<tr>
<td>ICU LOS</td>
<td>2.8(5.9)</td>
<td>10.8(21.9)</td>
<td>1.051 (1.03-1.07)</td>
<td>0.000</td>
<td>1.000 (0.98-1.02)</td>
<td>0.990</td>
</tr>
</tbody>
</table>

Table 19: Binary logistic regression analysis relating potential predictors of adverse outcome to vital outcome. N=2440.

Out of the study cohort of 2440 patients 56 (2.2%) died (Table 19). All the variables considered excluding CRP were statistically significantly associated with death ($p<0.05$). When analysed together to assess which were independently associated with an increased risk of death, in addition to age, maximum number of SIRS criteria fulfilled in the first 24 hours (odds ratio 2.95, 95% confidence intervals 1.75-4.98, $p = 0.000$) and SOFA score (odds ratio of 1.89 95% confidence intervals 1.64-2.19, $p = 0.000$) emerged as significant (Table 19).
The relationship between potential determinants and the binary outcome of greater than, or less than 1 day of ICU stay was assessed similarly (Table 20). ICU LOS is a continuous variable and may be best analysed as such [650], however dichotomizing is a common and accepted method of statistical analysis [651]. An ICU LOS of 1 day was the median and greater than, or less than, the median was used to dichotomize. All the variables considered excluding CRP were statistically significantly associated with an ICU LOS of greater than 1 day, p<0.05. When re-analysed together to assess which were independently associated with an increased risk of >1d ICU LOS, maximum number of SIRS criteria fulfilled in the first 24 hours (odds ratio 1.43, 95% confidence intervals 1.23-1.67, p = 0.000) and SOFA score (odds ratio of 1.60, 95% confidence intervals 1.52-1.69, p = 0.000) emerged as significant.
10.6. Discussion

The specific aims of the studies described in this chapter were fourfold. First, to demonstrate any relationship between patient age and SIRS criteria, and patient age and SOFA score. Second, to determine the relationship between patient age and markers of systemic inflammation, specifically WCC and CRP. Third, to establish the relationship between patient age and ICU LOS. Finally, to identify any predictors of fatal outcome or relatively prolonged ICU LOS.

In addressing these issues, data of general importance emerged. Thus, almost all (96%) patients enrolled met two or more SIRS criteria in the first 24h following snCPB, thereby calling into question the usefulness of the description. Similar findings have been reported in surgical ICU patients with an incidence of 93% [652]. Moreover, only a small minority (2.2%) of patients post snCPB developed severe manifestations of systemic inflammation and progressed to multiple organ dysfunction syndrome (MODS) and death. A definition that was less sensitive but which predicted this subset of patients could be of greater clinical utility. The term 'severe SIRS' has been used in such circumstances, based either on the presence of 3 or more SIRS criteria, or 2 or more in association with a high severity of illness score, such as APACHE II [653, 654]. Alternatively, refinements to the SIRS criteria, particularly cumulative consecutive hours scoring great than 2 or 3 criteria, have been developed by others and show improved discriminatory capacity for adverse outcome after snCPB [655].

Beyond these general results, in addressing the first study objective, a complex relationship was revealed between patient age and number of SIRS criteria met within the first 1h and up to 24h. In the first hour a consistent pattern of increasing SIRS with age was seen in all surgical groups. Up to 24h, there was a non-linear, ‘U-shaped’ relationship, the oldest (>80y) and youngest (<40y) cohorts having a higher number of SIRS criteria in the CABG group. The same pattern was seen in the valve group although without statistically significant increased SIRS at 24h in the >80y group compared to the other groups. No such pattern was seen in the numerically smallest group (Valve & CABG). Statistically significant increases in SOFA scores were seen with increasing age. Secondly, a consistent pattern was seen across all surgical categories, increasing age being associated with decreasing WBC and CRP. Thirdly, ICU LOS increased with age, with the exception of the <40y group who also had prolonged
LOS. Finally, two variables were independently associated with both mortality and prolonged ICU LOS: maximum number of SIRS criteria fulfilled in the first 24 hours and SOFA score.

A primary aim of these analyses was to demonstrate the relationship between patient age and markers of systemic inflammation. Compared to those aged between 40 and 80 years, the oldest patients (>80y) had higher SIRS criteria and SOFA scores but with lower CRP and WBC counts. SIRS criteria and SOFA scores had strong relationships to mortality and prolonged ICU stay in contrast to the lack of relationship of CRP and WBC.

Older patients may have a relatively exaggerated inflammatory response reflected in their higher SIRS and SOFA scores which could contribute to adverse outcomes. However, there are several alternative explanations. Firstly, the number of SIRS criteria met could have been higher in elderly patients due to the preference of treating physicians for cardiac pacing and ventilation settings. Thus, the elderly are at increased risk of developing perioperative atrial fibrillation and this may be ameliorated through prophylactic pacing [656]. Secondly, the SOFA score may be higher due to the incidence of other co-morbidities with age, such as chronic kidney disease. Age influencing physician preference in maintaining a higher arterial pressure requiring the use of higher doses of vasoactive drugs would also affect the score.

The lack of agreement between increased SIRS and SOFA score versus decreased WBC and CRP in those >80y compared to other groups is unexpected. Generally, one would expect inflammatory markers, such as WBC and CRP, to parallel changes in scores of inflammation and organ dysfunction in this setting. The most likely explanation is that CRP and WBC within the first 24h following snCPB do not represent a genuine reflection of systemic inflammatory state. CRP takes more than 24h to reach its peak following rise in the cytokines that lead to its synthesis and the WBC count early after snCPB will have had any increased production and decreased elimination counterbalanced by the haemodilution that occurs during CPB. Alternatively, SIRS criteria, SOFA score and ICU LOS are higher in the oldest group through mechanisms that are independent of systemic inflammation.

The patients over 80y in this study might not be representative of their age cohort. Not all patients with coronary or valvular heart disease in their 80s would agree to undergo snCPB. Therefore these patients represent a selected group who were referred and accepted for
snCPB. A 2009 UK report suggests that the average age of patients having snCPB continues to rise [8]. Indeed, by comparison with patients from the previous decade at the same institution, this cohort demonstrated increased age and APACHE II score, but unchanged ICU LOS and mortality [657].

Compared to patients between 40 and 80 years, younger patients (<40) had higher SIRS scores up to 24h, but lower SOFA scores with high WBC and CRP. This may reflect a propensity to mount a greater inflammatory response without the concomitant development of organ dysfunction, possibly reflecting increased physiological reserve. Alternately, younger patients may have a different range of co-morbidities and be confined to specific procedure groups. CABG operations vary in their complexity but are more homogeneous than ‘valve surgery’ as an operative category. The majority of younger patients in the ‘valve surgery’ group are likely to suffer from adult congenital heart disease, and to have a high incidence of pulmonary hypertension and previous sternotomy which are both associated with an increased incidence of peri operative complications.

The shortcomings of the SIRS criteria will always emerge in patients requiring intensive care, in that they present with physiological perturbations amenable to intervention – the heart rate and respiratory rate are examples. In these circumstances the decision to employ cardiac pacing or mechanical ventilation may reflect the choice of the treating physician rather than the severity of underlying systemic inflammation.

Other important limitations to these analyses should be mentioned. First, the data were collected prospectively although analysed retrospectively. Second, in a small number of cases, important data in the electronic record were missing. Third, the use of 1h epochs was necessary for analysis but could potentially lead to inaccurate calculation of SIRS criteria. Fourth, the proportion of patients within this cohort who received ‘off-pump’ cardiac surgery is not precisely known but is estimated to be 5%. Fifth, a group of patients with low risk for complicated recovery from CABG were not included within this cohort as only those who required ICU admission postoperatively were included. Sixth, the population was recruited from a single tertiary/quaternary referral centre admitting 1200 patients to ICU per annum; this casemix may not be comparable to that of other units. Finally, the absence of any estimation of the contribution of pre-operative co-morbidities, the severity of the
underlying condition necessitating requiring surgery and intra-operative details such as the
duration of CPB, to the development of SIRS criteria, SOFA score and outcomes is a
significant deficiency. Further, a technical limitation to the use of SIRS criteria in this study
is that WBC values in this study were mostly obtained pre-operatively. In ‘uncomplicated’
patients blood counts might not be drawn and analysed until the day following surgery. In
some a sample was analysed soon after their return from theatre; WBC values were higher
in these samples. Unlike other variables, WBC is relatively infrequently sampled and this can
result in artefact. Similarly, the blood that was analysed for CRP and WBC was not taken at a
uniform time following the operation which introduced variability.

The statistically significant relationship between the age of patient and the number of SIRS
criteria met in the first 24h following surgery was informative but has limited clinical
relevance for individual patients. The difference in SIRS scores between age groups were
numerically very small.

10.7. Conclusion
In this population, the oldest patients met more SIRS criteria in the first 1h and 24h
following snCPB than those aged 40-80 y. This was accompanied by higher SOFA scores,
reflecting more severe organ dysfunction. Meeting SIRS criteria in the first 24h and SOFA
score predicted adverse outcomes. Overall, these findings are supportive of the paradigm of
excessive systemic inflammatory responses contributing towards organ failure and death. In
support of our initial hypothesis, patients over 80y exhibited higher SOFA scores and
number of SIRS criteria met in the first 24h than patient between 40 and 80, possibly
reflecting predisposition to more severe inflammatory response. Future work in this area
should involve the conduct of a prospective study looking at relationship between age and
outcome. A homogeneous population, with similar indications for identical surgical
procedures and with similar co-morbidities, but from across a spectrum of ages; with clinical
management standardized before, during and after snCPB would be ideal. There are obvious
limitations to this approach, not least the prolonged recruitment time if conducted in a
single centre. A multi-centre study would make standardization of care difficult. Whether it
is age per se or age as a reflection of cumulative morbidities and loss of physiological
reserve that affects outcome is unclear. In any event the chronological age of the patient at
the time of any operative intervention does not represent the optimal measure of
senescence. A similar study would have greater value if paired with measures of biological aging and cell senescence. Thus, it has been postulated that senescence-associated attenuations in physiological function are caused by molecular oxidative damage, such as AGE modifications [658]. Others have shown that the length of regions of repetitive DNA on the ends of chromosomes (telomeres) diminishes with age, and that the rate of decrease in length can be affected by lifestyle factors and affects the onset of age-associated diseases [659]. Measuring macromolecular modifications and telomere length, or a surrogate thereof [660] could therefore add value to such an investigation.

The systemic inflammatory response syndrome (SIRS) is a useful concept which permits the identification of patients with more severe responses to their underlying condition, be it infection or injury, using simple and easily quantified parameters. However, when considering patients with more severe systemic inflammation, or following highly invasive surgery leading to ongoing physiological perturbations necessitating organ support, the use of the standard defining criteria has important limitations. The extent to which the RAGE axis contributes towards predisposition to excessive systemic inflammation is undetermined. Indeed, neither the abundance of the major ligands of RAGE nor the soluble receptor has been studied around snCPB in detail.
11. RELEASE OF LIGANDS AND SOLUBLE RAGE AROUND SNCPB

11.1. Introduction

In the previous chapter a relationship was demonstrated between advanced age and increased number of SIRS defining criteria and severity of organ failure (SOFA score) following snCPB. Furthermore, the maximum number of SIRS criteria and SOFA score in the first 24h following snCPB were statistically significantly related to mortality and prolonged ICU LOS.

The RAGE-axis may be up-regulated in response to chronic stimulation [285] and conditions that result in increased RAGE ligands, such as advanced age, have been associated with adverse outcome from critical illness (Sections 3.2.3 and 4.1). It is undetermined to what extent up-regulation of the RAGE-axis may contribute to predisposition to excessive inflammatory response.

The majority of RAGE ligands are damage-associated molecular pattern (DAMP) biomolecules and are released during tissue injury, such as occurs during snCPB. Ligands that have been particularly implicated in causing inflammatory responses in humans include three of the S100 proteins (S100B, S100A12, S100A8/9), HMGB1 and CML-albumin (Section 3.2).

The precise role of soluble RAGE (sRAGE) levels in plasma and their relation to tissue levels of the full membrane-spanning RAGE protein is uncertain in humans. sRAGE levels may vary in proportion to membrane levels, therefore acting as a gauge of RAGE-axis activity. Alternatively, sRAGE may act as a competitive antagonist binding RAGE ligands and preventing them from interacting with the cellular RAGE thus abrogating cell signalling events (Section 3.1.8).

sRAGE has been demonstrated to be elevated following elective general and pulmonary surgery, in ALI, sepsis and following snCPB [457, 461, 464, 470]. The elevations in plasma sRAGE following pulmonary surgery did not relate to clinical outcomes, although the sample size was small (n=30) [470]; similarly the elevations following snCPB were not related to duration of CPB, no comparison to clinical outcomes were described, and the sample size
was small (n=20) [457]. Patients with severe sepsis or septic shock were found to have elevated plasma sRAGE, with a positive association with mortality. However the interpretation of this association is limited by the co-existence of severe oxygenation impairment in this patient cohort, suggesting co-existent ALI/ARDS [461]. Plasma sRAGE was related to mortality in those with ALI who received high tidal (12ml/kg) volume ventilation but not those who received ‘lung protective’ ventilation [454, 459]. esRAGE is a soluble form of RAGE that is synthesised without a transmembrane domain, with an alternative C’ terminal, and is believed to have the same ligand binding capacity without signal transduction. Measurement of plasma esRAGE levels in acute systemic inflammation has not been previously reported and paired analyses with sRAGE (allowing calculation of shed RAGE) are rare.

Elevations in the levels of RAGE ligands following snCPB have been described, mostly in small cohorts with limited comparison to operative factors (such as duration of CPB) and clinical outcomes: HMGB1 [458, 661], S100B [662], and S100A8/9 [663-665].

11.2. Aims and objectives
To characterise the levels of relevant RAGE ligands and levels of soluble RAGE forms in plasma obtained around snCPB and their relationships to clinical variables.

Specific objectives:

i. Assay plasma obtained from patients around snCPB for S100B, S100A12, S100A8/9, CML-Albumin, HMGB1; and the soluble RAGE forms sRAGE and esRAGE

ii. Look for statistical relationships between assays and clinical variables

11.3. Specific methods
Patients presenting for snCPB at the Royal Brompton Hospital were approached and informed consent was obtained on the day prior to surgery. Blood samples were taken around snCPB as described in Methods (Section 6.7) and plasma isolated and stored at -80°C until analysis. Samples were obtained from patients in cohorts B and C, as defined in the methods section (Section 6.1).
Commercially available ELISA kits were used for all assays (Methods and Materials, Section 9.7) except endotoxin and CML-Albumin measurement. Endotoxin testing was performed by Lonza at their European endotoxin testing centre in Belgium. The CML-albumin measurement was performed by MicroCoat Biotechnologie GmbH using a custom ELISA technique. To calculate parts per million (PPM) of CML-albumin, the measured amount of total albumin (clinical laboratory) was divided by the measured amount of CML-albumin. All assays were conducted in duplicate and the mean result used.

A wide range of clinical variables were collated and relevant variables compared to assay results. The clinical variables, how they were measured, and their derived scores are described in methods (Section 6.3). Scoring systems used include Multiple Organ Dysfunction Score (MODS [90]), the New Simplified Acute Physiology Score (SAPS II [91]), and the Sequential Organ Failure Assessment (SOFA [92]) score.

Data were entered in Excel spreadsheets prior to statistical analysis using PRISM v4.0 (GraphPad software, La Jolla, CA, USA) and SPSS v17.01 (IBM, Somers, NY, USA).

11.3.1. Statistical methods

Samples were assayed at two times from the same individual and are thus paired, if parametric in distribution, a paired t test was used and mean/SEM displayed; if non-parametric, Wilcoxon Matched Pairs Test (WPMT) was used and median/IQR displayed.

For assessment of relationship between assays and each other or clinical variables; correlations were performed: if parametric in distribution, Pearson’s correlation is used and if non-parametric Spearman’s rank correlation. In both cases, a value is given for $r$ (degree of correlation) and $p$ (statistical significance), $r$ values of $>0.3$ or $<-0.3$ are considered significant when associated with a $p$ value of $<0.01$.

Binary logistic regression was used to assess which variables independently influence post-operative outcomes. Dependent variables are required to be parametric in distribution and need to relate to a binary outcome (i.e. prolonged ICU: yes/no). To permit this some variables required a log$_{10}$ transformation (Creatinine, BMI, and both sRAGE and S100A8/9 pre- and post-op) and binary outcomes were derived from continuous thus: duration of level 3 care was dichotomised into greater than median (L3_LONGER, yes/no), or 4$^{th}$ quartile
(L3_LONGEST, yes/no); similarly hospital LOS was dichotomised into greater than median (Hosp_LONGER, yes/no), or 4\textsuperscript{th} quartile (Hosp_LONGEST, yes/no); PaO2:FiO2 ratio (PFR) less than median (PFR_worse) or 1\textsuperscript{st} quartile (PFR_Worst); acute kidney injury at any time in first week (AKI, y/n); finally vital outcome (dead/alive).
11.4. Results

11.4.1. Changes in ligands and their correlates

11.4.1.1. S100B levels were elevated post-snCPB

![Graph showing concentration of S100B in plasma from patients pre-, immediately, 2h, 6h and 24h post snCPB.](image1)

Figure 43: Concentration of S100B in plasma from patients (n=2) pre-, immediately, 2h, 6h and 24h post snCPB. Concentrations of S100B were measured in plasma collected from patients pre-, immediately, 2h, 6h and 24h post snCPB.

To inform the choice of optimal time-point to measure the ligands and soluble RAGE forms in a larger number of patients, analysis of assays was conducted in two randomly-selected ‘pilot’ patients from Cohort B. Pre-operatively levels were barely detectable, peaked at the end of the operation, remained elevated at 2h and then continued to decline (Figure 43). The 2h time point was chosen and levels assayed in a larger number of patients (Figure 44).

![Graph showing concentration of S100B in plasma from patients pre and post-snCPB.](image2)

Figure 44: Concentration of S100B in plasma from patients pre and post-snCPB. Concentrations of S100B were measured in plasma collected from patients (n=36, cohorts B&C) pre- and 2h post-snCPB. Data are presented as median values with bars indicating IQR. Groups were compared using a Wilcoxon Matched Pairs Test; ***p<0.001

Pre-operative levels were undetectable in 26 of the 36 samples and low in the remainder. The lower limit of detection of the assay was 15pg/ml. 2h following snCPB levels were
significantly elevated with a median value of 280pg/ml (IQR 191-430pg/ml), p<0.001. There were no statistically significant correlations with clinical parameters.

11.4.1.2. S100A12 levels were elevated post-op

![Figure 45: Concentration of S100A12 in plasma from patients (n=2) pre-, immediately, 2h, 6h and 24h post snCPB. Concentrations of S100A12 were measured in plasma collected from patients pre-, immediately, 2h, 6h and 24h post snCPB.]

The levels of S100A12 for the two ‘pilot’ patients at five times are shown above (Figure 45). Pre-operatively levels were undetectable and remained low at the end of the operation, peaking 2h later and remaining elevated at 6h and 24h. The lower limit of detection of the assay was 56pg/ml. The 2h time-point was chosen and levels assayed in a larger number of patients (Figure 46).

![Figure 46: Concentration of S100A12 in plasma from patients pre and post-snCPB. Concentrations of S100A12 were measured in plasma collected from patients (n=39, cohorts B&C) pre- and 2h post-snCPB. Data are presented as median values with bars indicating IQR. Groups were compared using a Wilcoxon Matched Pairs Test; ***p<0.001]
Levels of plasma S100A12 significantly increased from a median 11.4ng/ml (IQR 0.0-21.5) to a median of 77.80 (IQR 40.8-112.0), p<0.001.

| Age of the patient and pre-op S100A12 levels | Spearman’s r=0.34, p=0.03 |
| Duration of CPB and ischaemia with post-operative S100A12 levels | Spearman’s r=0.73 p<0.0001 for both |
| Post-op A12 levels and A8/9 levels | Spearman’s r=0.76 p<0.0001 |

Table 21: Correlations between plasma levels of S100A12 and assays/clinical variables. (n=39, cohorts B&C).

A positive correlation was seen between pre-operative levels of S100A12 and the age of the patient and strong positive associations between duration of CPB and post-operative levels of S100A12. S100A12 was also highly correlated to levels of S100A8/9 (Table 21).

11.4.1.3. *S100A8/9 levels were elevated post-op*

![Graph showing concentration of S100A8/9 in plasma from patients](image)

*Figure 47: Concentration of S100A8/9 in plasma from patients (n=2) pre-, immediately, 2h, 6h and 24h post snCPB. Concentrations of S100A8/9 were measured in plasma collected from patients pre-, immediately, 2h, 6h and 24h post snCPB.*

The levels of S100A8/9 for the two ‘pilot’ patients at five times are shown above (Figure 47). Pre-operatively levels were low, becoming elevated by the end of the operation and only slowly declining. The 2h time-point was chosen and levels assayed in a larger number of patients (Figure 48).
Levels of plasma S100A8/9 were statistically significantly elevated from a median of 0.6mg/ml (IQR 0.5-0.8) to 4.0mg/ml (IQR 3.0-6.4mg/ml), p<0.001. S100A8/9 was also measured in a larger number of patients from cohort B (n=130) and a similar relationship was demonstrated: 0.4mg/ml (IQR 0.2-0.6) to 2.3mg/ml (IQR 1.7-3.1), p<0.0001.

A statistically significant positive correlation between duration of CPB (and ischaemic time) and post-operative levels of plasma S100A8/9 was demonstrated, Pearson’s r=0.57, p=0.0001 for both. No relationship between the age of the patients and their levels of post-operative S100A8/9 was seen.

11.4.1.4. **CML-albumin levels were elevated post-op**
Levels of plasma CML-albumin were statistically significantly elevated from a median of 13.9ppm (IQR 12.0-17.1ppm) to 23.7ppm (IQR 17.0-27.3ppm), p<0.001 (Figure 49).

11.4.1.5. **HMGB1 levels are elevated post-op**

![Graph showing HMGB1 levels](image)

**Figure 50:** Concentration of HMGB1 in plasma from patients (n=6, cohort B) pre-, immediately, 2h, 6h and 24h post snCPB. Concentrations of HMGB1 were measured in plasma collected from patients pre-, immediately, 2h, 6h and 24h post snCPB. Data are presented as mean values with bars indicating SEM. Repeated measures ANOVA p>0.05 for all comparisons.

The levels of HMGB1 for six patients randomly selected from cohort B and levels measured at five times are shown above (Figure 50). Pre-operatively levels were low, becoming elevated by the end of the operation and only slowly declining with a possible late increase in levels at 24h. The 2h time-point was chosen and levels assayed in a larger number of patients (Figure 51).

![Graph showing HMGB1 levels](image)

**Figure 51:** Concentration of HMGB1 in plasma from patients pre and post-snCPB. Concentrations of HMGB1 were measured in plasma collected from patients (n=25, 6 from cohort B & 19 from cohort C) pre- and 2h post-snCPB. Data are presented as median values with bars indicating IQR. Groups were compared using a Wilcoxon Matched Pairs Test; **p<0.01
Levels of HMGB1 significantly increased (Figure 51) and similar to the relationships seen between post-operative levels S100A12 and A8/9 to duration of CPB, HMGB levels were positively associated with duration of CPB (Pearson’s r=0.48, p=0.04).

11.4.1.6. Endotoxin was not detectable in plasma

Endotoxin levels have been reported to be elevated during and following snCPB [191-196]. Plasma was obtained from 3 patients pre-, during and post-snCPB (cohort B); following heat treatment and suitable dilution (according to Lonza instructions), samples were sent to Lonza’s European endotoxin testing facility in Belgium. Lonza used the highly sensitive kinetic chromogenic LAL method and no endotoxin was detected in any of the samples.

11.4.1.7. Patient characteristics and assays

<table>
<thead>
<tr>
<th>Assay</th>
<th>With the co-morbidity</th>
<th>Without</th>
<th>P value (t test)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ±standard deviation, n</td>
<td>Mean ±standard deviation, n</td>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
<td>Pre-op S100A8/9</td>
<td>1023.9±419.4, 5</td>
<td>636.4±296.6, 30</td>
</tr>
<tr>
<td></td>
<td>Pre-op CML-alb</td>
<td>11.0±2.5, 3</td>
<td>15.1±3.2, 16</td>
</tr>
<tr>
<td>Pulmonary hypertension</td>
<td>Pre-op S100A8/9</td>
<td>968.0±477.3, 7</td>
<td>622.4±262.7, 28</td>
</tr>
<tr>
<td></td>
<td>Pre-op CML-alb</td>
<td>18.4±3.4, 4</td>
<td>13.4±2.6, 15</td>
</tr>
<tr>
<td>Ischaemic heart disease</td>
<td>Change in S100A12</td>
<td>32.8±86.8, 18</td>
<td>122.5±129.7, 18</td>
</tr>
</tbody>
</table>

Table 22: Patient characteristics and relationship to assay values. Pulmonary hypertension (PHT) was determined on pre-operative echocardiography. Diabetes when treated with insulin or oral hypoglycaemic agents; Ischemic heart disease (IHD) when undergoing CABG or having had either CABG or percutaneous coronary intervention. Diabetes/S100A89 data was obtained from patients in cohorts B&C, unknown status in 4; CML-albumin from cohort C, no missing data. PHT/S100A89 data was obtained from patients in cohorts B&C, unknown status in 4; CML-albumin from cohort C, no missing data. IHD data was obtained from patients in cohorts B&C, no missing data.

Levels of ligands were related to clinical characteristics and the positive findings are shown above (Table 22). Patients with diabetes (either treated with insulin or oral hypoglycaemic agents) had statistically significantly higher levels of S100A8/9 pre-operatively, and lower levels of CML-albumin pre-operatively. Those with pulmonary hypertension (detected on pre-operative echocardiography) had statistically significantly higher levels of S100A8/9 pre-
operatively, and higher levels of CML-albumin pre-operatively. Those with ischaemic heart disease (either presenting for, or having previously had coronary artery revascularisation) had statistically significantly lesser increment in S100A12 levels between pre- and post-operation.

Comparisons of assay values without significant findings were also performed between other groups: LV function (from pre-op echo; normal, mildly impaired or moderately impaired), pre-op use of HMG-CoA reductase inhibitors (statins) and renal function as assessed by creatinine clearance.

11.4.1.8. Correlates between clinical characteristics and outcomes

Spearman’s correlations

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
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<th></th>
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</thead>
<tbody>
<tr>
<td>Age and Hospital LOS</td>
<td>r=0.47, p=0.003</td>
<td></td>
</tr>
<tr>
<td>CRP (48h) and Hospital LOS</td>
<td>r=0.46, p=0.004</td>
<td></td>
</tr>
<tr>
<td>SOFA score and ICU LOS</td>
<td>r=0.51, p=0.001</td>
<td></td>
</tr>
<tr>
<td>SOFA score and hospital LOS</td>
<td>r=0.52, p=0.001</td>
<td></td>
</tr>
<tr>
<td>SAPSII score and hospital LOS</td>
<td>r=0.38, p=0.017</td>
<td></td>
</tr>
<tr>
<td>MODSII score and ICU LOS</td>
<td>r=0.43, p=0.007</td>
<td></td>
</tr>
<tr>
<td>MODSII score and hospital LOS</td>
<td>r=0.44, p=0.005</td>
<td></td>
</tr>
</tbody>
</table>

Table 23: Correlations between clinical characteristics and outcomes, n=39 (cohort B&C).

Positive associations were seen between scores of severity of illness (SOFA and MODSII) and ICU LOS. Hospital LOS was positively associated with age, CRP (at 48h post-op), SOFA score, SAPSII score and MODS II score (Table 23).
11.4.2. Changes in soluble RAGE forms and their correlates

11.4.2.1. sRAGE measured around snCPB

Figure 52: Concentration of sRAGE in plasma from patients (n=6, cohort B) pre-, immediately, 2h, 6h and 24h post snCPB. Concentrations of sRAGE were measured in plasma collected from patients pre-, immediately, 2h, 6h and 24h post snCPB. Data are presented as mean values with bars indicating SEM. Repeated measures ANOVA, ** p<0.01.

The levels of sRAGE for six ‘pilot’ patients at five times are shown above (Figure 52). Pre-operatively levels were low, becoming elevated by the end of the operation and slowly declining until less than pre-operative by 24h. Levels at 6h and 24h were statistically significantly decreased when compared to levels immediately following CPB, p<0.01 ANOVA with Bonferroni correction. The 2h time-point was chosen and levels assayed in a larger number of patients (Figure 53).

Figure 53: Concentration of sRAGE in plasma from patients pre and post-snCPB. Concentrations of sRAGE were measured in plasma collected from patients (n=39, cohorts B&C) pre- and 2h post-snCPB. Data are presented as paired symbols. Groups were compared using a Wilcoxon Matched Pairs Test; ***p<0.001
Levels of sRAGE in plasma statistically significantly increased from a median of 1116pg/ml (IQR 907-1853pg/ml) to 1908pg/ml (IQR 1189-2478pg/ml), p<0.001. Data are presented as paired pre- and post-snCPB values to demonstrate how the majority increment but a small number decline. In a larger number of patients (n=130, from cohort B) sRAGE was measured in paired pre- and post-snCPB plasma and similar results were seen with a median of 1054pg/ml (IQR 717-1760pg/ml) incrementing to 1897pg/ml (IQR 1129-2641pg/ml), p<0.001. Plasma samples from patients obtained pre-, post- and also intra- CPB were obtained (n=13, cohort B) and the measured levels of sRAGE are presented (Figure 54).

![Figure 54: Concentration of sRAGE in plasma from patients pre-, intra- and immediately post-snCPB.](image)

sRAGE levels were statistically significantly higher intra- (6177±4653pg/ml) than pre- (1749±1546pg/ml), p<0.001, and higher post- (4098±2778pg/ml) than pre, p<0.001.

11.4.2.2. *shed RAGE from esRAGE and sRAGE*

Plasma from 19 of the 39 patients with sRAGE measured (Figure 53, from cohort C) also had esRAGE measured and the difference between represents shed RAGE, these data are presented below (Figure 55).
Figure 55: Concentration of soluble RAGE forms in plasma from patients pre- and 2h post-snCPB. Concentrations of sRAGE and esRAGE were measured in plasma collected from patients (n=19, cohort C) pre-, and 2h post-snCPB. Shed RAGE levels were calculated from the difference between sRAGE and esRAGE. Data are presented as mean values with bars indicating SEM. paired t test ** p<0.01.

As discussed previously, sRAGE was statistically significantly increased, in contrast esRAGE was not, from 530±299pg/ml to 653±337pg/ml, p=0.06. Shed RAGE was statistically significantly increased, from 747±408pg/ml to 1133±400pg/ml, p<0.01. There was a tight correlation between shed RAGE and esRAGE both pre-operatively (Pearson’s r=0.90 p<0.0001) and post-operatively (Pearson’s r=0.90 p<0.0001).

11.4.2.3. Correlations

Levels of soluble RAGE forms were related to clinical characteristics and the positive findings, and important negatives, are shown below (Table 24). Unless otherwise specified, 39 patients were studied (Cohorts B&C).

<table>
<thead>
<tr>
<th>Correlation</th>
<th>Method</th>
<th>Correlation Coefficient</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-op sRAGE and age of patient</td>
<td>Spearman’s r=0.18</td>
<td>p=0.25</td>
<td></td>
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<tr>
<td>Pre-op sRAGE and Diabetes</td>
<td>MWU</td>
<td>p&gt;0.05</td>
<td></td>
</tr>
<tr>
<td>Pre-op sRAGE and Creatinine clearance</td>
<td>Spearman’s r=0.21</td>
<td>p=0.19</td>
<td></td>
</tr>
<tr>
<td>Pre-op sRAGE and ICU LOS</td>
<td>Spearman’s r=0.35</td>
<td>p=0.03*</td>
<td></td>
</tr>
<tr>
<td>Pre-op sRAGE and hospital LOS</td>
<td>Spearman’s r=0.25</td>
<td>p=0.12</td>
<td></td>
</tr>
<tr>
<td>Post-op sRAGE and post-op CML-albumin</td>
<td>Pearson’s r=0.73</td>
<td>p&lt;0.001*** (n=19, cohort C)</td>
<td></td>
</tr>
<tr>
<td>Delta sRAGE and PFR post-op</td>
<td>Pearson’s r=0.36</td>
<td>p=0.03*</td>
<td></td>
</tr>
</tbody>
</table>

Table 24: Correlations between plasma levels of sRAGE pre- and post-operatively and assays/clinical variables, n=39.
The lack of statistically significant association between the age of the patient and the levels of sRAGE (either pre- or post-) was also seen in a larger cohort (n=127 from cohort B, Spearman's r=0.21, p=0.01). A strong positive correlation was found between post-op levels of sRAGE and CML-albumin Pearson’s r=0.73, p<0.001, n=19 from cohort C.

The observation of a positive correlation between pre-op sRAGE and ICU LOS (Spearman’s r=0.35, p=0.03) was investigated further in a larger cohort (Section 11.4.3). The positive correlation between change in sRAGE levels (delta sRAGE) and the PaO₂:FiO₂ (PFR) Pearson’s r=0.36, p=0.03, was not replicated in the larger cohort (n=127, Spearman’s r=0.12 p=0.19).

### 11.4.2.4. Patient characteristics and assays

<table>
<thead>
<tr>
<th>Assay</th>
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<th>Without</th>
<th>P value (t test)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ±standard deviation, n</td>
<td>Mean ±standard deviation, n</td>
<td></td>
</tr>
<tr>
<td>Impaired LV function</td>
<td>Pre-op sRAGE</td>
<td>2618.4±1012.0, 5</td>
<td>1262.3±744.3, 34</td>
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<tr>
<td>Pulmonary hypertension</td>
<td>Pre-op esRAGE</td>
<td>820.6±317.5, 4</td>
<td>451.9±249.0, 15</td>
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<tr>
<td>Ischaemic heart Disease</td>
<td>Post-op esRAGE</td>
<td>477.9±260.8, 11</td>
<td>892.8±283.9, 8</td>
</tr>
</tbody>
</table>

Table 25: Patient characteristics and relationship to assay values. Impaired LV function and pulmonary hypertension were determined on pre-operative echocardiography. Ischemic heart disease when undergoing CABG or having had either CABG or percutaneous coronary intervention.

Levels of soluble RAGE forms were related to clinical characteristics and the positive findings are shown (Table 25). Patients with impaired left ventricular function (on pre-operative echocardiography) had statistically significantly elevated levels of sRAGE pre-operatively (p=0.01). Those with pulmonary hypertension (on pre-operative echocardiography) had statistically significantly elevated levels of esRAGE pre-operatively (p=0.02). Those with ischaemic heart disease (either presenting for, or having previously had coronary artery revascularisation) had statistically significantly diminished levels of esRAGE post-operatively.
Neither renal function as assessed by creatinine clearance nor pre-op use of HMG-CoA reductase inhibitors (statins) had a relationship with assay values.

11.4.3. Pre-op sRAGE and ICU LOS

The relationship between pre-op sRAGE and ICU LOS was further investigated patients from Cohort B with paired pre- and 2h post- CPB plasma (n=124 from 127, due to missing data in 3). In preference to using ICU LOS, duration of Level 3 care was used as a more specific indicator of severity of post-operative SIRS (Methods Section 6.5.1).

ICU LOS (hours) was longer than L3 care duration (hours) reflecting the extent to which non-clinical factors influence ICU LOS (Table 26).

<table>
<thead>
<tr>
<th>Measure</th>
<th>Mean</th>
<th>Median</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level 3 care (hours)</td>
<td>11h1m</td>
<td>7h59m</td>
</tr>
<tr>
<td>ICU LOS (hours)</td>
<td>22h11m</td>
<td>15h37m</td>
</tr>
<tr>
<td>Hospital LOS (days)</td>
<td>11.7d</td>
<td>9.0d</td>
</tr>
</tbody>
</table>

Table 26: Comparison of level 3 care to ICU LOS and hospital LOS. The mean value was greater than the median for all measures due to skewing effect of the uncommon patient with a very prolonged length of stay. N=124.

For the purposes of illustrating the relationship between sRAGE and L3 care, the cohort was split into quartiles of pre-op sRAGE and plotted against duration of L3 care (Figure 56).

Figure 56: Difference in duration of level 3 care between quartiles of pre-op plasma sRAGE. Plasma sRAGE was measured in 124 patients and patients were split into quartiles of sRAGE, corresponding duration of level 3 care charted. Data are presented as median values with bars indicating IQR. Groups were compared using the Kruskal Wallis Test with Dunn’s Multiple Comparison’s test, *p<0.05, ** p<0.01, *** p<0.001. n=124.
The correlations between sRAGE measurements and duration of stay (Table 27).

<table>
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<tr>
<th></th>
<th>N</th>
<th>Pre-op sRAGE</th>
<th>Post-op sRAGE (2h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duration of L3 care, hours (ICU)</td>
<td>124</td>
<td>r =0.3, p&lt;0.001*</td>
<td>r =0.2, p&lt;0.05</td>
</tr>
<tr>
<td>Hospital stay (days)</td>
<td>124</td>
<td>r =0.32, p&lt;0.001*</td>
<td>r =0.26, p&lt;0.05</td>
</tr>
</tbody>
</table>

Table 27: Assessment of correlation between sRAGE levels and duration of stay using Spearman’s rank correlation

To optimally assess the relationship between pre-op sRAGE and clinical outcomes binary logistic regression was performed. Clinical variables (or assays) and their statistical relationship to the outcome variable are tabulated in the following tables (28-33) with one outcome of interest per table. The variables assessed including age, duration of aortic cross-clamp time (ischaemic time), marker of renal function (Log creatinine), indicator of obesity (Log body mass index, BMI), sRAGE measurements (Log), S100A8/9 measurements (Log) and Euroscore (Log). Euroscore is a risk stratification score that relates to risk of death.
<table>
<thead>
<tr>
<th></th>
<th>No</th>
<th>Yes</th>
<th>Univariate analysis</th>
<th>p</th>
<th>Multivariate analysis</th>
<th>p</th>
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</thead>
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<tr>
<td></td>
<td>Mean(std)</td>
<td>Mean(std)</td>
<td>Exp(B) 95% CI</td>
<td></td>
<td>Exp(B) 95% CI</td>
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</tr>
<tr>
<td>Age</td>
<td>68.4 (12.1)</td>
<td>70.2 (8.6)</td>
<td>1.02 (0.98-1.05)</td>
<td>0.340</td>
<td>1.00 (0.99-1.02)</td>
<td>0.326</td>
</tr>
<tr>
<td>Ischaemic time</td>
<td>85.9 (29.1)</td>
<td>95.7 (31.1)</td>
<td>1.01 (1.00-1.02)</td>
<td><strong>0.072</strong></td>
<td>1.00 (0.99-1.02)</td>
<td>0.326</td>
</tr>
<tr>
<td>Log Creatinine</td>
<td>1.97 (0.1)</td>
<td>1.97 (0.1)</td>
<td>1.55 (0.83-29.0)</td>
<td>0.768</td>
<td>1.00 (0.99-1.02)</td>
<td>0.326</td>
</tr>
<tr>
<td>Log BMI</td>
<td>1.43 (0.1)</td>
<td>1.42 (0.1)</td>
<td>0.34 (0.00-26.55)</td>
<td>0.624</td>
<td>1.00 (0.99-1.02)</td>
<td>0.326</td>
</tr>
<tr>
<td>Log sRAGE pre-op</td>
<td>2.97 (0.3)</td>
<td>3.13 (0.3)</td>
<td>6.38 (1.78-22.90)</td>
<td><strong>0.004</strong></td>
<td>7.97 (1.13-56.39)</td>
<td><strong>0.038</strong></td>
</tr>
<tr>
<td>Log sRAGE post-op</td>
<td>3.20 (0.3)</td>
<td>3.30 (0.3)</td>
<td>3.38 (0.87-13.09)</td>
<td><strong>0.079</strong></td>
<td>0.43 (0.05-3.71)</td>
<td>0.443</td>
</tr>
<tr>
<td>Log S100A8/9 pre-op</td>
<td>2.67 (0.23)</td>
<td>2.70 (0.3)</td>
<td>1.63 (0.35-7.67)</td>
<td>0.534</td>
<td>1.00 (0.99-1.02)</td>
<td>0.326</td>
</tr>
<tr>
<td>Log S100A8/9 post-op</td>
<td>3.34 (0.2)</td>
<td>3.42 (0.2)</td>
<td>10.62 (1.42-79.50)</td>
<td><strong>0.021</strong></td>
<td>6.20 (0.75-51.0)</td>
<td>0.090</td>
</tr>
<tr>
<td>Log Euroscore</td>
<td>0.73 (0.2)</td>
<td>0.77 (0.2)</td>
<td>2.21 (0.45-10.80)</td>
<td>0.327</td>
<td>1.00 (0.99-1.02)</td>
<td>0.326</td>
</tr>
</tbody>
</table>

Table 28: Multivariate binary logistic regression considering relationship between clinical and assay results to longer duration of L3 care. Log sRAGE pre-op is the only statistically significant result. N=124.
<table>
<thead>
<tr>
<th></th>
<th>No</th>
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<th>p</th>
<th>Multivariate analysis</th>
<th>p</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Mean(std)</td>
<td>Mean(std)</td>
<td>Exp(B) 95% CI</td>
<td></td>
<td>Exp(B) 95% CI</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>68.2(11.3)</td>
<td>72.7(7.2)</td>
<td>1.05(1.00-1.09)</td>
<td>0.047</td>
<td>1.04(0.99-1.10)</td>
<td>0.141</td>
</tr>
<tr>
<td>Ischaemic time</td>
<td>89.6(30.5)</td>
<td>93.6(30.0)</td>
<td>1.00(0.99-1.01)</td>
<td>0.523</td>
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<td></td>
</tr>
<tr>
<td>Log Creatinine</td>
<td>1.97(0.11)</td>
<td>1.98(0.14)</td>
<td>1.22(0.04-39.1)</td>
<td>0.910</td>
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</tr>
<tr>
<td>Log BMI</td>
<td>1.43(0.08)</td>
<td>1.40(0.08)</td>
<td>0.18(0.00-3.80)</td>
<td>0.141</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Log sRAGE pre-op</td>
<td>3.02(0.28)</td>
<td>3.14(0.36)</td>
<td>3.92(0.94-16.40)</td>
<td>0.061</td>
<td>3.74(0.66-21.25)</td>
<td>0.136</td>
</tr>
<tr>
<td>Log sRAGE post-op</td>
<td>3.22(0.25)</td>
<td>3.31(0.30)</td>
<td>3.38(0.67-17.01)</td>
<td>0.140</td>
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<tr>
<td>Log S100A8/9 pre-op</td>
<td>2.65(0.25)</td>
<td>2.76(0.26)</td>
<td>6.35(0.90-45.00)</td>
<td>0.064</td>
<td>3.19(0.38-26.73)</td>
<td>0.284</td>
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<tr>
<td>Log S100A8/9 post-op</td>
<td>3.36(0.18)</td>
<td>3.45(0.20)</td>
<td>15.04(1.52-149.30)</td>
<td>0.021</td>
<td>5.67(0.34-95.40)</td>
<td>0.228</td>
</tr>
<tr>
<td>Log Euroscore</td>
<td>0.75(0.24)</td>
<td>0.74(0.24)</td>
<td>0.80(0.13-4.74)</td>
<td>0.802</td>
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</table>

Table 29: Multivariate binary logistic regression considering relationship between clinical and assay results to longest duration of L3 care. N=124.
<table>
<thead>
<tr>
<th></th>
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<th>Yes</th>
<th>Univariate analysis</th>
<th>p</th>
<th>Multivariate analysis</th>
<th>p</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Mean(std)</td>
<td>Mean(std)</td>
<td>Exp(B) 95% CI</td>
<td></td>
<td>Exp(B) 95% CI</td>
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</tr>
<tr>
<td>Age</td>
<td>67.7(11.7)</td>
<td>70.5(9.5)</td>
<td>1.03(0.99-1.06)</td>
<td>0.141</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ischaemic time</td>
<td>83.3(26.3)</td>
<td>96.7(32.2)</td>
<td>1.02(1.00-1.03)</td>
<td><strong>0.015</strong></td>
<td>1.01(0.99-1.03)</td>
<td>0.253</td>
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<tr>
<td>Log Creatinine</td>
<td>1.98(0.12)</td>
<td>1.96(0.11)</td>
<td>0.39(0.21-7.31)</td>
<td>0.530</td>
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<tr>
<td>Log BMI</td>
<td>1.43(0.08)</td>
<td>1.42(0.83)</td>
<td>0.62(0.01-48.2)</td>
<td>0.829</td>
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<tr>
<td>Log sRAGE pre-op</td>
<td>2.97(0.28)</td>
<td>3.12(0.30)</td>
<td>5.76(1.62-20.4)</td>
<td><strong>0.007</strong></td>
<td>5.09(0.52-49.9)</td>
<td>0.162</td>
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<tr>
<td>Log sRAGE post-op</td>
<td>3.18(0.27)</td>
<td>3.30(0.25)</td>
<td>6.69(1.64-27.3)</td>
<td><strong>0.008</strong></td>
<td>2.02(0.17-23.9)</td>
<td>0.576</td>
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<tr>
<td>Log S100A8/9 pre-op</td>
<td>2.63(0.22)</td>
<td>2.72(0.27)</td>
<td>4.93(0.95-25.7)</td>
<td><strong>0.058</strong></td>
<td>4.05(0.70-24.5)</td>
<td>0.128</td>
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<tr>
<td>Log S100A8/9 post-op</td>
<td>3.36(0.17)</td>
<td>3.40(0.19)</td>
<td>2.34(0.35-15.7)</td>
<td>0.380</td>
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<td>Log Euroscore</td>
<td>0.73(0.27)</td>
<td>0.76(0.20)</td>
<td>1.52(0.32-7.26)</td>
<td>0.601</td>
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</table>

Table 30: Multivariate binary logistic regression considering relationship between clinical and assay results to longer duration of hospital care. N=124.
<table>
<thead>
<tr>
<th></th>
<th>No</th>
<th>Yes</th>
<th>Univariate analysis</th>
<th>p</th>
<th>Multivariate analysis</th>
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<td></td>
<td>Mean(std)</td>
<td>Mean(std)</td>
<td>Exp(B) 95% CI</td>
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<td>Exp(B) 95% CI</td>
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<td>Age</td>
<td>68.4(11.4)</td>
<td>71.3(8.3)</td>
<td>1.03(0.99-1.07)</td>
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<tr>
<td>Ischaemic time</td>
<td>85.1(25.5)</td>
<td>103.8(36.8)</td>
<td>1.02(1.01-1.03)</td>
<td><strong>0.003</strong></td>
<td>1.01(0.99-1.0)</td>
<td>0.186</td>
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<td>Log Creatinine</td>
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<td>1.97(0.12)</td>
<td>0.95(0.38-23.9)</td>
<td>0.976</td>
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<tr>
<td>Log BMI</td>
<td>1.43(0.08)</td>
<td>1.41(0.08)</td>
<td>0.11(0.00-14.1)</td>
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<td>Log sRAGE pre-op</td>
<td>3.00(0.29)</td>
<td>3.16(0.29)</td>
<td>6.61(1.64-26.6)</td>
<td><strong>0.008</strong></td>
<td>18.9(1.22-392.0)</td>
<td><strong>0.035</strong></td>
</tr>
<tr>
<td>Log sRAGE post-op</td>
<td>3.22(0.26)</td>
<td>3.31(0.26)</td>
<td>4.16(0.92-18.9)</td>
<td><strong>0.065</strong></td>
<td>1.3(0.06-27.74)</td>
<td>0.865</td>
</tr>
<tr>
<td>Log S100A8/9 pre-op</td>
<td>2.65(0.22)</td>
<td>2.74(0.31)</td>
<td>4.71(0.76-29.4)</td>
<td><strong>0.097</strong></td>
<td>1.8(0.21-14.32)</td>
<td>0.601</td>
</tr>
<tr>
<td>Log S100A8/9 post-op</td>
<td>3.35(0.17)</td>
<td>3.45(0.19)</td>
<td>19.6(2.16-177.9)</td>
<td><strong>0.008</strong></td>
<td>11.9(0.60-237.2)</td>
<td>0.105</td>
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<tr>
<td>Log Euroscore</td>
<td>0.75(0.25)</td>
<td>0.75(0.19)</td>
<td>1.04(0.19-5.62)</td>
<td>0.965</td>
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</table>

Table 31: Multivariate binary logistic regression considering relationship between clinical and assay results to longest duration of hospital care. Log sRAGE pre-op is the only statistically significant result. N=124.
<table>
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<th>Variable</th>
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<th>Multivariate analysis Exp(B) 95% CI</th>
<th>p</th>
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<tr>
<td>Age</td>
<td>67.9(12.1)</td>
<td>70.8(8.6)</td>
<td>1.03(0.99-1.06)</td>
<td>0.124</td>
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<tr>
<td>Ischaemic time</td>
<td>89.0(27.6)</td>
<td>92.3(33.2)</td>
<td>1.00(0.99-1.02)</td>
<td>0.549</td>
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<tr>
<td>Log Creatinine</td>
<td>1.98(0.11)</td>
<td>1.96(0.12)</td>
<td>0.21(0.01-4.05)</td>
<td>0.299</td>
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<tr>
<td>Log BMI</td>
<td>1.42(0.08)</td>
<td>1.43(0.08)</td>
<td>1.41(0.18-109.9)</td>
<td>0.876</td>
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<tr>
<td>Log sRAGE pre-op</td>
<td>3.01(0.28)</td>
<td>3.08(0.32)</td>
<td>2.15(0.66-7.01)</td>
<td>0.204</td>
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<tr>
<td>Log sRAGE post-op</td>
<td>3.22(0.26)</td>
<td>3.27(0.27)</td>
<td>2.33(0.62-8.82)</td>
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<tr>
<td>Log S100A8/9 pre-op</td>
<td>2.66(0.25)</td>
<td>2.69(0.26)</td>
<td>1.49(0.32-6.95)</td>
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<td>Log S100A8/9 post-op</td>
<td>3.39(0.21)</td>
<td>3.37(0.16)</td>
<td>0.67(0.10-4.36)</td>
<td>0.670</td>
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<tr>
<td>Log Euroscore</td>
<td>0.74(0.24)</td>
<td>0.76(0.23)</td>
<td>1.31(0.28-6.22)</td>
<td>0.735</td>
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</table>

Table 32: Multivariate binary logistic regression considering relationship between clinical and assay results to worse PFR post-operatively. N=124.
### PFR Worst (1st Quartile)

<table>
<thead>
<tr>
<th></th>
<th>No Mean(std)</th>
<th>Yes Mean(std)</th>
<th>Univariate analysis Exp(B) 95% CI</th>
<th>p</th>
<th>Multivariate analysis Exp(B) 95% CI</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td>69.1(11.3)</td>
<td>69.7(8.8)</td>
<td>1.01(0.97-1.04)</td>
<td>0.771</td>
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<tr>
<td><strong>Ischaemic time</strong></td>
<td>88.9(28.2)</td>
<td>94.8(35.5)</td>
<td>1.01(0.99-1.02)</td>
<td>0.334</td>
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</tr>
<tr>
<td><strong>Log Creatinine</strong></td>
<td>1.97(0.12)</td>
<td>1.98(0.12)</td>
<td>2.23(0.09-58.6)</td>
<td>0.630</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Log BMI</strong></td>
<td>1.42(0.07)</td>
<td>1.44(0.09)</td>
<td>14.87(0.11-2048)</td>
<td>0.283</td>
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<tr>
<td><strong>Log sRAGE pre-op</strong></td>
<td>3.04(0.28)</td>
<td>3.07(0.36)</td>
<td>1.46(0.39-5.43)</td>
<td>0.575</td>
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<tr>
<td><strong>Log sRAGE post-op</strong></td>
<td>3.25(0.26)</td>
<td>3.23(0.29)</td>
<td>0.85(0.20-3.71)</td>
<td>0.833</td>
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<tr>
<td><strong>Log S100A8/9 pre-op</strong></td>
<td>2.68(0.25)</td>
<td>2.68(0.27)</td>
<td>0.98(0.18-5.56)</td>
<td>0.980</td>
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<tr>
<td><strong>Log S100A8/9 post-op</strong></td>
<td>3.37(0.20)</td>
<td>3.40(0.15)</td>
<td>2.64(0.32-21.5)</td>
<td>2.643</td>
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<tr>
<td><strong>Log Euroscore</strong></td>
<td>0.74(0.23)</td>
<td>0.77(0.24)</td>
<td>1.81(0.30-10.8)</td>
<td>0.518</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 33: Multivariate binary logistic regression considering relationship between clinical and assay results to worst PFR post-operatively. N=124.
Multivariate binary logistic regression analyses demonstrated that levels of pre-operative sRAGE (transformed to $\log_{10}$) were independently associated with an ICU LOS of greater than median (odds ratio of 7.97 with 95% confidence intervals of 1.13-56.39, >19.1 hours, "ICU_LONGER") and with being in the 4th quartile of hospital LOS (odds ratio of 18.9 with 95% confidence intervals of 1.22-392.0, >13 days, "HOSP_LONGEST"). The PFR calculated from $\text{PaO}_2$ and $\text{FiO}_2$ in the first 24h immediately following snCPB did not relate to any of the considered characteristics.

11.4.4. Inflammatory markers and snCPB

11.4.4.1. CRP

C-reactive protein is routinely measured in ICU patients. Levels measured in 39 patients (from cohorts B&C) at 24h were 58 (41-92) mg/L, and 48h were 173 (132-216) mg/L, p<0.001 (Figure 57).

![Figure 57: Levels of CRP in patients at 24h and 48h following snCPB. Plasma CRP was measured in 39 patients (cohorts B&C). Data are presented as median values with bars indicating IQR. Groups were compared using the Wilcoxon Matched Pairs test, *** p<0.001](image)
11.5. Discussion

11.5.1. RAGE ligands

All measured RAGE ligands statistically significantly increased and the extent of increase of S100A12, S100A8/9 and HMGB1 was in proportion to the duration of cardiopulmonary bypass. Different S100 proteins appeared to show different temporal release patterns with S100B levels falling sharply after their initial increase in comparison to S100A8/9 and S100A12 levels that stayed elevated.

S100B plasma levels have been extensively studied as a marker of injury to the blood-brain barrier due to snCPB and in this context have been repeatedly reported to be elevated post-CPB [662] with levels correlating to duration of CPB in some studies [666]. As S100B is released from many injured tissues and there have been inconsistent associations between measured levels and neuropsychological outcomes, its use as a cerebral-injury biomarker remains controversial [667-669]. S100B has been found to be elevated in the plasma of the critically ill and a positive association with plasma lactate levels (and SOFA score) has led to the hypothesis that tissue hypoperfusion may result in S100B release [670]. Traumatic injury and non-cardiac operative surgery have been associated with rapid and short-lived increases in plasma S100B levels [466].

S100A12 plasma levels around snCPB have not been previously reported but an increase in gene expression for S100A12 (and S100A8) in circulating leukocytes has been demonstrated 4h following CPB [671]. S100A8/9 has been demonstrated to be elevated following snCPB in several small studies [663-665]. However, the strong positive associations between duration of CPB and levels of post-operative S100A8/9 and S100A12 are not previously described and may reflect a number of factors. Release of S100A12 could plausibly be due to cardiopulmonary ischaemia, systemic hypoperfusion and consequent reperfusion injury, or duration of CPB as an indirect marker of surgical trauma. The inter-relationship between S100A12 and S100A8/9 may indicate a shared aetiology of release, perhaps release from neutrophils, in contrast to S100B levels which may solely source from injured tissues. The correlation between S100A12 levels pre-op and the age of the patient has not been previously described and is weak and may be artefactual. S100A8/9 was higher in those
patients with diabetes and although this has not been previously reported, increased levels of S100A12 have been [344].

HMGB1 has been demonstrated to be released by leukocytes in response to surgery with maximal release at 24h [383], in contrast to victims of polytrauma with peak levels at 2-4h [400]. These different temporal relationships may reflect the two modes of release. The finding of elevated levels of HMGB1 and their positive correlation to duration of CPB is novel and is most likely to indicate release from tissue damaged during snCPB. HMGB1 measurements from six patients over a more prolonged time showed a possible second peak of release that may reflect immune signalling. Levels of HMGB1 measured in six patients around snCPB have very recently been reported by others in abstract form; elevations following surgery were also found [661]. In a study of 20 patients undergoing elective thoracic aortic aneurysm repair (requiring some form of cardiopulmonary bypass in all cases), HMGB1 levels peaked at day two and remained elevated; levels did not correlate with duration of CPB but a greater peak HMGB1 elevation was associated with more severe SIRS and a higher incidence of impaired oxygenation [458].

Acute AGE formation is facilitated by oxidative stress, acute hyperglycaemia and the actions of neutrophil myeloperoxidase, all conditions present during snCPB. Elevation in levels CML-albumin is not previously described in relation to snCPB.

In contrast to some published reports we were unable to detect any endotoxin within the plasma of our patients either pre-, during- or post- CPB. Severity of illness scores variably correlated with duration of stay outcomes, as did the levels of CRP at 48h.

**11.5.2. Soluble RAGE**

Pre-operative levels of sRAGE did not differ between patients with diabetes than those without, neither did it relate to their age nor renal function. However, of the 39 patients, only 5 had diabetes. There were large variations in pre-snCPB sRAGE levels and this may be partially explained by the range of conditions within the cohort: ischaemic heart disease requiring revascularisation and/or valvular heart disease requiring repair or replacement. Those undergoing valve surgeries had higher sRAGE levels and may have been more likely to have a degree of heart failure than those having coronary revascularisation. Although
clinical measures of heart failure were not assessed, plasma sRAGE levels (and HMGB1) have been reported to be elevated in those with heart failure [672, 673].

The levels of pre-operative sRAGE correlated with the duration of ICU stay and this was investigated further in a larger cohort of patients (n=124). Binary logistic regression revealed pre-op sRAGE was independently related to longer L3 care time and longest hospital stay. In preference to using ICU LOS we calculated the duration of Level 3 care, as a more specific indicator of severity of post-operative SIRS. Level 3 care duration was always shorter than ICU LOS. Levels of plasma sRAGE measured prior to surgery were more closely related to prolonged recovery from acute systemic inflammation (duration of level 3 care) and from overall hospital admission (time to hospital discharge) than variables including the age of the patient, a score of their operative risk (Euroscore), and the duration of pulmonary/myocardial ischaemic time (component of total CPB time). This striking finding can be explained if plasma sRAGE levels reflect tissue RAGE levels and therefore predisposition to excessive RAGE-mediated systemic inflammation following systemic inflammatory stimulus (snCPB). The systemic inflammation resulting in organ dysfunction or failure necessitating organ support is reflected in duration of level 3 care.

sRAGE levels statistically significantly increased during CPB and 2h post-CPB compared to pre-op levels. esRAGE levels also increased but did not reach statistical significance. Shed RAGE was very highly correlated with esRAGE. Elevation of sRAGE levels following snCPB had not been described until very recently [457]. During CPB, the pulmonary circulation is not receiving a flow of blood and the marked elevation of sRAGE levels at this time would be consistent with sRAGE sourcing from the systemic circulation. This is of particular interest as many authorities propose that circulating sRAGE predominantly sources from the pulmonary alveolar epithelial cells. During CPB the bronchial circulation continues to receive a variable supply of blood [674] and considering that it may anastamose as distal as the terminal bronchioles [675], it may make a small contribution to drainage of the alveolar epithelium. To what extent the bronchial circulation may be responsible is hard to know. This finding has not been previously described.

Levels of sRAGE and esRAGE were tightly correlated at all times and in all patients, either suggesting a commonality of regulation, contrary to accepted dogma, or suggesting a lack of
specificity of the enzyme immunoassays. In a large cohort of patients with chronic kidney disease, levels of sRAGE and esRAGE have been demonstrated to correlate very closely [266]. An absence of difference between antibodies directed towards the extracellular V domain of human RAGE (sRAGE) and the C-terminal (esRAGE) has been previously observed [304]. Assuming differential regulation of esRAGE and shed RAGE (the difference between sRAGE assay and esRAGE assay) then the increase in esRAGE being modest (25%) and the increase in shed RAGE more substantial (51%) suggests that the contribution of esRAGE to ligand quenching is minor in comparison to that of RAGE shed from plasma membranes.

The strongly positive correlation between post-op sRAGE and CML-albumin is consistent with the concept of RAGE ligands stimulating cleavage of sRAGE. This correlation has also been found in other studies [262].

The positive association between the change in sRAGE (delta sRAGE) and the PaO2:FiO2 ratio (PFR) immediately post-operatively seen in the cohort of 39 patients lost statistical significance when analysed in the larger cohort. Pulmonary dysfunction following snCPB is both common and multi-factorial, and although subclinical ALI is common, clinically evident ALI is not. The lack of relationship between initial post-operative PaO2:FiO2 ratio and sRAGE level is therefore not surprising.

An important limitation to these investigations is the absence of standardisation of peri-operative care. In common with the majority of similar observational cohort studies, the specific management by the anaesthetist, perfusionist and cardiac surgeon was not protocolised and thus was likely to differ between patients. The anaesthetic induction agent, neuromuscular blocking agent, choice of maintenance agent, and fluid resuscitation strategy all have potential to modulate systemic inflammatory responses. Given the purported role of sRAGE as a marker of alveolar injury and the potential for intra-operative ventilation to contribute to the incidence of ALI post-operatively, it would have been preferable to have a uniformly applied ventilatory strategy. Important variables pertaining to the role of the perfusionist and the extra-corporeal circuit include the choice of priming solution. This may influence systemic inflammatory responses and particularly the content of plasma AGE in those who received albumin (approximately half of those in which AGE was measured, cohort C). Furthermore, the degree of intra-CPB hypothermia and the
manner in which it was reversed may also be of relevance. Finally, the maintenance medications taken by the patients prior to snCPB (such as aspirin) certainly have the potential to influence inflammatory response and the absence of information about all pre-operative medication represents a shortcoming of this study.

There are limitations to the application of simple statistic correlations between clinical and biochemical parameters. The greater the number of comparisons between variables, the greater the risk of finding positive associations though chance alone. A correlation may be highly statistically significant but only be weak in extent; there is no consensus on what level of correlation is considered ‘strong’ or ‘weak’.
11.6. Conclusion

We have demonstrated substantial increases in circulating levels of the five best-characterised ligands for RAGE, with a strong relationship between severity of surgical injury (duration of CPB) and extent of S100 protein release; elevations in levels of circulating soluble RAGE forms, maximal during surgery; and a robust relationship between preoperative levels of sRAGE and duration of requirement of intensive care.

Plasma sRAGE deserves further study as a biomarker for systemic inflammatory response to tissue injury and as such, could offer superior risk stratification of patients presenting for major elective surgery than current methods. Plasma sRAGE likely reflects shedding from many cell types, possibly including leukocytes. Thus, measuring membrane-bound RAGE on circulating leukocytes will compliment these findings.
12. LEUKOCYTE RAGE EXPRESSION AROUND SNCPB

12.1. Introduction

In the previous chapter it was shown that levels of plasma sRAGE increase during and following snCPB. It was also shown that levels of RAGE ligands increase in the plasma, some in proportion to the duration of snCPB (Section 11.4.1). The cellular origin of plasma shed RAGE in humans has not, previously, been described. One possible source is leukocytes, with elevated levels of RAGE ligands in plasma causing up-regulation of leukocyte RAGE and subsequent shedding of the RAGE ectodomain. Levels of plasma sRAGE were independently related to prolonged duration of critical illness and therefore show promise as a biomarker for systemic inflammatory response to tissue injury (Section 11.4.2). Membrane-bound RAGE on circulating leukocytes potentially represents a complimentary assay of pro-inflammatory RAGE-axis activity and may therefore be a superior biomarker.

Relationship between RAGE expression on circulating leukocytes and concentration of RAGE ligands in chronic conditions has been described previously. Thus, in patients with diabetes, monocyte RAGE expression correlated with serum AGE; similarly, in patients with chronic kidney disease, monocyte RAGE expression correlated with serum AGE [296, 606]. RAGE expression on neutrophils obtained from patients with exacerbations of cystic fibrosis was assessed using flow cytometry and was increased in the airway in comparison to the circulation [463], but no other studies have reported leukocyte RAGE expression in acute inflammation. Measurement of RAGE at the mRNA level has not, previously, been reported in human primary leukocytes.

By contrast, RAGE expression has been studied in THP-1 cells, a monocyte cell line derived from peripheral blood of a 1 year-old patient with acute monocytic leukaemia [676]. Such immortalised cells retain some characteristics of their cellular origin (monocytes) but may lose or gain features related to their neoplastic transformation. At the time of starting these experiments, it had been shown by others that RAGE mRNA was present in THP-1 cells and RAGE protein levels on these cells were increased in response to AGE, an effect blocked by anti-RAGE antibodies [298]. Furthermore, AGE up-regulated NF-κB signalling and pro-inflammatory cytokine secretion, an effect blocked by competitive antagonists [677]. RAGE protein had not been identified on the cell surface of THP-1 cells but had been on
monocytes and tissue macrophages [678-680]. It was later observed that expression of RAGE protein on the cell surface was limited [250] and RAGE expressed on the plasma membrane was predominantly shed. Most recently, further evidence of an intact RAGE pathway has been observed in THP-1 cells: with S100B causing pro-inflammatory cytokine release [681] and signalling events triggered by AGEs and blocked with anti-RAGE antibody [682] or small interfering RNA to RAGE [683].

The very significant disadvantage of experimenting with immortalised cell lines such as THP-1 cells is that the findings may not be replicated in the equivalent human primary cells. Thus, alteration in RAGE-mediated signalling events and in surface RAGE expression that are demonstrated in THP-1 cells may not be reproducible in human primary monocytes.

Measuring changes in abundance of receptors and other biomolecules on the cell surface is a useful research tool and flow cytometric determination of leukocyte membrane expression is being developed for diagnostic and prognostic purposes (Section 1.3.4). Moreover, in some cases, measures obtained from membrane expression profiles are superior to plasma assays, for example the ratio of expression of monocyte membrane-bound TNF-α protein to its receptor TNFR was shown to correlate with scores of organ dysfunction better than plasma levels in ICU patients [684].

With regard to the RAGE axis, others have shown that neutrophil RAGE expression can be detected by flow cytometry [463]. Similarly, other putative RAGE ligand receptors such as TLR-4 and TLR-9 have been studied in other patient cohorts [685, 686]. However, neither RAGE nor TLR-9 have been quantitated in leukocytes pre- and post-snCPB. Alterations in the expression of the LPS receptor TLR-4 during surgery have been reported with inconsistent results [200, 685, 687, 688]. TLR4 receptor down-regulation during snCPB could be an important contributory factor to endotoxin tolerance. Toll-like receptor 9 (TLR-9) is an intracellular receptor for DNA and as such plays a role in detection of ‘foreign’ bacterial and viral DNA, but also responds to cellular DNA. The response to cellular DNA may be important in detection of damage to tissues in the host organism but has also been implicated in auto-immune diseases such as systemic lupus erythematosus. In this context, interaction between HMGB1 and DNA, RAGE and TLR-9 responses have been shown to augment immune signalling in antigen presenting cells [388]. Release of HMGB1 into the
circulation during snCPB has been demonstrated (Section 11.4.1) and tissue damage causing release of cellular DNA into the circulation is likely.

### 12.2. Aims and objectives

To develop flow cytometry (FC) and qRT-PCR assays to facilitate subsequent analysis of cellular RAGE before and after snCPB at both the protein and transcript levels.

Specific objectives:

i. To develop a FC assay of cell surface RAGE expression on leukocytes in whole blood.

ii. To develop and optimise quantitative reverse transcriptase real-time PCR measure of RAGE expression relative to stable reference genes.

iii. To obtain blood from patients around the time of snCPB and perform FC measurements for RAGE, TLR-4 and TLR-9; to extract mRNA from leukocytes (either as buffy coat or isolated subsets).
12.3. Specific methods

12.3.1. Protocol
Patients presenting for snCPB at the Royal Brompton Hospital were approached and informed consent was obtained on the day prior to surgery. Samples were obtained from patients in Cohorts B, as defined in the methods section. Blood was obtained (pre-op) within 10m of induction of anaesthesia and initiation of invasive ventilation. The sample was split into small aliquots (3ml) for FC and a large aliquot (30ml) for leukocyte preparation. The samples were processed for flow cytometry and mRNA extraction as per Methods (Sections 7.5.2 and 7.9.1). Following cessation of CPB, blood was obtained similarly (post-CPB) and within 30 mins after cessation of CPB. On the first post-operative day, the cells prepared for flow cytometry were washed and re-suspended prior to reading on the cytometer.

12.3.2. Flow cytometry
General principles of flow cytometry are discussed in Section 7.5. The development of the specific protocol used to stain whole blood for surface and intracellular RAGE follows in Section 12.4.1 and the complete protocol is presented in Section 7.5.2.

In brief, whole blood was obtained and the cellular constituents washed in an excess of PBS (with 0.5% EDTA). The cells were re-suspended in PBS to the original volume and 0.5µl of each undiluted antibody added to each 50µl of cell suspension; incubation was for 20 mins at 4°C in the dark. For each condition, one pair of samples was stained with the RAGE conjugated antibody and one pair without; permitting comparison between stained and the so-called FMO which will have no specific staining. Staining conditions were performed in duplicate and results averaged to account for variability in the assay. All samples were stained with the following markers: CD14, CD16 and the Live/Dead™ green assay, thereby permitting identification of leukocyte subsets and exclusion of dead cell events. Following staining, cells were washed in PBS prior to addition of excess a fix/lyse buffer that caused hypotonic lysis of erythrocytes and fixation that stabilised the staining and allowed acquisition at a later time. For intracellular staining the cells were then washed and permeabilized prior to a second round of staining, for those that were not for further staining they were washed and stored at 4°C in the dark until acquisition.
12.3.3. qRT-PCR

General principles of qRT-PCR are discussed in Section 7.9.3. The development of the specific protocol used follows in Section 12.5.1 and the complete protocol is presented in Section 7.9.4.

In brief, the whole blood was either processed to isolate leukocyte subsets or minimally processed to isolate the buffy coat. The buffy coat is the total leukocyte population, identified at the interface between plasma and erythrocytes following density gradient centrifugation. The cellular suspensions were mixed in a 1:3 ratio with TRI reagent, following vigorous mixing, were stored at -80°C. At a later time, samples were thawed and the further stages of choloform-phenol extraction (Section 7.9.1) resulted in a small volume of RNA dissolved in water. Following measurement of RNA concentration, 0.6µg of RNA was reverse transcribed resulting in cDNA formation (Section 7.9.2). Using primers specific for RAGE and the reference genes HPRT1, RPL13 and GUSB, qRT-PCR reactions were conducted. Data analysis took place using Rotor-gene 6000 (Corbett) and using the delta-delta-CT formula in Excel (Microsoft, Redmon, USA).
12.4. Results of flow cytometry

12.4.1. Development of flow cytometry protocol

12.4.1.1. Indirect staining

Antibodies directed against human RAGE with a directly conjugated fluorochrome were not available; therefore an indirect staining protocol was adapted from manufacturer’s protocols. Indirect staining uses an unconjugated primary antibody directly against the epitope of choice (RAGE) and following a period to allow antibody capture, the excess antibody is washed off and then a secondary antibody added (Figure 58). The secondary antibody is usually directly against the Fc portion of IgG in the animal species in which the primary antibody was raised. The theoretical advantage of this method is increased sensitivity as each primary can be bound by several secondary antibodies thus amplifying the signal. However, the major disadvantage is the increased background, non-specific, staining. A goat anti-RAGE antibody from Abcam (Cambridge, UK) was used with an anti-goat IgG-FITC conjugate.

![Diagram: Indirect antibody staining]

Figure 58: Indirect antibody staining

To determine the extent to which staining is specific for the epitope of interest it is common to compare the staining with a negative control. For an indirect protocol the negative control could be staining due to a primary isotype control (with secondary) or to secondary only. An isotype antibody is directed against an irrelevant epitope and any binding should be non-specific. A secondary antibody in the absence of any primary should result in very little staining (i.e. an Anti-goat IgG antibody will not bind to any part of a human monocyte).
Important variables within a staining protocol include the non-specific blocking content of the wash buffer (and/or a discrete blocking step), the conditions of the staining steps, the order of fixing/staining, the option of fixing the cells and at what conditions.

### 12.4.1.2. Experiments to determine optimal antibody concentration

THP-1 cells (2.5 ×10^5) were subjected to experimental conditions and then stained for RAGE prior to analysis (staining protocol 1, Figure 59). Experimental conditions included LPS (400ng/ml) or HMGB1 (400ng/ml) for 18 hours – both intended as positive controls.

Four different concentrations of primary antibody and three different concentrations of secondary antibody were used (Figure 60). The cells were not fixed and were analysed soon after the final wash stage.

![Experimental protocol diagram](image)

**Figure 59: Experimental protocol.** Cells were resuspended in medium before experimental conditions, which took place in a CO₂ incubator at 37°C. Fixing was in 1% paraformaldehyde and either took place directly after experimental conditions or prior to analysis. Cells were resuspended in PBS with 1% BSA until the final resuspension prior to analysis which was in FACSflow™ solution.
Figure 60: Fluorescence intensity (MFI) charted against increasing concentrations of primary antibody (x axis) and secondary antibody (different plots, 0, 2, 5 10 μg/ml). THP-1 cells were stimulated with LPS (400ng/ml) prior to staining for FC.

12.4.1.3. Experiments to determine specificity of antibody

Despite differing experimental conditions (to maximise RAGE expression) and many modifications to the staining protocol, the extent of staining obtained with the isotype control (IC) antibodies was indistinguishable from the RAGE-specific antibodies. Staining for both was greater than that for the secondary antibody alone. Conditions used to maximise RAGE staining may have been suboptimal but even in the absence of stimulation, RAGE should be detectable as basal levels.

To minimise background staining, particularly when working with cells that express immunoglobulin receptors (such as THP-1 [689]) it is important to block non-specific binding. 1% bovine serum albumin in the phosphate buffered saline was being used but may have been inadequate. Normal human serum (the cells were human) or goat serum (the secondary antibody was a goat antibody) were tried without improvement.

Failure to detect specific RAGE staining on the THP-1 cells was attributed to the lack of sufficient specificity for RAGE of the Abcam antibody and the possibility that THP-1 cells do not express RAGE on their cell surface. A presentation at conference (3rd International DAMPS, Endokines and Alarmins symposium, Pittsburgh, 2008) and publication [250] suggested that THP-1 cell surface expression of RAGE was limited.
**12.4.1.4. Whole blood staining protocols**

Whole blood (WB), offers a more analogous system to the *in vivo* state and is readily obtainable from healthy volunteers. A schematic demonstrating the WB staining protocol is shown (Figure 61). Cells can be fixed prior to primary staining or after primary staining. The fixing solution also lyses erythrocytes. An alternative primary antibody (rabbit anti-RAGE, Millipore/Fisher) was used.

![Diagram](image)

**Figure 61: Revised experimental protocol.** 100μl of whole blood plated out. Experimental conditions took place in a CO₂ incubator at 37°C. Fixing was in BD FACSLyse solution and either took place directly after experimental conditions or after primary antibody staining. Cells were resuspended in PBS with 1% BSA until the final resuspension prior to analysis which was in FACSflow™ solution.

Additional changes included S100A12 (500ng/ml) to up-regulate RAGE and blocking with 50% pooled normal human serum/matched donor serum. Pooled human immunoglobulin was not attempted as the IgG content of pooled serum was greater.

Despite these modifications, the signal attributable to membrane RAGE was almost indistinguishable when primary antibody was compared to IC. Furthermore, there was evidence of increased fluorescence in the absence of primary or isotype control antibody, possibly due to neutrophils binding the secondary antibody. On isolated occasions the results from the flow cytometric assay were as intended, Figure 62. However this result was not achieved consistently.
In the absence of any primary antibody there should be minimal fluorescence (green line); in the presence of an isotype control primary and a fluorescent secondary there should be background staining (blue); with primary antibodies present and unstimulated cells there should be increased fluorescence (purple); and finally with primary antibodies present and stimulated cells there should be maximal fluorescence (red).

To circumvent the background staining, an alternative to indirect staining protocols was found – antibody labelling technology. An IgG labelling kit (Invitrogen Zenon™ labelling kit with Alexa-fluor 488) was used: the anti-RAGE primary is incubated with a fluorophore-labelled Fc-specific anti-IgG Fab fragment which binds the Fc portion of the primary antibody. Excess binding fragment is neutralised by addition of excess IgG then the primary antibody-conjugate is ready for use.

Additional changes included the use of a cell marker antibody to improve discrimination of neutrophils events from non-neutrophil events. Selecting neutrophils based on expression of characteristic markers is superior to using purely physical characteristics as assessed by forward scatter/side scatter. An anti-CD16 fluorochrome-conjugated antibody was purchased. A flow cytometry facility with improved infrastructure and support was used. To distinguish flow cytometry events related to intact cells from cell remnants, cell viability assays were planned.

However, the assay did not achieve sufficiently reproducible quantitation of membrane RAGE expression. Work from investigators at the Herzenberg Laboratories, Stanford University was published at this time and included the first description of a flow cytometric assay for membrane RAGE performed on whole blood [463] (Figure 63). A visit to the lab was arranged and following the trip a protocol was developed (Section 7.5.2).
Figure 63: Histograms of RAGE expression on neutrophils from 3 representative patients with inflammatory airways disease (filled black airway neutrophils, filled grey circulating neutrophils), compared to unstained control (unfilled). Image adapted from paper by Stanford group ([463]).

12.4.1.5. Stanford protocol
The protocol includes membrane staining and intracellular staining; to stain proteins within the cell the membrane must be permeabilized using saponin. A stage was introduced prior to the intracellular staining whereby the cells are fixed and the erythrocytes lysed. To improve discrimination of leukocyte subsets a large array of cluster differentiation (CD) markers are available. CD16 is characteristically on neutrophils and CD14 on monocytes. A cell viability marker that is unaffected by the fixation process was used (Invitrogen Live/Dead). Bead controls were introduced to help calibrate the flow cytometer to improve intra-assay comparability. To avoid the problems of background staining a protein conjugation kit was used (Innova Lightning link™) with a well characterised anti-RAGE antibody (Santa Cruz). This allowed a single staining stage. Lastly, whole blood was used but the non-cellular components were washed away prior to staining and the cells re-suspended in cold PBS.

12.4.1.6. Reporting fluorescence
When reporting the fluorescence intensity for an experimental sample it is standard practice to express in relation to a control sample staining. In order of increasing complexity: the fluorescence intensity can be used without comparator; the fluorescence intensity can be compared between the stained sample and the unstained, where the unstained could be either the secondary antibody alone or a sample without antibody in the fluorescent
channel of interest (fluorescence minus one, FMO); the fluorescence intensity can be compared between the stained sample and an identically treated sample apart from the substitution of an isotype control antibody for the primary antibody. There is no consensus within the flow cytometry community and the details are beyond the scope of this thesis (readers referred to reviews [690, 691]).

Within this thesis all reported measure of extent of staining are given as the mean fluorescent intensity (MFI) and relate to the difference in MFI between the sample with relevant antibody present and the FMO; all measures were taken in duplicate and averaged.

12.4.1.7. Gating strategy

Acquisition of flow cytometry data provides a huge amount of information that needs to be logically represented to extract the relevant information. This may be achieved through successive ‘gating’ populations of events until the events of interest can be measured in isolation. In this case we wish to select live (L/D low) neutrophils (CD16+CD14-), Figure 64 for details.
Figure 64: Gating strategy for selective consideration of live neutrophils. Dead cells were not considered by excluding events that were either excessively small (low forward scatter) or had excessive staining in the FL1 channel that corresponded to high staining for the live/dead marker [as dead cells lack of membrane integrity and bind more of the marker (top left)]. The second gate shown (top right) was around neutrophils which had high values in the Pacific Blue channel, corresponding to the CD16 marker, and low values in the PE-Cy7 channel, corresponding to the CD14 marker. The histogram shown (bottom left) represents the range of fluorescence intensities in the gated population, it is parametrically distributed and can be expressed as a mean fluorescence intensity (MFI) which appears to be $10^3$, or 1000.
12.4.2. RAGE protein expression on leukocytes around snCPB

The extent of RAGE expression upon the surface and within circulating neutrophils and monocytes was compared (Figure 65).

12.4.2.1. Differences in RAGE expression between leukocyte populations

![Figure 65: Expression of RAGE on surface (left) and intracellular (right) of neutrophils and monocytes from blood obtained pre-operatively. Blood was obtained pre-operatively (n=8) and stained for RAGE using a standardized protocol. Extent of staining is shown as the mean fluorescence intensity (MFI). Data are presented as mean with bars indicating SEM. Unpaired t tests, p>0.05 for both.]

The extent of monocyte surface RAGE expression (mean MFI 2296±2463) was not significantly different from that on neutrophils (mean MFI 501±232; p=0.06). Likewise, intracellular monocyte RAGE expression (mean MFI 8533±3774) was not significantly different from that in neutrophils (mean MFI 12480±5144; p>0.05), as seen in Figure 65.
12.4.3. Difference between intracellular and membrane staining

The expression of RAGE was compared between the surface and the intracellular space of neutrophils and monocytes (Figure 66).

Figure 66: Expression of RAGE on surface (s) and intracellular (i) of neutrophils (left) and monocytes (right) from blood obtained pre-operatively. Blood was obtained pre-operatively (n=8) and stained for RAGE using a standardized protocol. Extent of staining is shown as the mean fluorescence intensity (MFI). Data are presented as mean with bars indicating SEM. Unpaired t tests, ** p < 0.01 for both.

Figure 67: Overlay of two histogram representing distribution of fluorescence intensity for APC (RAGE) on cells staining for surface staining (red) and permeabilized cells for intracellular staining (blue)

For both cell types the quantity of intra-cellular RAGE protein was greater than the surface expression (unpaired t test p<0.01), Figures 66 and 67.
12.4.4. Changes related to snCPB

The change in extent of RAGE expression upon the surface of circulating neutrophils was compared pre- and post-snCPB (Figure 68, left). Similarly, the extent of RAGE expression within the circulating neutrophils was compared pre- and post-snCPB (Figure 68, right).

Figure 68: Expression of RAGE on surface (left) and intracellular (right) of neutrophils from blood obtained pre- and post-snCPB. Blood was obtained pre- and post-snCPB (n=8) and stained for RAGE using a standardized protocol. Extent of staining is shown as the mean fluorescence intensity (MFI). Data are presented as mean with bars indicating SEM. Paired t-tests, * p <0.05.

Expression of RAGE on the surface of neutrophils fell significantly following snCPB (mean MFI pre 501±232 vs. post 346±172, p=0.025); there was no change in the extent of intracellular RAGE expression, mean pre MFI 12480±5144 vs. post 15850±5144, Figure 68.

The change in extent of RAGE expression upon the surface of circulating monocytes was compared pre- and post-snCPB (Figure 69, left). Similarly, the extent of RAGE expression within the circulating monocytes was compared pre- and post-snCPB (Figure 69, right).
Expression of RAGE on the surface of monocytes did not change significantly following snCPB (mean MFI pre 2296±2463 vs. post 4749±5251, p=0.08). Likewise, there was no change in the extent of intracellular RAGE expression (mean MFI pre 8533±3774 vs. post 7242±6233), Figure 69.

### 12.4.5. Expression of Toll-like receptors

#### 12.4.5.1. TLR-4

Levels of expression of TLR-4 were not consistently detectable on neutrophils or monocytes.

#### 12.4.5.2. TLR-9

TLR-9 is an intracellular toll-like receptor and levels of expression were measured in neutrophils and monocytes pre-snCPB (Figure 70).
Expression of intracellular TLR-9 pre-operatively was higher on monocytes (mean MFI 1543±522) than neutrophils (mean MFI 684±508), p<0.001, Figure 70.

The change in extent of TLR-9 expression within neutrophils was compared pre- and post-snCPB (Figure 71). Similarly, the extent of TLR-9 expression within monocytes was compared pre- and post-snCPB (Figure 72).

Expression of intracellular TLR-9 was not statistically significantly different between pre- and post-snCPB in neutrophils (mean MFI pre 460±89 vs. post 684±508, p>0.05), Figure 71.
Figure 72: Expression of intracellular TLR-9 within monocytes from blood obtained pre- and post-snCPB. Blood was obtained pre- and post-snCPB (n=8) and stained for TLR-9 using a standardized protocol. Extent of staining is shown as the mean fluorescence intensity (MFI). Data are presented as mean with bars indicating SEM. Paired t test, p >0.05.

Expression of intracellular TLR-9 was not statistically significantly different between pre- and post-snCPB in monocytes; mean MFI pre 8533±3774 vs. post 7242±6233, p>0.05, Figure 72.
12.5. Measurement of RAGE mRNA in leukocytes around snCPB using qRT-PCR

12.5.1. Method development - reference gene experiments

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) allows quantification of levels of mRNA from cells or tissues and is described in detail (Section 7.9). Levels are necessarily expressed in relative terms. For each sample analysed by the real-time PCR machine and software, a result is given as a CT value which corresponds to the cycle number at which the fluorescence emission exceeds a fixed threshold. The higher the initial amount of DNA, the sooner accumulated product is detected in the PCR process, and the lower the CT value. Errors and inconsistencies in measurement and pipetting can result in different amounts of mRNA entering the reverse transcription reaction, and this may be compounded by different efficiencies of reverse transcription, resulting in variability in amount of cDNA going into the PCR reaction. Thus the results of PCR may vary according to initial amount of mRNA (the signal) but also due to unwanted variability (noise). To minimise the noise, experiments were conducted in duplicate and were compared to reference gene expression.

Reference gene expression remains constant under experimental or clinical conditions. However, traditional reference genes (β-actin and GAPDH) may vary unacceptably and give rise to erroneous results [692]. To ensure that the selected reference genes are unaffected and remain relatively constant, the chosen reference genes will have been validated for the cells and experimental conditions under consideration. Several candidates genes were identified as being suitable for use in monocytes and neutrophils [693, 694] (Table 34).

<table>
<thead>
<tr>
<th>Reference Gene</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACTB</td>
<td>β-actin</td>
</tr>
<tr>
<td>B2M</td>
<td>β-2-microglobulin</td>
</tr>
<tr>
<td>CSF3</td>
<td>Colony stimulating factor 3</td>
</tr>
<tr>
<td>GNB2L1</td>
<td>Guanine nucleotide-binding protein subunit beta-2-like 1</td>
</tr>
<tr>
<td>GUSB</td>
<td>β-K glucuronidase</td>
</tr>
<tr>
<td>HPRT1</td>
<td>Hypoxanthine phosphoribosyltransferase 1</td>
</tr>
<tr>
<td>RPL13A</td>
<td>60S ribosomal protein L13a</td>
</tr>
<tr>
<td>RPL32</td>
<td>60S ribosomal protein L32</td>
</tr>
<tr>
<td>SDHA</td>
<td>Succinate dehydrogenase complex subunit A</td>
</tr>
</tbody>
</table>

Table 34: Potential reference genes
Blood from three patients pre- and post- snCPB was obtained and neutrophil and monocyte populations isolated. Primers were obtained for seven potential reference genes: SDHA, B2M, RPL13, GUSB, CSF3, HPRT1, and ACTB. qRT-PCR was conducted for each sample in duplicate (both values used) and Ct values obtained for neutrophils and monocytes (Figures 73 and 74).

Figure 73: Neutrophil expression of reference genes. Neutrophils were isolated from patients pre- and post-snCPB, qRT-PCR for reference genes (SDHA, B2M, RPL13, GUSB, CSF3, HPRT1, and ACTB) was performed and box and whisker plot of percentage change in CT values are shown, comparing pre- to post-snCPB mRNA expression of reference genes in neutrophils (n=3 in duplicate with both values used).
Figure 74: Monocyte expression of reference genes. Monocytes were isolated from patients pre- and post-snCPB, qRT-PCR for reference genes (SDHA, B2M, RPL13, GUSB, CSF3, HPRT1, and ACTB) was performed and box and whisker plot of percentage change in CT values are shown, comparing pre- to post-snCPB mRNA expression of reference genes in neutrophils (n=3 in duplicate with both values used).

Together these graphs demonstrate the large extent that reference gene expression changes. Of the seven reference genes, HPRT1, RPL13 and GUSB had the least variability (Table 35) and were chosen for future experiments.

<table>
<thead>
<tr>
<th>Mean % variability</th>
<th>HPRT1</th>
<th>RPL13</th>
<th>GUSB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophil</td>
<td>6.9%</td>
<td>10.7%</td>
<td>16.9%</td>
</tr>
<tr>
<td>Monocyte</td>
<td>4.1%</td>
<td>4.1%</td>
<td>0.5%</td>
</tr>
</tbody>
</table>

Table 35: Mean percentage variability of the three best reference genes for neutrophils and monocytes
12.5.2. Expression of RAGE mRNA in leukocytes from patients around snCPB

12.5.2.1. Isolated leukocyte populations

Neutrophils and monocytes were isolated from patients pre- and post-snCPB (Section 7.2.3). The fold-change in quantity of mRNA for RAGE in relation to changes in reference genes was determined in neutrophils and monocytes (Figure 75).

![Figure 75: Fold change in expression of RAGE relative to reference genes in neutrophils and monocytes relating to snCPB. Neutrophils and monocytes were isolated from patients pre- and post-snCPB (n=4, measured in duplicate and mean value charted) and using qRT-PCR their fold-change in expression of RAGE relative to reference genes was calculated. Data are presented as mean with bars indicating SEM, paired t test p>0.05.](image)

The fold-change in expression of RAGE mRNA relative to the mean change in three reference genes was 2.1±1.6 in neutrophils and 1.4±0.9 in monocytes. Although methodologically superior, this method does not permit traditional statistical analysis in this instance. An alternative means of analysis allows such statistical testing and relies upon comparative estimates of mRNA content through comparison of the expression relative to the standards; the results from this analysis are presented in Figure 76.
Neutrophils and monocytes were isolated from patients pre- and post-snCPB (n=4, measured in duplicate and mean value charted) and using qRT-PCR their expression of RAGE relative to reference genes was calculated. Data are presented as mean with bars indicating SEM, paired t test p>0.05.

When analysed using comparative estimates of mRNA content these differences did not reach statistical significance, p >0.05.

12.5.2.2. Buffy coat

Buffy coat was isolated from patients pre- and post-snCPB (Section 12.3.3). The fold-change in quantity of mRNA for RAGE in relation to changes in reference genes was determined (Figure 77).

The fold-change in expression of RAGE mRNA relative to the mean change in three reference genes was 23.2±32.8 in cells from the buffy coat.
Results from the alternative method of analysis are presented in Figure 78.

Figure 78: Expression of RAGE relative to reference genes in leukocytes relating to snCPB. Neutrophils and monocytes were isolated from patients pre- and post-snCPB (n=5, measured in duplicate and mean value charted) and using qRT-PCR their expression of RAGE relative to reference genes was calculated. Data are presented as mean with bars indicating SEM, paired t test p>0.05.

When analysed using comparative estimates of mRNA content these differences did not reach statistical significance, p >0.05.
12.6. Discussion

The first specific objective was to develop an assay of cell surface RAGE expression on leukocytes in whole blood using flow cytometry. Performing the staining in whole blood as opposed to processing to remove non-leukocyte components was intended to shorten the duration of the protocol and more importantly, avoid activating the leukocytes through isolation procedures prior to staining [695, 696]. This objective was met after protracted systematic experimentation and having sought assistance from international experts. Significant problems encountered during the development of the methods were lack of antibody specificity and high levels of non-specific staining; use of THP-1 cells, that were later found not to express RAGE; uncertainty regarding optimal conditions to up-regulate expression of RAGE; and lack of manufactured primary conjugated antibody and issues related to consequent indirect staining techniques which were resolved when antibody conjugation was used.

Quantification of extent of staining with flow cytometry requires correction for background staining. As discussed in section 12.4.1.6, there is no consensus on the optimum method of performing this correction. Consistent with the method adopted from the Stanford group, the raw value of mean fluorescence intensity (MFI) had the ‘fluorescence-minus one’ (FMO) value subtracted. The FMO represents background staining. By contrast, some authorities recommend the use of an isotype control antibody and the subtraction of this MFI instead of the FMO. It is not possible to know how the use of an isotype control antibody might have affected the results of the assays reported within this thesis.

The second objective was to develop and optimise quantitative reverse transcriptase real-time PCR measures of RAGE expression relative to stable reference genes. qRT-PCR results are expressed in relation to changes in standard “reference” genes and if these genes vary in response to the experimental conditions (or clinical interventions) then the results may be erroneous [692, 697]. Having established which reference genes varied the least around snCPB the assay was used to measure the extent of change in RAGE mRNA expression.

Initial plans included a study of the expression of cellular RAGE expression within a large cohort of patients around snCPB and use of these data to gain insight into the regulation of RAGE expression and its relation to sRAGE and esRAGE levels in the plasma; to assess the
relation to measures of systemic inflammation, related organ dysfunction, and clinical outcomes. Unfortunately these objectives could not be met as experimental methodology took considerably longer to develop than anticipated and thus, the sample size was too low to permit these detailed analyses.

Nonetheless, these data represent the first account of alterations in the expression of leukocyte RAGE due to acute systemic inflammation in humans. RAGE expression on circulating leukocytes was previously related to the abundance of RAGE ligands in chronic conditions. Having shown increased RAGE ligands during snCPB, increased leukocyte RAGE expression following snCPB was anticipated.

Contrary to these expectations, the extent of RAGE expression on circulating neutrophils significantly decreased. Plasma soluble RAGE levels increase in acute inflammatory conditions, including snCPB, and a large proportion of soluble RAGE is believed to result from cleavage of the full-length membrane bound form. Data presented in this chapter are consistent with the idea that RAGE expression on circulating neutrophils decreased due to increased shedding, concomitantly increasing plasma sRAGE levels.

Which leukocytes are the predominant sources of sRAGE in plasma? No statistically significant difference in surface RAGE expression between monocytes and neutrophils was observed. This is in contrast to the recent observation of greater RAGE expression on neutrophils than monocytes in the circulation of mice [311]. In humans, neutrophils are present in a far greater number than monocytes and thus, are likely to represent the major source of sRAGE. In recently published work, very low plasma sRAGE levels were reported in patients with neutropenic sepsis [454] supporting this conjecture.

TLR-9 expression was consistently measurable within the cytoplasm of neutrophils and monocytes and was present to a greater extent on monocytes than neutrophils. Comparative expression of TLR-9 at the protein level has not been previously reported but mRNA for TLR-9 was reported to be equivalent between neutrophils and monocytes [698]. Expression of TLR-9 was not significantly altered by snCPB but the small sample size might, at least in part, contribute to the lack of statistical significance.
The assessment of expression of mRNA for RAGE in leukocytes in relation to reference genes showed increases associated with snCPB. In retrospect, in order to establish statistical significance of the fold-changes it would have been ideal to collect blood from a matched control group at two similar times to the pre- and post-snCPB; then compare the fold change between the two times between the two groups. An alternative method of showing statistical significance is to compare the estimated amount of RNA for RAGE (relative to reference genes) at each time and compare using a paired t test; results from this method are shown and although they do not reach statistical significance it is easily appreciated that they might if the sample size were larger. The fold-change result stems from the use of the ‘delta delta CT method’ which is mathematically more appropriate and as it does not rely on the standard curve, potentially less susceptible to variation [699].

12.7. Conclusion

RAGE is expressed on human neutrophils and monocytes and snCPB was associated with a significant decrease in RAGE protein on neutrophils, possibly relating to shedding of the ectodomain increasing soluble RAGE in the plasma. snCPB was associated with increased expression of mRNA for RAGE. Consistent with this, intracellular RAGE protein was higher post-operatively, though with such a low sample size and high degree of assay variability, this did not reach statistical significance.

As the relationships between leukocyte RAGE and soluble RAGE, and between leukocyte RAGE and systemic inflammation and the consequence thereof, remain incompletely understood, future work might include a prospective observational study. The study could recruit patients with systemic inflammation (± lung inflammation) and detailed clinical characterisation combined with sampling of plasma and cells would contribute to our understanding of these questions. Populations of interest would include those having snCPB, but also acute lung injury and acute sepsis/septic shock. Cells that could be used for flow cytometric measurement of RAGE could include circulating leukocytes and also airway leukocytes and peripheral venous endothelial cells [700, 701]. A further quantity of blood could be obtained for RNA extraction and subsequent analysis of the ‘riboleukogram’ with gene expression arrays (Section 1.3.4); also DNA could be extracted for genetic polymorphism analysis.
Thus far we have established that systemic inflammation is an important determinant of outcome from snCPB, RAGE ligands including S100B and S100A12 are released during snCPB and that leukocytes express RAGE. The inflammatory potential of RAGE ligands on human leukocyte populations and the potential to inhibit these responses will be investigated in the following chapter.
13. EX VIVO ASSESSMENT OF PRO-INFLAMMATORY EFFECTS OF RAGE LIGANDS

13.1. Introduction

In the previous chapters it was established that systemic inflammation is an important determinant of outcome from snCPB (Section 10); RAGE ligands including S100B, S100A12, and HMGB1 are released during snCPB (Section 11); and that leukocytes express RAGE (Chapter 12). However, the extent to which RAGE ligands might activate leukocytes, triggering cytokine release that could contribute to the systemic inflammatory response, has not been well-investigated.

Thus, in this chapter, the pro-inflammatory effects of the RAGE ligands S100B, S100A12, and HMGB1 on leukocytes, specifically cytokine release, and the efficacy of a range of putative inhibitors will be determined. Whilst the effects on RAGE ligands has been investigated in various cell types and with different means of inhibition evaluated (Table 36), there have been few studies of the effects of RAGE ligands on leukocyte inflammatory responses in whole blood models or primary human neutrophils. The efficacy of inhibition of RAGE-mediated responses in reducing cytokine release has yet to be demonstrated in these models.

Of the two models, whole blood (WB) models offer distinct advantages over the use of isolated leukocyte subsets, not least for ease and efficiency of screening multiple experimental conditions. Moreover, leukocyte isolation techniques cause activation of sensitive cells such as neutrophils, thereby affecting the inflammatory state of the leukocytes prior to experimental conditions. Finally, the WB model is more clinically analogous as the actions of RAGE ligands on leukocytes in vivo occur in whole blood, not on isolated cells (Section 7.2.2).
Table 36: Studies of effects of S100B, S100A12 or HMGB1 on pro-inflammatory effects on human cells and their inhibition, with focus on cytokine release, preferentially including leukocyte studies.

Nevertheless, WB and isolated neutrophil assays were utilised for the experiments presented in this chapter to allow comparison between the two models. The main cytokine measured was IL-8 because, as discussed (Section 2.1.1), it is an archetypal chemokine, produced by neutrophils and monocytes. The functions of IL-8 include chemoattraction and neutrophil activation, and it is raised in plasma of patients following snCPB [707]. However, for comparison other cytokines were also determined; IL-1β, IL-10, IL-6, IL-8 and TNF-α.
Also, cytokine release from WB of healthy volunteers (HV) was compared to that released from WB from patients undergoing CPB to determine how release might change as a consequence of snCPB.

13.2. Aims and objectives

The investigations reported within this chapter aim to compare the effects of the RAGE ligands S100B, S100A12 and HMGB1 on release of cytokines from whole blood and isolated neutrophil suspensions from healthy volunteers and where possible patients undergoing CPB.

Specific objectives:

i. To compare the effects of RAGE ligands (S100B, S100A12 and HMGB1) on leukocyte cytokine release in 2 models: whole blood and isolated neutrophils obtained from snCPB patients and from healthy volunteers (HV).

ii. To determine the effect of snCPB on responsiveness to S100B in comparison to anticipated endotoxin hypo-responsiveness.

iii. To investigate the effect of putative inhibitors on this cytokine release

iv. To establish the pattern of cytokine release in response to RAGE ligands
13.3. Specific methods

Patients presenting for snCPB at the Royal Brompton Hospital were approached and informed consent was obtained on the day prior to surgery. Blood samples were taken around snCPB as described in Methods (Section 6.7) and plasma isolated and stored at -80°C until analysis. Samples were obtained from patients in Cohorts B, as defined in the methods section.

Following whole blood acquisition or neutrophil isolation (Section 7.2.3) cell suspensions (250µl) were incubated with or without stimuli/inhibitors in 96-well plates for 4h; where possible, assay conditions were set up in duplicate. Cells were centrifuged (400g, 4°C, 10 mins), supernatant aspirated and placed in a new microplate for storage at -20°C until assay. ELISA assays were always carried out in duplicate and the mean value used. In selected experiments, the cell pellet remaining after removal of the supernatant was re-suspended and cytocentrifuged (450g, 3 mins) onto slides and air-fixed prior to staining (Section 7.2.4), photographed and the cell number counted.

When presenting results, the number of samples (n) was either the number of individuals, or the number of measurements in total from fewer individuals, for example 8 measurements from 4 individuals. Where the number of samples equals the number of individuals, it is written as n=x; conversely where the number of measurements is used from a lesser number of individuals, it is written as n=x from y. This is justifiable as the cells are not clonal and therefore each experimental result valid per se. This compromise was arrived at due to the expense of experiments and/or limited number of cells available.

The sequence of adding ligands, inhibitors and cell suspensions could impact on the findings. Addition of all three components (cells, inhibitors and ligands) simultaneously was deemed neither clinically analogous to a treatment, nor providing optimum conditions for inhibitors to act (A, Figure 79). Addition of inhibitors to the ligands prior to addition of cells might increase the effectiveness of the inhibitor, particularly if the inhibitor is thought to bind directly to the ligand (B, Figure 79). Finally, adding the inhibitor following the addition of stimulus to cell suspensions (C, Figure 79) was deemed most clinically analogous to a therapy. Thus, unless otherwise stated, inhibitors were co-incubated with stimuli for 1h prior to addition of both to cell suspensions (B, Figure 79).
Figure 79: Three methods of combining stimulus, inhibitor and cell suspensions. A represents adding the stimulus and inhibitor at the same time to the already-plated-out cell suspension ‘co-incubation’; B represents combining the stimulus and inhibitor together for a duration prior to addition to cell suspension ‘co-pre-incubation’; C represents adding the stimulus, then the cell suspension, then the inhibitor, ‘treatment’.
13.4. Results

13.4.1. Healthy volunteers

13.4.1.1. S100B caused release of IL-8 from WB and neutrophils from healthy volunteers

Initial experiments suggested that the optimum duration of incubation to assess IL-8 release was 4h. Earlier time points (30 or 60 mins) did not provide equivalent range of cytokine release and later times (24h) showed substantially elevated cytokine release (>15,000pg/ml of IL-8 in the absence of stimuli) without experimental stimuli making it impossible to assess effects of stimuli/inhibitors.

Figure 80: IL-8 release from whole blood in response to S100B. WB was obtained from healthy volunteers (n=6) and incubated with S100B (0.1-2.0µg/ml) or negative control for 4h. IL-8 content of supernatant was measured with ELISA. Data are presented as mean with bars indicating SEM. ANOVA with Bonferroni correction * p<0.05, ** p<0.01, *** p<0.001.

IL-8 levels at 4h in supernatants of whole blood were elevated when S100B was present and increases were concentration-dependent (Figure 80). IL-8 levels were undetectable in the absence of stimulus, increased with 0.1µg/ml of S100B to 145±163pg/ml, with 1.0µg/ml to 763±265pg/ml (p<0.01) and further still with 2.0µg/ml to 1113±590pg/ml IL-8 (p<0.001)
**Figure 81**: IL-8 release from neutrophils in response to S100B. Neutrophils were isolated from healthy volunteers (n=6) and suspended at 1x10⁶/ml prior to incubation with S100B (0.1-2.0µg/ml) or negative control for 4h. IL-8 content of supernatant was measured with ELISA. Data are presented as mean with bars indicating SEM. ANOVA with Bonferroni correction * p<0.05.

Similar results were seen in isolated neutrophil suspensions (as seen in Figure 81). Mean IL-8 levels in the absence of stimulus were 112±205pg/ml, with 0.1µg/ml of S100B 449±300pg/ml and 1241±789pg/ml and 1601±1146pg/ml of IL-8 with 1.0 and 2.0µg/ml, respectively.

The quantities of IL-8 released from WB and neutrophils are not directly comparable as the neutrophil concentration in WB was not determined.
13.4.1.2. Lack of inhibition of S100B-stimulated IL-8 release

**Figure 82**: IL-8 release from neutrophils in response to S100B with inhibitor. Neutrophils were isolated from healthy volunteers (n=8 from 4) and suspended at 1x10⁶/ml prior to addition of the combined S100B (0.1µg/ml) and inhibitor (having been co-incubating at 37°C for >1h). IL-8 content of supernatant was measured with ELISA. Data are presented as mean with bars indicating SEM. ANOVA with Bonferroni correction p>0.05 for all. PBS phosphate buffered saline, sRAGE (0.5µg/ml), TTP (1µg/ml), mAB to RAGE (1µg/ml), AB to S100B (1µg/ml) or isotype control antibody (1µg/ml).

In experiments conducted on both WB and isolated neutrophils and despite several protocol modifications, alterations in concentrations, and adding components in different sequences, there was no consistent statistically significant reduction in IL-8 release following co-incubation of S100B with any putative inhibitors. Representative data from select neutrophil experiments are shown (Figure 82).

S100B was used at 0.1µg/ml in these experiments and those described in section 13.4.2 onwards. Figures 81 and 82 do not show a significantly increased quantity of IL-8 released in response to S100B at this concentration in comparison to the negative control. However, in allied experiments without the dose range but with higher sample numbers, this concentration was consistently found to induce a significantly different release of IL-8 than negative control. Furthermore, it is of greater clinical relevance than higher concentrations, being closer to levels observed in vivo, and maximised the number of experiments that could be performed for a given quantity of expensive recombinant protein.
13.4.1.3. *S100A12* caused release of IL-8 from WB and neutrophils from healthy volunteers

![Graph showing IL-8 release from whole blood](image)

Figure 83: IL-8 release from whole blood in response to S100A12. WB was obtained from healthy volunteers (n=4 from 2) and incubated with S100 A12 (0.01-1.0µg/ml) or negative control for 4h. IL-8 content of supernatant was measured with ELISA. Data are presented as mean with bars indicating SEM. ANOVA with Bonferroni correction ** p<0.01, *** p<0.001.

IL-8 levels in the supernatant of whole blood were significantly increased with S10012; increases were concentration-dependent (Figure 83). IL-8 was undetectable in the absence of stimulus and with 0.01µg/ml of S100A12. With 0.1µg/ml the mean IL-8 release was 236.9±101pg/ml (P<0.01) and with 1.0µg/ml 2157±1040pg/ml (p<0.001)
Figure 84: IL-8 release from neutrophils in response to S100A12. Neutrophils were isolated from healthy volunteers (n=4 from 2) and suspended at 1x10⁶/ml prior to incubation with S100 A12 (0.01-1.0µg/ml) or negative control for 4h. IL-8 content of supernatant was measured with ELISA. Data are presented as mean with bars indicating SEM. ANOVA with Bonferroni correction *** p<0.001.

A similar profile of IL-8 release from isolated neutrophil suspensions (Figure 84) to that from whole blood was seen, but the amounts were less. Thus, basal release was 42±28pg/ml of IL-8 increasing to 55±40pg/ml with 0.01µg/ml, 139±16pg/ml with 0.1µg/ml but only reaching significance from basal with 542±133pg/ml IL-8 release with 1.0µg/ml (p<0.001).
13.4.1.4. Lack of Inhibition of S100A12-related cytokine release

![IL-8 release from neutrophils in response to S100A12 with inhibitor](image)

**Figure 85:** IL-8 release from neutrophils in response to S100A12 with inhibitor. Neutrophils were isolated from healthy volunteers (n=6 from 3) and suspended at 1x10⁷/ml prior to addition of the combined S100A12 (0.1µg/ml) and inhibitor (having been co-incubating at 37°C for >1h). IL-8 content of supernatant was measured with ELISA. Data are presented as mean with bars indicating SEM. ANOVA with Bonferroni correction p>0.05 for all. PBS phosphate buffered saline, sRAGE (0.5µg/ml), TTP (1µg/ml), mAB to RAGE (1µg/ml).

In experiments conducted on both WB and isolated neutrophils and despite several protocol modifications, alterations in concentrations, and adding components in different sequences, there was no consistent statistically significant reduction in IL-8 release following co-incubation of S100A12 with any putative inhibitors. Representative data from select neutrophil experiments are shown (Figure 85).
13.4.1.5. HMGB1 does not cause release of IL-8

**Figure 86**: IL-8 release from neutrophils in response to S100A12. Neutrophils were isolated from healthy volunteers (n=6 from 3) and suspended at 1x10⁶/ml prior to incubation with PBS (negative control) vs. HMGB1 at 0.5µg/ml, S100B (B) at 0.1µg/ml vs. both s100B and HMGB1 (B+H), S100A12 (A) at 0.1µg/ml vs. both S100A12 and HMGB1 (A+H). IL-8 content of supernatant was measured with ELISA. Data are presented as mean with bars indicating SEM. Unpaired t test correction p>0.05 for each pair.

In contrast to the findings with S100B and S100A12, HMGB1 did not alter release of IL-8 from neutrophils of healthy volunteers (Figure 86). As it has been suggested that HMGB1 may lack inherent pro-inflammatory activity, but might augment effects of other mediators, HMGB1 was co-incubated with S100B and S100A12, but no significant change in IL-8 release compared with S100B or S100A12 alone was seen.
LPS caused concentration-dependent release of IL-8 in WB

Figure 87: IL-8 release from whole blood in response to LPS. WB was obtained from healthy volunteers (n=4 to 8, from 2 to 4) and incubated with LPS $1\times10^{-10}$ g/L (0.1µg/ml) to $1\times10^{-3}$ g/L (1µg/ml) for 4h. IL-8 content of supernatant was measured with ELISA. Data are presented as mean with bars indicating SEM.

LPS was used as a positive control in experiments and exhibited a concentration-dependent release of IL-8 from whole blood (Figure 87).
13.4.2. Patients pre/post snCPB

Whole blood was obtained from ten snCPB patients pre- and post-CPB and co-incubated with S100B/controls following 1h co-incubation with putative inhibitors or controls.

13.4.2.1. Effect of S100B (with and without inhibitors) on IL-8 release pre- vs. post-snCPB

Figure 88: IL-8 release from whole blood obtained pre-snCPB in response to stimuli in the presence or absence of putative inhibitors. WB was obtained from patients pre-snCPB (n=18 from 9) and incubated with S100B (0.1µg/ml), LPS (1ng/ml) or negative control (PBS) for 4h in the presence/absence of inhibitors sRAGE (1µg/ml, SR) or TTP-4000 (1µg/ml, TTP) that had been co-incubating at 37°C for >1h with the S100B or LPS respectively. IL-8 content of supernatant was measured with ELISA. Data are presented as mean with bars indicating SEM. ANOVA with Bonferroni correction p>0.05 for effect of inhibitors on IL-8 release.

S100B (0.1µg/ml) increased IL-8 release in comparison to PBS; LPS (1ng/ml) caused a greater release of IL-8 compared to S100B. Co-incubation with putative inhibitors sRAGE (1 µg/ml) and TTP (1 µg/ml) did not significantly alter IL-8 (Figure 88), p>0.05.
Figure 89: IL-8 release from whole blood obtained post-snCPB in response to stimuli in the presence or absence of putative inhibitors. WB was obtained from patients post-snCPB (n=18 from 9) and incubated with S100B (0.1µg/ml), LPS (1ng/ml) or negative control (PBS) for 4h in the presence/absence of inhibitors sRAGE (1µg/ml, SR) or TTP-4000 (1µg/ml, TTP) that had been co-incubating at 37°C for >1h with the S100B or LPS respectively. IL-8 content of supernatant was measured with ELISA. Data are presented as mean with bars indicating SEM. ANOVA with Bonferroni correction p>0.05 for effect of inhibitors on IL-8 release.

In blood obtained from patients post-snCPB, the amount of IL-8 released in response to S100B or LPS was decreased (Figure 89). Also, neither sRAGE nor TTP decreased IL-8 production from WB.
Figure 90: IL-8 release from whole blood in response to identical stimuli but comparing pre- vs. post- snCPB. WB was obtained from patients pre- and post-snCPB (n=18 from 9) and incubated with S100B (0.1μg/ml), LPS (1ng/ml) or negative control (PBS) for 4h. IL-8 content of supernatant was measured with ELISA. Data are presented as mean with bars indicating SEM. Paired t test ** p<0.01.

When IL-8 release pre and post bypass were compared, directly, release post-CPB was significantly decreased following stimulation with either LPS (P<0.01) or S100B (P<0.01), Figure 90.
13.4.2.2. Decreased cytokine release with S100B following snCPB

In samples obtained from 3 patients IL-6, IL-8, TNF-α, IL-1β, and IL-10 were measured using the MSD system. Measurements were in duplicate on supernatants from whole blood, obtained pre- and post-CPB, following 4h of experimental conditions.

In WB obtained pre-snCPB, levels of IL-6 and IL-8 were elevated following stimulation with S100B, in comparison to stimulation with PBS, p<0.01. By contrast, in WB obtained following snCPB, levels of IL-6 and IL-8 were non-significantly elevated following stimulation with S100B, in comparison to stimulation with PBS (Figure 91).
Figure 92: TNF-α, IL-1β or IL-10 release from whole blood in response to S100B or negative control pre- (graphs on left) and post- snCPB (graphs on right). WB was obtained from patients pre- and post-snCPB (n=18 from 9) and incubated with S100B (0.1µg/ml) or negative control (PBS) for 4h. IL-8 content of supernatant was measured with ELISA. Data are presented as mean with bars indicating SEM. Unpaired t test ** p≤0.01.
In WB obtained pre-snCPB, levels of TNF-α, IL-1β and IL-10 were elevated following stimulation with S100B, in comparison to stimulation with PBS (p≤0.01). By contrast, on WB obtained following snCPB, levels of TNF-α and IL-1β were non-significantly elevated following stimulation with S100B, in comparison to stimulation with PBS (Figure 92). IL-10 levels were high following snCPB, irrespective of stimulating conditions with S100B or PBS, Figure 92.

![Graphs showing IL-6 and IL-8 release from whole blood in response to identical stimuli but comparing pre- vs. post-snCPB.](image)

**Figure 93:** IL-6 and IL-8 release from whole blood in response to identical stimuli but comparing pre- vs. post-snCPB. WB was obtained from patients pre- and post-snCPB (n=18 from 9) and incubated with S100B (0.1µg/ml) or LPS (1ng/ml) for 4h. IL-8 content of supernatant was measured with ELISA. Data are presented as mean with bars indicating SEM. Paired t test ** p<0.01.
Figure 94: TNF-α, IL-1β or IL-10 release from whole blood in response to identical stimuli but comparing pre- vs. post-snCPB. WB was obtained from patients pre- and post-snCPB (n=18 from 9) and incubated with S100B (0.1µg/ml), LPS (1ng/ml) or PBS (IL-10 only) for 4h. IL-8 content of supernatant was measured with ELISA. Data are presented as mean with bars indicating SEM. Paired t test ** p<0.01.
Comparing cytokine release due to identical experimental conditions but using whole blood acquired post- compared to pre-snCPB, statistically significant decreases in response to both S100B and LPS were seen (Figures 93 and 94), p<0.01 for all.

IL-10 levels in the post-op whole blood supernatants were uniformly high post-snCPB, irrespective of stimulation/inhibiting conditions (without stimulus: 1.17±0.3pg/ml pre vs. 129.8±28.7pg/ml post). This probably reflected high levels in the plasma of the patients following snCPB.

The negative control (PBS) was not associated with significant cytokine release and the positive control (LPS at 1ng/ml) was associated with a relatively large release of all measured cytokines: Mean values pre-snCPB, IL-6 11254±3833 pg/ml, IL-8 8923±1262 pg/ml, TNF-α 12765±4683 pg/ml, IL1-β 636±295 pg/ml, IL-10 47.62±23.6 pg/ml.

Supernatant of WB incubated with HMGB1 (20ng/ml) for 4h showed no difference in levels of cytokines compared to supernatant incubated with negative control, Figure 95.

Figure 95: Extent of cytokine release from whole blood (pre-snCPB) in response to HMGB1. WB was obtained from patients pre- snCPB (n=6 from 3) and incubated with HMGB1 (10ng/ml, black) or negative control (PBS, grey) for 4h. Cytokine content of supernatant was measured with MSD. Data are presented as mean with bars indicating SEM. Paired t test p>0.05 for all.
13.4.2.3. Lack of efficacy of TTP-4000 at reducing S100B-mediated cytokine release from WB obtained pre- and post-snCPB

Figure 96: Extent of cytokine release from whole blood (pre-snCPB) in response to S100B following co-incubation with TTP-4000 (black) or PBS (negative control, grey). WB was obtained from patients pre-snCPB (n=6 from 3) and incubated with S100B (0.1µg/ml) for 4h following co-incubation with TTP-4000 (black) or PBS (negative control, grey). Cytokine content of supernatant was measured with MSD. Data are presented as mean with bars indicating SEM. Paired t test p>0.05 for all.

Figure 97: Extent of cytokine release from whole blood (post-snCPB) in response to S100B following co-incubation with TTP-4000 (black) or PBS (negative control, grey). WB was obtained from patients post-snCPB (n=6 from 3) and incubated with S100B (0.1µg/ml) for 4h following co-incubation with TTP-4000 (black) or PBS (negative control, grey). Cytokine content of supernatant was measured with MSD. Data are presented as mean with bars indicating SEM. Paired t test p>0.05 for all.
For each of the four archetypal pro-inflammatory cytokines, and for the anti-inflammatory cytokine IL-10, the pre-incubation of TTP-4000 (1µg/ml) compared to negative control (PBS), with S100B (0.1µg/ml) for 1h prior to co-incubation in whole blood for 4h, was not associated with a significant difference in release either pre- (Figure 96) or post- (Figure 97) snCPB, p>0.05 for all.

The TTP-4000 inhibitor was not associated with significant cytokine release in the absence of stimulus, with mean IL-8 release of 37.1±20.7 versus 39.3±23.2 for PBS (n=6 from 3), p>0.05; similar lack of difference from negative control was seen for IL-6, TNF-α, IL-1β and IL-10.

### 13.4.3. Assessment of S100B for contamination

The S100B used was a recombinant protein manufactured in *Escherichia coli* (Randox Laboratories) and was not certified endotoxin-free. The failure to eliminate the possibility of bacterial contamination has caused incongruous and conflicting results in related areas of investigation (Section 3.2.2). Therefore it was necessary to establish to what extent, if any, LPS might contribute to the S100B response. The S100A12 and HMGB1 were certified free from endotoxin contamination. sRAGE was not formed in bacterial cells. TTP was not certified free from endotoxin but did not elicit any inflammatory responses.

#### 13.4.3.1. Polymyxin did not diminish IL-8 response

*Figure 98: IL-8 release from whole blood in response to S100B in the presence or absence of polymyxin B (PMX).* WB was obtained from healthy volunteers (n=4) and incubated with S100B (1µg/ml) for 4h in the presence or absence of polymyxin B (0.1µg/ml or 10µg/ml). IL-8 content of supernatant was measured with ELISA. Data are presented as median with bars indicating IQR. Kruskal Wallis test, p>0.05.
Figure 99: IL-8 release from neutrophils in response to S100B in the presence or absence of polymyxin B (PMX). Neutrophils were isolated from healthy volunteers (n=8 from 4) and incubated with S100B (0.1µg/ml) for 4h in the presence or absence of polymyxin B (0.1µg/ml). IL-8 content of supernatant was measured with ELISA. Data are presented as median with bars indicating IQR. Mann Whitney U test, p>0.05.

Had the cytokine-releasing attributes of the S100B been due to endotoxin contamination then the IL-8 release from the cells incubated without polymyxin (PMX) would have been expected to be higher than that from cells incubated with PMX. No significant difference was seen in WB or neutrophil suspension, p>0.05 (Figures 98 and 99).

13.4.3.2. Endotoxin assay

S100B (1mg/ml) was diluted in Lonza’s endotoxin-free water to reach the minimum testing volume (500µl) and placed into endotoxin-free glass vials and the most accurate available test, the kinetic chromogenic LAL assay, was conducted at Lonza’s European endotoxin testing centre. Endotoxin contamination was measured to be 2810 European Units/ml. Having considered the dilution, the S100B 1mg/ml solution was estimated to have 16860 EU/ml. One Endotoxin Unit (EU) has the endotoxin activity of 0.2 ng of reference Endotoxin Standard. The extent of contamination may be expressed as 3.372 µg/ml endotoxin in a 1mg/ml solution, or 0.34%.

13.4.3.3. Comparing known with estimated endotoxin

To further validate the results of the external test, the cytokine release between whole blood and isolated neutrophils exposed to either S100B, or the amount of LPS that should be within that quantity of S100B was compared; using the estimated degree of contamination provided by Lonza.
Had the cytokine-releasing attributes of the S100B been entirely due to endotoxin contamination and the assay accurate, the amounts would have been expected to be similar; had the S100B had inherent pro-inflammatory activity above and beyond any LPS contamination then the release from S100B would have been predicted to be greater. However, in this case the LPS was associated with significantly more release of IL-8 (Figures 100 and 101). This is likely to be due to inaccurate estimation of endotoxin contamination and/or increased potency of our LPS relative to reference LPS.

**13.4.3.4. S100B-stimulated cytokine release profile**

Had the cytokine-releasing properties of the S100B be attributable to contaminating LPS then the pattern of cytokine release would be identical to that due to pure LPS. However,
S100B provokes a substantially different pattern with S100B having the greatest release of IL-8 then IL-6 then TNF-α, the complete opposite of LPS with TNF-α > IL-6 > IL-8 (Table 37).

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>S100B, pg/ml</th>
<th>LPS, pg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>55.03</td>
<td>635.77</td>
</tr>
<tr>
<td>IL-6</td>
<td>1,275.56</td>
<td>11,253.62</td>
</tr>
<tr>
<td>IL-8</td>
<td>1,492.26</td>
<td>8,923.44</td>
</tr>
<tr>
<td>IL-10</td>
<td>7.05</td>
<td>47.62</td>
</tr>
<tr>
<td>TNF-α</td>
<td>1,119.43</td>
<td>12,765.38</td>
</tr>
</tbody>
</table>

Table 37: Mean cytokine levels in supernatant from whole blood (n=3) obtained pre-operatively and co-incubated with either S100B (100ng/ml) or LPS (1ng/ml) for 4h.
13.5. Discussion

13.5.1. Effect of RAGE ligands

Our first specific objective was to compare the effects of the RAGE ligands S100B, S100A12 and HMGB1 on cytokine release in two models, WB and neutrophils. The RAGE ligands S100B and S100A12 resulted in release of the pro-inflammatory cytokine IL-8 from whole blood and neutrophil suspensions in a concentration-dependent manner. Whole blood and neutrophil assays invariably showed similar patterns of response.

S100B was used as there is strong evidence that it binds to RAGE [339], has pro-inflammatory activity in leukocytes [287, 350-353], and is known to be increased in the plasma of patients following snCPB. Although RAGE-mediated inflammatory signalling is likely to result in the release of a range of mediators including IL-8, until very recently this had only been shown in human endothelial cells and chondrocytes [702, 703]. One report of concentration-dependent release of IL-8 from neutrophils in response to S100B measured by Western Blot has previously been was published in abstract form. No attempts at inhibition were reported [353].

S100B-induced inflammatory response investigations in leukocytes have been predominantly performed in cell lines. Neutrophil-like cells (HL-60) were used to investigate the effect of hyperglycaemia and S100B on oxidative burst and the effects of MAPK signalling inhibitors [287]; monocyte-like cells (THP-1) and primary human monocytes showed increased expression of cyclo-oxygenase enzymes (enzymes with pro-inflammatory products) in response to S100B and AGE. Successful inhibition experiments with anti-RAGE antibody and NF-κB and MAPK pathway inhibitors were performed in THP-1 cells [351].

S100A12 was also used on the basis of strong evidence of ligation to RAGE, pro-inflammatory activity in leukocytes [339, 340] and is increased in the plasma of patients following snCPB. S100A12 has not been previously shown to induce IL-8 release from leukocytes, indeed there was a lack of IL-8 release shown in a study of THP-1 cells [704]. Others have shown a chemo-attractant effect diminished by the presence of sRAGE [339, 708].
Concentrations of S100A12 and S100B used in these experiments (mostly 0.1 µg/ml but up to 2µg/ml) were higher than those measured in plasma samples from patients post-snCPB but were similar to, or significantly less than, amounts described in the literature. In defence of this, levels at sites of inflammation are far higher than those found in the circulation and these experiments are models to facilitate experimental investigations rather than attempts to accurately recreate the *in vivo* state.

In our models, the HMGB1 neither induced IL-8 release alone or in combination with S100B or LPS. As discussed (Section 3.2.2), the inflammatory activity of HMGB1 is controversial. HMGB1 was used both at concentrations close to those observed clinically (10ng/ml), and at higher concentrations (0.5µg/ml). By contrast, others have shown induction of IL-8 release in WB when used at 5µg/ml. This effect was not found to be inhibited by anti-RAGE antibodies, but by anti-TLR-4 antibodies used at 100µg/ml [387]. The same investigators used HMGB1 at 1.15 µg/ml and demonstrated release of TNF-α from human monocytes, with inhibition by the selective cholinergic agonist GTS-21 [706]. In both these studies the HMGB1 was recombinant but not of bacterial origin. Therefore, we might have used insufficient HMGB1 to induce cytokine release from WB or neutrophils. However, responses induced by very high concentrations of HMGB1 and their inhibition by extremely high concentrations of inhibitors bear little relevance to the clinical state and therefore lack translational relevance.

13.5.2. Hypo-responsiveness

The second objective was to investigate -responsiveness of leukocytes before and after snCPB. Leukocytes were hypo-responsive to S100B in terms of cytokine release (IL-1β, IL-6, IL-8, and TNF-α) from whole blood obtained post compared with pre- snCPB. This has not been previously described. Also, findings of leukocyte LPS hypo-responsiveness concur with previous findings showing that *ex vivo* cytokine production is suppressed early after the onset of CPB [199, 202].

Postulated mechanisms of endotoxin tolerance include the effects of increased circulating anti-inflammatory cytokines. Increased expression of the anti-inflammatory cytokine IL-10 was shown however the sample size was insufficient to robustly investigate any relationship between extent of IL-10 release and degree of change in cytokine response to S100B or LPS.
Another potential mechanism that has been implicated is altered NF-κB subunit composition. In an endotoxin tolerance model conducted using a leukocyte cell line (Mono-Mac-6) a shift from p50/p65 heterodimers to p50 homodimers resulted in impaired ability to transcribe mRNA for pro-inflammatory cytokines [709, 710]. As NF-κB is a major RAGE signal transduction pathway, such changes might also mediate the S100B tolerance observed.

In support of the hypothesis that excessive leukocyte-mediated inflammatory responses contribute to organ failure, pre-operative ex vivo production of the pro-inflammatory cytokine IL-6 was previously correlated with impaired post-snCPB oxygenation [202]. By contrast, ex vivo endotoxin hypo-responsiveness in an undifferentiated group of surgical ICU patients was associated with prolonged ventilation [711]. The latter findings are likely to reflect the deleterious effects of immunoparesis and its relationship to nosocomial infections and consequent prolonged critical illness.

13.5.3. Inhibition

The third objective was to investigate the effect of putative inhibitors on cytokine release. In this regard, S100B and S100A12-mediated cytokine release from either whole blood from healthy volunteers and patients, or neutrophil suspensions from healthy volunteers could not be inhibited using the protocols utilised in this study. The failure to inhibit RAGE-mediated cytokine release in whole blood and isolated neutrophils could be ascribed to problems with the models and/or the inhibitors.

Firstly, the models employed were human leukocytes ex vivo as WB or suspension of isolated neutrophils. None of the putative inhibitors used in this study have previously been used in whole blood models, or neutrophil suspensions. Certainly, pre-clinical efficacy data of the synthetic sRAGE analog (TTP-4000) comes from murine, in vivo studies.

Secondly, the inhibitors; there are no published studies of inflammation-suppressing effects of sRAGE on human leukocytes. In fact, following reports of sRAGE induced production of inflammatory cytokines from murine splenocytes [480], studies on human monocytes reported induction of IL-6, TNF-α by recombinant (endotoxin-free) sRAGE [481]. Both sRAGE and TTP-4000 should bind S100B and S100A12 with high affinity and prevent binding to cellular RAGE, thus abrogating RAGE-mediated IL-8 secretion. TTP-4000 is a synthetic sRAGE
analogue and would have been expected to have similar inhibitory effects to sRAGE (Section 9.8.5). The monoclonal antibody to RAGE should bind and block interaction with its ligands; the antibody to S100B should similarly bind to S100B and prevent interaction with RAGE. The possible reasons for the failure of these agents include failure to bind their targets; failure to eliminate binding to cellular RAGE; or the presence of inherent pro-inflammatory activity. The latter possibility is unlikely as sRAGE and TTP in isolation did not increase IL-8 released compared with negative control.

In general terms, mediator-binding proteins may vary in their capacity to block mediator-receptor interaction depending upon factors including the relative concentrations of binding proteins and mediator; indeed, there are examples of the opposite effect occurring with inhibitors both prolonging the duration of mediator activity and having agonist activity [712].

The sRAGE from Prospec was a human recombinant protein produced in HEK (Human Embryonic Kidney) cells as a single, glycosylated, polypeptide chain containing 331 amino acids and having a molecular mass of 35.2 kDa. It is fused to a Flag tag at C-Terminus and purified by proprietary chromatographic techniques. Investigators have previously found reduced inhibition of RAGE-mediated inflammation when using recombinant sRAGE compared to tissue-derived sRAGE due to differences in glycosylation that affect ligand binding [252, 253]. Therefore, the dissimilarities between this protein and endogenous human sRAGE may significantly affect its ability to bind ligand. Furthermore, structural differences may affect its ability to interact with membrane-bound RAGE, another hypothesised mechanism by which sRAGE could interfere with RAGE-mediated signal transduction [275]. Finally, despite adhering to manufacturer’s guidance on storage, there is evidence that sRAGE degrades into several peptide species during storage [713] and this might also affect activity. Alternatively, sRAGE could have bound the S100B but ineffectively inhibited its binding to membrane-bound RAGE protein. Similarly, the investigational drug TTP-4000 might lack affinity or unsuccessfully block interaction with the receptor.

The S100B antibody was synthesised in the murine peritoneal cavity and is provided at 80-90% purity. As a mixture of monoclonal antibody to S100B and undetermined murine proteins it may offer more pro-inflammatory stimuli than inhibitory effect. Although it was shown to be
effective at inhibiting human RAGE-S100B interactions, the model used was very different, specifically, cultured rectal epithelial cells from patients with ulcerative colitis and assessment of the reduction in protein production of inducible nitric oxide synthetase after 24h [714].

The HMGB1 used was sourced from R&D (catalogue number 1690-HM) and derived from a murine myeloma cell line, with a predicted molecular mass of 24.9kDa. This is dissimilar to endogenous human HMGB1 (Molecular mass 30kDa) and is likely to have different or absence post-translational modifications (Section 3.2.2) which might account for the absence of effect demonstrated.

13.5.4. Cytokine release pattern
The final objective was to establish the pattern of cytokine release from WB obtained from patients pre- and post-snCPB in response to RAGE ligands. In a limited number of patient samples we measured release of IL-6, IL-8, TNF-α, IL-1β, and IL-10 in response to stimulation. The pattern of cytokine release from whole blood from patients pre- and post-snCPB differed in response to S100B compared to LPS. The S100B used is of recombinant bacterial origin and therefore likely to have a degree of LPS contamination, however this is difficult to accurately quantify. Any contaminating LPS is unlikely to account for the observed activity for several reasons: firstly, the pattern of cytokine release differs from that observed for LPS; secondly, the cytokine response is unaltered following co-incubation with the LPS-binder polymyxin.

13.5.5. Future work
There are many options for taking this work forward. Firstly, other effects of RAGE ligands on leukocyte function (chemotaxis, phagocytosis, intracellular killing) could be investigated. Further inhibition experiments with pharmacological agents might include short peptide sRAGE fragments [713], inhibitors of cell signalling pathways, or inhibition of toll-like receptors. Also, it would be essential to establish that RAGE-inhibitory effects of some of the putative antagonists could be established in models that have been better characterised, such as alveolar epithelial cell lines (A549s, [715]) or endothelial cells (HUVECs [716]).

Although the advantages of using primary cell lines are considerable, characterisation of the responses in leukocyte cell lines including THP-1 for monocytes, HL-60 for neutrophils would
facilitate siRNA experiments to knock-down RAGE, TLR-2 and TLR-4 to determine which has the greater effect on S100 protein-mediated responses. Also, larger numbers of cells could be used which would facilitate collection of mRNA for qRT-PCR. Although siRNA techniques are possible in primary leukocytes and have been used to knock-down RAGE in monocytes [290], experience from our laboratory suggests that the techniques are difficult to reproduce consistently.

13.6. Conclusion

S100B and S100A12 are DAMPs released during tissue injury or infection. Such proteins cause secretion of canonical inflammatory cytokines from neutrophils. In the context of localised release this will cause acute inflammation and may be beneficial in leading to resolution of injury or infection. However, when high levels are present in the circulation, disseminated activation and recruitment of inflammatory cells is likely to be disadvantageous. The data presented within this chapter supports the hypothesis that release of the RAGE ligands, S100B and S100A12, during snCPB has pathophysiological significance.

Successful inhibition of RAGE-mediated leukocyte cytokine release was elusive in our investigations and has yet to be demonstrated by other investigators but might yet prove to be valuable therapy.
14. GENETIC POLYMORPHISMS IN THE GENES FOR RAGE AND ITS LIGANDS

14.1. Introduction

Activation of the RAGE-axis predisposed patients to excessive systemic inflammation, manifest as prolonged ICU LOS (Section 11). The extent of systemic inflammation was related to adverse outcome, including death (Section 10). Leukocytes expressed RAGE and possibly contribute to circulating sRAGE (Section 12). RAGE ligands were released during snCPB and caused pro-inflammatory responses in leukocytes (Section 11 and 13). This chapter describes investigations into the role of genetic variation in the RAGE-axis and how genetic variants may be associated with more severe systemic inflammation and organ dysfunction following snCPB.

Variation in the genes of the inflammatory response has been investigated in the context of post-snCPB SIRS with mixed results (Section 2.4). SNPs affecting expression of pro-inflammatory cytokines such as IL-6, IL-8 and TNF-α have been related to greater elevations in circulating levels of cytokines post-operatively than their allelic variants [222, 224, 226]. These elevated cytokines were variably related to adverse clinical outcomes such as impaired oxygenation, impaired renal function, prolonged ventilation, and prolonged ICU and hospital LOS [220, 225, 227, 228]. More generally, elevated levels of circulating inflammatory cytokines were associated with adverse outcome [110].

The RAGE gene has been studied in relation to onset of diabetes and the development of diabetic complications (Section 3.1.6). However, there are very few studies that investigated the role of genetic variation in the RAGE gene in chronic inflammatory disease; and none in acute critical illness. One particular study showed associations between SNPs in the HMGB1 gene and adverse outcome from SIRS [271].

Previous genetic association studies of RAGE genes have identified SNPs which bear functional significance and also some candidate SNPs that could potentially have implications on outcomes from acute critical illness. These studies pave the way for the investigation of genetic variation in the genes of the RAGE axis and their influence on outcome from snCPB, as an exemplar of acute critical illness.
14.2. Aims and objectives
The studies reported within this chapter aimed to investigate genetic variation in select genes of the RAGE axis and their influence on outcome from snCPB.

Specific objectives:

i. To select appropriate SNPs in the RAGE, S100A8 and HMGB1 genes, then design primers for these SNPs and test them using the SSP-PCR technique

ii. To clinically characterise a large cohort of snCPB patients and genotype their DNA using SSP-PCR

iii. To verify genotype and distribution

iv. To measure sRAGE and S100A8/9 in the plasma of patients in the cohort

v. To analyse the relationship between genotype and clinical outcomes, initially using data mining

14.3. Specific methods

14.3.1. Genes of the RAGE axis
These investigations focus on RAGE and in particular its ligands HMGB1, S100B, S100A8/9, S100A12 and AGEs. As AGE modifications do not result from gene products the influence of genetic variability is limited.

The National Center for Biotechnology Information maintains a SNP database (www.ncbi.nlm.nih.gov/SNP) which was used to determine the nucleic acid sequences of the SNP, necessary for primer design. The Ensembl project (www.ensembl.org) and SNPer (snpper.chip.org) provided further detailed information.

On the basis of published reports and choice of genotyping methodology, 8 SNPs were selected for analysis, Table 38.
<table>
<thead>
<tr>
<th>Gene</th>
<th>refSNP cluster ID numbers</th>
<th>SNP name</th>
<th>Location</th>
<th>Change in nucleotide (common, variant)</th>
<th>Change in amino acid</th>
<th>Functional significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAGE</td>
<td>rs1800624</td>
<td>-374 T/A</td>
<td>Promoter region</td>
<td>T to A</td>
<td>N/A</td>
<td>Increased transcriptional activity of RAGE[255]</td>
</tr>
<tr>
<td></td>
<td>rs1800625</td>
<td>-429 T/C</td>
<td>Promoter region</td>
<td>T to C</td>
<td>N/A</td>
<td>Increased transcriptional activity of RAGE[255]</td>
</tr>
<tr>
<td></td>
<td>rs2070600</td>
<td>Gly82Ser G/A</td>
<td>Ligand binding domain – exon3</td>
<td>G to A</td>
<td>Glycine to Serine</td>
<td>Missense, results in damage to structure and function of RAGE[269]</td>
</tr>
<tr>
<td></td>
<td>rs184003</td>
<td>1704 G/T</td>
<td>Intron 7</td>
<td>G to T</td>
<td>N/A</td>
<td>Unknown function but with clinical associations (diabetes)</td>
</tr>
<tr>
<td></td>
<td>rs3134940</td>
<td>2184 A/G</td>
<td>Intron 8</td>
<td>A to G</td>
<td>N/A</td>
<td>Unknown function but with clinical associations (diabetes)</td>
</tr>
<tr>
<td>S100A8</td>
<td>rs3806232</td>
<td>5’ near gene</td>
<td>C to T</td>
<td>N/A</td>
<td>Unknown function but with clinical associations (periodontitis[270])</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rs3795391</td>
<td>-</td>
<td>Intron</td>
<td>C to T</td>
<td>N/A</td>
<td>Unknown function but with clinical associations (periodontitis[270])</td>
</tr>
<tr>
<td>HMGB1</td>
<td>rs1060348</td>
<td>982C/T</td>
<td>Exon 2</td>
<td>C to T</td>
<td>None</td>
<td>Unknown function[717] but with clinical associations[271]</td>
</tr>
</tbody>
</table>

Table 38: SNPs in the RAGE axis that were genotyped. Adenine (A), Cytosine (C), Thymine (T), Guanine (G)
From the 5 SNPs of the RAGE gene studied, there were eight possible haplotypes, based on computationally calculated haplotypes [718], Table 39.

<table>
<thead>
<tr>
<th>HAPLOTYPE</th>
<th>-429T/C</th>
<th>-374T/A</th>
<th>G82S</th>
<th>1704G/T</th>
<th>2184A/G</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>T</td>
<td>T</td>
<td>G</td>
<td>G</td>
<td>A</td>
</tr>
<tr>
<td>2</td>
<td>C</td>
<td>T</td>
<td>G</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>3</td>
<td>T</td>
<td>A</td>
<td>G</td>
<td>G</td>
<td>A</td>
</tr>
<tr>
<td>4</td>
<td>C</td>
<td>T</td>
<td>G</td>
<td>G</td>
<td>A</td>
</tr>
<tr>
<td>5</td>
<td>T</td>
<td>T</td>
<td>G</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>6</td>
<td>C</td>
<td>T</td>
<td>-</td>
<td>-</td>
<td>G</td>
</tr>
<tr>
<td>7</td>
<td>C</td>
<td>T</td>
<td>G</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>T</td>
<td>A</td>
<td>G</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 39: Construction of 8 haplotypes from the variants for the 5 SNPs of interest

Further details of the primer design and methods of genotyping are in Methods (Section 7.8).

14.3.2. Clinical variables

187 UK Caucasian patients undergoing snCPB (Cohort B) were genotyped for the eight SNPs listed in Table 40. Clinical parameters relating to the severity of the inflammatory response and degree of organ dysfunction were collected: hospital and intensive care unit length of stay (ICU LOS), duration of level 3 care, neutrophil and white blood cell (WBC) count, C-reactive protein (CRP), PaO$_2$:FiO$_2$ ratio and alveolar-arterial oxygen difference. Additional clinical variables include plasma levels of sRAGE, S100A8/9; analysis of ten pro-inflammatory cytokines is current.

14.3.3. Data mining

Data regarding genotype, clinical outcomes and sRAGE and S100A8/9 assays were entered into an Excel (Microsoft) spreadsheet. Data were imported into KnowledgeStudio 4.1 (Angoss Software, Guildford, UK); a decision tool used to ‘data mine’ or extract previously unknown information from large databases.

Data mining was necessary to efficiently analyse the possible combinations of comparisons. For example, for each of the SNPs (8), there are several permutations (5) of genotype and allelic distribution, and each of these must be compared to each of the clinical outcomes (9)
at each of the times (4); representing over 1000 analyses. RAGE haplotypes were also compared to outcome variables.

Prior to the data mining processing, the variables were appropriately categorised. Of the data mining tools available within KnowledgeSTUDIO (decision trees, neural networks, clustering algorithms and affinity analysis methods) the ‘decision tree’ tool was the most appropriate and was used to investigate the relationship between several independent variables and one dependent variable.

Variables of interest were individually selected as the dependent variable and the in-built algorithm (KnowledgeSEEKER) was run to identify a list of correlated variables. The lists of potentially related variables were further investigated by selecting them as the dependent variable and verifying that the relationships remained. KnowledgeSTUDIO was used as a preliminary step in the analysis of databases to select variables for further traditional statistical analysis using PRISM v4.0 (GraphPad software, La Jolla, CA, USA) and SPSS v17.01 (IBM, Somers, NY, USA).

14.3.4. Statistical methods
There remain unresolved issues relating to statistical analysis of genetic association studies, particularly how to correct for the risk of false positive results stemming from the use of multiple comparisons. Purely as a function of statistical testing and the use of a P value of ≤0.05 as signifying significance, analysing associations with a large number of SNPs, and/or with a large number of outcomes will inevitably result in false positives. One way of circumventing this is the Bonferroni method, in which the threshold P value is divided by the number of tests. However, this may be overly conservative and stringent. Although there have been many suggested methods, there is no consensus on which is optimal [719]. Despite recognition of the risk of false positive findings, given the uncertainties of correction for multiple comparisons, genetic association studies are particularly susceptible to publication bias – with strongly positive results finding their way into press more easily than negative results. Within this thesis the Bonferroni method is used, the p value acquired from comparisons between variable of interest and genotype is multiplied by the number of SNPs of that gene that were typed for.
The influence of genetic associations on complex disease is usually small with odds ratios of <2.0 and therefore single studies with several hundred participants are usually underpowered [720, 721] and even sizeable studies may fail to detect associations, resulting in a substantial risk of false negative results [719].

14.3.5. Data presentation

As described above, there are a vast number of possible combinations of genetic and clinical data. The data displayed and/or commented on represents the highlights - positive results and important negatives.

The genetic variation of a gene within a study population should not differ from the variation found in other populations. It is standard practice to compare the observed genotypic and allelic distribution to that found by other investigators in order to confirm the maintenance of what is known as Hardy–Weinberg equilibrium. The distributions found by other investigators are stored within the online repository, the HapMap project; this aims to create a complete haplotype map (HapMap) of the human genome. Of the populations selected for inclusion in the HapMap project the most phylogenetically similar to the patients included within our cohort (Caucasian patients in London) are either from U.S. residents of northern and western European ancestry (CEU), or Tuscans in Italy (TSI). When there were more than one set of HapMap CEU data, either the largest sample size, or the most recently submitted were used.
14.4. Results

14.4.1. Single nucleotide polymorphisms in the RAGE gene

14.4.1.1. Genotypic distribution

The distribution of genotypes and alleles in the cohort studied and that recorded within the HapMap project are shown in Table 40.

<table>
<thead>
<tr>
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<th>TSI</th>
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Table 40: The observed distribution of genotype and allele for 5 SNPs of the RAGE gene compared with those recorded in on-line databases of genomic distribution for two populations of relevance (CEU and TRI).
The observed distribution of RAGE genotypes was comparable to the distributions recorded in online repositories of genetic epidemiological data, as shown in Table 40. A $\chi^2$ test confirmed Hardy-Weinberg equilibrium, $p<0.05$. i.e. the observed frequencies of each genotype were not statistically significantly different to those expected.

14.4.1.2. Relationship to endpoints

14.4.1.2.1. Clinical

14.4.1.2.1.1. Systemic inflammation

Graphical representation of selected relationships between genotype distribution within SNPs of the RAGE gene and markers of systemic inflammation (neutrophil count and CRP) are presented in Figures 102, 103, and 104).

As seen in Figure 102, there was a consistent trend towards a lower neutrophil count in those with the minor (A, in grey) allele and at post-operative day 2 (POD2) this became statistically significant: minor allele carrier’s median neutrophil count $7.6$ (IQR 5.8-9.0) $\times 10^9$/l versus 8.9 (IQR 7.2-12.0) $\times 10^9$/l in those without, $p_c=0.026$. 
As seen in Figure 103, the levels of CRP measured immediately post-operatively (POD0), and on each of the following days, were not statistically significantly different between those patients carrying the minor allele and those without.

Figure 103: CRP in post-snCPB patients, grouped by RAGE G825-A carriage status on each post-operative day (POD). snCPB patients were genotyped and comparison of CRP made between those carrying the minor allele (A, grey, n=17) and those without the minor allele (no A, black, n=168). Data are presented as median with bars indicating IQR. Mann Whitney U test \( p_c >0.05 \) for all.

Figure 104: CRP in post-snCPB patients, grouped by RAGE -374 genotype on each post-operative day (POD). snCPB patients were genotyped and comparison of CRP made between those with AA genotype (in white, n=7), AT genotype (in grey, n=62) and TT genotype (in black, n=106). Data are presented as median with bars indicating IQR. Kruskal Wallis test with Dunn’s Multiple Comparison test * \( p_c <0.05 \).
As seen in Figure 104, there was a pattern of those homozygous for the minor allele (AA, shown in white) having lower CRP at each time considered. At POD1 this reached statistical significance: median CRP in those genotyped AA 39 (IQR 24-48) mg/L, vs. AT 67 (IQR 49-91) mg/L, vs. TT 63 (IQR 46-94) mg/L, \( p_c = 0.02 \) for both comparisons.

14.4.1.2.1.2. Pulmonary dysfunction

Graphical representation of selected relationships between genotype distribution within SNPs of the RAGE gene and markers of pulmonary dysfunction (PFR and D(A-a)) are presented in Figures 105 and 106.

As seen in Figure 105, there was a trend towards a lower PFR in those with the minor (A) allele but at no point did this result become statistically significant.

Figure 105: PFR in post-snCPB patients, grouped by RAGE G82S-A carriage status on each post-operative day (POD). snCPB patients were genotyped and comparison of PFR made between those carrying the minor allele (A, grey, \( n=17 \)) and those without the minor allele (no A, black, \( n=168 \)). Data are presented as mean with bars indicating SEM. Unpaired t test \( p>0.05 \) for all.
Figure 106: D(A-a) in post-snCPB patients, grouped by RAGE G825-A carriage status on each post-operative day (POD). snCPB patients were genotyped and comparison of D(A-a) made between those carrying the minor allele (A, grey, n=17) and those without the minor allele (no A, black, n=168). Data are presented as median with bars indicating IQR. Mann Whitney U test p>0.05 for all.

As seen in Figure 106, no statistically significant differences or apparent trends were seen.

14.4.1.2.1.3. Length of stay

A relationship between RAGE genotype and the composite endpoint of ICU LOS was of primary importance within these investigations. Data mining did not identify any statistically significant relationships with the exception of possible relationship between a 1704-T RAGE variant and prolonged advanced respiratory support.

Figure 107: Hours of advanced cardiovascular support in post-snCPB patients, grouped by RAGE 1704-T carriage status. snCPB patients were genotyped and comparison of hours of advanced cardiovascular support made between those carrying the T allelic variant (grey, n=6) and those not (black, n=74). Data are presented as median with bars indicating IQR. Mann Whitney U test p>0.05.
As seen in Figure 107, those carrying the minor variant at 1704 (T, in grey) had a median duration of requiring CV support of 27.7 (IQR 19.0-208) hours vs. those without the minor variant 37.3 (IQR 20.0-65.5) hours, p>0.05. Therefore the possible association was found not to have statistical significance.

14.4.1.2.2. Plasma assays

Graphical representation of relationships between G82S genotype and sRAGE levels pre- and post-snCPB are presented in Figures 108.

14.4.1.2.2.1. RAGE axis

As seen in Figure 108, pre-operative plasma levels of sRAGE were lower in those patients carrying the minor allelic variant, median(IQR): 1030(522.0-1318) pg/ml vs. 1092(724-1763) pg/ml. The effect was more pronounced post-operatively, median(IQR): 1365 (633.7-2794) pg/ml vs. 1937 (1284-2681) pg/ml. However, the differences did not meet statistical significance.

14.4.1.2.2.2. Cytokine

The relationships between genotype distribution within SNPs of the RAGE gene and cytokines are yet to be determined. Multiplex assays for IL-6, IL-8, IL-10, MCP-1, TNF-α, TNF-β, GRO, Eotaxin, VEGF and IP-10 are ongoing.
14.4.2. Single nucleotide polymorphisms in the S100A8 and HMGB1 genes

14.4.2.1. Observed allelic frequencies

S100A8

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<th>Observed</th>
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</thead>
<tbody>
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Table 41: The observed distribution of genotype and allele for 2 SNPs of the S100A8 gene compared with those recorded in on-line databases of genomic distribution for two populations of relevance (CEU and TRI).

Linkage disequilibrium analysis revealed the two SNPs rs3795391 and rs3806232 in the S100A8 gene to be in tight linkage disequilibrium and therefore co-inherited. Our observations were consistent with this.

HMGB1

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Table 42: The observed distribution of genotype and allele for one SNP of the HMGB1 gene compared with those recorded in on-line databases of genomic distribution of the MITOGPOP6 population (mixed population including Asian ancestry).

The observed distribution of S100A8 and HMGB1 genotypes was comparable to the distributions recorded in online repositories of genetic epidemiological data, as shown in Tables 41 and 42. A $\chi^2$ test confirmed Hardy-Weinberg equilibrium, p<0.05.
14.4.2.2. Relationship to endpoints

14.4.2.2.1. Clinical

14.4.2.2.1.1. Systemic inflammation
Data mining did not identify any potential relationships between genotypic variants of the S100A8 or HMGB1 genotypes and white blood cell count, neutrophil count or CRP.

14.4.2.2.1.2. Pulmonary dysfunction

![Figure 109: PFR in post-snCPB patients, grouped by S100A8 (rs3806232) genotype on each post-operative day (POD). snCPB patients were genotyped and comparison of PFR made between those carrying the minor C allele (white, n=47) and those not (black, n=138). Data are presented as mean with bars indicating SEM. Unpaired t test * p<0.05.

As seen in Figure 109, carriers of the minor allele (white) had lower PFR (equating to worse oxygenation) on post-operative days 1 and 2, reaching statistical significance at post-operative day 3; mean PFR for minor allele carriers 160±73mmHg vs. 232±86mmHg, p<0.05.
Figure 110: D(A-a) in post-snCPB patients, grouped by S100A8 (rs3806232) genotype on each post-operative day (POD). snCPB patients were genotyped and comparison of D(A-a) made between those carrying the minor C allele (white, n=47) and those not (black, n=138). Data are presented as mean with bars indicating SEM. Unpaired t test ** p<0.01.

<table>
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<tr>
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<th>POD2</th>
<th>POD3</th>
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</thead>
<tbody>
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<td>22</td>
<td>10</td>
</tr>
<tr>
<td>TT</td>
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<td>129</td>
<td>41</td>
<td>26</td>
</tr>
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</table>

Table 43: Numbers of patients on each day in the two groups: Those with carriage of the minor C allele and those without.

As seen in Figure 110, carriers of the minor C allele (white) had higher D(A-a) (equating to worse oxygenation) on post-operative days 1 and 2, although not statistically significantly, but reached statistical significance at post-operative day 3: mean D(A-a) for minor allele carriers was 33.9±20.3kPa vs. 20.5±10.0kPa, p=0.01.

On each passing day a proportion of patients will have recovered sufficiently to have been extubated or even left ICU and therefore the numbers in each group diminish, as can be seen in Table 43.

When comparing the total duration of advanced respiratory support (in hours) between the TT [median (IQR): 15.8 (11.5-21.7)] and CT/CC groups [median (IQR): 16.3(8.5-19.7)], there was no significant difference (p>0.05 MWU).
14.4.2.2.1.3. Renal dysfunction

Renal dysfunction following snCPB is associated with adverse patient outcome, even when mild [69]. Clinical assessments of renal dysfunction included changes in plasma creatinine but of greater clinical relevance is the need for renal replacement therapy or the worse acute kidney injury (AKI definitions in Section 6.4) score within the first week. The distribution of worst AKI scores within the first week against S100A8 genotype (Figure 112) showed dissimilarity.

**Figure 111:** Histogram of distribution of post-snCPB acute kidney injury (AKI) score grouped by carriage of the minor C allele at S100A8 (rs3806232). snCPB patients were genotyped and comparison of worse AKI score in the first week post-snCPB compared between those with the minor allele (Not_TT, top image) and those without (TT, lower image).

This pair of histograms (Figure 111) demonstrate the distribution of worst AKI scores in the first week following snCPB, with the top figure (Not_TT) representing those carrying the minor allele and the lower figure, those homozygous for the major variant (TT).
To investigate this further, the worst AKI score was entered into a contingency table and Fisher’s exact test calculated (Table 44).

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<tr>
<td>≥3</td>
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Table 44: Table of p values from the Fisher’s exact test comparing distribution of AKI between the two groups: not_TT and TT.

Patients carrying the minor allele had a statistically significantly greater incidence of AKI of greater than or equal to stage 2 (an increase of 200-300% in serum creatinine from baseline) than those with the TT genotype.

14.4.2.2.1.4. Length of stay
Data mining did not identify any potential relationships between genotypic variants of the S100A8 or HMGB1 genotypes and duration of Level 3 (ICU) or hospital stay.

14.4.2.2. Plasma assays

14.4.2.2.1. Plasma levels of S100A8/9

![Figure 112: Plasma S100A8/9 levels in patients pre- and post snCPB, grouped by carriage of the minor C allele at S100A8 (rs3806232). snCPB patients were genotyped and comparison of S100A8/9 levels made between those carrying the C allelic variant (grey, n=29) and those not (black, n=96). Data are presented as mean with bars indicating SEM. Unpaired t test p>0.05.](image)
No relationship was found between S100A8 genotype and levels of circulating S100A8/9 either pre-operatively: mean levels of minor allele carriers 484.2±378.7pg/ml vs. 449.9±385.9pg/ml; or post-operatively 2915±1486pg/ml vs. 2561±1234pg/ml, p>0.05 (Figure 112).

14.4.2.2.2.2. Cytokine

The relationships between genotype distribution within SNPs of the S100A8 and HMGB1 genes and cytokines are yet to be determined. Multiplex assays for IL-6, IL-8, IL-10, MCP-1, TNF-α, TNF-β, GRO, Eotaxin, VEGF and IP-10 are ongoing.
14.5. Discussion

Our primary hypothesis was that genetic variation within the RAGE axis would influence clinical outcomes from snCPB, particularly duration of requirement for level 3 care; a more specific marker than ICU LOS. Accordingly, SNPs were identified in the RAGE, S100A8 and HMGB1 genes. Furthermore, the DNA of a large cohort of snCPB patients was genotyped using SSP-PCR. The distribution of studied genotypes was found to be consistent with those reported in the Hapmap database. However, none of the genetic variations studied showed statistically significant relationships to prolonged duration of ICU or hospital stay. Nonetheless, there were notable relationships found between the RAGE and S100A8 SNPs with other end-points, which will be discussed.

Before discussing the positive findings it is important to explain why we should not accept the null hypothesis – that there is no relationship between genetic variation in the RAGE axis and outcome from snCPB. This study did not recruit sufficient numbers of patients to be adequately powered to confidently accept or reject the null hypothesis. This shortcoming is common in such trials, with the majority of trials assessing genetic variation in inflammatory genes and their effect on outcomes from sepsis recruiting no more than 200 patients [215].

Of the SNPs studied here, the G82S variant of RAGE was arguably the most likely to have an association with clinical outcome given the repeated associations of G82S genotype with plasma sRAGE levels and predisposition to inflammatory conditions. Unfortunately, as in other studies, the low prevalence (5%) of the minor allele of the G82S SNP might have compounded the effect of small sample size [722]. The duration of requirement for level 3 care reflect the extent of respiratory, cardiovascular, neurological and renal dysfunction and the effects of variation in one inflammatory pathway would have to be very large to significantly influence this. As the extent of the effect is likely to be small, a very large sample size would be required. For example, it has been estimated that for a study assessing the influence of candidate SNP on outcome from septic shock to be suitably powered it would need to recruit 2000 patients [216].

14.5.1. RAGE

Polymorphisms in the RAGE gene were related to differences in systemic inflammation, as assessed by neutrophil count and CRP (Figures 102-104). Being heterozygous for the
functional RAGE G82S minor allele (genotype AG) was associated with lower neutrophil counts post-operatively, reaching statistical significance at day 2 post-operatively. No patients were homozygous for the minor allele of G82S.

The RAGE G82S SNP (rs2070600) has a common and a minor variant; the later is associated with a change in amino acid glycine to serine within the ligand-binding domain of RAGE. The 82Serine variant is associated with enhanced ligand affinity and up-regulated RAGE signalling with hyper-responsiveness to S100A12 resulting in increased pro-inflammatory signalling and cytokine secretion in monocytes [265]. Carriage of this minor allele has been associated with increased risk of type 2 diabetes, development of nephropathy in patients with existing diabetes and retinopathy, and increased risk of rheumatoid arthritis; as well as relatively lower levels of plasma sRAGE in several populations [256, 260, 265, 267, 268].

The association between carriage of G82S SNP with lower neutrophil count was therefore unexpected. As the G82S minor variant has been associated with increased inflammatory signalling, the development of RAGE-mediated complications and low sRAGE levels, we might have expected an association with increased inflammatory markers. Neutrophilia reflects increased neutrophil formation as well as prolonged lifespan from delayed apoptosis, a mechanism proposed to mediate tissue damage in systemic inflammation. The relatively less elevated neutrophil counts associated with the G82S SNP following snCPB may reflect a diminished response.

The crucial question when interpreting these data is; to what extent does neutrophilia vary in proportion to inflammation? Extent of leukocytosis (or neutrophilia) has not been assessed in isolation in relation to outcome from systemic inflammation but is an integral part of prognostic severity of illness scores such as APACHE II [518]. On a population basis there may be a strong relationship between leukocytosis and outcome but in individuals, a higher WBC does not necessarily indicate a more severe infection. Indeed the SIRS criteria have high, or low, WBC as defining criteria. Furthermore, very high WBC counts are more likely to be due to non-infectious or inflammatory conditions [723].

Those who were homozygous for the minor allele for the RAGE promoter region SNP (-374) had a lower CRP at day 1 post-operatively but not on other days (Figure 104). This SNP has been associated with increased transcriptional activity of the RAGE gene and with the
abolition of a nuclear protein binding site, the functional significance of which is unclear [255].

The physiological delay of 24-36h between inflammatory stimulus and peak concentration of CRP means that the levels on post-operative day 1 may represent the best indicator of initial inflammatory response to surgery. Levels of plasma CRP do not necessarily correlate with RAGE ligands such as S100A8/9 [724] and therefore the RAGE axis might function in parallel and independently to the inflammatory axis reflected in CRP levels. The lack of relationship between CRP and neutrophil count during the first 24h and either the vital outcome or ICU LOS demonstrated in Section 10.5.6, suggests that they are not the optimal variables to be used as a measure of systemic inflammation. Studies of the predictive value of CRP for adverse outcome in patients with systemic inflammation suggest that isolated measurements have some value but are inferior to serial measurements [648].

Plasma sRAGE levels has been reported to be lower in healthy subjects carrying the minor allelic variants at G82S and although the levels were lower both pre- and post- operatively in these patients, these differences were not statistically significant (Figure 108). It is possible that the association between G82S and lower sRAGE levels and lower WBC counts are related but this requires further investigation.

The small numbers of patients who were carriers of the minor variants combined with a large degree of variability of sRAGE values post-operatively might have contributed towards the lack of statistical significance. There were no associations between SNPs in the RAGE gene and post-operative pulmonary function or duration of ventilation.

14.5.2. S100A8 and HMGB1

Polymorphisms in the S100A8 gene were related to differences in pulmonary and renal dysfunction. The minor allelic variants of the S100A8 genes were consistently associated with impaired oxygenation as assessed by PFR and A-a difference, becoming statistically significant on day 3 post-operatively. There were no significant differences between the measured levels of S100A8/9 either pre- or post-operatively between the genotypes.

The S100A8 SNPs are in strong LD and can be considered together. Neither are in coding regions of the S100A8 gene with rs3806232 lying in the promoter region and rs3795391 in
an intron, yet they may still exert influence over gene function (Section 2.4). There are no studies of these SNPs in Caucasian populations and the associations from other populations (Chinese [270]) may not be valid in Caucasians. These SNPs were chosen as the only SNPs in the S100A8 gene with association with an inflammatory condition, albeit the minor allele with reduced incidence of aggressive periodontitis. The reported study included small numbers of patients, did not report power calculations, made multiple comparisons without appropriately statistical correction and the findings are yet to be replicated. Given the lack of robust data to suggest whether the S100A8 minor allele was associated with greater or lesser inflammatory activity, we had no expectations regarding any associations between carriage of the minor allele and outcomes following snCPB.

Post-snCPB oxygenation impairment is multi-factorial and includes non-inflammatory sequelae such as cardiogenic pulmonary oedema, bronchospasm and pleural collections. However, snCPB induces lung inflammation on a spectrum from subclinical lung injury to ARDS [725, 726]. The chosen measures of oxygenation are both accepted and clinically useful but are interrelated. PFR reflects extent of hypoxaemia for a given concentration of inspired oxygen and the alveolar-arterial oxygen difference indicates the severity of impairment of the alveolar membrane function. A potentially superior measure would have been the ‘oxygenation index’ – a measure of oxygenation impairment that is specific for ventilated patients as it incorporates the mean airway pressure, which broadly reflects how much effort is being put into ventilation [727].

During the successive days following snCPB, an increasing proportion of patients have recovered sufficiently to be extubated and discharged from the ICU; such patients do not have oxygenation data and were not represented within the analysis. The difference in oxygenation by genotype only reached significance by post-operative day 3, when the numbers in the groups had significantly decreased (26 vs. 10 carrying the minor allele). The mean incidence of ARDS post-snCPB is 5 days, therefore the differences in oxygenation at these later times might relate more to the influence of inflammatory responses than during the earlier times [726]. Perhaps the most clinically significant measure of pulmonary dysfunction is duration of mechanical ventilation and there was no statistically significant difference in this measure between the genotypes.
There was an association between minor allelic variants of the S100A8 genes and a higher incidence of acute kidney injury (AKI) of stage 2 or worse in the first week (an increase of 200-300% in serum creatinine). AKI following snCPB is associated with increased mortality, even if it does not require renal replacement therapy [728, 729]. Indeed, AKI complicating admission to ICU for any cause is associated with adverse outcome in proportion to stage [52, 730]. The main contributory determinants of AKI post-snCPB are cellular ischaemia and systemic inflammation. Furthermore, S100A6 and S100B have been implicated in the pathogenesis of experimental AKI [731].

The minor alleles of these S100A8 SNPs are associated with impaired oxygenation following snCPB and we hypothesise that this could be due to increased pulmonary inflammation due to altered S100A8/9-mediated effects. Although plasma levels did not vary (Figure 112), pulmonary levels (perhaps via broncho-alveolar lavage, BAL sampling) may have. Similarly, the increased risk of AKI may relate to aberrant inflammatory signalling within the injured kidney.

No associations were found between the HMGB1 SNP studied (rs1060348) and clinical outcomes. This is likely to be due to the low prevalence of the minor variants of this SNP within the cohort, with only 6 heterozygous and no homozygous. The SNP is in a coding region (exon 4) but not associated with a change in the protein structure. However, it is in close LD with a SNP in the promoter region (−1615 A/G SNP, 1412125) [717] and despite not being a known binding or splice site, may affect transcriptional activity of HMGB1. The minor allele of the HMGB1 SNP has been associated with increased number of SIRS criteria, greater organ failure and impaired oxygenation in 239 patients on an adult ICU. In the same study a promoter variant (-1377delA) was associated with inferior longer-term (4y) outcome.

There are significant limitations to genetic association studies (Section 2.4.1) and of particular relevance to these investigations are those that stem from a small sample size, such as risk of type I and II errors.

14.5.3. Future work

In order to avoid false positive associations and falsely accept the null hypothesis a significantly larger sample size would be necessary. To facilitate this, the study could be
extended to all types of major surgery. Studying patients at higher-risk for the development of organ dysfunction, such as those with diabetes, chronic kidney injury and of increased age might increase the sensitivity of the study. Using more sensitive markers of organ dysfunction would also be advantageous. In order to ascertain the role of the S100 proteins in pulmonary and renal inflammation during and following snCPB, acquisition of clinical samples such as BAL and urine would be informative.

Alternatively the systemic inflammatory response to infection could be studied in patients with sepsis, perhaps in a cohort similar to the GenoSept study. This International study has recruited patients with sepsis and septic shock from across Europe and intends to identify novel candidate genes which are then used in genetic association epidemiological studies of sepsis related adverse outcomes [732].

The SNPs considered in this study were selected from a far larger range of possibilities and we were restricted by our choice of genotyping technology (SSP-PCR). Given the opportunity, further SNPs of the RAGE, S100A8 and HMGB1 genes could have been assessed, in addition to SNPs in the genes for S100A12, and S100B.

14.6. Conclusion

Genetic association studies have the potential to offer valuable insights into pathogenesis of diseases states. However, for the findings to be reliable and reproducible the studies need to be large. The risks of false positive associations and accepting the null hypothesis are significant and cannot be overlooked.

The complexities of the immune response to injury should not be underestimated and for a study of this size, even if a genetic variation has a potent and profound effect within the pathway of interest, it is hard to demonstrate. This is particularly the case when the outcome is a clinical outcome such as duration of requirement for intensive care that reflects many processes, of which only a proportion will relate to extent of systemic inflammatory response.

The role of genetic variation in the RAGE axis in clinical response to acute inflammatory insult, such as snCPB, is an unexplored area, as are the functional significance of many of the SNPs studied. The associations identified within this study are supportive of the hypothesis
that the RAGE axis is important in determining extent of systemic inflammatory response and may play an important role in the development of organ dysfunction.
Concluding remarks

Summary of results
The relationship between systemic inflammation following snCPB and the age of the patient had not been previously investigated. Extent of systemic inflammation was assessed using C-reactive protein, neutrophil count and the number of SIRS-defining criteria fulfilled within the first hour or first 24h. Extent of organ dysfunction was assessed with the SOFA score and ICU LOS was used as a surrogate of severity of illness. The largest patient group was those who underwent CABG, and also represents the most homogeneous surgical category. In this surgical category, the mean number of SIRS-defining criteria was significantly higher in the >80y group compared with the 40-60y or 60-80y groups both in the first hour and the first 24h. Similarly, the SOFA score and ICU LOS were significantly higher in the >80y group compared with the 40-60y or 60-80y groups. However, these markers of increased systemic inflammation, organ dysfunction and severity of illness were not reflected in higher CRP or WBC; in fact, these were significantly lower in the >80y group than the younger groups. These incongruous findings may be reconciled either through acknowledgement that CRP and WBC within the first 24h do not represent a genuine reflection of systemic inflammatory state; or that SIRS criteria, SOFA score and ICU LOS are higher in the oldest group through mechanisms that are independent of systemic inflammation. However, considering all the patients who had snCPB, the maximum number of SIRS criteria in the first 24h and the SOFA score were demonstrated to be independently associated with risk of mortality and prolonged ICU LOS, itself an undesirable outcome. This clearly supports the paradigm of excessively severe systemic inflammatory responses contributing towards organ failure and death.

To what extent might the RAGE-axis participate in, determine the extent of, and outcome from, the acute systemic inflammatory response? RAGE ligands are damage-associated molecular pattern (DAMP) biomolecules and are released during tissue injury, such as occur during snCPB. The release of some RAGE ligands following snCPB has been reported by others in small series without detailed comparison to clinical characteristics [661, 663-666]. Soluble RAGE levels in plasma have been demonstrated to be elevated in patients with
acute systemic inflammatory conditions and were recently reported in a small cohort of patients following snCPB [457]. However, reports have not featured paired assays of sRAGE with esRAGE, have not studied large groups of patients and have not measured levels of sRAGE during CPB. We demonstrated significant elevations in plasma levels of S100B, S100A8/9, S100A12, HMGB1 and CML-albumin, with a strong relationship between severity of surgical injury (duration of CPB) and extent of S100 protein release – supporting the concept of surgery and CPB resulting in release of RAGE ligands. sRAGE plasma levels bore a strikingly consistent relationship to esRAGE levels – casting doubt on the prevailing understanding of differential regulation. The maximal extent of sRAGE abundance in the plasma was observed during CPB, a time when the lungs are solely dependent on the bronchial circulation for perfusion – casting doubt on the current dogma of the pulmonary alveoli representing the predominant source of sRAGE in the circulation. In the chronic state, plasma sRAGE levels may reflect extent of RAGE-mediated signalling and thereby propensity to RAGE-mediated inflammation. Thus, in patients prior to snCPB the levels were found to have a robust relationship to duration of requirement of intensive care post-snCPB. Plasma sRAGE could represent a biomarker for systemic inflammatory response to tissue injury and as such, could offer advantages over current methods of risk stratification for patients presenting for major elective surgery.

Having demonstrated elevations of RAGE ligands and soluble RAGE in the plasma following snCPB we sought to determine the expression of leukocyte RAGE expression both at the level of transcription and protein. Membrane-bound RAGE on circulating leukocytes could represent a more direct assay of pro-inflammatory RAGE-axis activity. Development of a reproducible method for the quantitative assay of RAGE on live leukocyte populations of whole blood was facilitated by collaboration with a group with an established protocol. The extent of RAGE expression on neutrophils was shown to decrease following snCPB. Extent of intracellular RAGE expression was high relative to membrane expression, but was not demonstrated to significantly alter following snCPB. Change in expression of RAGE when measured at the level of transcription, and in relation to the expression of reference genes, was non-significantly increased in neutrophils, monocytes and buffy coat when comparing post-snCPB to pre-snCPB. Together these findings indicate that snCPB affects the regulation of leukocyte RAGE. It seems probable that RAGE ligands up-regulate RAGE expression and
following RAGE protein expression on the cell surface, there is cleavage of the extracellular domain resulting in increased sRAGE levels paired with decreased RAGE on leukocytes.

To investigate the effects of RAGE ligands on leukocyte inflammatory responses and how snCPB may alter these, samples were obtained from healthy volunteers and patients for ex vivo experiments. Stimulation of both whole blood and isolated neutrophils with S100B and S100A12 resulted in a concentration-dependent release of the archetypal chemokine IL-8. We were unable to demonstrate pro-inflammatory responses or augmentation of such responses with HMGB1. Pre-incubation of either the cells or the ligands with either a recombinant sRAGE protein or an experimental analogue (TTP-4000) was not associated with any diminution of cytokine release. Similarly, use of anti-S100B or anti-RAGE antibodies was not associated with diminution of cytokine release. Endotoxin tolerance has been reported to occur following snCPB and in addition to replicating this phenomenon we demonstrated tolerance to S100B. Identical stimulation of whole blood obtained post-versus pre-snCPB with S100B resulted in significantly lower release of the pro-inflammatory cytokines IL-1β, IL-6, IL-8, and TNF-α.

Genetic variation in the genes of the inflammatory response has been associated with altered levels of pro-inflammatory cytokines and adverse clinical outcomes following snCPB. Although the RAGE gene has been studied in relation to development of diabetes and its complications, there had been no studies in acute critical illness. In order to investigate the hypothesis that genetic variation within the RAGE axis would influence clinical outcomes from snCPB, the DNA of a large cohort of snCPB patients was genotyped for genes of the RAGE axis using SSP-PCR. Of the studied genetic variations in the genes for RAGE, S100A8 and HMGB1 there were no significant associations with prolonged duration of ICU or hospital stay. Unfortunately, the study did not recruit sufficient numbers of patients to be adequately powered to confidently accept or reject the null hypothesis and therefore we cannot conclude that there is no relationship between genetic variation in the RAGE axis and outcome from snCPB. Carriage of the G82S SNP within the RAGE gene was associated with lower neutrophil counts post-operatively than the common variant. Homozygous carriage of minor variant of the promoter -374 of the RAGE gene was associated with a lower CRP on the first post-operative day. The clinical significance of these relationships is uncertain. The association between the minor variant of an S100A8 SNP with impaired
oxygenation and acute kidney injury post-snCPB could indicate the influence of RAGE-mediated inflammation on the development of organ dysfunction.

**Clinical relevance**

Increased age predisposes to adverse outcomes from a range of acute systemic inflammatory conditions including snCPB. The novel finding of an association between number of SIRS criteria met within the first 24h and both mortality and prolonged ICU LOS supports the concept of systemic inflammation being an important influence on outcome. Immune responses are altered with advanced age but the extent to which these alterations are important in the evolution of acute systemic inflammation and development of complications remain undetermined. Similarly, the extent to which increased abundance of RAGE ligands and up-regulation of the RAGE axis contributes is unknown.

RAGE ligands are elevated in the plasma following a range of acute systemic inflammatory conditions including snCPB, and also at sites of localised inflammation. S100 proteins and HMGB1 are archetypal DAMPs and, as such, are released from injured or inflamed tissues. The measurement of RAGE ligands as biomarkers of specific tissue injury or inflammation has entered clinical practice with varying degrees of adoption: S100B has yet to become a standard of care in brain injury [733], S100A8/9 has a well recognised role in inflammatory bowel disease [734], and use of plasma S100A12 assays remains highly specialised in some arthritides and auto-immune diseases [735].

The measurement of sRAGE has been proposed as a marker of acute lung injury and several studies have reported its use in this context [454, 459, 482]. Close correlations between physiological and radiographic markers of ALI with sRAGE levels, combined with *ex vivo* experimental data from human lungs, provides particularly compelling evidence for the role of sRAGE as a marker of alveolar damage [454, 489]. Major limitations include the lack of specificity and the differences in measured values between control populations [294, 454].

The novel finding of a statistically significant relationship between pre-snCPB plasma levels of sRAGE and prolonged duration of level 3 care is potentially of great clinical significance. The levels of pre-snCPB sRAGE were more closely related to prolonged recovery from acute systemic inflammation (duration of level 3 care) and to overall hospital admission (time to hospital discharge) than variables including the age of the patient, a score of their operative
risk (Euroscore), and the duration of pulmonary/myocardial ischaemic time (component of total CPB time). Measurement of sRAGE pre-operatively could be incorporated into clinical practice, improving provision of accurate prognostic information for patients and identifying those requiring closer monitoring, or even those who might benefit from RAGE inhibition.

The physiological function of sRAGE in humans remains to be determined. The meaning of elevated levels of sRAGE may depend upon the clinical situation. In the setting of acute critical illness, elevated sRAGE levels could stem from several sources including injured alveoli with their constitutively high levels of membrane-bound RAGE being shed, activated inflammatory cells up-regulating RAGE and shedding the ectodomains, and other, as yet unidentified sources that may include the vascular endothelium. Outside the setting of acute critical illness, elevated sRAGE levels probably reflect the ‘tone’ of the RAGE-axis, that is, the abundance of ligands and consequent activity of cellular RAGE.

RAGE inhibition in animal models of the sepsis syndromes has been reported to improve outcomes including mortality. Whether this might translate into clinical benefit in patients is not yet known and before such trials can be seriously contemplated there are fundamental questions that need to be answered. Primarily, does inhibition of RAGE-mediated signalling reduce excessive systemic inflammation without compromising vital anti-microbial defences in animals that are biologically closer to humans than mice, and in models that are more clinically relevant than caecal ligation and puncture? For example, inhibition of RAGE in established models of pneumonia in pigs would potentially provide more relevant information [736]. It may be that RAGE inhibition does compromise microbial defences and the benefits of inhibition are only seen in ‘sterile’ SIRS. Therefore investigation in models of ‘sterile’ SIRS would also be valuable; for example, use of established porcine haemorrhagic shock and resuscitation models of systemic inflammation [737].

Survivors of critical illness suffer not just increased morbidity, but also increased mortality compared to matched controls for years following hospital discharge [738]. Indeed, severity of acute illness (assessed by APACHE II score) and extent of organ failure are independently associated with increased risk of long term mortality [738, 739]. The physiological mechanisms that mediate this phenomenon remain undetermined. Although it is highly speculative, it is possible that episodes of acute systemic inflammation cause long-standing
alterations in immune responses that contribute towards these observations. Up-regulation of the RAGE axis could participate in this, with increased ligands during inflammatory episodes causing widespread up-regulation of RAGE that persists after resolution of the acute illness.

**Future work**

To determine the influence of age of the RAGE axis it would be desirable to measure RAGE expression on a variety of cells and tissues from patients across a wide range of ages. In addition to extent of expression, the inflammatory response consequent upon RAGE ligation and particularly its relation to the age of the leukocyte donor would further elucidate the influence of age on the RAGE axis. Measurements of AGE content of tissues as well as AGE-modified plasma proteins would be novel and informative, particularly if paired with measures of RAGE expression and responsiveness.

To establish the relationship between leukocyte RAGE expression before, during and after snCPB to both clinical characteristics and to sRAGE levels, the work presented in Section 12 could be continued in a larger cohort of patients. Were such observations to be paired with measures of soluble RAGE forms it would be possible to gain further understanding of their relationship. Indeed, such studies would be valuable in other populations with acute systemic inflammation including trauma and severe sepsis.

To further characterise the alterations in leukocyte function consequent upon RAGE ligand binding we could assay chemotaxis, phagocytosis, or intracellular killing instead of cytokine release. Inhibition experiments could be conducted in better-characterised models such as alveolar epithelial cell lines (A549 [715]) or endothelial cells (HUVEC [716]) prior to the use of novel inhibitors such as sRAGE fragments [713]. Moving from *in vitro* models to *in vivo* models but with greater relevance to the human immune response, ‘humanized’ mice could be used to study the effects of RAGE inhibition on outcomes from experimental SIRS [740].

Genetic association studies require large sample sizes to have adequate power to reject the null hypothesis. Therefore, a far larger cohort of patients would be required to take this work further. One approach would be to use DNA from a biobank of previously collected samples, such as the previously described GenoSept study. Furthermore, use of semi-
automated sequencing technologies instead of SSP-PCR would permit genotyping of a wider range of SNPs more efficiently and accurately.

**Conclusions**

These investigations provide evidence supporting the role of the RAGE axis in acute systemic inflammation following snCPB. The implications of these findings reach beyond the immune response to cardiac surgery and are likely to apply similarly to other causes of SIRS. Further investigations are required to gain a deeper understanding of the role of RAGE and its soluble form sRAGE in human inflammatory disease, prior to the evaluation of RAGE inhibitors in this setting.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>A549 cells</td>
<td>Human Type II Alveolar Epithelial Cell Line</td>
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<td>ABG</td>
<td>Arterial Blood Gases</td>
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<tr>
<td>ACCP/SCCM</td>
<td>American College Of Chest Physicians/Society Of Critical Care</td>
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<tr>
<td>ACE</td>
<td>Angiotensin Converting Enzyme</td>
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<tr>
<td>ADAM</td>
<td>A Disintegrin And Metalloprotease</td>
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<tr>
<td>AECC</td>
<td>American-European Consensus Conference</td>
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<tr>
<td>AGE</td>
<td>Advanced Glycation End-Products</td>
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<tr>
<td>AKI</td>
<td>Acute Kidney Injury</td>
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<tr>
<td>ALI</td>
<td>Acute Lung Injury</td>
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<tr>
<td>ANOVA</td>
<td>Analysis Of Variance</td>
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<tr>
<td>AP-1</td>
<td>Activator Protein-1</td>
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<tr>
<td>APACHE</td>
<td>Acute Physiology And Chronic Health Evaluation</td>
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<tr>
<td>ARB</td>
<td>Angiotension Receptor Blocker</td>
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<tr>
<td>ARDS</td>
<td>Acute Respiratory Distress Syndrome</td>
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<tr>
<td>BAL</td>
<td>Bronchoalveolar Lavage</td>
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<tr>
<td>BALF</td>
<td>Bronchoalveolar Lavage Fluid</td>
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<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<tr>
<td>CABG</td>
<td>Coronary Artery Bypass Graft</td>
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<tr>
<td>CAD</td>
<td>Coronary Artery Disease</td>
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<tr>
<td>CARS</td>
<td>Compensatory Anti-Inflammatory Response Syndrome</td>
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<tr>
<td>CD</td>
<td>Cluster Of Differentiation</td>
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<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
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<tr>
<td>CF</td>
<td>Cystic Fibrosis</td>
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<tr>
<td>CKD</td>
<td>Chronic Kidney Disease</td>
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<tr>
<td>CLP</td>
<td>Caecal Ligation And Puncture</td>
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<tr>
<td>CML</td>
<td>Nε Carboxymethyl-Lysine</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic Obstructive Pulmonary Disease</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclo-Oxygenase</td>
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<tr>
<td>CPB</td>
<td>Cardiopulmonary Bypass</td>
</tr>
<tr>
<td>CRP</td>
<td>C-Reactive Protein</td>
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<tr>
<td>Ct</td>
<td>Cycle Threshold</td>
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<tr>
<td>CURB</td>
<td>Confusion, Urea, Respiratory Rate, Blood Pressure</td>
</tr>
<tr>
<td>CV</td>
<td>Cardiovascular</td>
</tr>
<tr>
<td>CXR</td>
<td>Chest X-Ray</td>
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<tr>
<td>D(A-a)</td>
<td>Alveolar-Oxygen Difference</td>
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<tr>
<td>DAMP</td>
<td>Damage Associated Molecular Pattern</td>
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<tr>
<td>DM</td>
<td>Diabetes Mellitus</td>
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<tr>
<td>DMCT</td>
<td>Dunn’s Multiple Comparisons Test</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl Sulphoxide</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<tr>
<td>ECMO</td>
<td>Extracorporeal Membrane Oxygenation</td>
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EDTA
Ethylenediaminetetraacetic Acid
ELISA
Enzyme Linked Immunosorbent Assay
EP
Ethyl Pyruvate
ERK
Extracellular Signal Related Kinase
esRAGE
Endogenous Secretory Receptor For Advanced Glycation End-Products
FC
Flow Cytometer
FCS
Fetal Calf Serum
FEV1
Forced Expiratory Volume In 1 Second
FiO2
Inspiratory Oxygen Fraction
FITC
Fluorescein Isothiocyanate
FITC
Fluorescein Isothiocyanate
FSC
Forward Scatter
G-CSF
Granulocyte Colony-Stimulating Factor
h
Hour
Hb
Haemoglobin
HFOV
High Frequency Oscillatory Ventilation
HLA
Human Leukocyte Antigen
HMGB1
High Mobility Group Box 1
HMGB1
High Mobility Group Box 1
HRP
Horse Radish Peroxidase
HV
Healthy Volunteers
IC
Isotype Control
ICAM
Intercellular Adhesion Molecule
ICU
Intensive Care Unit
IFN
Interferon
IKK
IkB Kinase
IL
Interleukin
IQR
Interquartile Range
JNK
C-Jun N-Terminal Kinase
KS
Kolmogorov-Smirnov
KWT
Kruskal-Wallis Test
L3C
Level 3 Care
LAL
Limulus Amoebocyte Lysate
LOS
Length Of Stay
LPS
Lipopolysaccharide
LR
Logistic Regression
mins
Minutes
Mac-1
Macrophage-1 Antigen
MAPK
Mitogen Activated Protein Kinase
MFI
Mean Fluorescence Intensity
MHC
Major Histocompatibility Complex
MMP
Membrane Metalloprotease
MODS
Multiple Organ Dysfunction Score
mRNA
Messenger Ribonucleic Acid
MSD
Meso-Scale Discovery
MV
Mechanical Ventilation
MW
Molecular Weight
Appendix

Patient information sheets and consent forms for the patients in cohort C ("Does albumin reduce inflammation following cardiac surgery and reduce length of time patients spend on the intensive care unit?"); followed by the information sheets and consent forms for cohort B ("Cardiac surgery and the effect of haem release on the development of post operative lung related complications (Genetic polymorphisms in lung injury)").
DOES ALBUMIN REDUCE INFLAMMATION AFTER CARDIAC SURGERY AND REDUCE THE LENGTH OF TIME PATIENTS SPEND ON THE INTENSIVE CARE UNIT?

PATIENT INVITATION AND INFORMATION SHEET

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Talk to others about the study if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

1. What is the purpose of the study?

Some patients who have had an operation on their heart become very inflamed after their operation. This inflammation can cause problems with their kidneys, liver, lungs, and heart. It often means that they need to stay on the Intensive Care Unit (ICU) longer. The inflammation is related partly to the heart and lung machine, that is needed for the surgeon to do the operation. We have blood results that suggest the heart and lung machine causes inflammation by damaging red blood cells and causing their contents (such as haemoglobin and haem) to spill into the circulation.

The aim of this study is to "mop" up the red cell contents and reduce the amount of inflammation in patients. We hope that this will result in patients having fewer problems with their kidneys, lungs, liver and heart. This may in turn mean that they will be well enough to leave ICU sooner.

We will use a dose of human albumin in the heart and lung machine to "mop" up the red cell contents. Albumin is a normal part of blood and can stick to haem making it less toxic. Albumin levels fall during an operation and it is possible that this normal defence mechanism may be overwhelmed during heart surgery.

2. Why have I been chosen?

You have been chosen because you will need to go on a heart and lung machine so the surgeon can do your operation.

3. Do I have to take part?

No. It is up to you to decide whether or not to take part. If you do, you will be given this information sheet to keep and be asked to sign a consent form. You are still free to withdraw at any time and do not need to tell us why.

If you do decide not to take part, or change your mind, then this will not affect the standard of care you receive.
4. What will happen to me if I take part?

If you want to take part in this study we will ask you to sign a form to show that you agree to take part and are happy with our explanations of what will happen. We will give you a copy of the form and this leaflet to keep.

When we don't know which way of treating patients is best, we do a randomised trial. This means that we compare patients receiving one treatment to patients receiving another. We then work out which treatment is better. To try to make sure the groups are the same to start with, each patient is put into a group by chance (randomly). This means you will have a one in two chance of being given albumin. If you are not given albumin then you will receive a synthetic compound (gelofusine) that is used in all our patients at the moment.

We will also take some samples of your blood before and after your operation to look for signs of inflammation and measure how well your kidneys, liver, and lungs are working. We will take about two tablespoonfuls of blood each time. We will consider this blood as a gift from you. We will take this once you are asleep for your operation. It will not need any extra needles since we can take blood from the tube that is placed in an artery (usually your wrist) to monitor your blood pressure during the operation. All patients have this tube which is called an arterial line, even if they are not in a study.

We will measure how well your liver is working by giving you a small dose of a substance called ICG and measuring how quickly it is removed from your blood. We measure this using a probe that is placed on your finger. This test is used in many Intensive Care Units routinely. ICG has no known side effects.

The study will not alter any decisions that your doctors want to take about your care.

5. What if I change my mind?

We understand that people change their mind. You can change your mind at any time during the study. You do not have to give us a reason for doing this. If you do not want to remain in the study then we will treat you exactly as we treat all our patients. It will not affect the quality of care you will receive. Any information that we have collected about you will not be discarded.

6. What will happen to me if I do not take part?

You will be treated exactly as we do for all our patients at the Royal Brompton Hospital.

7. What are the side effects of any treatment received when taking part?

Albumin is frequently given to patients with minimal side effects. Its safety was confirmed in a large study of nearly 7,000 patients in Australia. Severe allergic reactions are much less common than for the synthetic version (gelofusine) that we use at the moment.

Albumin is made from plasma given by blood donors. We would like to reassure you that all blood donors are checked for known viruses such as HIV and hepatitis C. We also pasteurise albumin (like milk) which is thought to kill any known virus or bacteria. Indeed, albumin is considered to be very safe by the drug regulatory authorities who do not ask us to tell patients that albumin is used to make many drugs.
8. What are the possible benefits of taking part?

If you take part and are in the group that receives albumin then you may have less inflammation and fewer problems with your kidney, liver, heart and lungs. This may mean you are better sooner and allow you to leave the ICU earlier.

Finally, if albumin does help reduce inflammation after cardiac surgery then this improvement in our care will benefit future patients.

9. What if there is a problem?

If you are worried about any aspect of this study, you should ask to speak with the researchers who will do their best to answer your questions. Their contact details are on page 4. If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure. The Patient Advisory and Liaison Services (PALS) will tell you how to do this. Their telephone number is 020 7351 7715.

In the event that something does go wrong and you are harmed during the research study there are no special compensation arrangements. If you are harmed and this is due to someone’s negligence then you may have grounds for a legal action against the Royal Brompton and Harefield NHS Trust, but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you.

10. Will my taking part in the study be kept confidential?

Yes. All information which is collected about you during the course of the research will be kept strictly confidential. Your consent form will be the only piece of paper that will have your name and address on it. We will store this in a locked filing cabinet in a locked office at the Royal Brompton Hospital. A copy will be put in your medical file, which is also stored safely. All other forms and computer files will only contain a code number that represents you. All the data in this study will be destroyed ten years after it has finished.

Occasionally we are asked to show our data and consent forms to the regulatory authorities that check that we are doing research properly. We will do this since they also have a duty of confidentiality to you as a research participant.

We will also write a letter to your GP explaining that you have taken part in this study.

11. What will happen to any samples I give?

Your blood samples will be considered as a gift to the Hospital for use in the research. At the moment we plan to analyse them to see how well your blood will clot. We will also store some of the blood in case we want to measure any of the things that help your blood to clot. We will not test the blood for HIV. All of the samples will be destroyed ten years after the study is completed.

12. What will happen to the results of the research study?

We plan to publish the results of this study in a medical journal. It will not be possible to identify you from our report.

13. Who is organising and funding the research?

The study is being funded by the Moulton Charitable Foundation through Imperial College, London. We will not be paid for including you in this study.

Study number: 07/H0712/137
Version: 3.0 (1st June 2009)
14. Who has reviewed the study?

All research in the NHS is looked at by independent group of people to protect your safety, rights, wellbeing and dignity. This study was given a favourable ethical opinion by the Medicine and Healthcare Products Regulatory Agency (MHRA) and the National Research Ethics Service.

15. Contact Details:

Thank you for thinking about taking part in this study. If you want more information about this study please contact Dr Mark Griffiths (Consultant of Intensive Care) or Dr Simon Finney (Consultant in Intensive Care) in the Department of Critical Care at the Royal Brompton Hospital, Sydney Street, London. SW3 6NP. The hospital's telephone number is 020 7352 8121.

If you want to talk to someone independent of the study about being involved in research then contact the Patient Advisory and Liaison Services (PALS). Their telephone number is 020 7351 7715.
CONSENT FORM

Title of Project: Does albumin reduce inflammation after cardiac surgery and reduce the length of time spent on the Intensive Care Unit?

Names of researchers: Dr Mark Griffiths, Dr Simon Finney and Natalie Dormand

1. I confirm that I have read and understand the information sheet dated 1st June 2009 (version 3.0) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.

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

3. I understand that relevant sections of any of my medical notes and data collected during the study, may be looked at by responsible individuals from the Royal Brompton Hospital, from regulatory authorities. I give permission for these individuals to have access to my records.

4. I agree to take part in the above study.

Name of Patient __________________________ Date ____________ Signature __________________________

Name of Person taking consent (if different from researcher) __________________________ Date ____________ Signature __________________________

Researcher __________________________ Date ____________ Signature __________________________

When completed: Original to be kept in medical notes, 1 copy for patient, 1 copy for researcher site file

Version 3.0 (1st June 2009)
Royal Brompton & Harefield NHS Trust & National Heart and Lung Institute

INFORMATION and CONSENT FORM

ADULT PATIENT

TITLE OF PROJECT:
Cardiac surgery and the effect of haem release on the development of post operative lung related complications (Genetic polymorphisms in lung injury)

EXPLANATION OF PROJECT:

Lung injury sometimes complicates cardiac surgery involving cardiopulmonary bypass (CPB). It is important that you understand that people who are having CPB surgery, such as yourself, do not normally experience this complication. We are asking your help so that we can compare what normally happens, with people who do develop acute lung injury. Sometimes this injury is temporary, and needs 12-24 hours intensive care, but around 1 in 100 people develop a much more severe form of lung damage, called Acute Respiratory Distress Syndrome (ARDS). ARDS is also a complication of a wide variety of medical and surgical conditions, not all of which directly involve the lung, including major trauma, septic shock and pneumonia. We know that the extent of lung damage depends upon the nature of the underlying illness, and varies from patient to patient. We also know that ARDS starts because of the uncontrolled production in the body of poisonous chemical substances called oxidants. Normally the body’s own defence system removes these by producing other chemicals called anti-oxidants, but we believe that people may develop ARDS after bypass surgery because their defence systems are not working properly. We also believe that it may not be working properly because of a defect in susceptible patients’ immune response. Our immune responses are regulated by our body’s ability to produce a group of hormones called cytokines.

We would like to find out why a minority of people having CPB surgery react differently and develop lung injury when most people do not. We would do this by examining samples of blood, before and after you have your surgery. Whilst we cannot offer you any other direct benefit, it is through research of this kind that we are able to improve the treatment we offer, and others may benefit in the future.

Signed by the person in charge of the Project

Date 31/10/06

The Ethics Committee of the Royal Brompton & Harefield NHS Trust/National Heart & Lung Institute has approved the above statement.

Signed by the Chairman/Representative of the Committee

Date 31/4/06

DOCTOR'S DECLARATION

I have explained the project to the Participant as outlined above in the presence/absence* of a Witness**

Signed ___________________________ Date ___________________________ Time __________

* delete as appropriate

** every effort should be made to give the explanation in the presence of a witness

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If you agree to take part, we would need:

Five small blood samples (about 4 tablespoonsfuls in total). These will be taken at the same time as routine blood samples taken before and just after your operation and at intervals of 24 and possibly 48 hrs (if ICU stay is prolonged) thereafter. No extra needle will be necessary.

The tests on your blood involve genetic research. We wish to look at gene polymorphism. This means we will look at groups of genes which are found in everybody, but which in certain combinations make it more likely (or less likely) that certain characteristics can occur. We would like to stress that this research does not involve genetic tests for particular diseases on any of your samples, and need not be mentioned in (for example) insurance or mortgage applications. All information we obtain would of course be strictly confidential. Your name would not be disclosed in any reporting of results.

We would also like to examine certain white blood cells isolated from blood and also measure certain changes in some blood borne substances.

The samples would be treated as gifts to the Hospital/Institute. They will be under direct control of nominated custodians (Prof. Evans, Dr. Quinlan and Dr. Lagan), who would ensure that strict confidentiality was maintained. We would like to keep the samples after we have completed these tests, and use them for possible further research in the future. We would not ask your permission again before doing this, but any future research would require Prof. Evans, Dr. Quinlan or Dr. Lagan to have received ethical committee approval. All tests will be carried out in our laboratories. If you wish to take part in the study but are NOT happy for us to keep your blood for future research, this would be perfectly acceptable.

You are of course quite free to take part in this research or not, as you choose. Whatever you decide will have no effect upon your care and treatment, nor on the timing of your operation, and if you agree to take part, you may change your mind at any time up to the operation, and need give no reason for doing so.

Nevertheless we would be most grateful for your help, because it is only through the kindness of people such as yourself that we are able to improve medical care. We expect that this research will increase our understanding of the way lung injury can develop after surgery and might also help us to explore the causes of other lung diseases such as asthma, pulmonary hypertension and smoking related conditions.

Thank you very much for your consideration.

Signed by Chairman/Representative of the Royal Brompton & Harefield NHS Trust/N.H.I. Ethics Committee.

Protocol Reference Number: 00-033 version 1
NOTES FOR PATIENTS

1. Please do not hesitate to ask the Doctor any questions you may have about this project before you decide whether you wish to participate.

2. If you decide, now or at any other stage, that you no longer wish to participate in the project, this will not in any way prejudice your future treatment.

3. If you decide to take part in this project, you will be given a copy of this form for your information.

FORM OF CONSENT

(for use by adult patients undergoing investigations connected with clinical research)

______________________________

of

______________________________

give my consent to undergo the research procedures described overleaf. The nature, purpose and possible consequences have been fully explained to me.

Signed_________________________ Date_________________________

Patient's Hospital Number ________________________________

**WITNESS (See Note 1)**

I am satisfied that the procedures have been fully explained to the Patient. The Patient has given consent in my presence.

Name of Witness_________________________ Position_________________________

Address______________________________

Signed_________________________ Date_________________________

NOTES

1. "**WITNESS:** Wherever possible, a witness should be present whilst the doctor/spONSOR explains the procedures to the patient. If this is impractical, complete the "Doctor's Declaration" accordingly. In the case of a patient, the witness should not be a doctor if the sponsor is a doctor. If the witness is a nurse, he or she must be a ward sister or senior nurse in charge of the ward who is not undertaking a post-basic course.

2. "SAFE-KEEPING OF THIS FORM: In the case of a patient, a copy of this signed form must be kept in the patients case-notes, and the sponsor should retain a copy for subsequent examination if required by the Ethics Committee.
References


Inflammatory processes: the role of redox in the different outcomes.


338.


441. Rezzonico, R., V. Imbert, R. Chicheportiche, and J.M. Dayer, *Ligation of CD11b and CD11c beta(2) integrins by antibodies or soluble CD23 induces macrophage inflammatory protein


with sepsis


