Investigations into low-dose aspirin as an adjunct therapy for sepsis and device-related biofilm infections

A thesis submitted in fulfilment of the requirements for the degree of
Doctor of Philosophy

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General Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and, ethics procedures and guidelines have been followed.

Jacinta Marie Ortega

Date: November 27, 2015
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Jacinta
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List of Abbreviations

7-AAD | 7-Aminoactinomycin D
---|---
AA | Arachidonic acid
ACCP | American College of Chest Physicians
AKI | Acute kidney injury
ALI | Acute lung injury
ALX | ATL/Lipoxin receptor
APACHE | Acute Physiology and Chronic Health Evaluation
ATL | Aspirin-Triggered Lipoxin
BHIA | Brain Heart Infusion Agar
BHIB | Brain Heart Infusion Broth
BSA | Bovine serum albumin
CLP | Caecal ligation and puncture
CO\textsubscript{2} | Carbon dioxide
COX | Cyclooxygenase
CRP | C-reactive protein
DABCO | 1,4-diazabicyclo [2.2.2] octane
DHA | Docosahexaenoic acid
DMEM | Dulbecco's Modified Eagle Medium
DMSO | Dimethylsulfoxide
DNA | Deoxyribonucleic acid
ECM | Extracellular Matrix
eDNA | Extracellular DNA
EDTA | Ethylenediaminetetraacetic acid
EGDT | Early Goal Directed Therapy
EIA | Enzyme immunoassay
ELISA | Enzyme-linked immunosorbent assay
EPA | Eicosapentaenoic acid
EPS | Extracellular polymeric substance
FCS | Foetal calf serum
FPR | Formyl peptide receptor
GM-CSF | Granulocyte-macrophage colony-stimulating factor
H & E | Haematoxylin and eosin
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<thead>
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<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intercellular adhesion molecule-1</td>
</tr>
<tr>
<td>IE</td>
<td>Infective Endocarditis</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>LOX</td>
<td>Lipoxygenase</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>L-selectin</td>
<td>Leukocyte-selectin</td>
</tr>
<tr>
<td>LxA$_4$</td>
<td>Lipoxin A$_4$</td>
</tr>
<tr>
<td>MaR1</td>
<td>Maresin 1</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemotactic protein-1</td>
</tr>
<tr>
<td>MIF</td>
<td>Macrophage migration inhibitory factor</td>
</tr>
<tr>
<td>MODS</td>
<td>Multiple organ dysfunction syndrome</td>
</tr>
<tr>
<td>MPV</td>
<td>Mean platelet volume</td>
</tr>
<tr>
<td>MQH$_2$O</td>
<td>MilliQ Water</td>
</tr>
<tr>
<td>MRSA</td>
<td>Methicillin-resistant Staphylococcus aureus</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
<td>Sodium bicarbonate</td>
</tr>
<tr>
<td>NFkB</td>
<td>Nuclear factor kappa beta</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non-steroidal anti-inflammatory drug</td>
</tr>
<tr>
<td>O$_2^-$</td>
<td>Superoxide</td>
</tr>
<tr>
<td>ONOO$^-$</td>
<td>Peroxynitrite</td>
</tr>
<tr>
<td>P-selectin</td>
<td>Platelet-selectin</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate buffered saline with Tween-20</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PCT</td>
<td>Pre-calciton</td>
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<tr>
<td>PD-1</td>
<td>Protectin D1</td>
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<tr>
<td>PIA</td>
<td>Polysaccharide intracellular adhesin</td>
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<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
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### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>PMN</td>
<td>Polymorphonuclear neutrophils</td>
</tr>
<tr>
<td>PSGL-1</td>
<td>P-selectin glycoprotein ligand-1</td>
</tr>
<tr>
<td>PUFA</td>
<td>Polyunsaturated fatty acid</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative real-time PCR</td>
</tr>
<tr>
<td>rhAPC</td>
<td>Recombinant human activated protein C</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>Rv</td>
<td>Resolvin</td>
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<tr>
<td>SA</td>
<td>Salicylic acid</td>
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<tr>
<td>SCCM</td>
<td>Society for critical care medicine</td>
</tr>
<tr>
<td>SIRS</td>
<td>Systemic inflammatory response syndrome</td>
</tr>
<tr>
<td>SOFA</td>
<td>Sequential organ failure assessment</td>
</tr>
<tr>
<td>SPM</td>
<td>Specialised pro-resolving mediator</td>
</tr>
<tr>
<td>s/s</td>
<td>Stainless steel</td>
</tr>
<tr>
<td>SSCC</td>
<td>Surviving sepsis campaign</td>
</tr>
<tr>
<td>TAC</td>
<td>Tris-ammonium chloride</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TMB</td>
<td>Tetramethylbenzidine</td>
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<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor alpha</td>
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<tr>
<td>Treg</td>
<td>Regulatory T lymphocyte</td>
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<td>vWF</td>
<td>von Willebrand Factor</td>
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### Units of Measurement

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<tr>
<td>%</td>
<td>Percentage</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
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<tr>
<td>°F</td>
<td>Degrees Fahrenheit</td>
</tr>
<tr>
<td>&lt;</td>
<td>Less than</td>
</tr>
<tr>
<td>&gt;</td>
<td>Greater than</td>
</tr>
<tr>
<td>≥</td>
<td>Equal to or greater than</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony-forming unit</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>fL</td>
<td>Femtolitre</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>kg</td>
<td>Kilogram</td>
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<tr>
<td>L</td>
<td>Litre</td>
</tr>
<tr>
<td>mg</td>
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<td>Nanogram</td>
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<td>nm</td>
<td>Nanometre</td>
</tr>
<tr>
<td>ns</td>
<td>Not significant</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>pg</td>
<td>Picogram</td>
</tr>
<tr>
<td>pH</td>
<td>Negative algorithm of hydrogen ion concentration</td>
</tr>
<tr>
<td>pmol</td>
<td>Picomoles</td>
</tr>
<tr>
<td>RFU</td>
<td>Relative fluorescence units</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolution per minute</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>s</td>
<td>Second</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight per volume</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume per volume</td>
</tr>
<tr>
<td>\times g</td>
<td>Gravitational acceleration</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>µL</td>
<td>Microlitre</td>
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<td>µm</td>
<td>Micrometre</td>
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Abstract

Sepsis is a deadly condition that develops when the host immune system is ill equipped to respond to infection. Delays in the appropriate identification and management of sepsis in health care facilities can lead to organ failure, which is associated with high mortality rates (40-70%). Current therapies of sepsis provide support and target the source of infection, but do not address the inflammatory pathways that have shown to contribute to increased disease severity. As such, there is an urgent need for an effective adjunct therapy for sepsis.

There is increasing evidence for the use of low-dose aspirin as an adjunct therapy in sepsis. Aspirin offers a cost-effective alternative to other adjunct therapies currently under investigation, and at low-doses the proposed mechanism of action includes the production of specialised pro-resolving mediators; including aspirin-triggered lipoxins (ATL), that mediate the resolution of inflammation by inhibiting the activation of NFκB, which has been shown to improve outcomes in other inflammatory conditions. The purpose of this study was therefore to investigate the anti-inflammatory effects of aspirin in clinical sepsis, as well as experimentally in mouse models of gram-positive sepsis, using ATL and NFκB as primary read out parameters and device-related biofilm infection.

Chapter 3 of this thesis investigated of the mechanisms of aspirin-therapy in participants with sepsis enrolled into the Mechanism of Aspirin Therapy in Sepsis (MATHS) randomised control trial. In keeping with the hypothesis, low-dose aspirin therapy (100 mg/day) was shown to induce the increased production of ATL in plasma, and inhibition of NFκB in PBMC (not previously shown with this dose). Aspirin therapy did not appear to significantly reduce the production of previously reported pro-inflammatory cytokines, but did impact the proportion of circulating innate and adaptive immune cell subsets. While these results were encouraging, it remained unclear whether these effects extended beyond circulation. In order to address this question, a mouse model of systemic bloodstream infection was established.

As *Staphylococcus aureus* is the most frequent source of sepsis, Chapter 4 of this thesis investigated the effect of aspirin therapy in a wild-type mouse model of staphylococcal-induced sepsis. The model was designed following the preliminary analysis of data from
the MATHS trial. Further, this study permitted analysis of target organs that tend to be damaged in the early development of sepsis. Unlike the participants in the trial, aspirin at an equivalent low-dose (1 mg/kg/day) over two days did not significantly impact ATL and NFκB in mice. Nevertheless, low-dose aspirin-treated mice did demonstrate a complete clearance of bacteria from all target organs within 24 h of the second aspirin dose, and showed considerable improvements in clinical outcomes; in the absence of supportive antibiotic therapy. Redevelopment of the model timeline in future studies hope to provide a more accurate comparison with human trial subjects.

Contamination of indwelling medical devices can lead to the formation complex biofilms on device surfaces, which are difficult to treat. Further, replacement of contaminated devices can result in spread of bacteria to other sites in the body, leading to the development of sepsis. Aspirin and its major metabolite Salicylic acid (SA) have been shown to diminish biofilm formation in vitro in a number of pathogens, however the exact mechanism of action against staphylococcal biofilm formation is poorly understood.

Chapter 5 in this study aimed to elucidate the mechanisms of SA inhibition on growing staphylococcal biofilms, both in vitro and in an in vivo mouse model of device-related biofilm infection. Results from in vitro studies revealed that SA treatment significantly diminishes two key mechanisms of biofilm formation including: reducing the amount of biomass produced and extracellular DNA (eDNA) released, reducing the structural integrity of biofilm. This combined effect suggests weakened biofilms have an increased susceptibility to both immune attack and antimicrobial therapies.

Of the studies investigating novel therapies of device-related biofilm in vivo, none thus far have investigated the potential therapeutic benefit of low-dose aspirin. In this study, low-dose aspirin (1 mg/kg/day) over five days lead to the reduction of surface biofilm in stainless steel implanted mice, however this wasn’t seen with catheter implants. Aspirin-treated mice, regardless of implant type appeared to have accelerated wound healing as shown by reduced epithelial thickness compared with non aspirin-treated counterparts, and in some cases an increased local presence of CD11b+ cells. This suggested that the inhibitory effect of aspirin in vivo is inducing CD11b+ cell-mediated clearance of biofilms; however further investigation is required to confirm this.
In summary, this thesis has contributed to the understanding of immunological pathways activated following localised and systemic infection, leading to the early development of sepsis. Further, this study has elucidated a potential role for low-dose aspirin (100 mg/day or equivalent in animal studies) as an adjunct pro-resolution therapy in both device-related biofilm infection and sepsis. Completion of the MATHs clinical trial will be required to provide definitive evidence to support advancing to a prospective trial in septic patients. Further research is required in to develop animal models that will more accurately represent human disease.
Chapter 1

Introduction
and review of the literature
1.1. General introduction

Sepsis is the leading cause of infection-related death worldwide, with one death occurring every 3-4 seconds [1]. Despite every effort in the last two decades to improve outcomes in critically ill patients, mortality rates of sepsis continue to rise [2]. The significance of this severe inflammatory condition is often under-reported, due to limited available data from the developing world. As symptoms of this condition are non-specific, it is difficult for clinicians to clearly distinguish sepsis. This often results in a delay in correct diagnosis and contributes to increased mortality rates of sepsis. Following infection, the host immune response triggers a series of pro-inflammatory and anti-inflammatory responses. Sepsis is characterised by uncontrolled immune response and occurs when infection fails to clear; resulting in widespread inflammation, organ failure and death [3]. The predominant causative pathogen of sepsis has changed in the past fifteen years there has been from gram-negative bacteria, to gram-positive bacteria; with increasing incidence of Staphylococcus aureus (S. aureus) infections causing sepsis [4].

*S. aureus* is a well-recognised nosocomial pathogen. This bacterium is commonly the cause of a number of deep-seated invasive infections including endocarditis [5] and device-related biofilm infections [6]. These infections are a frequent source of sepsis, as transiently bound bacteria detaching from local sites of infection, disseminate into the bloodstream and other sites of the body. The increasing prevalence of sepsis due to antibiotic-resistant strains of *S. aureus* is becoming a cause for concern, with a need for novel adjunct therapies.

Early identification and intervention provides the best potential for limiting progression of sepsis. Considerable interest has surrounded the use of biomarkers for rapid recognition and diagnosis of sepsis, permitting early intervention and potentially reducing the risk of death [7]. While many have been proposed, no individual biomarker has shown to be useful in diagnosis of sepsis. It has been suggested however, that the use of several biomarkers in combination (targeting both pro-inflammatory and anti-inflammatory factors) could be more successful [8, 9].

There is little evidence to suggest that currently employed therapies of sepsis (bundled therapies comprising of fluid resuscitation, oxygen delivery; achieving target acid-base
levels and empiric antibiotic delivery) have the capacity to reduce mortality rates alone [10, 11], thus the search for an alternative adjunct therapy to improve outcomes in sepsis continues. To be considered suitable, novel adjunct therapies should be cost-effective and aim to address the uncontrolled inflammatory response in sepsis. While many therapeutics avenues have been investigated, none have proved successful in reducing absolute levels of mortality. Other anti-inflammatory therapies previously suggested for sepsis target the inflammatory pathways via complete inhibition, which can delay the natural resolution of inflammatory pathways; associated with worsened outcomes [12].

More recently it has been determined that “resolution” of inflammation, via the synthesis of specialized “pro-resolution” lipid mediators (SPMs) including lipoxins, resolvins, protectins and maresins [13-15] leads to improved outcomes. Following the selective inhibition of pro-inflammatory mediators, rapid activation of “pro-resolving” pathways leads to a return to homeostasis as shown in both animal models [16-18] and clinical studies [12, 19-21] of inflammation.

Increasing evidence suggests there may be a potential role for low-dose aspirin in the adjunct treatment of sepsis. Not only is aspirin a cost effective therapeutic with multiple applications, low-dose aspirin therapy was shown to trigger the enhanced biosynthesis of these SPMs [14, 15]. The central hypothesis for the studies described in this thesis is that low-dose aspirin therapy produces immuno-modulatory effects in inflammation in both clinical and animal models of sepsis, as well as device-related biofilm infections. The rationale for this study is described further in the following review of the literature.
1.2. Sepsis

1.2.1. Defining sepsis

Sepsis is described as the interaction between the pathogen responsible for the infection and the host immune response; including but not limited to innate/adaptive immune systems, inflammatory and coagulation responses [3].

Traditional consensus definitions of sepsis include: the presence of at least two signs of systemic inflammatory response syndrome (SIRS), and a suspected or known source of infection. This was determined in 1992, by the American College of Chest Physicians (ACCP) and the Society for Critical Care Medicine (SCCM) conference panel [22]. The pathway from sepsis to severe sepsis (sepsis with hypotension and evidence of organ dysfunction) arises when the host immune system is ill equipped to respond to the infection resulting in uncontrolled inflammation [23]. Septic shock is a most severe form of sepsis often accompanied by multiple-organ dysfunction syndrome (MODS). Patients presenting with septic shock and MODS have increased mortality rates of 40-70% [24]. In 2001, the consensus panel reconvened to evaluate the relevance of these definitions. It was concluded that the criteria for SIRS were still not specific enough for clinicians to distinctly identify the syndrome [25]. Clinicians continue use a range of different criteria to identify sepsis in the Intensive Care Unit (ICU) of hospitals, including patient demographics, source of infection, symptoms present, Acute Physiology and Chronic Health Evaluation (APACHE II or III) and Sequential Organ Failure Assessment (SOFA) physiological scoring systems and hospital mortality [26].
Table 1.1: Consensus conference definitions of sepsis, severe sepsis and septic shock.
Adapted and reproduced with permission from the American College of Chest Physicians (ACCP) and the Society for Critical Care Medicine (SCCM) [22].

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| **Systemic inflammatory response syndrome (SIRS)** | Systemic inflammatory response syndrome may or may not be the result of infection. Clinical manifestation includes two or more of the following conditions:  
- Temperature >38˚C or <36˚C  
- Heart rate >90 beats/min  
- Respiratory rate >20 breaths/min OR PaCO$_2$ <32 mm Hg  
- White blood cell count >12,000/mm$^3$ OR <4,000/mm$^3$ OR >10% immature (band) forms |
| **Sepsis** | The systemic inflammatory response to infection*. Clinical manifestations as described above. |
| **Severe Sepsis** | Sepsis and organ dysfunction, hypoperfusion OR hypotension. Manifestations of hypoperfusion may include but are not limited to:  
- Lactic acidosis  
- Oliguria  
- Acute alterations in mental status |
| **Septic shock** | Sepsis induced hypotension, persisting despite adequate fluid resuscitation, and the manifestations as described above** |
| **Multiple Organ Dysfunction Syndrome (MODS)** | Presence of altered organ functions in an acutely ill patient such that homeostasis cannot be maintained without intervention. |

* Inflammatory response to the presence of microbes or invasion of normally sterile tissues by these organisms;  
** Patients receiving inotropic or vasopressor agents may no longer be hypotensive by the time they manifest hypoperfusion abnormalities or organ dysfunction, yet they would still be considered to have septic shock; PaCO$_2$, Partial pressure of carbon dioxide.
1.2.2. Epidemiology of sepsis

An estimated 28% of fatalities occurring in the United States (US) alone are due to severe sepsis. As a result, sepsis is now the leading cause of infection-related death worldwide [3]. For clinicians, often the consensus definitions are not specific enough to clearly identify a patient as being septic. As a result, data records for the number of sepsis cases are generally lower than estimated figures predict [27]. A US National Hospital Discharge Survey data brief in 2008 noted that the rate of hospitalisations associated with sepsis had more than doubled since 2000 (as shown in Figure 1.1).

![Figure 1.1: Increase in rates of sepsis reported in the US between 2000 and 2008.](image)

Documented hospitalisation rates per 10,000 people in the US with or as a result of septicaemia or sepsis. Blue line indicates the rate of hospitalisation for patients with septicaemia or sepsis (as a first-listed, principal, or secondary diagnosis, including: patients hospitalised for septicaemia or sepsis, patients hospitalized for another diagnosis but also had septicaemia or sepsis at the time they were admitted, and those who acquired septicaemia or sepsis during their hospital stay); Green line indicates patients who were hospitalised for septicaemia or sepsis (as a first-listed or principal diagnosis only). Figure reproduced, with permission from [28].

This rising incidence of sepsis in the US is attributed to the continuously ageing population, and increased prevalence of co-morbidities such as cancer, diabetes and the human immunodeficiency virus (HIV) [1, 4, 26, 29]. A study by the Centre for Disease
Control and Prevention (CDC) showed that the likelihood of hospitalisations associated with sepsis were higher in those older than 65 years of age, with patients 85 years and above approximately 30 times more likely to be hospitalised [28]. Similar with the CDC studies, other retrospective studies have shown the highest mortalities associated with those above 80 years of age [1, 26, 29, 30]. Other factors contributing to the likelihood of development and severity of sepsis include the site and source of initial infection.

Finfer et al. conducted a study in Australian and New Zealand, showing that pulmonary (50.3%) and the abdomen (19.3%) were the most common sites in sepsis patients [30]. The average age of the participants was 55.9 years, and majority of the patients were male. Alongside this study, a more focused study on sepsis in the state of Victoria showed that of those admitted to ICU, again the majority of patients identified as having sepsis were male, and the predominant systems where organ dysfunction occurred were respiratory (41.9%), renal (41.5%) and cardiovascular systems (37.4%), respectively [29].

The association between race/ethnicity and likelihood of sepsis development has also been studied, with increased incidence of sepsis observed in minority groups, however this does not always translate to higher mortality rates [4, 31, 32]. Two recent Australian studies also showed in tropical regions of Australia, that indigenous populations had a higher prevalence of infectious and chronic diseases, and an increased likelihood of developing sepsis, but again mortality rate was not affected [33, 34]. It has also been shown, that when identified the most common source of infection was bacterial. Prior to 1987, gram-negative bacterial infections were the majority cause of sepsis. Since then, the incidence gram-positive bacterial infection has risen rapidly to represent the primary cause of sepsis. There has also been a dramatic increase in the proportion of sepsis cases caused by fungal infections [26, 29, 30].

1.2.3. Sepsis pathophysiology

Infections caused by pathogenic bacteria initiate the activation of the innate and adaptive immune responses. These immune responses collectively are aimed at controlling microbial growth in order to protect the host [35]. The involvement of both the innate and adaptive immune systems has been illustrated in both human trials and experimental animal models. An understanding of immunological contributions in regard to severe sepsis and septic shock is still yet to be fully elucidated [36].
Figure 1.2: Immune response to bacterial infection, including communication between innate and adaptive immune cell types. Following ingestion of bacteria, Macrophages produce pro-inflammatory cytokines via activation of NFκB. Activation of NFκB will also induce activation of cyclooxygenase-2 (COX-2), which metabolises arachidonic acid to yield pro-inflammatory lipid derivatives, which contribute to vasoconstriction/vasodilation of endothelial tissues. Macrophages also secrete IL-12 to activate CD4⁺ T lymphocytes, which also contributes to cytokine production. Bacteria also affect activation of neutrophils and platelets contributing to exacerbation of endothelial inflammation. Excessive damage to the endothelium results in bacterial and neutrophil spread to tissues, leading to tissue damage, organ dysfunction and death. Figure adapted and reproduced with permission from [3], Copyright Massachusetts Medical Society.
1.2.3.1. Innate immune response in sepsis

The mechanism by which the inflammatory cascade is instigated by the innate immune system has been studied extensively. The cell subsets of the innate immune system comprise monocytes (such as macrophages and dendritic cells), granulocytes (such as neutrophils), and large granular lymphocytes (such as natural killer cells) [23].

Activated endothelial cells also release cyclooxygenase (COX) and inducible nitric oxide synthase (iNOS), which contribute to septic shock by dilating blood vessels and eliciting a strong cardio-depressive effect [37]. COX enzymes convert essential fatty acids into pro-inflammatory eicosanoids such as prostaglandins, prostacyclin and thromboxane (shown in Figure 1.8A) [38]. Two isoforms of COX exist; COX-1, which is constitutively expressed in most tissues, and COX-2; whose activation is reliant on external pro-inflammatory stimuli [39]. Production of cytokines during the sepsis inflammatory cascade leads to the activation of COX-2 and the production of prostanoids, resulting in vasoconstriction and/or vasodilation and platelet aggregation [40]. Further, cytokine production induces synthesis of nitric oxide (NO), via iNOS in endothelial cells, as well as activated macrophages and neutrophils. This leads to the increased formation of reactive oxygen and nitrogen species like hydrogen peroxide (H$_2$O$_2$) and peroxynitrite (ONOO$^-$), which leads to stress-induced organ damage (shown in Figure 1.3) [41].

The activation of these pathways in sepsis can result in the development of MODS, resulting in mortality rate increase of up to 90% (with failure of 4 or more organs), irrespective of treatment methods used [24]. In addition to initiating the inflammatory response, innate immune cells also have functions in phagocytosis of pathogens, as well as antigen presentation to initiate the adaptive immune response [42]. The roles of innate immune cells are discussed in more detail below.
Figure 1.3: Generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in stimulated macrophages, neutrophils and endothelial cells. Following pro-inflammatory stimulus (lipopolysaccharide, peptidoglycan, and cytokines) macrophages, neutrophils and endothelial cells convert L-arginine into nitric oxide (NO) by inducing nitric oxide synthase (iNOS) activation. Pro-inflammatory stimuli also activate nicotinamide adenine dinucleotide phosphate (NADPH) production of superoxide radicals (O$_2^-$). The presence of these radicals and water molecules generates ROS such as hydrogen peroxide (H$_2$O$_2$), likewise presence of oxygen radicals and NO results in production of RNS such as peroxynitrite (ONOO$^-$). Production of ROS and RNS leads to DNA, protein and lipid damage contributing to mitochondrial dysfunction and stress-induced organ damage. Figure adapted, with permission from [43].
1.2.3.2. The role of platelets in sepsis

Platelets are essential components in homeostasis and one of the first responding cell types in systemic infections. Bacteria, such as streptococci and staphylococci mediate the activation of platelets by binding plasma proteins such as von Willebrand factor (vWF) [44, 45]. Platelet activation leads to increased surface expression of platelet (P-) selectin in patients with sepsis, and increases in plasma levels of soluble factors including soluble P-selectin (sP-selectin), following cleavage from activated platelet surfaces [46, 47]. Soluble P-selectin triggers signalling via receptors present on myeloid cells and can result in disseminated intravascular coagulation (DIC) events [48, 49].

Activated platelets intrinsically interact with a number of other immune cells during sepsis, including peripheral blood mononuclear cells (PBMC). Platelet interactions have been shown to induce the activation of B and T lymphocytes as well as monocytes in vitro [50]. An increase in formation platelet-neutrophil aggregates has also been shown in sepsis, with activated complexes showing a greater ability to phagocytose bacteria [51, 52]. On the other hand, production of pro-inflammatory cytokines from this complex can be deleterious leading to increased inflammation [53, 54]. In contrast, complexes formed between platelets and macrophages inhibit the production of pro-inflammatory mediators during sepsis, preventing development of septic shock in a COX-dependent manner [55].

Platelets have also been investigated as prognostic markers in sepsis. The study conducted by Aydemir et al. in 2015 evaluated whether the kinetics of platelet counts and mean platelet volumes (MVP) differed in adult patients with nosocomial sepsis [56]. Patients admitted to ICU had platelet count and MPV measured daily, over a 5 day period. A significant reduction in platelet count (<150,000/mm$^3$) was noted for the first 3 days in patients with gram-positive sepsis, 4 days in patients with gram-negative sepsis and 5 days in patients with fungal sepsis. These decreases in platelet count were also associated with an increase in MPV (defined as MPV of >10.4/fL), in the first 3 days in patients with gram-positive sepsis, and 5 days in patients with fungal sepsis. The study concluded that fungal sepsis induced a more significant effect on platelet count and function.
Decreased platelet count in septic patients are associated with higher mortality rates of up to 40%, compared with those with higher platelet count (~10%) [57]. Decreased platelet count is also associated with activation of coagulation pathways, with complications including DIC events and development of MODS [48]. A recent study by de Stoppelaar et al. (2014), showed similar results in a mouse model of pneumonia-induced gram-negative sepsis [58]. When intranasally infected with $10^3$ colony-forming units (CFU) of *Klebsiella pneumoniae*, platelet-depleted C57BL/6 mice showed a significantly higher level of bacterial burden in primary sites of infection (lung), blood and other distant organs (spleen and liver), compared with control mice. Further, evidence of haemorrhage at the primary site of infection was seen in platelet-depleted mice, as well as a significant increase in pro-inflammatory cytokines released (TNF-α and IL-6), both *in vivo* and when further stimulated *in vitro*. Combined, these complications lead to impaired survival in platelet-depleted mice, suggesting that decreased platelet count contributes to increased sepsis mortality [58].

Excess accumulations and aggregation of platelets are also shown to be key contributing factors to the development of acute lung injury (ALI) [59] and acute kidney injury (AKI) [60]; organ damage syndromes resulting from severe sepsis. As such, platelet count is often used as an independent predictor of mortality in ICU clinical settings [48].

Inhibition of platelet activation and function in sepsis patients appears to be important in the modulation of the host immune response to systemic infection. Several animal and clinical studies appear promising, supporting the potential for anti-platelet therapies improving outcomes in sepsis [61-64], which will be discussed further in section 1.4.2.

1.2.3.3. The role of monocytes/macrophages in sepsis

Monocytes and macrophages are an essential part of the innate immune response, and along with neutrophils, provide the first line of defence against infection (see Figure 1.2). Produced from hemopoietic stem cells in the bone marrow, monocytes enter the bloodstream and then differentiate into macrophages or dendritic cells, with functions in phagocytosis [65] and antigen presentation [66]. In contrast with what has been described for lymphocyte subsets, sepsis does not appear to affect levels of monocyte/macrophage populations, further suggesting that these cells are key drivers in regulating immune responses in sepsis.
Monocyte and macrophage populations are thought to play a dual role in sepsis [67]. Following infection, pattern-recognition receptors such as Toll-like receptors (TLRs) are expressed on these innate immune cell surfaces and activate the inflammatory cascade upon interaction with pathogenic microorganisms. Toll Like Receptor-2 (TLR-2) is one of the most studied receptors in the recognition of gram-positive specific molecular patterns. Peptidoglycan (PGN) and Lipotechoic acid (LTA) from the cell wall of bacteria such as *S. aureus* are recognised by TLR-2 on circulating monocyte/macrophages. This initiates signalling cascade via the activation of nuclear factor κB (NFκB), leading to the production of both pro-inflammatory (such as tumour necrosis factor α (TNF-α), macrophage migration inhibitory factor (MIF) interleukin-1β (IL-1β) and interleukin-6 (IL-6)), and anti-inflammatory cytokines (such as interleukin-10 (IL-10); shown in Figure 1.2). As a result, levels of pro-inflammatory cytokine release (TNFα and IL-6) increase, contributing to destruction of tissues and promoting organ failure [68].

Monocyte/macrophage cells were also shown to be key regulators of systemic coagulation and inflammatory pathways in a mouse model of abdominal sepsis [69]. Mice were depleted of monocytes and then abdominal sepsis was induced via CLP, following which plasma and lung levels of inflammatory and coagulation markers were determined. As a result of monocyte/macrophage depletion, there were notable decreases in levels of plasma prothrombin, and coagulation factors x and v. Further, depletion of monocyte/macrophages lead to a decrease in plasma levels of IL-6 and CXC chemokines, which in control CLP sepsis mice were increased as expected, due to inflammation. Together these decreases in pro-inflammatory and coagulation pathways resulted in reduced kidney injury and further understand the role of monocytes/macrophages in pathology of sepsis, however it is unclear if this also extends to clinical sepsis [69]. Activated macrophages (stimulated by Interferon-γ (IFNγ)) also accumulate in and around infected tissues, and increase in number as inflammation progresses [70].

When infected with $10^7$ colony-forming units (CFU) *S. aureus*, mice with septic arthritis with depleted peripheral blood monocytes showed impaired pro-inflammatory cytokine production. This affected recruitment of leukocytes to tissues resulting in increased weight loss and likelihood of mortality [67]. Further, a lack of phagocytosing monocyte/macrophage population resulted in increased bacterial infiltration into organs [67] contributing to development of sepsis and organ failure. This was also similarly
shown in the recent study by Ngo et al. (2013) [71], in mice infected with $10^5$ CFU of *Candida albicans*, following depletion of monocytes. Compared with the control mice, monocyte-depleted mice had significantly increased fungal burden in kidneys, and histological analysis showed abscess development in kidney tissues; containing neutrophils and cellular debris. Additionally, adoptive transfer of monocytes to depleted mice in this study restored some antifungal phagocytic responses. Macrophages also play a role in the NO synthesis; reacting with superoxide (produced by activated neutrophils) to form peroxynitrite, with cytotoxic and bactericidal effects [72] (Figure 1.3).

1.2.3.4. The role of neutrophils in sepsis

Neutrophils are another important part of the innate immune response against infection, and are the first innate immune cell to arrive at sites of inflammation. Derived from haemopoetic stem cells in bone marrow in a similar manner to monocyte/macrophages, neutrophils also have functions in regulating inflammation via phagocytosis [73]. It is reported that the human body produces $10^{11}$ neutrophils daily, and that this number increases ten-fold with inflammatory stimuli such as cytokines or bacterial surface components [74]. Neutrophils are central to the sepsis inflammatory cascade, however there is continued debate in the effect on neutrophil phagocytosis of bacteria during sepsis; some studies reported no change [75, 76] others showing increased phagocytosis [77-80], or decreased phagocytosis [81-85].

Maturation of neutrophils is a multi-stage process progressing from myeloblast, promyelocyte, myelocyte, metamyelocyte, band cell, to mature polymorphonuclear (segmented) cells [86]. One of the criteria for identifying sepsis is the presence of immature neutrophils (band cells) [22]. These are prematurely released from bone marrow in response to severe infections and have impaired phagocytosing ability compared to mature counterparts in both sepsis infected and healthy individuals [87]. Increased numbers of immature neutrophils were shown in a recent clinical trial in patients with sepsis, compared with SIRS patients and healthy volunteers [88]. While these cells are not considered to be fully functional, it was argued in another study that this did not negatively affect the phagocytic abilities of immature neutrophils in septic patients, when compared with healthy controls [89].
While the host employs neutrophils in a protective role, in the presence of pro-inflammatory cytokines and pathogenic stimuli these innate immune cells contribute to exacerbation of inflammation [90] (Figure 1.2), by binding endothelium and interfering with coagulation and fibrinolysis pathways (as shown in Figure 1.4), and migrate to other sites of inflammation. Recruitment of activated leukocytes to the vascular endothelium for migration to tissues occurs via a regulated multi-step cascade, including capture, rolling and firm adhesion [91]; mediated by surface molecules. Mature neutrophils express a number of surface markers following stimulation including CD66b; a carcinoembryonic antigen [92], and CD11b; also known as integrin alpha M (ITGAM) [93]. The study by Schmidt et al. (2015) recently showed that human peripheral neutrophils in whole blood became dysfunctional when exposed to *S. aureus* components *in vitro*, with reduced phagocytic ability and subsequently decreased anti-bacterial activity [94]. The study also noted a decrease in surface expression complement factor c5a, accompanied by an overexpression of CD66b and CD11b on the neutrophil surfaces and elevated aggregate formation, which contributed to the dysregulated phenotype of neutrophils. These activation markers, in conjunction with other cell surface molecules like selectins mediate the rolling and firm adhesion of neutrophils to the endothelial surfaces.

Leukocyte (L-) selectin is the most ubiquitously expressed selectin subtypes, present on the cell surface of macrophages, neutrophils and T lymphocytes, with the primary role of trafficking leukocytes to local sites of acute inflammation [95]. During sepsis, L-selectin is often prematurely shed from neutrophil surfaces to reduce the level of injury at local sites [96]. Another adhesion molecule involved in migration of leukocytes is P-selectin glycoprotein ligand-1 (PSGL-1). PSGL-1 is a well-described ligand of L-selectin [97], and an integral surface adhesion molecule for recruitment of neutrophils to tissues [98].

Inflammation is generally resolved by way of apoptosis; as neutrophils are generally considered constitutively apoptotic [99], however during sepsis stimulatory signals inhibit apoptosis of neutrophils. A study by Taneja et al. [100] confirmed this, showing that only 6.2% of neutrophils extracted from whole blood from critically ill patients with sepsis became apoptotic after 24 hours. Delayed apoptosis of neutrophils leads to exacerbated inflammation, which is a key-contributing factor for organ failure.
None of the current therapies for sepsis target the delayed apoptosis of neutrophils. In a recent study, neutrophils extracted from humans were cultured in the presence of serum amyloid A (an acute-phase protein that is increased during inflammation; SAA). Serum amyloid A binds to the formyl peptide receptor-like 1/lipoxin A4 (LxA4) receptor (ALX) on the surface of neutrophils, leading to suppressed apoptotic signalling pathways and prolonged neutrophil survival. However, incubation with pro-resolving lipid mediator 15-epi-lipoxin A4 (15-epi-LxA4; 2 µmol/L) prior to incubation with SAA, resulted in restored neutrophil apoptosis by re-instating signalling pathways [101]. The implications of this study are that induction of pro-resolving mediators such as lipoxins may also be beneficial in sepsis (discussed further in section 1.4.6).

1.2.3.5. Adaptive immune response in sepsis

Sepsis also affects a number of key adaptive immune cell sub-populations, including B and T lymphocytes [23]. Sepsis causes a decrease in the proportion of B lymphocytes and both CD4+ and CD8+ T lymphocytes, proposed to be in conjunction with an increase in CD4+ CD25+ Regulatory T lymphocytes [102]. The combined effects of sepsis on the adaptive immune response are discussed below.

1.2.3.6. The role of B lymphocytes in sepsis

B lymphocytes are an integral component of the adaptive immune system. They are the only cell type that produces the immunoglobulins (Ig), which recognise antigens of pathogenic microorganisms such as bacteria and facilitate the delivery of these antigens to neutrophils and natural killer cells for bacterial degradation [103, 104]. Two recent studies have highlighted a potential role for a newly identified subset of B-lymphocytes named innate response activator (IRA) B lymphocytes, which in mice were shown to protect against sepsis and septic shock [105, 106].

The 2012 study by Rauch *et al.* noted an increase in both mortality rate and overall number of fatalities in B lymphocyte and GM-CSF deficient mice (GM/µMT−/−), when subjected to CLP to induce sepsis [105]. These deficiencies resulted in pronounced inflammation characterised by increased presence of neutrophils, as well as elevated levels of IL-1β, IL-6 and TNF-α in both the peritoneum and in serum, leading to severe bacteraemia and development sepsis before MODS, and death. In contrast, GM-CSF
produced by IRA B lymphocytes limited infection and inflammation, which was associated with improved outcomes. Following this, another study determined the mechanism of protection by B lymphocyte derived GM-CSF in a mouse model of pneumonia [106]. It was shown in vitro that wild type B-1a B lymphocytes produced IgM in response to IRA B lymphocyte-derived GM-CSF, leading to speculation that IgM recognises bacterial components, targeting them for phagocyte-mediated elimination, reducing the probability of uncontrolled inflammatory response. The study also showed that B lymphocytes migrate from the pleural space into the lung in order to protect against Escherichia coli and Streptococcus pneumoniae-induced pneumonia. Mice with B lymphocyte and GM-CSF deficiencies had increased bacterial burden and subsequent increased mortality rate, in a similar fashion to that of the Rauch et al. study [105].

Apoptosis of B lymphocytes during sepsis can lead to a state of immunosuppression. In a recent clinical study, septic patients demonstrated a significant loss of B lymphocytes in spleen (via immuno-histological and flow cytometry analysis), compared to non-infected trauma patients [107]. This was similarly shown in a mouse study, where caecal ligation and puncture (CLP) was performed to induce polymicrobial sepsis in B lymphocyte deficient µMT−/− mice. B lymphocyte deficient µMT−/− mice were not able to generate a sufficient inflammatory cytokine/chemokine response for bacterial clearance, leading to increased mortality compared with wild-type mice. Survival rates could be partially restored by transferring wild-type serum containing B lymphocytes [103]. In the same study, decreased Type-1 interferon-induced chemokine production was seen in µMT−/− mice, contributing to increased severity of disease. This suggests a role for B lymphocytes in regulation of early immune responses by way of Type-1 interferon signalling.

1.2.3.7. The role of T lymphocytes in sepsis

Cytotoxic T lymphocytes (CD8+) are not generally considered to be of importance in acute sepsis; however one study did note an initial increase in CD8+ T lymphocytes in septic patients compared with trauma patients [107]. In contrast with other chronic infection and inflammatory states, sepsis is an acute inflammatory condition. As such, it has been debated as to whether subsets of Helper T lymphocytes (CD4+) are directly involved in early sepsis development. A number of studies have suggested that CD4+
T lymphocytes mediate innate immune responses in animal models of sepsis. In these studies, CD4-deficient mice had reduced capacity to clear bacteria, and decreased neutrophil activity compared with wild-type mice, which contributed to an increased likelihood of mortality [108, 109]. Conversely, one study argued that CD4⁺ T lymphocytes do not impact inflammatory pathways in sepsis [110]. Depletion of CD4 from wild-type mice in this study had no significant effect on bacterial load, pro-inflammatory cytokine production or overall survival; contradicting what was shown in the above two studies.

Similar to B lymphocytes, the main impact of sepsis on T lymphocytes is induced apoptosis (in lymphatic tissues), causing impaired antimicrobial responses and immunosuppression. Surviving CD4⁺ and CD8⁺ T lymphocytes in circulation either switch to anti-inflammatory (Th2) phenotypes [111], or become “exhausted” [112]. T lymphocyte exhaustion is characterised by decreased cytokine production and reduced T lymphocyte receptor diversity, resulting in increased apoptosis [113]. Extended “paralysis” of the adaptive immune system contributes to increased mortality rates in sepsis.

Upon activation, naïve CD4⁺ T lymphocytes differentiate into one of: Th1, Th2, Th17 and induced regulatory T lymphocytes (iTreg) populations. Treg are also derived directly from the thymus naturally (nTreg) [114]. Regulatory T lymphocytes (CD4⁺CD25⁺ Treg) have a number of proposed roles in regulation of both adaptive and innate immune responses in sepsis [115, 116]. One such function is in mediating immunosuppression, by inhibiting effector responses mediated by both CD4⁺ and CD8⁺ T lymphocytes and the innate immune response. Initially observed in trauma patients [117], decreases in CD4⁺CD25⁻ cells were accompanied with an increase in CD4⁺CD25⁺ Treg. This has also been shown in patients with sepsis and septic shock [102], and in a mouse model of polymicrobial sepsis [118]. Given that an immunosuppressive role of Tregs has been shown in sepsis, a recent review proposed that inhibition of Tregs could potentially restore immune function in sepsis [119].

As sepsis can result in immunosuppression via apoptosis of B and T lymphocytes (via the suppressive activity of Tregs), it is of both interest and importance that novel potential
therapies of sepsis aim to restore the activity of these cells for improved immune responses.

1.2.4. Biomarkers as predictors of sepsis

A number of biomarkers have been proposed in the identification of sepsis, as well as prognosis, however debate continues over their usefulness. Novel potential biomarkers should provide information that is additional to standard clinical assessments and investigations [7]. Optimally, detection of biomarkers could be done in a rapid and cost-effective manner, and determine between the different pathogenic sources of sepsis and non-infective SIRS.

C-reactive Protein (CRP) and Pre Calcitonin (PCT) are currently the two most frequently used biomarkers in the clinical diagnosis of sepsis. CRP is an acute-phase reactant produced in the liver, stimulated by IL-6 [120]. The exact role of CRP in inflammation is unclear, however in critically ill patients in Australia CRP was shown to be a reliable marker of bloodstream infection [121]. The assay to detect CRP is inexpensive and easy to perform. PCT is the precursor to calcitonin hormone synthesised in serum in low levels, with increased levels peaking early in inflammation (8-24 hours vs. 36 hours for CRP) potentially allowing for more rapid identification. While PCT is more specific and more sensitive (88% sensitive compared with 75% sensitivity for CRP) in determining bacterial sepsis [122]. Levels of PCT are more stable than CRP, and the assays to detect have a short turn-around time of 2 hours, however PCT does not accurately differentiate between other non-bacterial sources of sepsis and non-infectious inflammatory states (i.e. trauma, surgery) [123]. Thus, the best use for PCT is in ruling out bacterial infection, in order to reduce unnecessary administration of antibiotics.

Early response cytokines such as TNF-α, IL-1β and IL-6 have also been proposed as biomarkers for identification of sepsis. These cytokines are released predominantly by macrophages and lymphocytes that have been activated by inflammatory stimuli, and rapidly increase following onset of sepsis (see Figure 1.2) [124]. Both TNF-α and IL-1β activate cells of the vascular endothelium, resulting in the recruitment of neutrophils to the site, continuing to perpetuate inflammation. Further, the release of TNF-α and IL-1β activates coagulation pathways and initiates the sepsis inflammatory cascade that leads to the production of other pro-inflammatory cytokines (like IL-6 and IL-8), lipid mediators,
and ROS and RNS; contributing to organ damage [24]. As levels of TNF-α were not significantly altered in clinical studies following anti-TNF therapy [125] and the role for IL-1β in sepsis has been debated, neither have been considered a suitable biomarker for sepsis diagnosis, however as increased levels are associated with poor outcomes, they could be useful as prognostic markers of sepsis.

IL-6 is similarly produced by a wide variety of immune cells (in response to TNF-α and IL-1β), and is elevated in a wide variety of acute inflammatory conditions. A number of biological functions have been described for IL-6 in sepsis including the activation of B and T lymphocytes [126]. Other key functions of IL-6 include the induction of fever and loss of appetite, as well as increased production of acute-phase reactants (like CRP) in the liver, and signalling the release of immature neutrophils (hallmark of SIRS and sepsis). IL-6 appears to be a more reliable prognostic marker for sepsis than TNF and IL-1β, as elevated levels of plasma IL-6 are more stable (allowing for detection). Further, elevated IL-6 levels in sepsis are associated with highest mortality rates [127], and recent evidence suggests that low-dose aspirin therapy could potentially reduce IL-6 levels [128]. As such, an intervention study has been initiated (discussed in section 1.4.2), to investigate the effect of low-dose aspirin therapy with primary read-out parameters including levels of IL-6, measured over a 48 hour time frame.

Activation markers on the surface of innate immune cell populations (in particular neutrophils and macrophages) have also been proposed as potential biomarkers of sepsis. CD64 expression was shown to be elevated on the surface of neutrophils in the presence of bacterial infection [129, 130], and has The expression of CD64 was shown in one study to have a higher specificity (95%) and sensitivity (96%) in detecting sepsis compared with PCT (70% and 94%, respectively) [130]. However, another concurrent study disputed the usefulness of CD64, as expression cannot distinguish between viral and bacterial infections reliably, nor could it distinguish between localised infection and systemic inflammation [131]. Further, a recent cross-sectional study by Gámez-Díaz et al. showed that CD64 expression on neutrophils were not significantly specific or sensitive enough to diagnose sepsis in patients admitted to the emergency department (cohort of 631 patients) [132]. CD11b is expressed on macrophages and neutrophils, and facilitates binding to intracellular and vascular adhesion molecules (ICAM-1 and VCAM-1) on the endothelium, for recruitment to inflamed tissues, and one study therefore suggested that
the combined use of CD64 and CD11b could be beneficial biomarkers in diagnosing sepsis [133].

The generation of ROS and RNS and the downstream pathways of apoptosis and organ damage have also recently become a point of interest as potential prognostic biomarkers of sepsis [134]. Markers of extrinsic apoptosis pathways such as cell surface death receptor Fas and their ligands (like FasL), were shown in a number of studies to correlate with the development of sepsis and high clinical scores (SOFA and APACHE II) [135, 136]. The 2012 study by Huttenen et al. evaluated the prognostic value of soluble Fas (sFas), FasL and sFas/FasL ratio in the plasma patients with bacteraemia [137]. Patients presenting with systemic S. aureus, S. pneumoniae, beta-haemolytic streptococci or E. coli infections, were enrolled and plasma was taken over 4 days, following positive bacteraemia identification. The study showed that patients with increased concentration of sFas (ng/mL) and sFas/FasL ratio, and a decrease in FasL were associated with a higher SOFA score. Additionally, increased concentration of sFas (ng/mL) and sFas/FasL ratio were also indicative of hypotension, however these values did not predict 14- or 28-day mortality. Similarly, the 2015 study by Mikić et al. evaluated the predictive value of sFas on APACHE II scores in patients with sepsis, severe sepsis and septic shock [135]. The study showed that sFas concentrations in plasma were elevated in 54.4% of all patients enrolled (with 76.9% of this being patients with MODS and 84.6% of mortalities) and this was correlated with higher APACHE II scores. The study concluded that in combination, sFas and APACHE II score could be used to predict poor outcomes in sepsis.

To date, no individual biomarker has been successful in the identification of sepsis, however many have been useful as prognostic markers, allowing for precise monitoring and treatment. It has been suggested that a panel of useful biomarkers (both pro- and anti-inflammatory) could serve as a better diagnostic tool for identification or potential development of sepsis [8, 9].

Although microbiological confirmation of infection is required for sepsis diagnosis, the use of modern molecular technologies (such as amplified molecular methods, and whole blood assays) for rapid identification of causative organism are now also being considered, alongside a panel of biomarkers. In combination, these novel diagnostic
approaches would decrease turn-around time for positive identification, permitting more rapid therapeutic intervention [138]. The LightCycler SeptiFast test is a multiplex real-time PCR assay, that can positively identify up to 25 of the most common sepsis-causing pathogens (10 different bacteria identified to species level, other bacteria to genus level, and 5 Candida species) produced by Roche Molecular Systems (USA), and has previously been evaluated in patients with endocarditis and suspected sepsis [139-141]. The basis for this technique is fluorescently labelled probes, which target the internal transcribed spacer (ITS) region, specific for each bacterium. The study by Wallet and colleagues evaluated the SeptiFast test in patients with suspected sepsis over a 6-month period [140], with the test producing reliable results; with increased specificity (93.5%) and sensitivity (85%) up to 57 hours sooner than conventional blood cultures. Some limitations reported with the assay; chiefly relating to some unreliability in accurate detection of pathogens in the system database, and that the test does not allow for identification of organisms not in the database. Further, as this method does not rely on growth prior to amplification, there is no discriminating between amplification of dead organisms, and like other highly sensitive molecular methods there is the possibility of contamination, and false positive results.

These newer advancements in modern molecular detection methods have also allowed for identification of diagnostic and prognostic biomarkers of sepsis including microRNAs (miRNA). These small endogenously produced RNAs (approximately 22 nucleotides) were shown to be important in targeting mRNA for translational repression or cleavage [142]. The study by Wang and colleagues in 2012, highlighted 6 serum miRNAs with potential prognostic value in sepsis [143]. Using Next-generation Solexa sequencing techniques, which had similarly been useful in identifying biomarkers in patients with other conditions like cancer [144, 145], the miRNA expression profiles in sera of surviving and non-surviving patients of sepsis were screened. Quantitative RT-PCR was then used to confirm that 6 miRNAs were differentially expressed in non-survivors (miR-223, miR-15a, miR-16, miR-122, miR-193*, and miR-483-5p). Further, when combined with sepsis stage, APACHE II and SOFA scores, these seven variables were strongly associated with sepsis mortality, were not useful from a diagnostic perspective. These approaches are yet to be evaluated in multi-center trials.
There is recent interest in the pro-inflammatory marker NFκB and anti-inflammatory lipid mediators such as lipoxins, as levels of both markers are shown to rapidly increase following onset of inflammation (within 8 hours) [146, 147]. Not only could these markers be useful in rapid identification of inflammation, both NFκB and Lipoxin A₄ pathways are impacted by low-dose aspirin therapy with therapeutic benefit seen in other conditions (discussed further in section 1.4.3). A majority focus of studies in this thesis is the production of NFκB and Lipoxin A₄, as potential surrogate markers of sepsis. As well as this, the potential therapeutic benefit of low-dose aspirin therapy will be determined by measurable levels of these markers in both patients with sepsis (Chapter 3) and in a murine model of staphylococcal-induced sepsis (Chapter 4).

1.2.5. Treatments of sepsis

Current guidelines for sepsis therapy suggest that a successful therapy should be directed towards rapid identification and effective clearance of infectious foci [2]. Following this, the delivery of appropriate antimicrobial therapy and symptomatic organ support (via fluid resuscitation and vasopressor support) should be instigated (as outlined in Table 1.2). However, these therapies do not target the uncontrolled inflammatory response by the host immune system, contributing to sepsis mortality. Since 1982, over 60 phase 2 (P2) or phase 3 (P3) randomised control trials of potential therapeutic targets for sepsis have been initiated [148]. Despite this, no significant advance has been made in reducing the mortality rates associated with sepsis. The following treatments represent some of the most notable current and potential target therapies that have been investigated extensively for their use against sepsis.

1.2.5.1. Early goal directed therapy

Early goal directed therapy (EGDT) was pioneered in a study conducted by Rivers et al. in 2001, following a randomised single-centre clinical trial [149]. The rationale for the study was that oxygen delivery would improve outcomes in sepsis. Patients were enrolled following admission to the emergency department (ED) or ICU, with severe sepsis as well as decreased systolic blood pressure (less than 90 mmHg) or blood lactate concentration equal to or greater than 4 mmol/L. Patients randomly assigned to EGDT, had central venous catheter measurements for levels of oxygen saturation ($S_{VO_2}$) taken, following which “goal directed” treatment protocol was employed.
The trial was initially deemed a success with an absolute reduction in sepsis mortality of 16%. Although some groups adopted the EGDT protocol; conducting similar single and multi-centre trials [150-153], other studies have argued that the major limitation of the study (data analysed were from a single centre study), imply the results were not indicative of an absolute mortality rate that was comparable with a broader subset of patients [11, 154]. An investigation conducted by the Australasian Resuscitation in Sepsis Evaluation (ARISE) Investigators and the Australian and New Zealand Intensive Care Society (ANZICS) Adult Patient Database (APD) Management Committee was initiated in 2007, questioning the validity of EGDT, following the success of the pilot study [11, 149]. This study showed, although the incidence of sepsis continued to increase over a 9 year period, associated mortality rates decreased from an estimated 36% in 1997 to 21.2% in 2005 [11]. Similar declines in hospital mortality were shown in a concurrent epidemiological study in the US by Martin et al., with a 9.9% reduction in mortality over a 22 year period [4]. This drop in mortality rates over time were not taken account in the design of the EGDT pilot study [155]. As a result, further research is currently underway to permit evidence-based decisions on the therapeutic benefit of EGDT in sepsis.

1.2.5.2. Antimicrobial therapies

1.2.5.2.1. Source control and empirical antibiotic therapy

Identification of causative pathogens of sepsis is usually made via blood culturing, which has a turn-around time of 24-28 hours. Empirical therapy employs broad-spectrum antibiotics, targeting a variety of both gram-positive and gram-negative organisms [156]. During the first “golden” hours in sepsis identification, empiric antibiotic therapy is administered along with fluid resuscitation, to treat the unknown infection, while blood culture results are pending (Table 1.2) [157]. Patients are assessed according to the guidelines for early empiric-antibiotic delivery based on parameters including: recent antibiotic therapy, previous infection or colonisation with antibiotic resistant organisms, potential harm of antibiotic treatment, polymicrobial infection, organ failure status, risk of antibiotic resistant infection and recent surgeries (that may contribute to risk of infection) [158]. Once this information is determined, patients are then assessed for neutrophil counts (as this is a key indicator of infection severity), in which case a separate set of guidelines is consulted before antibiotics can be prescribed.
Table 1.2: Initial treatment regimen for septic patients, according to the 2012 Surviving Sepsis Campaign guidelines, reproduced with permission from [2].

<table>
<thead>
<tr>
<th>Strong recommendations*</th>
<th>Suggestions*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Initial resuscitation (first 6 hours)</strong></td>
<td></td>
</tr>
<tr>
<td>Begin Resuscitation immediately if hypotension or arterial lactate &gt; 4 mmol/L</td>
<td></td>
</tr>
<tr>
<td><strong>Resuscitation Goals:</strong></td>
<td><strong>If venous saturation target not achieved:</strong></td>
</tr>
<tr>
<td>• CVP 8-12 mmHg</td>
<td>• Consider increasing fluids</td>
</tr>
<tr>
<td>• MAP ≥ 65 mmHg</td>
<td>• Transfuse packed red blood cells to maintain haematocrit ≥ 30% and/or</td>
</tr>
<tr>
<td>• Urine Output ≥ 0.5 mL/kg/h</td>
<td>• Begin dobutamine infusion (maximum 20 µg/kg/min)</td>
</tr>
<tr>
<td>• SVC ( \text{O}_2 ) ≥ 70% or mixed venous saturation ≥ 65%</td>
<td></td>
</tr>
<tr>
<td><strong>Diagnosis</strong></td>
<td></td>
</tr>
<tr>
<td>Obtain appropriate cultures before antibiotic delivery (including blood cultures and other sites as clinically indicated, consider candidiasis)</td>
<td></td>
</tr>
<tr>
<td><strong>Antibiotic therapy</strong></td>
<td></td>
</tr>
<tr>
<td>Begin broad spectrum intravenous antibiotics within 1 hour</td>
<td>Consider combination therapy if <em>Pseudomonas spp.</em> infection is suspected or if patient is neutropenic</td>
</tr>
<tr>
<td>Re-assess antimicrobial regimen daily</td>
<td>Combination therapy for 3-5 days, then de-escalation</td>
</tr>
<tr>
<td>Limit therapy to 7-10 days unless patient is immunosuppressed or has undrivable focus</td>
<td></td>
</tr>
<tr>
<td>Initiation of antiviral therapy as quickly as possible if suspected viral origin</td>
<td></td>
</tr>
<tr>
<td>Antimicrobial therapy should not be used in patients with severe inflammation of non-infectious cause</td>
<td></td>
</tr>
<tr>
<td><strong>Source identification and control</strong></td>
<td></td>
</tr>
<tr>
<td>A specific source of infection should be rapidly identified</td>
<td></td>
</tr>
<tr>
<td>Formally evaluate patient for infection focus amenable to source control measures (i.e. drainage, debridement)</td>
<td></td>
</tr>
<tr>
<td>Remove intravascular devices if infected</td>
<td></td>
</tr>
</tbody>
</table>

* Recommendations are classified according to GRADE of evidence system [172, 173]. CVP: central venous pressure; MAP: mean arterial pressure; SVC \( \text{O}_2 \): superior vena cava saturation.
Debate continues as to whether this type of therapy does in fact have any impact on the reduction of mortality in patients with severe sepsis and/or septic shock. Several studies have demonstrated that treatment with adequate empirical antibiotics within the first few hours following identification lead to lower mortality rates (Table 1.2) [10, 159, 160], however this was excluding patients who have been transferred from a non-hospital setting who may have been taking antibiotics previously.

1.2.5.3. Recombinant human activated protein C

Human activated protein C (hAPC) is an endogenous plasma protein activated in the presence of thrombin/thrombomodulin complex to form a serine protease, and is involved in modulating coagulation and inflammation; interfering with thrombotic signalling (as shown in Figure 1.4) [161, 162]. The pilot study of recombinant hAPC (rhAPC) in sepsis (PROWESS trial) was conducted in 2001 [163]; showing a 6.1% absolute reduction in mortality compared with placebo-treated patients, as well as improved cardiovascular and respiratory function. Following this, rhAPC became the first pharmacological therapeutic approved for use in over two decades in the treatment severe sepsis in the US [164] between 2002 and 2010. The use of rhAPC was included as a recommended therapeutic for critically ill sepsis patients with a high risk of death in the first edition of the Surviving Sepsis Campaign guidelines in 2004 [162, 165].

Not only has debate arisen over the costs associated with rhAPC; with short-term treatment estimated to be AUD$15,000 per 4 day course [166], treatment with rhAPC was also associated with increased bleeding (especially in patients with low risk of death) [163, 164, 167-169]. The efficacy of rhAPC was questioned after other randomized trials failed to reproduce outcomes seen in the initial study [168-170]. In 2011, the Cochrane collaboration reviewed five of the largest clinical trials involving treatment with rhAPC against severe sepsis [171]. The study concluded that rhAPC showed no significant beneficial effect on the 28-day mortality in sepsis patients compared to the placebo group; leading to the withdrawal of rhAPC from the market in October 2011, as it was not a reliable therapeutic against severe sepsis and septic shock [170, 171].
Figure 1.4: Systemic inflammatory, pro-coagulant, and fibrinolytic pathways and the proposed mechanisms of action by Recombinant Human Activated Protein C (rhAPC). Following recognition of pathogens in circulation, monocyte/macrophages produce cytokines and chemokines to induce inflammatory response. The uncontrolled inflammatory response (due to reactive oxygen species produced by neutrophils) results in local cellular damage of the vascular endothelium and development of coagulopathy. TNF and IL-1 induce monocyte production of tissue factor leading to thrombin formation and formation of a fibrin clot. Both cytokine production and thrombin induces release of plasminogen-activation inhibitor 1 (PAI-1), interfering with fibrinolysis resulting in oedema; contributing to diffuse endovascular injury, micro-vascular thrombosis, organ ischemia, development of MODS, and death. RhAPC treatment was shown to intervene at multiple within the vascular endothelium, reducing inflammation. While these pathways were impacted with RhAPC treatment, no significant therapeutic benefit has been observed in clinical outcomes of sepsis patients. Figure reproduced with permission from [163], Copyright Massachusetts Medical Society.
1.2.5.4. Novel anti-complement therapies for sepsis

Of the complement factors that drive innate immune responses in sepsis, C5 is becoming of significant interest. Cleavage of this complement factor leads to the production of C5a; a powerful anaphylatoxin that triggers the pro-inflammatory responses, contributing to MODS and septic shock [174]. The other products of the cleavage of C5 include C5b, which is then converted to C5b-9 (by the C5 convertase enzyme), forming the membrane attack complex (MAC), which lyses gram-negative bacteria [175, 176]. As complement pathways are activated rapidly following onset of sepsis, investigations in targeting complement pathways have shown potential for neutralising antibodies as adjunct therapy for sepsis.

The use of anti-C5 therapy was also shown to have benefits in other terminal illnesses which interfere with the complement system such as paroxysmal nocturnal haemoglobinuria (PNH) [177, 178] and atypical haemolytic uraemic syndrome (aHUS) [179]. In both of these conditions, administration of a humanised monoclonal anti-c5 antibody (Soliris: Eculizumab marketed by Alexion Pharmaceuticals) was found to be safe and effective in reducing the number of thrombotic events and intravascular hemolysis [180]. There are some limitations that have been noted with this therapy in patients with aHUS. Once given, this therapy is life-long, making it the most expensive drug currently on the market (€430,000 annual spend in Europe, $409,500 USD/year) [181]. Also, adverse side effects including minor headaches, nausea and anaemia have been reported, however most concerning was the increased risk of developing sepsis, shown in aHUS patients treated with anti-C5 therapies [182]. Further, use of anti-complement pathways to inhibit C5a, and in particular C5b-9 MAC could result in unfavourable outcomes, due to the impairment of other general immuno-protective mechanisms provided by the complement system.

Recent studies in animal models of sepsis have also shown therapeutic benefit of anti-C5 antibody therapies, both alone and in combination inhibiting other markers of inflammatory responses in sepsis [183, 184]. Investigations into anti-complement therapies in sepsis do in fact date back as far as the 1980’s; where septic monkeys treated with anti-C5 antibody, showed attenuation of E. coli-induced septic shock and had decreased presence of acute respiratory distress syndrome (ARDS) [185, 186]. The 2015
study by Huber-Lang and colleagues, showed that by blocking both C5 and CD14 (a Toll-like receptor family protein that recognises LPS of gram-negative bacteria) with neutralising antibody therapy, reduced morbidity and mortality mice with CLP-induced sepsis [184]. More recently, a phase 2 randomised-control human-intervention trial has just been completed at the Paul-Erlich Institut, Germany (Trial ID: NCT02246595), to further evaluate the safety and efficacy of anti-C5 therapies in patients with early or newly developing sepsis. Data from this trial may show whether or not the potential seen in animal models are translated into a human therapeutic potential.

1.2.5.5. Corticosteroids in sepsis therapy

Corticosteroids are synthetic hormone drugs related to cortisol, a classically produced hormone in the adrenal gland [187]. The most recognised corticosteroids with known anti-inflammatory properties class are Glucocorticoids (GC) [188]. Therapeutic benefit of GC therapy have been demonstrated in both infectious and non-infectious sources of inflammation [189], bronchial inflammation (e.g. asthma) [190], inflammatory bowel diseases [191] and traumatic brain injuries [192]. Schumer et al. first noted success in the treatment of sepsis with corticosteroids in 1976, with significant decrease in mortality observed in patients treated with dexamethasone or methylprednisolone compared with placebo treated patients [193]. Studies following this failed to reproduce the effect seen in the preliminary study, with the exception of reversal of symptoms in cases of septic shock [194-198]. Also noted, in non-life threatening cases, administration of corticosteroids was more harmful than beneficial, resulting in increased risk of hyperglycaemia (85% of patients) or super-infection (33% of patients) [199]. Currently corticosteroid therapy is only recommended for use (as a last resort) in sepsis patients with a high risk of death and is used in conjunction with other antimicrobial therapies.

1.2.5.6. Acetaminophen (Paracetamol)

Hyperthermia is one of the key markers in identifying severe infection. It was suggested in one study that targeting the internal body temperature with anti-pyretic therapy could potentially improve mortality rates [200]. Paracetamol (acetaminophen) is a widely used over-the-counter analgesic and anti-pyretic agent. Initial studies in children with viral illnesses supported the use of paracetamol treatment in a randomised study [201], leading to a number of intervention trials with adults and other inflammatory states. One study
showed the use of paracetamol in febrile patients (650 mg every 6 hours), lead to a significantly higher mortality rate [202]. Differences in mortality rates were so extreme that the study was terminated following completion of the first preliminary analysis. This was consistent with other studies showing increased risk in mortality in infected patients with suppressed fever, due to anti-pyretic medication [203, 204]. Currently a multi-centre randomised study is underway in Australia and New Zealand called the HEAT trial (permissive HyperthErmia Through Avoidance of paracetamol in known or suspected infection in the ICU) [205]. The outcomes of which are to determine the safe and effective use of paracetamol in treating fever in sepsis patients within ICU.

1.2.5.7. Non-steroidal anti-inflammatory drugs (NSAIDs)

As the name suggests, NSAIDs are non-steroid based drugs that provide analgesic and anti-pyretic effects and at higher doses also confer anti-inflammatory effects. The main mechanisms of action are by inhibition of COX-1 and COX-2, which reduces synthesis of pro-inflammatory eicosanoids (prostaglandins and thromboxane). There are a number of family groups of NSAIDs classified on their structure including: salicylates (including aspirin and salicylic acid), propionic acid derivatives (including Ibuprofen), acetic acid derivatives (including indomethacin and diclofenac), among others (reviewed in [206]). There have been several studies elucidating the potential for NSAID treatment of sepsis, the most widely studied being ibuprofen and aspirin as discussed below. Some research into indomethacin has been done, however only in an animal model of sepsis [207].

1.2.5.7.1. Ibuprofen

Ibuprofen (from isobutylphenylpropanoic acid) is a NSAID derived from propionic acid, commonly prescribed for use as an analgesic, with anti-pyretic and anti-inflammatory effects [208]. The mechanism of action of ibuprofen is similar to other NSAID therapies via the non-selective inhibition of both COX-1 and COX-2, blocking pro-inflammatory prostanoid synthesis (i.e. prostaglandin, thromboxane). Initial investigations of ibuprofen treatment showed promise against severe inflammatory conditions, however sample sizes in these studies were not large enough to confer significance [40, 209, 210]. A larger study was conducted in 1997 to determine what the effects of ibuprofen intervention against sepsis [211] finding that in patients treated with ibuprofen did in fact have reduced levels of pro-inflammatory lipid mediators and decreased fever, but did not
Chapter 1

prevent the development of shock or respiratory distress. Further, the study was underpowered, showing that ibuprofen therapy did not significantly impact mortality rates of septic patients, with only 3% of patients showing any improvement.

In contrast with aspirin, a recent study also showed that ibuprofen therapy in healthy volunteers with induced inflammation also suppressed leukotriene responses, leading to reduced lipoxygenase (LOX) activity [12]. Impairment of these pathways results in diminished production of pro-resolution lipid mediators such as lipoxins, resolvins and protectins (discussed further in section 1.4.6), and failure to resolve inflammation is associated with poor outcomes. Given that sepsis is characterised by uncontrolled inflammation, and resolution of inflammation, returning to homeostasis are key pathways for improved outcomes, it is not surprising that ibuprofen therapy did not appear to confer any therapeutic advantage.

1.2.5.7.2. Aspirin

Aspirin is a salicylate-derived NSAID with analgesic, anti-pyretic and anti-inflammatory properties. Similar to Ibuprofen, the mechanisms of aspirin’s anti-inflammatory effects are by COX inhibition (previously described in section 1.1.3.1), leading to inhibition of platelet aggregation and reduced prostaglandin synthesis [212]. Unlike other NSAIDs, aspirin at low-doses not only inhibits COX-1, but also acetylates COX-2, activating a biosynthetic pathway triggering the epimeric production of anti-inflammatory lipid mediators including lipoxins, resolvins and protectins. These lipid mediators have “pro-resolving” characteristics, which contribute to resolution of inflammation and return to homeostasis (discussed further in section 1.4.6) [19, 20]. Evidence for the potential benefit of aspirin as an adjunct therapeutic of sepsis is increasing, with a number of animal studies [16-18, 213, 214] and clinical studies [20, 61, 101, 215-220] showing support for this hypothesis. In addition to being cost-effective, aspirin therapy triggers resolution of inflammatory pathways (at low-doses), and recently it has been suggested that aspirin directly impacts bacteria such as S. aureus [221]; the most frequent source of sepsis. The significance of aspirin and its potential as a therapeutic agent for sepsis is further discussed in section 1.4.3.
1.2.6. Animal models of sepsis

One of the major obstacles in the development of novel adjunct therapies of sepsis is the lack of animal models that are a true representation of the clinical syndrome. The most popular animals used for pre-clinical studies of sepsis are mice, as inbred strains (such as C57BL/6 and BALB/c), largely because of the availability of reagents, markers and knockout strains. A recent review on animal models of sepsis remarked that of the pharmacological agents that show promise in animal models, none have shown significant therapeutic benefit in human clinical sepsis [148]. This has become a significant discussion point, with continued debate over the usefulness of animal models for this syndrome. In 2013, a meta-analysis performed by Seok et al. [222] comparing transcription profiles of inflammatory genes in PBMC found a poor correlation between mouse models and human systemic inflammation. The authors stated that on this basis, mouse models were not useful for investigating sepsis. More recently, another group analysing the same subset of data via different methods found the opposite conclusion, with high correlations observed between the two models of infection [223]. A closer look at the design of these analyses, raised questions about the assumptions made and what constituted an equivalent study; as “blood from an inbred mouse, 4 hours after staphylococcal infection compared with uninfected control mice” is not a valid comparison to “whole blood from patients with sepsis as a result of community-acquired infection compared with healthy controls”. This question was also raised in a recently published letter by Shay et al. [224]. As such, the use of and limitations of animal models of sepsis and human clinical sepsis remain controversial.

There are a number of established animal models of systemic infection and sepsis including; Lipopolysaccharide (LPS) stimulation by intravenous injection; to mirror gram-negative endotoxin infection [225-228], caecal ligation and puncture (CLP) [229-232]; to induce polymicrobial infection, or direct intravenous infection with either gram-negative [17] or gram-positive bacteria [233]; to induce bloodstream infection and development of sepsis.

In contrast with what has been described for human sepsis, the clinical definitions for sepsis in veterinary medicine has not been well defined. Parameters commonly measured in rodent animal models of sepsis are based on responses seen in human sepsis, and
include changes in physical appearance, weight loss/gain, measurable clinical signs; such as temperature, cardiac or respiratory rate change, and unprovoked/stimulated behavioural responses [234-236]. However, not all of these responses are seen in in vivo models of sepsis, and response can depend on the dose administered. A 2005 study by Copeland et al. compared the responses to endotoxemia in humans and in a mouse model. Human subjects injected with 2 ng/kg of endotoxin derived from E. coli were monitored for physiological changes every 2 hours for 24 hours, demonstrating elevated temperature, heart rate, and blood pressure. In contrast physiological changes were not seen in C57BL/6 mice, even with a higher dose (500 ng/kg) of endotoxin [237]. Indeed, mice have been shown to be significantly more resistant to infection, requiring doses 1,000 to 10,000-fold greater than doses inducing severe symptoms of human inflammation [238]. At these high doses, intravenous bacterial infection in mice can also lead to rapid cardiovascular collapse and death (in a matter of hours), which is not commonly seen in human sepsis.

Other measurable parameters used in animal models of sepsis include the analysis of immunological responses. Aside from the lack of physiological responses seen in mice in the Copeland et al. study, similar immunological responses were seen; in changes to the proportions of circulating neutrophils and lymphocytes in both humans and mice over a 24 hour period; and peak response times for increases in plasma IL-6 and TNF-α similarly seen in humans and mice within 2 hours of infection; signifying in this model, that immunological responses in mice were a valid representation of human inflammatory disease [237].

Coinciding with the increased prevalence of gram-positive sepsis, there has been an increase in the number established mouse models of gram-positive sepsis [67, 239-241]. A recent study by von Köckritz-Blickwede et al. showed differences in the levels of susceptibility to bloodstream infection with S. aureus in a variety of inbred mouse strains including C57BL/6, C57BL/10, C3H/HeN, CBA, BALB/c, DBA/2, and A/J mice [241]. Mice were infected with $4 \times 10^7$ CFU S. aureus, and monitored survival over a 14 day period. Over this time, C57BL/6 mice appeared more resistant to infection and had higher survival rates (15% mortality rate) compared with BALB/c, DBA/2 and A/J mice, all of which showing 100% mortality within 7 days of infection. This was similarly shown in other studies of staphylococcal sepsis [242, 243]. Additionally, C57BL/6 mice limited
bacterial growth more effectively in target organs within 24 hours of infection, with no histological sign of tissue damage observed in these organs [241]. As such, C57BL/6 mice could provide a robust mouse model for the extended study of sepsis development. Although mouse models of systemic staphylococcal infection have been established, comparative studies of responses of mice in parallel studies of human sepsis are limited.

A major consideration is the variations in responses observed in clinical infections (due to previous or current infection or colonisation), compared to the inbred species of laboratory animals that have not previously been exposed to systemic infection [148].

Sepsis is a complex inflammatory condition that has proved difficult to replicate in animal models, to address this a number of considerations should be made when designing animal models of sepsis studies:

i. The animal model should aim to mirror the timelines of infection with that of human clinical sepsis (days not hours)

ii. Measurement of the same parameters where possible during infection in animal models to determine whether similarities/differences are observed in the mechanism of immune responses with human clinical sepsis.

iii. Animals of both genders in wild-type strains, and in more susceptible strains of mice (including immuno-deficient/transgenic mice) should be investigated to model patients with comorbidities with increased risk of mortality in sepsis.

The study described in Chapter 4 of this thesis represents a preliminary investigation into the response of C57BL/6 wild-type mice to staphylococcal-induced sepsis as well as the potential of low-dose aspirin therapy to resolve inflammatory states, in parallel with the clinical study outlined in Chapter 3. The C57BL/6 mouse strain was selected on the basis of previous studies showing increased resistance to staphylococcal infection; in order to facilitate the measurement of clinical parameters during the onset of early sepsis following bloodstream infection, and permitted analysis of target organs. Additionally, this model took into consideration the humane endpoints required for approval by the institutional ethics committee, as death as an endpoint was not permissible.
Parameters indicating development of sepsis seen in clinical patients will also be determined in mice: including activation of NFκB and production of specialised pro-resolving mediators, as well as effects to innate and adaptive immune systems. The design of the study included similar timeline of infection, and permitted a detailed analysis of the mouse model as an accurate representation of human clinical sepsis.
1.3. *Staphylococcus aureus*

Discussed below are general characteristics of *S. aureus* as an important nosocomial pathogen and the virulence and pathogenicity factors involved in the development of invasive staphylococcal infections (e.g. endocarditis and device-related biofilm infections). These invasive infections are a frequent source of bloodstream infections leading to the development of sepsis.

1.3.1. General classification and characteristics

*Staphylococcus species* were first identified in the early 1880’s by Scottish surgeon Sir Alexander Ogston [244]. Initial classification of staphylococci was given within the common genus of Micrococcus, but was later reclassified as *Staphylococcus* with species determined according to colony colour with *S. aureus* (from the Latin word for gold) compared with *S. epidermidis* to signify its consistent colonisation on human skin.

*S. aureus* is differentiated from other bacterial species on the basis of biochemical testing for catalase and coagulase production, mannitol fermentation, and acid production of D-trehalose [245]. Catalase production also distinguishes *S. aureus* from other *Enterococcus* and *Streptococcus* species, and majority (but not all) of *S. aureus* isolates will produce coagulase, which clots blood plasma of the host [246]. Those staphylococcal species that are not coagulase-positive are classified as Coagulase Negative Staphylococci (CoNS). Traditional bacterial identification methodology carries the disadvantage of the prolonged time required for a definitive result. As this contributes to the delay in diagnosis and severity of sepsis, rapid identification of causative organisms potentially improve response time for targeted antibacterial therapy. Since the 1970’s, advancement in identification methods have improved the detection time of *S. aureus* by molecular methods such as PCR [247] and semi-automated systems like Vitek and matrix-assisted laser desorption/ionisation time of flight of mass spectrometry (MALDITOF-MS) [248]. However as these methods still rely on growth of colonies from blood cultures, reductions in the detection time remain at approximately 8 hours.
1.3.2. Emergence of methicillin-resistant *S. aureus* (MRSA)

Records of penicillin and methicillin resistant variants of *S. aureus* date as far back as 1948 and 1961, respectively [249, 250]. The introduction of these antibiotics into the routine treatment of infections, gives bacteria the pressure to adapt in order to survive. Initial resistance to penicillin by staphylococci was due to the production β-lactamases that could hydrolyse β-lactam. Methicillin was synthesised with modifications to resist the hydrolysis of β-lactam. Methicillin resistance is attributed not to β-lactamase production, but to the expression of an additional penicillin binding protein (PBP2a) encoded by the gene conferring methicillin resistance (*mecA*), acquired from another species [251].

Initially the epidemiology of MRSA was limited to the hospital-acquired infections (HA-MRSA), however in the last decade the incidence of skin and soft tissue infections due to community-acquired MRSA (CA-MRSA) have risen dramatically [252]. Molecular typing studies conducted in both Australia and the US have shown that CA-MRSA infections are more strongly associated with strains carrying the Panton-Valentine leukocidin (PVL) virulence factor and the presence of SCCmec type IV allele, and appear more susceptible to some non- β-lactam antibiotics than HA-MRSA strains [253]. Of interest HA-MRSA infections did not appear to posses PVL and were usually related to other SCCmec alleles [253, 254]. The persistent increase in these multi-drug resistant strains and their spread from hospital environment into the community had increased the likelihood of a potentially serious infection to the general public.

Vancomycin is the last resort for antibiotic treatment of MRSA the emergence of such strains is of great concern for global health, with increases in the number of strains of MRSA with reduced susceptibility to vancomycin being reported [255, 256]. Strains of vancomycin-intermediate *S. aureus* (VISA) were first reported in 1997 in Japan, with moderate increases in resistance to glycopeptide therapy [255]. Further, there are *S. aureus* strains reported to have a vancomycin minimum inhibitory concentration (MIC) within a susceptible range, however upon more detailed investigation (by either prolonged incubation or higher inoculum) show a resistant subpopulation with an increased MIC [257]. These strains are now referred to as heterologous-VISA (hVISA).
These vancomycin intermediate strains are more commonly associated with HA-MRSA, and can result in vancomycin treatment failure; contributing to extended hospitalisation, due to prolonged infection [258]. Although VISA and hVISA strains are not typically associated with high mortality rates, infections with these strains are difficult to treat. As vancomycin is at present a last resort, antibiotic resistance poses a significant challenge for treatment of complex infections. The use of alternative therapies have been suggested in the treatment of complex VISA and hVISA infections such as endocarditis and joint infections, with aspirin and major metabolite salicylic acid showing potential benefit (discussed further in section 1.4.3).

1.3.3. Epidemiology of staphylococcal disease

1.3.3.1. Colonisation and infection

While colonisation rates of *S. aureus* vary, approximately 20% of the general population consistently carry at least one strain of *S. aureus* [259, 260]. Epidemiological studies have shown general carriage rates among the general population are similar to that of hospital staff and other patients (both on admission and already hospitalized) [261]. Patients already colonised with *S. aureus* are at also a higher risk of developing infections, by transmission via contact with hospital staff that are transiently colonised with a different strain. The rise in antibiotic resistant strains of *S. aureus* (MRSA) within the hospital and community settings also increases the risk of spread to other patients.

There are several known risk factors for infection with *S. aureus*. Individuals who are immuno-compromised (including infants, the elderly and those with previous infections or pre-existing conditions such as diabetes, patients on haemodialysis and intravenous drug users) presented with higher carriage rates than healthy counterparts [259, 262, 263]. High carriage rates in immuno-compromised patients are also associated with increased risk of developing infection. Patients who had previously had surgery or indwelling catheters other medical devices (such as pacemakers and artificial joints) inserted are also high potential risk factors for hospital-acquired infection [264-266].
1.3.4. Pathogenesis of staphylococcal disease

In order to initiate infection, *S. aureus* enters the bloodstream or other adjacent tissues following damage to skin or mucosal barriers. With a host of virulence factors, and the ability to avoid the innate immune response [267], *S. aureus* infection can cause result in the destruction of host lymphocytes and serious inflammation, giving rise to a symptomatic response. Figure 1.5 illustrates the mechanisms of staphylococcal pathogenesis. The virulence factors and associated bacterial genes that play a role in staphylococcal pathogenesis are also summarised in Table 1.3; the most significant virulence and pathogenicity factors for this study are discussed below.

1.3.4.1. Adhesins as virulence factors

Adhesion is the first step in initiation of *S. aureus* infection. Adhesins are molecules produced in order to facilitate bacterial attachment to a variety of host substrates including epithelial and endothelial cells, and other host protein surfaces. Adhesins may be anchored to the surface of staphylococci like the family of proteins known as Microbial Surface Recognising Adhesive Matrix Molecules (MSCRAMMs) [268], or secreted adhesins known as Secretable Expanded Repertoire Adhesive Molecules (SERAMs) [269]. Both of these types of adhesins are proteinaceous, however polysaccharide intercellular adhesin (PIA) [270] and techoic acids [271] have also been shown to behave as adhesins.

Major MSCRAMMs of *S. aureus* include clumping factors (ClfA and ClfB), fibronectin-binding proteins (FnBPA and FnBPB), and collagen-binding protein (Cna). These proteins represent the largest class of cell wall anchored (CWA) proteins [272]. FnBPs initiate a bond between fibronectin from the host and the surface of the bacteria, allowing attachment to host integrins via the extracellular matrix proteins [273]. ClfA binds fibrinogen on the surface of implanted medical devices to promote biofilm formation [274], and also interferes with complement pathways [275]. Both ClfA and ClfB are involved in platelet aggregation by binding host fibrinogen [276]. ClfB on the other hand is also involved in the attachment of staphylococci to desquamated epithelial cells by binding to cytokeratin-8 [277]. Attachment to host collagen by staphylococci is mediated by Cna, facilitating deeper invasion into the tissues [278]. Further, Cna behaves as a virulence factor in experimental models of endocarditis [279] and arthritis [280].
1.3.4.2. Staphylococcal protein A

An important virulence factor of \textit{S. aureus} is staphylococcal protein A (SpA), with significant roles in including inflammation in lung infection, development of abscess formation and severe sepsis \cite{234, 281, 282}. This 45-kDa secreted and cell-anchored protein is present in majority of clinical isolates and is unique in its N-terminal structure; unlike other proteins its signal sequence is comprised of five adjoining immunoglobulin G (IgG) binding domains that fold into repeated triple helical bundles \cite{283, 284}. Another noted function of SpA is in facilitating staphylococcal evasion of phagocytosis by polymorphonuclear leukocytes (PMN) \cite{285}. By binding this the Fc region of non-specific IgG, SpA interferes with the signalling cascade; preventing recognition of \textit{S. aureus} by PMN, avoiding opsonisation and Fc-mediated phagocytosis \cite{286, 287}. Surface-anchored SpA also instigates B lymphocyte proliferation and apoptosis, in a T lymphocyte independent manner, by increasing B lymphocyte sensitivity to allow cell-wall binding of staphylococcal LPS to TLR-2 \cite{288}. Soluble SpA (sSpA) has also been suggested to be involved B lymphocyte activation, in the presence of T lymphocytes, as well as behaving as a T-cell mitogen \cite{289, 290}. Its also been shown that SpA mediates bacterial binding to human vWF \cite{291}, interfering with platelet-binding at sights of endothelial damage further promoting endovascular infection \cite{292}. Moreover, SpA was shown to activate TNF-\alpha by binding its receptor (TNFR1), contributing to inflammation, in a mouse model of \textit{S. aureus}-induced pneumonia \cite{293}. 
Figure 1.5: Pathogenesis of staphylococcal invasion of tissue (sequence beginning left to right). Staphylococci bind damaged vascular cells via MSCRAMM-mediated mechanisms or bind endothelial cells directly by either adhesion-receptor or ligand bridging interactions. Heparin sulfate proteoglycan on the surface of the extracellular matrix (ECM) facilitates the staphylococcal attachment and increases susceptibility of endothelial cells to infection [294]. In order to facilitate spread of infection, staphylococci excrete proteolytic enzymes and translocate to the bloodstream to infect adjoining tissues. The inflammatory response triggered by infection in sub-epithelial tissues results in abscess formation, which can increase pathogenesis of endocarditis in the cardiac endothelium. Inflammatory cytokines are released by tissue-based macrophages and circulating monocytes, triggering the recruitment of T lymphocytes. Interferon-γ (IFN-γ) released by T lymphocytes activates neighbouring macrophages. The continued release of inflammatory cytokines contributes to symptoms of systemic staphylococcal infection and severe sepsis. PMN denotes polymorphonuclear leukocytes. Figure reproduced with permission from [295], Copyright Massachusetts Medical Society.
1.3.4.3. Evading host defences

*S. aureus* adheres to host lymphocytes and can also invade, in order to initiate infection. Once *S. aureus* enters host tissues deeper than the mucosal layer, secretable proteins interact with local tissues and trigger the host immune response. These exoproteins include cytolytic toxins and other pathogenicity factors that protect the bacteria from elimination (Table 1.3). Of these, PVL is an important virulence factor for *S. aureus*, frequently responsible for necrotic lesions found in infected skin of patients [296]. PVL is only produced in 2-3% of strains, however of those strains producing PVL, the majority are MRSA isolates [297]. The two subunits of the toxin assemble in the membrane of monocytes, macrophages and lymphocytes, forming a pore [298]. In contrast with pvl-negative staphylococci, the release of PVL from pvl-positive staphylococci lead to rapid cell death in both human and rabbit PMN, with greater implications in staphylococcal pathogenesis [299].

1.3.4.3.1. Interaction of *S. aureus* with Toll-like receptors

The role of TLRs in the innate immune system has previously been discussed in the context of sepsis and similarly applies in regards to staphylococcal infection. Recognition of *S. aureus* LTA by TLR2/TLR6 complexes, results in a signalling cascade and the activation of pro-inflammatory mediators, such as NFκB. This promotes the production of inflammatory cytokines and chemokines in order to initiate phagocytosis by PMNs [300].

1.3.4.3.2. Inhibition of neutrophil chemotaxis

*S. aureus* can persist within epithelial cells, endothelial cells and macrophages, however neutrophils provide a challenge for *S. aureus* survival [301]. Patients with severe neutrophil dysfunction disorders (such as Leukocyte Adhesion Deficiency and Chronic Granulomatous Disease) have increased susceptibility to *S. aureus* infection, further confirming the importance of neutrophils in the clearance of staphylococcal infection [302, 303]. In order to evade neutrophil killing, *S. aureus* secretes two molecules: Chemotaxis Inhibitory Protein (CHIP) and Extracellular adherence protein (Eap). Sixty percent of *S. aureus* strains secrete CHIP, which binds to both the formyl peptide receptor (FPR) and the C5a receptor (C5aR), blocking ligand binding and signal transduction of neutrophils [304]. Likewise, Eap directly interacts with intracellular adhesion molecule 1
(ICAM-1), fibrinogen or vitronectin, effectively disrupting β₂-integrin mediated adhesion and migration of neutrophils to the infected tissues [305].

**Table 1.3: Pathogenicity factors associated with *S. aureus* infection and biofilm formation.**

<table>
<thead>
<tr>
<th>Virulence Factor</th>
<th>Function</th>
<th>Genes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibronectin binding protein (FnBPA/FnBPB)</td>
<td>Fibronectin binding</td>
<td>fnbA, fnbB</td>
<td>[45]</td>
</tr>
<tr>
<td>Clumping factor A/ B (ClfA/ClfB)</td>
<td>Fibrinogen binding</td>
<td>clfA, clfB</td>
<td>[276]</td>
</tr>
<tr>
<td>Collagen Binding protein (Cna)</td>
<td></td>
<td>cna</td>
<td>[280]</td>
</tr>
<tr>
<td>Extracellular cellular matrix binding protein (ECM)</td>
<td>Extracellular matrix binding</td>
<td>ebh</td>
<td>[306]</td>
</tr>
<tr>
<td>Extracellular adherence protein (Eap)</td>
<td>Extracellular adherence</td>
<td>eap</td>
<td>[305]</td>
</tr>
<tr>
<td>Protein A</td>
<td>Evasion of opsonisation and</td>
<td>spa</td>
<td>[282]</td>
</tr>
<tr>
<td>Capsular polysaccharide type 1, 5 &amp; 8</td>
<td>phagocytosis</td>
<td>cap1, cap5,</td>
<td>[307]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>cap8</td>
<td></td>
</tr>
<tr>
<td>Aureolysin</td>
<td>Metalloproteinase involved in</td>
<td>aur</td>
<td>[308]</td>
</tr>
<tr>
<td></td>
<td>complement inactivation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Staphylokinase</td>
<td>Enzyme involved in complement</td>
<td>sak</td>
<td>[309]</td>
</tr>
<tr>
<td></td>
<td>inactivation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-haemolysin</td>
<td></td>
<td>hla</td>
<td></td>
</tr>
<tr>
<td>β-haemolysin</td>
<td>Haemolytic toxins</td>
<td>hlb</td>
<td>[310]</td>
</tr>
<tr>
<td>γ-haemolysin</td>
<td></td>
<td>hlg</td>
<td></td>
</tr>
<tr>
<td>δ-haemolysin</td>
<td></td>
<td>hld</td>
<td></td>
</tr>
<tr>
<td>Panton-Valentine Leukocidin (PVL)</td>
<td>β-pore forming cytotoxin</td>
<td>lukF, lukS</td>
<td>[311]</td>
</tr>
<tr>
<td>Polysaccharide intracellular adhesin (PIA)</td>
<td>Non-proteinaceous factors</td>
<td>ica</td>
<td>[312]</td>
</tr>
<tr>
<td></td>
<td>including pro-inflammatory</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>cell wall components</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipoteichoic acid, Cell wall teichoic acid</td>
<td></td>
<td>ltaS, wta</td>
<td>[313]</td>
</tr>
</tbody>
</table>
1.3.5. Staphylococcal infections in humans

1.3.5.1. Skin-related infections

Any minor breach of mucosal barriers or the skin gives staphylococci the opportunity to invade local and adjacent tissues. As an abscess forms with a core of pus containing a combination of bacteria, macrophage and PMN influx [314]. Skin-related infections including, carbuncles, cellulitis, folliculitis, furuncles, impetigo, keratitis, mastitis and psoriasis are often caused by *S. aureus*. Localised infections like these have the potential to become life-threatening infections and inflammatory diseases including bacteraemia, sepsis, endocarditis, pneumonia and device-related biofilm infections. The significance of these invasive infections is discussed in more detail below.

1.3.5.2. Bacteraemia and sepsis

As blood is generally considered sterile the presence of the bacteria is considered abnormal and serious, as often there is no symptomatic response. *S. aureus* is the most frequent source of bacteraemia, with nosocomial causes including the use of indwelling medical devices such as pacemakers and catheters. A recent study by the Australian Institute of Health and Welfare reported moderate decreases in hospital-associated staphylococcal bacteraemia cases between 2010 and 2013; coinciding with implemented MRSA screening and early identification of bacteraemia-like infections [315]. What this study did not take into consideration was the *S. aureus* bacteraemia events occurring as a result of indwelling medical devices prior to hospitalisation, thereby underestimating the total number of cases of bacteraemia, and the seriousness of infection. Untreated bacteraemia can result in an uncontrolled inflammatory response, leading to the development of severe sepsis; associated with a high mortality rate and gross economic burden (discussed in section 1.2.2).

The relationship between staphylococcal disease and sepsis has been studied since the early 1880’s, with the initial identification of *S. aureus* from an abscess that had become septic [244]. Over the last two decades, the incidence of *S. aureus*-related sepsis has risen; now the majority cause of severe bloodstream infections, with the high associated mortality rates [4]. With this in mind, invasive staphylococcal infections known to be a frequent source of sepsis are discussed below.
1.3.5.3. Persistent complex infections (Biofilms)

In nature, biofilms tend to contain complex communities of different bacterial and/or fungal species. The history and significance of biofilm forming organisms dates back to Van Leeuwenhoek’s observation of dental plaque formation by various microbes [316]. Following this, it has been shown that biofilm-forming organisms form multi-dimensional structures [317], and changes in phenotype (from planktonic to biofilm-forming) can result in a change in gene expression and growth patterns [318]. The stages of biofilm formation by staphylococci are outlined in Figure 1.6.

1.3.5.3.1. Components of the biofilm

The composition of biofilm consists of multiple biopolymers, termed extracellular polymeric substances (EPS). Originally thought to be comprised solely of polysaccharide matter, EPS also includes peptides (such as pili and flagella peptides in the case of motile bacteria including Pseudomonas aeruginosa, Salmonella species and Escherichia coli [319, 320]); nucleic acids (extracellular DNA), which were shown to be integral to staphylococcal biofilms [321, 322]; lipids, and other non-cellular components (such as blood or water), depending on the environment.

A number of general functions have been determined for the EPS matrix of staphylococcal biofilms. EPS allows quorum sensing (signalling between adjacent cells) by immobilising the cells closely next to one another [323-325]. The matrix also recycles lysed components of dead microbial cells such as extracellular DNA, for horizontal gene transfer and EPS degradation [326]. This regulated lysis process also provides an alternative source of nutrition for remaining biofilm cells [327-330].
Figure 1.6: Stages of biofilm formation by *S. aureus*. Attachment represents the initiation of the biofilm and there are multiple factors to consider. Microbes will often bind preferentially to an interface between the surface and an aqueous medium. Following attachment, microbes further adapt to grow within a biofilm environment, increasing the production of extracellular polymeric substances (EPS), this is known as biofilm maturation. This results in increased structural integrity of biofilm (via crosslinking of extracellular DNA and polypeptides the EPS matrix), leading to increased resistance to antibiotic therapy. Dispersal of the biofilm is the final stage of its development. Cells begin to detach from the biofilm, then disseminate to a new site, initiating attachment to a new surface; perpetuating infection. Figure reproduced and adapted with permission from [331].
1.3.5.3.2. Quorum sensing

Quorum sensing also commonly known as intracellular signalling is an important regulatory aspect of staphylococcal biofilm production. This mechanism is also seen in biofilms of *Pseudomonas aeruginosa* [332-334] and *Streptococcus mutans* [335, 336], for example. Quorum sensing in *S. aureus* is mediated by the protein encoded by accessory gene regulator (agr) locus [337, 338]. The agr locus consists of two operons (P2 and P3) that are governed by separate promoters. These operons encode the RNAII and RNAIII regulatory molecules, which then are responsible for the transcription and translation of various staphylococcal virulence factors. Agr-mediated signalling is regulated by the Staphylococcal accessory regulator (SarA) [339, 340], which increases cellular concentrations of RNAII and RNAIII. This signalling mechanism is critical to the generation and enhanced survival of staphylococcal biofilms, and impairment in signalling can result in reduced capacity to form biofilms.

1.3.5.3.3. Extracellular DNA in staphylococcal biofilms

*S. aureus* produces extracellular DNA (eDNA) in excess in both standard broth culture and biofilm via autolysis [341]. When produced within a staphylococcal biofilm, eDNA is what provides the integral and structural support. When bound to bacterial cells, eDNA provided a more viscous and adhesive surface enabling the enhanced attachment to hydrophobic substrata (such as plastic/glass/metal surfaces) [342]. Crosslinking of eDNA within the EPS matrix provides strength and stability to the biofilm, contributing to increased resistance to antimicrobial therapies and phagocytosis by macrophages. Cell lysis is a tightly regulated process by which eDNA is produced, released into the environment [343]; facilitated by autolysins from Cid (positive regulators) and Lrg (negative regulators) operons [328, 329]. These operons are regulated by quorum sensing molecules agr and SarA, thus impairment to quorum sensing could impact regulation of autolysis [344]. Reduced eDNA production impacts structural integrity of staphylococcal biofilms, potentially increasing susceptibility of weakened biofilms to immune attack (phagocytosis) [345].
1.3.5.3.4. Biofilms as a source of infection

The clinical relevance of staphylococcal biofilms is becoming more apparent with the increased incidence of complex infection associated with the use of indwelling medical devices and observed increases antimicrobial resistance within the last 15 years [346, 347]. Discussed below are a range of infectious diseases that can develop following staphylococcal biofilm formation.

Periodontitis is the inflammation of bone and ligaments that surround teeth bones. While not commonly caused by *S. aureus*, the formation of staphylococcal biofilms on teeth will then promote a host of other oral infections [348]. Similarly, Osteomyelitis is the presence of bacterial infection in bone and surrounding tissues. At present, *S. aureus* is the leading cause of bone infections, commonly introduced following surgery or trauma, bloodstream infection or as a result of other underlying conditions or infections [349]. Bacterial stimulation of host adhesive matrix proteins (fibronectin, fibrinogen, collagen), facilitates bacterial attachment to the damaged or dead bone [350]. As the host cannot resorb dead bone, the severity of infection of bone becomes more difficult to treat, with debridement required to resolve infection [351]. Mature staphylococcal biofilms are resistant to PMN-mediated phagocytosis, interfering with PMN motility. Evasion of phagocytosis leads to extended tissue damage and provides a perfect niche for continued bacterial attachment and survival. Intervention during early stages of biofilm formation, can result in effective clearance of biofilms by phagocytosis [352].

Patients with indwelling medical devices such as catheters [6, 353, 354], pacemakers [265] medical prostheses [355] and those patients requiring haemodialysis are at an increased risk for development of a biofilm infection. Breaks in mucosal membranes provide opportunities for nosocomial bacteria like *S. aureus* to migrate across impaired mucosal membranes, attach to the device surface and form biofilms. In both osteomyelitis and infective endocarditis (discussed in section 1.3.5.4), persistence of biofilms are dependent on the recruitment of host matrix proteins; providing an optimal surface for attachment [274]. Complex vegetations formed on the surface of indwelling devices, comprise of both host matrix proteins and biofilm components, and demonstrate enhanced antimicrobial resistance.
1.3.5.3.4.1. Animal models of device-related biofilm infections

While in vitro models of biofilm-infections provide useful information on the characteristics of biofilm development, their usefulness is limited, as they do not allow the investigation of the complex strategies employed by biofilms to evade host immune responses in vivo [356, 357]. Animal models have allowed researchers to bridge the gap, replicating biofilm and device-related infections in humans; with rodents (such as rats and mice) and rabbits being popular choices for pre-clinical and comparative animal model studies (as similarly described in section 1.2.6) [358, 359]. Discussed below are the advantages and disadvantages of animal-models of biofilm-related infections, including sub-cutaneous implant infections and prosthetic-joint infections, which were investigated in Chapter 5 of this study.

The association between intravascular- and urinary-catheter insertion, biofilm growth and subsequent development of bloodstream infection have been examined in a number of in vivo animal models [323, 360-362]. The sub-cutaneous infection model in this study (Chapter 5) represents a static infection, allowing the investigation of both biofilm development and local immune response, following insertion of the contaminated or infected foreign body implant. An intravascular venous catheter-model first developed by Rupp et al. in 1999 is one of the most popular models, used to investigate colonisation and infection with $10^6$-$10^8$ CFU S. epidermidis in rats [360]. This model has been successfully used to identify the important bacterial components that contribute to establishment of biofilm, and in more recent studies elucidated the effects of complex biofilm-related infections on the innate immune system [357, 360, 363]. Sub-cutaneous catheter models of biofilm-related infection are often employed to investigate the potential of novel anti-bacterial compounds (such as anti-microbial coatings), to reduce or prevent adhesion of biofilm formation, or as a pre-clinical model prior to trials or application in clinical setting [364, 365]. As similarly discussed in section 1.2.6, these studies do have the capacity to identify promising compounds that can reduce bacterial attachment, biofilm formation or reduced inflammation, however these benefits are not always seen in clinical biofilm-related infections [366].

The connection between human orthopaedic devices and joint-implants with biofilm formation was made in the clinical setting, following the use of rabbit models to study a foreign body inserted in the tibia [367-369]. In this model, a number of different materials
such as stainless-steel (s/s), titanium, other metals and plastics have been used to study \textit{in vivo} biofilm formation (reviewed in [370]). The most recently reported models use contaminated pins, which are then inserted into a mouse tibia [356]. This model similarly allows for visual investigation by imaging, as well as bacterial enumeration and histological analysis. It has been debated whether this model is suitable for determining antimicrobial activity, or pharmacokinetics of a potential therapy or investigating immune responses. However the study by Prabhakara \textit{et al.} (2011) did in fact show that an adaptive immune response of Th2/Treg cells were protective against a chronic \textit{S. aureus} implant infection [356]. Further, antibiotic-coated implants have been developed as a preventative measure against biofilm attachment and formation, with promising results in both an animal model and in humans [371, 372].

As similarly described for other human infections (section 1.2.6), there are considerations that must be made when developing models for device-related biofilm infections. While these models have provided researchers with valuable information, a major concern remains, with the promising results shown in both \textit{in vitro} and \textit{in vivo} models of device-related infection, have not translated to any therapeutic/prophylactic benefit in clinical trials [366, 373]. This is often due to the limitations of \textit{in vivo} models themselves. As similarly described in section 1.2.6, there are differences between human and murine in responses to infection (for example the response to different concentrations of LPS) that can influence conclusions made in these models [374]. Moreover, as so many \textit{in vivo} models of device-related biofilm infections have been developed; investigating different surface characteristics of implants, or novel therapeutic/preventative approaches, there is no standard model to allow reliable comparisons to be made between different device-related infection studies. Other suggestions have been made in an effort to improve animal models of device-related infections. These include, consideration of the effects of environmental factors that could affect outcomes in infection [375], and the study of polymicrobial biofilms, to more accurately represent the infections seen in clinical setting [376]. With these considerations in mind, development of novel \textit{in vivo} models of device-related biofilm infection will allow for a more reliable representation of human infection, and further our understanding of the complex nature of biofilm infections.
1.3.5.4. Endocarditis

Endocarditis is described as the inflammation of the endocardium (the inner surface) of the heart, more commonly affecting the heart valves. Host matrix proteins (such as fibrin and fibrinogen) and platelets bind to damaged valves to form non-infective thrombotic endocarditis. Extracellular matrix growth provides a perfect environment for bacterial colonisation. Infective endocarditis (IE) occurs when bacteria adhere to damaged heart valves, via interactions with fibrin; inducing platelet-aggregation, leading to enhanced complex vegetation growth. While infective endocarditis is ordinarily uncommon, *S. aureus* is the majority source of surgical treated IE, with high associated mortality rates [377, 378]. Increased incidence of *S. aureus*-induced IE is due to clinical risk factors such as indwelling medical devices, invasive diagnostic or therapeutic procedures and intravenous drug use [379].

Treatment of *S. aureus*-induced IE relies on long-term intravenous antibiotics at high-doses. As complex vegetations on heart valves have limited blood supply, antibiotic therapy is not always effective and more severe vegetations require surgical removal, which often leads to secondary infection [380]. Untreated vegetations carry an increased risk in complications such as congestive heart failure (CHF) and systemic embolism (SE) which contribute to a higher likelihood of death [381]. SE is the most commonly reported complication of IE, with majority of effects observed in neurological systems [382], however emboli may also affect major arteries and organs with large vascular supplies such as lungs, spleen and liver. Transiently colonised bacteria on the surface of emboli also present a complication with dissemination through the bloodstream often resulting in sepsis [383].

Studies in animal models of *S. aureus*-induced IE have shown that aspirin in various doses had the capacity to reduce bacterial vegetation size [384]. Further, retrospective analysis showed a decreased incidence of *S. aureus*-induced IE and reduced requirement for valve replacement, in patients on long-term aspirin therapy [385]. Additional supporting evidence for the potential use of aspirin as an adjunct therapy against staphylococcal-based infections is discussed further in the next sub-chapter.
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1.4. Aspirin

Aspirin is a salicylate derived NSAID. The active natural form; salicylic acid (SA), found in the bark of willow and myrtle tree species have documented medicinal uses dating as far back as 3000 BCE, in some Ancient Egyptian communities [386]. While the credit for production of modern acetylated aspirin was given to chemist Dr Felix Hoffman (while working at Bayer Pharmaceuticals) in 1897, aspirin (by acetylating traditional SA) was first made by Charles Frederic Gerhardt 14 years earlier [387]. Since then, aspirin has risen to become the most regularly consumed medication globally. Clinical responses differ depending on the dose of aspirin administered, providing a wide variety of therapeutic applications, some of which are discussed below.

1.4.1. Medical applications of aspirin

1.4.1.1. Aspirin as an analgesic and an anti-pyretic

Aspirin and aspirin-like drugs are classed as weak analgesics (when compared to stronger narcotics like morphine), with effective action against clinical pain of low to moderate intensity such as some headache, postoperative pain, rheumatoid arthritis and osteoarthritis [388]. However, the preferred NSAID given for pain relief is ibuprofen. Ibuprofen is considered a safer alternative, to aspirin with no apparent side effects (like gastrointestinal bleeding) [389]. As previously outlined in section 1.1.5.6, all NSAIDs elicit their anti-inflammatory effects by binding and inhibiting COX-1 and COX-2, interfering with prostaglandin synthesis. Aspirin however is the only NSAID known to irreversibly acetylate and inhibit COX [390]. While this treatment has been well documented in adults, the use of aspirin has been strongly discouraged in combating fever in children by medical societies and regulatory agencies. This is due to an increased risk of development of Reye’s syndrome) [391, 392].

1.4.1.2. Aspirin in the management of cardiovascular diseases

General physician Lawrence Craven was the first to identify the anti-thrombotic effects of aspirin in 1953 [393]. At this time, the beneficial effects of aspirin therapy in preventing cardiovascular events such as myocardial infarction (MI) had not been described. Craven observed more bleeding in patients taking analgesic gum treated with aspirin following oral surgery, showing that aspirin prevented MI. Within 10 years of this initial discovery,
connections had been made between cardiovascular diseases including coronary atherosclerosis, thrombosis and acute MI [394], and evidence suggested that prevention of cardiovascular events in these diseases were due to anti-platelet effects of aspirin therapy [395].

The pilot study in the treatment of cardiovascular disease with aspirin was at a dosage of 300 mg/day. At this dose of aspirin treatment, early maximum levels of platelet inhibition were also seen [396]. Initially the prescribed doses in a number of studies ranged between 500 mg and 4000 mg daily, however these high-dose treatments over long periods of time resulted in a significant increase in adverse bleeding [397, 398]. In the last three decades, an increasing trend towards low-dose aspirin treatment for cardiovascular disease has been adopted. Inhibition of COX-1 and decreases in gastric prostaglandin levels have been observed in healthy subjects with doses as low as 30 mg/day [399]. Thromboxane production is persistently increased in patients with cardiovascular disease; therefore a minimum of 50 mg/day aspirin treatment is required for optimal inhibition [400, 401]. At present, the recommended dose of aspirin for the prevention of cardiovascular disease is between 75 mg and 150 mg daily.

1.4.1.3. Aspirin in the prevention of cancer

The initial link between aspirin and cancer prevention was made in the late 1980’s with one study reporting a 50% reduction of colorectal cancer risk in patients that regularly took aspirin-like drugs [402]. Early studies into the protective effects elicited by aspirin and other NSAIDs were supported by literature, experimentally observed in the reduction of tumour growth in animal model studies [403, 404]. Within the last decade however, debates have risen in the effectiveness of aspirin to prevent cancers, with one study showing no benefit of long-term aspirin (100 mg/day) in preventing total breast, colorectal or other cancers in healthy women [405]. In contrast, recent studies have shown support for the anti-cancer effects of aspirin, with modest decreases in the risk of developing colorectal [406-408], oesophageal [409, 410], stomach [411], prostate [412, 413], breast [414, 415], pancreatic [416], skin melanomas [417], and ovarian cancers [418]. The mechanism of action of aspirin in its prevention of some cancers appears to be again the result of COX-2 inhibition and subsequent inhibition of prostaglandin production, which is often increased in a variety of cancer cell types [419, 420].
1.4.2. Adverse effects of aspirin therapy

The most commonly reported adverse effect of aspirin therapy is damage to the gastrointestinal (GI) tract [421-423]. Although administration of aspirin has been associated with GI injury, the level of damage observed depends on several factors including: dosage, term of administration, use of other medications, and pre-disposition from other conditions.

The adverse GI symptoms induced by low-dose aspirin (<325 mg daily) that have been reported range from symptoms without mucosal lesions, to more serious GI toxicity (with prolonged use), including bleeding, ulceration, with rare instances of perforation, resulting in death [424]. Upper GI symptoms including gastro-oesophageal reflux (regurgitation or heartburn), and dyspepsia (including bloating, gastric discomfort and ingestion-related nausea) are most frequently seen in patients (up to 20%) on long-term aspirin therapy [425, 426]. Additionally, low-dose aspirin therapy over a long period of time can lead to small bowel bleeding and protein loss, which can contribute to iron-deficiency, anaemia and hypoalbuminaemia (64.5% low-dose aspirin taking patients presenting with mucosal and ulcerated lesions) [427]. The exact mechanism of aspirin-induced ulcer formation in intestines is not yet clear, however it is known that following aspirin-induced COX inhibition, the resulting decreases in prostanoid synthesis contribute to loss of cells from the gastric epithelium, causing superficial mucosal injury [428].

The majority of studies reporting on gastric mucosal injury have only evaluated the effect low-dose aspirin therapy over a prolonged period of time. Although there is limited data available, there is some debate as to whether short-term administration may also cause minor mucosal inflammation. A 2009 pilot study in healthy volunteers (n=10 subjects) showed a higher incidence (30%) of mucosal injury in 100 mg aspirin-taking participants, compared with placebo control participants over a 2-week period, however this was not statistically significant [429]. It is still unclear whether the adverse effects described can also be expected to occur in patients treated with low-dose aspirin for a shorter period of time (100-325 mg daily dose, for less than 1 week).
1.4.3. Evidence for aspirin as an adjunct therapy in sepsis

Low-dose aspirin therapy has been shown to impact inflammatory pathways in the inflammatory conditions described above prompting the question, could aspirin be a useful adjunct therapeutic in sepsis?

As previously mentioned, evidence is accumulating for the potential of aspirin-therapy in sepsis, as shown in both animal models and clinical studies. Studies from mouse models of inflammation dating back to 2004 have proposed therapeutic benefits of aspirin therapy, via the triggered synthesis of aspirin-triggered lipoxins (ATL). In a mouse model of IL-1β induced inflammation, aspirin therapy (200 mg/kg) resulted in increased plasma ATL production, as well as decreased peritoneal influx of neutrophils and inhibition of leukocyte adherence, rolling and migration [18]. This was also seen in a rat model of myocardial inflammation. Rats stimulated with LPS showed decreased prostaglandin synthesis and increased myocardial ATL production, when pre-treated with 200 mg/kg aspirin [213]. In mouse models of both acute lung injury [16] and induced peritonitis [17], post-infection ATL therapy showed anti-inflammatory “pro-resolution” effects including reduction of pro-inflammatory cytokine and chemokine production, reduced influx of neutrophils. In conjunction with antibiotic therapy, ATL given to mice with E. coli induced peritonitis lead to decrease bacterial loads and improved survival [17]. While these data from animal studies are encouraging in supporting aspirin therapy in sepsis, comparisons between animal models of inflammation and human inflammation remain controversial (as discussed in section 1.2.6).

More recently, a number of observational studies have investigated the potential therapeutic benefit of aspirin therapy in both healthy volunteers and clinical inflammatory states. The pro-resolving benefits of aspirin therapy were first seen in a randomised double-blind trial investigating the anti-inflammatory effects of aspirin at different doses (81, 325, and 650 mg) in healthy volunteers [20]. Levels of plasma ATL and thromboxane B2 were analysed both at baseline and after 8-weeks of aspirin therapy, with a daily dose of 81 mg significantly increasing ATL production with a subsequent decrease in production of thromboxane B2, suggestive of a pro-resolution anti-inflammatory response. This was also shown in a study by Morris et al., investigating the effect of aspirin-therapy in healthy volunteers with inflammation by inducing skin blisters.
In this study, low-doses of aspirin (75 mg daily) over 10 days not only increased the localised production of ATL and reduced thromboxane and prostaglandin levels within the blister, but also modulated the migration of PMN and macrophages site of inflammation. This effect was also shown in mice with zymosan-induced peritonitis, when treated with aspirin at daily doses of 2 mg/kg for the same period of time, in the same study.

Both studies noted increases in ATL production and decreases in pro-inflammatory pathways. One study also suggested that because long-term aspirin therapy dampens inflammatory responses, this could contribute to the delay in diagnosis of sepsis and delivery of appropriate antibiotic therapy. However, this did not increase the risk of developing severe sepsis or septic shock [430]. Aside from this, recent cohort studies of ICU patients with SIRS and/or sepsis (with low-risk of active bleeding) have shown reduced mortality rates following low-dose aspirin therapy [61, 63]. Of these studies, the retrospective study by Eisen et al. in 2012 examined the differences in in-hospital mortality rates of patients with SIRS and/or sepsis in a single centre cohort from 2000 to 2009, on the basis of previous aspirin therapy [61]. When compared with non-aspirin takers, aspirin-therapy reduced the likelihood of mortality by 6.2%, suggesting that aspirin could be a suitable adjunct therapy of sepsis. Likewise, in clinical studies of patients with community-acquired pneumonia, aspirin therapy was associated with shorter length of hospital stay [64], as well as reduced mortality rates [217].

There is also increased evidence for the potential therapeutic benefit of aspirin in other inflammatory states including device-related infection. Patients previously taking aspirin had fewer incidences of both mitral valve regurgitation and congestive heart failure, resulting in a decreased requirement for early valve replacement surgery in a clinical study of *S. aureus*-induced IE [385]. This was also shown in an experimental rabbit model of *S. aureus*-induced IE, resulting in reduced vegetation, bacterial burden and embolic events in animals treated with 4-12 mg/kg aspirin [384]. Similarly aspirin use was shown to reduce the risk of *S. aureus* bacteraemia in patients undergoing haemodialysis with tunnelled catheters [218]. From these studies it has also been suggested that aspirin might have anti-bacterial action in infection (both directly and indirectly) [221, 431], with one study showing that aspirin and salicylic acid impacted *S. aureus* global regulatory pathways, affecting cell-signalling [221].
There has been limited experimental analysis of the immunological impact of aspirin therapy in clinical sepsis, however a number of clinical trials have been initiated to determine these effects worldwide including; Aspirin for Treatment of Severe Sepsis (currently recruiting patients, sponsored by the Federal University of São Paolo, Brazil Trial ID; NCT01784159), The PROTECTIN study (not yet recruiting, Austin Hospital, Australia; ACTRN12614001165673). The data presented within this study (see Chapter 3) is in part contributing to a current ongoing clinical study from a single centre in Melbourne, Australia observing the Mechanism of Aspirin THERapy in Sepsis (The MATHS clinical trial; ACTRN12611000649910). This intervention study is currently recruiting, analysing immunological parameters over a 48 hour period in patients with SIRS and sepsis; randomized to 100 mg aspirin, 300 mg aspirin therapy or placebo therapy.

### 1.4.4. Mechanisms of aspirin therapy in sepsis

Aspirins anti-inflammatory induced mechanism of action has been previously elucidated in general [212] and for cardiovascular events and to some degree cancer prevention, via the inhibition of COX [403, 404], prostaglandin synthesis [38], and platelet inhibition [396]. The effects of aspirin within the context of systemic infection derived inflammation, have been hypothesised to occur via a number of mechanisms including; the inhibition of inflammatory pathways via the inhibition of NFκB [432, 433] and an increase the production of anti-inflammatory lipid mediators including lipoxins [14, 18, 434], resolvins and protectins now grouped in a family of “specialised pro-resolving mediators” (SPMs) [13, 435].

### 1.4.5. Inhibition of NFκB signalling in inflammatory pathways

An integral signalling molecule, transcription NFκB is known for its role in regulation of the inflammatory cascade and the exacerbation of severe symptoms of sepsis and other serious inflammatory conditions (as shown in Figure 1.7) [436, 437]. Under ordinary inflammatory conditions, NFκB is bound to Iκβ in the cytoplasm, Ικκα and Ικκβ (Ικκ complex) will migrate and bind to Iκβ, phosphorylating it and releasing NFκB to move into the nucleus and transcribe genes encoding pro-inflammatory cytokines and chemokines (Figure 1.7A) [438]. However in the presence of aspirin and other salicylate based drugs (1 mmol/L to 20 mmol/L concentrations), the active salicylic acid component
binds the Iκκ complex, preventing phosphorylation of Iκβ and subsequent activation of NFκB, outlined in Figure 1.7B [433]. The minimal dose for this effect has not yet been defined.

**Figure 1.7**: Representation of the canonical NFκB activation pathway. A) Under normal inflammatory conditions. Pro-inflammatory stimulation will result in Iκκ complex phosphorylating Iκβ and the subsequent release of activated NFκB to enter the nucleus and further transcribe pro-inflammatory cytokines to prolong inflammatory states. B) Following treatment with aspirin and salicylates. The active metabolite salicylic acid binds the Iκκ complex preventing Iκβ phosphorylation and keeping NFκB bound and inactive, decreasing the transcription of pro-inflammatory cytokines and reducing inflammation, adapted and reproduced with permission from [437].
1.4.6. Specialised pro-resolving mediators (SPMs)

1.4.6.1. Production and role of lipoxins in inflammation

The lipid mediator Lipoxin A₄ (LxA₄) is a well-known SPM with both synthesis and applications studied. Derived from the metabolism of arachidonic acid (AA), there are three biosynthetic pathways that result in lipoxin production. Platelet/leukocyte interactions trigger leukocyte 5-lipoxygenase (5-LOX) conversion of AA to epoxide Leukotriene A₄ (LTA₄). Leukotriene A₄ is metabolised further generating LxA₄ in leukocyte-adherent platelets [439]. Alternatively, 15-LOX catalyses the conversion of AA (on mucosal surfaces) generating 15S-hydroxyleicosatetraenoic acid (15S-HETE). 15S-HETE is internalised by PMN and converted by 5-LOX to produce LxA₄ (Figure 1.8A) [440, 441]. Low-dose aspirin therapy (75 mg daily) was recently shown to trigger the increased production of epimeric lipoxin isomers (15-epi-lipoxin A₄ also termed aspirin-triggered lipoxins (ATL), similar to the “classical” lipoxin biosynthesis pathways as described above (shown in figure 1.8B) [442].

There is also evidence that statins including atorvastatin and pioglitazone can promote the production of ATL, via the S-nitrosylation of COX-2 in animal models of inflammation [443, 444]. Human studies of statin therapy also showed similar responses, however increases in ATL as a result of low-dose statin therapy over a 4-week period were much lower compared with increased levels shown with low-dose aspirin [445]. Not only can ATL be induced by other pharmacological agents like statins, previous studies investigating ATL production showed detectable levels of this epimeric lipid mediator even in the absence of aspirin. Given this, the term “aspirin-triggered” and subsequent abbreviation of ATL do not give an accurate description, and for the remainder of this thesis will be referred to as 15-epi-lipoxin A₄ (15-epi-LxA₄). Both LxA₄ and 15-epi-LxA₄ play important roles in improving innate immune responses and reducing inflammation as discussed below.[19].

Both LxA₄ and 15-epi-LxA₄ used in both in vitro studies and animal models of infection have showed therapeutic benefit. A 2011 in vitro study by Prescott & McKay showed that when stimulated with low-doses of 15-epi-LxA₄ (100 nmol/L) over 6 hours, THP-1 differentiated macrophages showed an enhanced capacity to phagocytose both fluorescently labelled beads (quantified by fluorescence microscopy), as well as non-
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pathogenic *E. coli* (quantified by the number of internalised bacteria) [446]. In conjunction with antibiotics, 15-epi-LxA₄ therapy not only reduced systemic inflammation, but also prevented bacterial dissemination into organs, leading to improved survival mice with gram-negative sepsis [17]. Further, in a model of induced acute lung injury, post-treatment with 15-epi-LxA₄ lead to decreased TNF-α and NO production in lungs, and histology showing a decrease in the proportion of neutrophils infiltrating into lungs, accompanied by reduced oedema, and alveolar injury. Combined, these effects lead to attenuated lung damage, as well as increased survival in 15-epi-LxA₄ treated mice, compared with untreated mice [16].

The pro-resolution mechanisms of LxA₄ and 15-epi-LxA₄ include the inhibition of pro-inflammatory cytokines (via inhibition of NFκB), and increased immune response (by inhibition of prostaglandin E₂ and 5-LOX derived leukotrienes) similarly seen with anti-inflammatory flavonoid compounds [230]. In both humans and mouse models, 15-epi-LxA₄ production impairs leukocyte migration (particularly neutrophils) measured as total cells in exudates in local inflammatory models, preventing progression of systemic inflammation [18, 19, 215]. Reduced or impaired lipoxin production (including 15-epi-LxA₄), can result in an uncontrolled inflammatory state; consistent with responses in severe sepsis [447]. These findings support the clinical relevance of LxA₄ and 15-epi-LxA₄ and their potential as an adjunct therapeutic treatment. Clearance of inflammation is a complex process, and these effects have also been shown with other SPMS like resolvins and protectins [13, 448]. As such, it is more likely that resolution of inflammation is the combined action of lipoxins with other SPMs; resolvins, protectins and maresins [449].
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Figure 1.8: A) Simplified pathways of biosynthesis of pro- and anti-inflammatory lipid mediators from their parent polyunsaturated fatty acids (PUFA). Enzymatic hydrolysis of PUFA such as Arachidonic acid (AA), Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA) yields specialised pro-resolving lipid mediators with functions in clearance of infection and resolution of inflammation. B) Detailed pathways and effects of aspirin-triggered lipoxin synthesis. Aspirin acetylates and inhibits COX-2 derived from AA in epithelial, endothelial cells and monocytes. Remaining AA generates 15R-hydroxyeicosatetraenoic acid (15R-HETE), which is metabolised by leukocyte 5-lipoxygenase (5-LOX) to produce 15-epi-lipoxin A₄ (15-epi-LxA₄). Synthesis pathways of 15-epi-LxA₄ mirror the pathway for classical lipoxin synthesis, with similar anti-inflammatory and pro-resolving functions, including the inhibition of plasma-derived growth factor (PDGF), epidermal growth factor (EGF) and leukotriene D4 (LTD₄)-mediated signaling in neutrophils and macrophages. Figure adapted and reproduced with permission from [450] and [451].
1.4.6.2. The production role of other SPMs in inflammation

Derived from polyunsaturated fatty acids (PUFA) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), resolvins, protectins and newly identified lipid mediators termed maresins represent the other SPMs with proposed roles in resolution of inflammation (Figure 1.8A).

E-series resolvin production occurs via a similar synthesis pathway to that of 15-epi-LxA4. Following aspirin treatment or cytochrome P450 mono-oxygenase stimulation, acetylation of COX-2 leads to the conversion of EPA to 18R-hydro(per)oxy-EPE, which is reduced to form resolvin E2 (RvE2). Alternatively an intermediate by product of this 18R-hydro(per)oxy-EPE (18R-HEPE), is converted to 5,6-epoxy fatty acid within human PMNs and resolvin E1 (RvE1) is produced via the 5-LOX pathway [452]. Recently a third E-series resolving was identified, produced via a novel alternative pathway of 18R-HEPE conversions via 12/15-LOX [453]. E-series resolvin treatment in vivo in murine infection models resulted in increased bacterial clearance, a decrease in pro-inflammatory cytokine production, an decreased PMN infiltration due to inhibition of chemotaxis; improving survival in a model of bacterial induced lung injury [448, 453]. There was also an observed increased in PMN apoptosis, with enhanced macrophage efferocytosis in vitro with RvE1 exposure at concentrations as low as 1 nmol/L [454, 455].

Resolvin D series, protectins and maresins, are produced as a result of DHA metabolism. D-series resolvins are the product of two lipoxygenation steps. Firstly, conversion of DHA to 17S-hydro(peroxy)-DHA (17S-HpDHA), which is then metabolised to generate resolvin D1 (RvD1) or epimeric resolvin D2 (RvD1). At present, 6 D-series resolvins have been identified through natural biosynthetic pathways. Mice treated with RvD1 showed suppressed NFkB activation, by impairing peroxisome proliferator-activated receptor γ (PPARγ) signalling pathways [456]; protecting against organ damage, leading to increased survival [13, 457]. Resolution of inflammation mediated by RvD2 is multifaceted; with roles in modulating leukocyte-endothelium interactions both directly and indirectly, similarly impacting cytokine production and promotion of bacterial clearance by phagocytosis [458]. Aspirin also triggers epimeric resolvin production similarly to 15-epi-LxA4, resulting in AT-RvD1, AT-RvD2 and others; with similar pro-resolving functions as described above.
Protectins are another class SPMs produced in a number of human cell types and in murine skin and brain tissue and exudates [450]. These SPMs are synthesised in a similar manner to RvD1 and RvE3, as a result of DHA conversion via the 15-LOX pathway to 17S-HpDHA. This is then converted once by an epoxide to form 16S, 17S-epoxy-protectin and hydrolysed to form protectin D1 (PD1) also known as neuroprotectin D1 (NPD1) when formed in the nervous system [459], with implications in Alzheimer’s disease (reviewed in [460]). The key described mechanism of action of protectins is in the reduction of PMN infiltration and enhanced efferocytosis of apoptotic neutrophils by macrophages [461]. Aspirin also able to trigger the epimeric synthesis of PD1/NPD1 (AT-PD1/NPD1), which was shown to have more potent pro-resolution effects, compared to their naturally biosynthesised counterpart [15].

Maresins are the newest family of SPMs to be identified, produced exclusively by macrophages. Synthesised via DHA conversion to 14S-HpDHA via the 12-LOX pathway and then similarly to PD1, epoxidation and hydrolysis events yield maresin 1 (MaR1) [462]. Like other described SPMs, MaR1 is a potent inhibitor of PMN infiltration and stimulator of phagocytosis and efferocytosis by both human and mouse macrophages (with treatment as low as 0.1 ng per mouse in a zymosan induced model of peritonitis). In the same study, MaR1 was shown in Planaria to stimulate tissue repair (although the mechanism is unclear) and impairs pain recognition in mice, by inhibiting expression of transient receptor potential V1 (TRPV1) currents on the surface of sensory neurons [463]. The findings from this study proposed a potent analgesic role for MaR1, on the basis of resolution of inflammation and associated pain.

In the case of critical inflammatory illnesses such as sepsis, it is unlikely that the actions of any of these SPMs are independently responsible for clearance of inflammation as production of these SPMs are intricately connected. Moreover, the proposed mechanism of combined action occurs over the course of inflammation; with a triggered “class switch” away from COX-derived pro-inflammatory mediators like prostaglandins and leukotrienes and to SPMs including lipoxins, resolvins, protectins and maresins (see figure 1.8A) [464], which in a number of studies was reported to be more potent following aspirin treatment [13, 15, 465].
1.5. Summary

Despite advances made in both medical and supportive care, we are no closer to reducing mortality rates associated with sepsis. Sepsis-related deaths occur more frequently than any other infection in clinical setting [1]. Reductions of mortality rates of sepsis rely heavily on the rapid identification of the condition and initiation of appropriate supportive therapies, which is difficult and often prolonged. Antibiotic therapy targets the pathogenic source of sepsis, with fluid resuscitation providing systemic organ support, however there is no approved adjunct therapy to target the uncontrolled inflammatory response that characterises the sepsis syndrome. Potential therapeutics identified in animal models of sepsis often fail to confer therapeutic benefit in human clinical trials; fuelling the debate over the validity of animal models as accurate comparisons of human inflammatory disease [148, 224]. A successful adjunct therapy should not only be cost-effective, the therapy should aim to target the previously described chaotic inflammatory responses, by resolving inflammation and promoting a return to homeostasis.

Of the many therapeutics investigated for potential use in sepsis, support is increasing for aspirin therapy. Aspirin is already produced on a global scale for the vast number of other medical applications, meeting the criteria for a cost-effective therapeutic. Low-doses of aspirin were shown in a number of inflammatory states (infectious and non-infectious alike) to trigger the production of anti-inflammatory lipid mediators that mediate resolution of inflammation, resulting in improved outcomes in both animal models and human clinical studies [18, 19, 219, 434, 447].

The present study will investigate the potential of low-dose aspirin therapy in critically ill patients with sepsis, as well as mouse models of staphylococcal-induced sepsis and device-related biofilm infection. It is hypothesised that low-dose aspirin therapy will produce immuno-modulatory effects in inflammation as seen in both clinical and animal models of sepsis, as well as device-related biofilm infections.
1.6. Statement of aims

The central aims of this study were to:

i. Analyse samples from the MATHS clinical study of low-dose aspirin in sepsis and to determine the impact on key surrogate measures of 15-epi-LxA₄ and NFκB and the subsequent impact on inflammatory pathways.

ii. Establish a murine model for staphylococcal-induced sepsis and perform parallel analyses to the human into low-dose aspirin therapy in wild-type C57BL/6 mice, to permit extended analysis of organs and other systemic effects.

iii. Investigate the effects of low-dose aspirin and its metabolite salicylic acid on staphylococcal biofilm formation in vitro and in an in vivo model of device-related biofilm infection.
Chapter 2

General Materials & Methods
### 2.1. General laboratory procedures

Unless otherwise stated, all solutions were prepared with reverse osmosis double distilled water (MQH₂O), obtained from the Millipore Milli-Q® water system (Millipore, USA). Chemicals used were analytical or molecular grade and stored at ambient temperature unless otherwise stated. Sterilisation of all media and glassware by autoclaving was performed at 121°C (15 lbf/in²) for 20 min.

### 2.2. Chemicals and media

#### 2.2.1. Bacterial media

Media used in bacterial culture studies were Brain Heart Infusion broth (BHIB; Oxoid, UK) and Brain Heart Infusion agar (BHIA; Oxoid). BHIB was prepared by dissolving 37 g dehydrated media in 1 L MQH₂O; BHIA was prepared by dissolving 47 g dehydrated media in 1 L MQH₂O. BHIB and BHIA were sterilised by autoclaving as described above.

#### 2.2.2. Aspirin and salicylic acid (SA) stock solutions

Stock solutions of SA (1 mol/L) were prepared by dissolving lyophilised SA (Sigma-Aldrich, USA) in 70% (v/v) ethanol (Merck KGaA, Germany). Once dissolved, stock solutions were filter sterilised (Filtopur 0.22 µm, Sarstedt, Germany) and stored at RT for up to one year.

Aspirin was prepared as follows: 0.2 mg/mL and 2 mg/mL solutions were prepared by dissolving 2 mg and 20 mg lyophilised aspirin (Sigma-Aldrich), respectively in a total of 10 mL sterilised MQH₂O containing 2% (w/v) sodium bicarbonate (NaHCO₃; BDH, Germany). Once dissolved, stock solutions were filter sterilised as described above and stored at 4°C.

#### 2.2.3. Cell culture media and reagents for histology and flow cytometry

Cells thawed at 4°C for flow cytometry analysis were resuspended in Dulbecco’s Modified Eagle Medium complete (DMEM; high glucose (4.5 g/L), GlutaMAX™ (4 mmol/L); Life Technologies, USA), supplemented with 10% (v/v) heat-inactivated foetal calf serum (FCS; Bovogen Biologicals, Australia).
Phosphate Buffered Saline (PBS; Bioline, UK) for histological and flow cytometry wash steps was prepared by dissolving one PBS tablet in 1 L MQH2O, and sterilised by autoclaving as described. FACS wash buffer (PBS supplemented with 2% (v/v) FCS) was used for wash steps in flow cytometry procedures. FACS freeze media (heat-inactivated FCS supplemented with 5-10% (v/v) dimethyl sulfoxide (DMSO; Sigma-Aldrich) was used to freeze all immune cell aliquots for flow cytometry analysis.

2.3. Bacteria

2.3.1. Bacterial strains

All bacterial strains used in Chapter 4 and Chapter 5 of this study are listed in Table 2.1.

Table 2.1: Bacterial strains used in this study.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Characteristics</th>
<th>Chapter</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus aureus</em> ATCC 25923</td>
<td>Methicillin sensitive</td>
<td>4</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> Rosenbach ATCC-BAA 1680</td>
<td>Methicillin resistant (Non-biofilm former)</td>
<td>5</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> Rosenbach ATCC-BAA 1698</td>
<td>Methicillin resistant (Biofilm former)</td>
<td>5</td>
</tr>
<tr>
<td><em>Staphylococcus epidermidis</em> ATCC 12228</td>
<td>Methicillin sensitive (Non-biofilm former)</td>
<td>5</td>
</tr>
<tr>
<td><em>Staphylococcus epidermidis</em> RP62A; ATCC 35984</td>
<td>Methicillin sensitive (Biofilm former)</td>
<td>5</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> Clinical isolate #5 (UoM)</td>
<td>Methicillin resistant (Non-biofilm former)</td>
<td>5</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> Clinical isolate #6 (UoM)</td>
<td>Methicillin resistant (Non-biofilm former)</td>
<td>5</td>
</tr>
<tr>
<td>RMIT University Clinical isolate PM#2</td>
<td>Methicillin sensitive from pacemaker (Biofilm former)</td>
<td>5</td>
</tr>
<tr>
<td>RMIT University Clinical isolate JO#2</td>
<td>Unknown staphylococcal isolate from wound (Biofilm former)</td>
<td>5</td>
</tr>
</tbody>
</table>
2.3.2. Bacterial growth conditions

2.3.2.1. General culture conditions

Strains of *S. aureus* and *S. epidermidis* frozen in glycerol stocks were plated onto BHIA plates and incubated at 37°C under aerobic conditions overnight. For preparation of broth cultures, a single bacterial colony was isolated from BHIA plates, resuspended in 5 mL of BHIB and incubated at 37°C under aerobic conditions, either shaking for 4-6 h in a shaking incubator at 180 rpm, or static conditions overnight. To determine the growth phase, the absorbance of broth cultures were measured at a wavelength of 595 nm. Colony-forming units (CFU)/mL were determined by plating 10-fold serially diluted staphylococcal broth cultures on BHIA and incubating overnight at 37°C, under aerobic conditions.

2.3.2.2. Growth in the presence or absence of SA

Stock solutions of SA (1 mol/L prepared in 70% (v/v) ethanol; described in section 2.2.2) were diluted in appropriate volumes of BHIB to achieve a final concentration of 1-20 mmol/L. Vehicle controls were prepared by diluting 70% (v/v) ethanol in appropriate volumes of BHIB to achieve equal concentrations described for SA. For biofilm growth experiments, broth cultures grown at 37°C overnight were diluted in BHIB/SA or BHIB/vehicle in a suspension of 10⁵ CFU.

2.4. Animal experiments

2.4.1. Mice

All experiments involving animals were carried out with the approval of the RMIT Animal Ethics Committee (AEC Permit Number 1213; Chapter 4, or 1426; Chapter 5).

Specific pathogen free, 6-8 week-old female C57BL/6 mice used in experiments were sourced from the Animal Resource Centre (ARC; Australia). Prior to experimentation, mice were acclimatised for a minimum of seven days within the RMIT Animal facility (RMIT University, Australia). A maximum of four mice were housed in a single housing unit; mice were fed standard rodent chow and had access to sterilised-water *ad libitum*. For the duration of experiments, infected mice were monitored for signs of weight loss, fatigue and behavioural abnormalities according to criteria as outlined in Table 2.2.
These criteria were used to determine the progression of inflammation following infection in Chapter 4 and Chapter 5 studies.

**Table 2.2: Clinical parameters measured and expected scores for mice with local and systemic inflammation, modified from [235].**

<table>
<thead>
<tr>
<th>Score</th>
<th>Variable</th>
<th>Expected score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Body Weight Changes</strong></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>&lt;10% Weight loss</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>10-15% Weight loss</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>&gt;15% Weight loss</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Physical Appearance</strong></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Lack of Grooming</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Rough Coat, Nasal/Ocular Discharge</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Very Rough Coat, Abnormal Posture, Enlarged Pupils</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Measurable Clinical Signs</strong></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Small changes of potential significance</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Temp change 1-2°C, cardiac &amp; respiratory rates increased up to 30%</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>Temp change &gt;2°C, cardiac &amp; respiratory rates increased up to 50%, or markedly reduced</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Unprovoked Behaviour</strong></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Minor Changes</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>Abnormal/reduced mobility, decreased alertness, inactive</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Unsolicited vocalisations, self-mutilations either very restless or immobile</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Behavioural Responses to External Stimuli</strong></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Minor depression/exaggeration of response</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>Moderately abnormal responses</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Violent reactions, or comatose</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Total Score</strong></td>
<td>9</td>
</tr>
</tbody>
</table>
Experimental designs of staphylococcal-induced sepsis and device-related biofilm implant models in mice are described in Chapter 4 and Chapter 5, respectively. Aspirin therapy (prepared as described in section 2.2.2) was administered by oral gavage at a dose of either 1 mg/kg (equivalent to 100 mg daily dose; Chapter 4 and Chapter 5) or 10 mg/kg (equivalent to 1000 mg daily dose; Chapter 4).

At the end of each experiment, mice were euthanised via CO\textsubscript{2} inhalation. Blood (Chapter 4 and Chapter 5) was collected for bacterial load and flow cytometry analysis of immune cell proportions; bacterial exudates (Chapter 5) and organs (spleen, kidney, liver; Chapter 4) were collected for bacterial load; spleen (Chapter 4) and skin (Chapter 5) were harvested for histology and immunohistochemistry.

**2.4.2. Determination of bacterial load in blood and organs**

After mice were euthanised by CO\textsubscript{2} inhalation, blood was collected immediately by cardiac puncture into syringes pre-loaded with 200 μL sterile Alsever’s solution (0.0055% (w/v) citric acid, 0.8% (w/v) sodium citrate, 2.05% (w/v) D-Glucose, 0.42% (w/v) sodium chloride dissolved in 100 mL MQH\textsubscript{2}O). Alsever’s solution is an isotonic balanced salt solution, routinely used (where citric acid/sodium citrate act as an anticoagulant) allowing the storage of blood at 2-8°C for up to 10 weeks [466]. Once collected, an aliquot of blood was diluted 1:2 in BHIB and a volume of 100 μL was spread plated onto BHIA plates and incubated as described. Swabs of bacterial exudates surrounding contaminated implants (Chapter 5) were collected into 1 mL BHIB, mixed by vortexing vigorously for 5 s. Exudates were serially diluted, plated onto BHIA plates and incubated as described above. Viable bacterial counts for blood, organs and exudates were performed following incubation at 37°C for 24 h, and colony counts for organs were expressed as CFU/g of tissue.
2.4.3. Determination of bacterial-induced damage in organs

Tissues harvested following euthanasia of mice were embedded in Tissue-Tek® O.C.T™ medium (Sakura Finetek, Japan), snap frozen over liquid nitrogen and stored at -80°C. Sections were cut at 10 µm thickness on the Leica CM1950 cryostat (Leica, Germany) and mounted onto StarFrost® superclean slides (ProSciTech Pty Ltd, Australia) and stored at -80°C.

2.4.3.1. Haematoxylin and eosin staining

Frozen 10 µm sections of skin, or spleen were fixed in 4% neutral buffered formalin (4% (v/v) formaldehyde solution containing 4 g/L NaH₂PO₄ and 6.5 g/L Na₂HPO₄; Thermo Fisher Scientific, USA). Sections were washed in PBS once, before being stained with haematoxylin and eosin. Histological sections were observed under light microscopy to determine the extent of damage and inflammation to tissues. Images were captured using a Leica DM2500 microscope and Leica Application Suite software (Version 4.3.0; Leica), and scoring was performed according to parameters described in each of Chapter 4 and Chapter 5, respectively.

2.4.3.2. Immunohistochemical detection of *S. aureus* and CD11b in organs

Frozen 10 µm sections of spleen (Chapter 4) or skin (Chapter 5) were fixed in ice-cold acetone (Merck KGaA). Sections were air dried and rehydrated in PBS for 10 min, then permeabilised with PBS containing 0.2% Triton X-100 (Sigma-Aldrich). Fc Receptor blocking was performed by incubation with PBS/1% bovine serum albumin (BSA; Sigma-Aldrich) and serum from the host species of the secondary antibody (10% (v/v) normal goat serum (NGS) and purified anti-mouse CD16/32 antibody (1:100; BioLegend, USA) for 1 h at RT. Slides were inverted to remove excess blocking solution and primary antibodies; rabbit anti-*S. aureus* (1:200; Abcam, UK), and rat anti-mouse CD11b (1:100; BioLegend) diluted in PBS/1% BSA, were added and sections were incubated overnight at 4°C. Sections were washed three times for 10 min in PBS containing 0.05% Tween-20 (PBST) and an anti-rabbit Cy3® (Abcam) and anti-rat Alexa Fluor® 488 (Life Technologies) conjugated secondary antibodies were added at 1:1000 and 1:800, respectively in PBS/1% BSA. Slides were incubated for 1 h at RT. Sections were washed three times for 10 min in PBST then slides were incubated with DAPI nuclear stain (2 µg/mL; Sigma-Aldrich) in PBS/1% BSA for 20 min at RT (in the dark). Slides were
washed frequently in PBST over 30 min, mounted in Mowiol (10\% (w/v) Mowiol 4-88 (Calbiochem, USA), 25\% (v/v) Glycerol (Chem-Supply, Australia), 0.1 mol/L Tris, 2.5\% (w/v) (Merck KGaA), 1,4-diazobicyclo-[2.2.2]-octane (DABCO; Sigma-Aldrich), pH 6.8). Images were captured using a Leica DM2500 microscope and Leica Application Suite software (Version 4.3.0; Leica). Images were overlayed for display using the Adobe Photoshop program (Version CC2015.16.0; Adobe, USA).

2.5. Immunological analysis

2.5.1. Blood processing

Human blood samples were centrifuged in the Heraeus Multifuge 1S-R centrifuge (Thermo Fisher Scientific); mouse blood samples were centrifuged in the Heraeus Fresco 17 refrigerated micro-centrifuge (Thermo Fisher Scientific). All centrifugation steps were performed at 500 × g for 5 min at 4°C. Plasma fractions were dispensed into either cryovials (Sarstedt) or 1.7 mL micro-centrifuge tubes (Interpath Services, Australia) and stored at -80°C. Following this, erythrocytes were lysed in tris-ammonium chloride (TAC) red cell lysis buffer (155 mmol/L ammonium chloride (Sigma-Aldrich), 0.1 mmol/L EDTA (Calbiochem) and 10 mmol/L potassium bicarbonate (BDH), pH 7.2) at 37°C for 5 min. Cells were centrifuged, and lysed erythrocytes were discarded. Cells were washed in FACS wash buffer (section 2.2.3) twice. Cells (including peripheral blood mononuclear cells; PBMC) resuspended in FACS wash buffer were dispensed in 500 μL into microcentrifuge tubes (Interpath Services) and centrifuged at maximum speed (16,000 × g) for 5 min at 4°C. Excess FACS wash was removed and PBMC pellets were stored at -80°C. Remaining cells were centrifuged, excess FACS wash buffer was discarded and aliquots (500 μL) were made in FACS freeze media (section 2.2.3) and slow frozen in the Nalgene® Mr Frosty freezing container (Sigma-Aldrich) at -80°C.

2.5.2. Spleen processing

Spleens (Chapter 4 and Chapter 5) were collected from mice into ice-cold DMEM complete media (section 2.2.3). Cells were placed in petri dishes and were gently teased through a 40 μm cell strainer (Biologix, China). Cells were centrifuged at 500 × g for 5 min at 4°C, resuspended in TAC lysis buffer (section 2.5.1) and incubated at 37°C for 5 min to lyse contaminating erythrocytes. Cells were washed in FACS wash buffer
(section 2.2.3), centrifuged and resuspended in 500 µL FACS freeze media (section 2.2.3), and slow frozen in the Nalgene® Mr Frosty freezing container at -80°C.

### 2.5.3. Flow cytometry

For flow cytometry, 10⁶ cells from blood (all studies) or spleen (Chapter 4 and Chapter 5) were thawed at 4°C and resuspended in DMEM complete media (section 2.2.3), centrifuged and resuspended in FACS wash buffer (section 2.2.3). Cells were incubated with blocking solution as follows: human studies (Chapter 3); cells were incubated with Human TruStain FeX™ (BioLegend), according to manufacturer’s specifications (1:20 diluted in FACS wash buffer containing 5% NGS), mouse studies (Chapter 4 and Chapter 5); Fc receptor block (Purified anti-mouse CD16/32 Antibody; BioLegend), at 4 μg/mL in 50 µL FACS wash buffer for at least 30 min on ice.

Antibodies for staining of human blood are listed in Table 3.2 (Chapter 3). Antibodies for staining of mouse blood and spleen are listed below in Table 2.3. All staining was performed in 100 µL FACS wash buffer for at least 30 min on ice. Cells were washed twice in FACS wash buffer and if required, incubated with a fluorescently conjugated secondary antibody diluted in FACS wash buffer. Cells were washed twice in FACS wash buffer, before being resuspended in PBS for data acquisition.

#### Table 2.3: Antibodies used in mouse flow cytometry analysis.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone (host)</th>
<th>Conjugate</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-CD3ε</td>
<td>145-2C11 (rat)</td>
<td>Brilliant Violet 421</td>
<td>1:800</td>
<td>BioLegend</td>
</tr>
<tr>
<td>α-CD3ε</td>
<td>145-2C11 (rat)</td>
<td>APC/Cy7</td>
<td>1:800</td>
<td>BioLegend</td>
</tr>
<tr>
<td>α-CD4</td>
<td>RM4-5 (rat)</td>
<td>eFluor 450</td>
<td>1:1600</td>
<td>eBioscience</td>
</tr>
<tr>
<td>α-CD8a</td>
<td>53-6.7 (rat)</td>
<td>PE/Cy7</td>
<td>1:800</td>
<td>BioLegend</td>
</tr>
<tr>
<td>α-CD11b</td>
<td>M1/70 (rat)</td>
<td>APC/Cy7</td>
<td>1:800</td>
<td>BioLegend</td>
</tr>
<tr>
<td>α-CD62L</td>
<td>MEL-14 (rat)</td>
<td>PE/Cy7</td>
<td>1:800</td>
<td>BioLegend</td>
</tr>
<tr>
<td>α-CD162</td>
<td>2PH1 (rat)</td>
<td>PE</td>
<td>1:800</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>α-Ly6G</td>
<td>1A8 (rat)</td>
<td>PerCP/Cy5.5</td>
<td>1:800</td>
<td>BioLegend</td>
</tr>
</tbody>
</table>
Compensation was performed using UltraComp eBeads (eBioscience, USA) based on the single staining of each individual fluorochrome and corrected for auto-fluorescence with unstained beads and cells.

Data was collected using the FACSCanto II flow cytometer (Beckton Dickinson, USA). A five-decade logarithmic scale was used to process fluorescence signals and a linear scale was used to process light scattering signals. Data analysis was performed using FlowJo software (Version 9.7.1: FlowJo, LCC, USA). Single cells were gated and then populations of cells were defined as being T lymphocytes (CD3+, CD4+, CD8+), monocytes/macrophages (Cd11b+) and neutrophils (Ly6G+ (mice)/CD66b+(human)) as well as other cell surface markers.

2.5.4. Detection of 15-epi-LxA₄ in plasma

2.5.4.1. Enrichment of plasma proteins and lipids

Plasma was enriched for proteins and lipid mediators including 15-epi-LxA₄, using the Bond Elute C₁₈ OH solid phase extraction column (Agilent technologies, USA) prior to quantification using an immunoassay. Frozen aliquots of plasma were thawed and acidified to pH 3.5 with 1 mol/L hydrochloric acid (HCl; Merck KGaA). Columns were pre-conditioned with 2 mL methanol (Merck KGaA) followed by 2 mL of MQH₂O. Samples were diluted in sterile MQH₂O and added to the column (specific dilutions for human and mouse plasma are described in section 3.2.2.3.1 and 4.2.4.1). The flow-through was collected from columns after centrifugation at 500 × g for 5 min at 4°C. The eluate was discarded, and columns were washed in 1 mL MQH₂O, followed by 1 mL petroleum ether (Sigma-Aldrich). Finally, purified plasma proteins and lipid mediators were eluted with 2 mL methyl formate (Sigma-Aldrich), and the solvent was evaporated under a stream of nitrogen gas. Dried pellets were resolubilised in an appropriate volume of 1× 15-epi-LxA₄ extraction buffer, supplied in the 15-epi-LxA₄ Enzyme Immunoassay (EIA) kit (Oxford Biomedical Research Inc, USA), prior to quantification.
2.5.4.2. Quantifying 15-epi-LxA4 in plasma via enzyme linked immunosorbent assay (ELISA)

Purified proteins and lipid mediators enriched from plasma were further diluted (1:10, human analysis or 1:5, mouse analysis) in 1× 15-epi-LxA4-Extraction Buffer (prepared in MQH2O) supplied in the 15-epi-LxA4 EIA kit (Oxford Biomedical Research). Quantification of plasma 15-epi-LxA4 was performed as follows: standards used for quantification were prepared in EIA Buffer with final concentrations ranging from 0 to 2 ng/mL. Prepared standards (50 µL) or pre-diluted plasma (50 µL) were added in duplicate to wells of a 96-well Costar® microplate (supplied in the 15-epi-LxA4 EIA kit), pre-coated with anti-15-epi-LxA4 rabbit antibody. Next, 50 µL of diluted 15-epi-LxA4-horseradish peroxidase (HRP) conjugated antibody (1:50 dilution in EIA buffer) was added to each well, plates were mixed by shaking gently on a plate shaker for 10 s, covered and incubated at RT for 1 h, under static conditions. Following incubation, plates were washed three times with 300 µL 1× 15-epi-LxA4-Wash Buffer (prepared in MQH2O) and 150 µL 3,3', 5,5' tetramethylbenzidine (TMB) with hydrogen peroxide (H2O2) substrate solution was added to each well, plates were mixed on a plate shaker and incubated at RT for 30 min. Absorbance was measured at a wavelength of 650 nm using the POLARstar Optima multi-detection microplate reader (BMGlabtech, Germany). Concentrations of 15-epi-LxA4 were determined using standard curves generated from 0 to 2 ng/mL, and adjusted for dilution factor and starting volume of plasma.

2.5.5. Detection of NFκB p65 activation

2.5.5.1. Preparation of nuclear and cytoplasmic extracts

Nuclear and cytoplasmic extracts were prepared from PBMC pellets with the use of the NE-PER nuclear and cytoplasmic extraction kit (Pierce, USA), according to the manufacturer’s specifications. PBMCs were thawed and washed once in FACS wash buffer. Cell pellets were centrifuged at the maximum speed (16,000 × g) for 5 min at 4°C and excess FACS wash buffer was removed. Cells were washed once in PBS and were centrifuged at 500 × g for 5 min at 4°C. Cells were resuspended in ice-cold CER I buffer; supplemented with 1× cOmplete ULTRA protease inhibitor cocktail (Roche AG, Switzerland), vortexed vigorously for 15 s and incubated on ice for 10 min. Ice-cold CER II buffer was added and cells were vortexed vigorously for 5 s and incubated on ice.
for 1 min. Cells were again vortexed vigorously for 5 s and centrifuged, then supernatants (cytoplasmic extracts) were then transferred to a clean pre-chilled microcentrifuge tube and stored at -80°C. Remaining pellets (containing nuclei) were resuspended in ice-cold NER buffer (supplemented with 1× cOmplete ULTRA protease inhibitor cocktail; Roche AG), vortexed vigorously for 15 s and incubated on ice for 10 min. This process was repeated every 10 min for a total of 40 min. Cells were centrifuged and supernatants (nuclear extracts) were transferred to a clean pre-chilled microcentrifuge tube and stored at -80°C.

2.5.5.2. Chemiluminescent detection of NFκB p65 activation

The activation of the NFκB p65 in nuclear extracts (section 2.5.5.1) was detected using the NFκB p65 Transcription factor assay (Pierce) according to the manufacturer’s specifications. Working Binding Buffer (1×) was prepared in MQH2O containing 50 µg/mL Poly dI•dC, and 50 µL was added to each well of the 96-well NFκB Assay Plate provided. Nuclear extracts were added (10 µL) in duplicate to wells and plate sealer was used to cover the plate. Plates were incubated gently shaking on a plate shaker at RT for 1 h. Plates were washed three times with 200 µL, 1× NFκB Assay Wash Buffer (prepared in MQH2O) and 100 µL NFκB p65 primary antibody (1:1000; prepared in supplied Antibody Dilution Buffer) was added to each well. Plates were then incubated at RT for 1 h, under static conditions. Following incubation, plates were washed three times with 200 µL, 1× Wash Buffer and 100 µL HRP-conjugated secondary antibody (1:10,000 in Antibody Dilution Buffer) was added. Plates were then incubated at RT for 1 h, under static conditions. Following incubation, plates were washed four times with 200 µL, 1× Wash Buffer and 100 µL substrate working solution (prepared by combining equal volumes of supplied Luminol/Enhancer and Stable Peroxide solutions) was added to each well. Chemiluminescence expressed as relative fluorescence units (RFU) was detected using the luminometer function on the POLARstar Optima multi-detection microplate reader (BMGlabtech).
2.5.6. Determination of inflammatory cytokine profile by LEGENDplex™ multi-analyte flow assay kit

Measurements of pro-inflammatory cytokines in plasma samples were taken using the LEGENDplex™ Human Inflammation panel (13-plex) (Chapter 3) and Mouse Inflammation panel (13-plex) (Chapter 4) flow assays (BioLegend). All procedures were performed according to manufacturer’s specifications. Cytokine standards and samples were prepared in LEGENDplex™ Assay Buffer and were added to a 96-well U-bottom plate (Nunc A/S, Denmark). Sonicated pre-mixed beads and detection antibodies were added to each well and then plates were incubated (in the dark) on a plate shaker at 600 rpm for 2 h at RT. Following incubation, streptavidin–phycoerythrin (PE) solution was added to each well and incubated for 30 min at RT. Samples were analysed and data collected on the FACSCanto II (Beckton Dickinson). Data were analysed using the LEGENDplex™ Data Analysis Software (BioLegend) with standard curves generated from 0 to 50,000 pg/mL, and samples adjusted for dilution factor.

2.6. Statistical analysis

All statistical analyses were performed using GraphPad Prism version 6.0 for Macintosh (GraphPad Software Inc, USA). The D’Agostino and Pearson omnibus test was applied to data to determine normality.

Where the test result revealed that data were not normally distributed or the value of n was too small to be able to determine normality, non-parametric statistical analyses were applied. A two-tailed Mann-Whitney Test was used to analyse the differences observed between two means at a single time point, where p<0.05 was considered significant. A Kruskal-Wallis test was used to analyse the differences observed between multiple groups, where p<0.05 was considered significant.

Where the test result revealed data were normally distributed, parametric statistical analyses were applied. A one-way analysis of variance (ANOVA) test was used to analyse the differences observed between multiple groups, where p<0.05 was considered significant.
Chapter 3

Investigating the effects of low-dose aspirin as a potential adjunct therapy for patients with sepsis
3.1. Introduction

Sepsis is a worldwide healthcare concern. Despite continued advances in both medical and supportive intervention, sepsis remains the leading cause of infection-related death, with associated mortality rates of 20-40% [29, 30]. A major reason for the continued high morbidity and mortality rates of sepsis are due to delayed identification, as early symptoms of this syndrome are indistinguishable from other inflammatory conditions. Meta-analyses of sepsis epidemiology have revealed that men develop sepsis more frequently than women [1, 4, 29, 30], and incidences of sepsis are often higher in minority groups [31-33]. Further, elderly patients and individuals with pre-existing comorbidities such as cardiovascular disease, diabetes, renal disease and cancer have increased probability of developing sepsis, presumably as they are more susceptible to infection in general [26, 29]. The individual response to sepsis is dependent upon the causative organism, as well as numerous host factors. It is noteworthy that since 1987, there has been an overwhelming increase in the percentage of gram-positive, and to a lesser extent fungi-associated sepsis [4, 29].

Sepsis develops when the body’s response to infection is not adequately controlled. This dysregulated response triggers a cascade of inflammatory responses; mediated by the innate immune system [36]. Recognition of pathogens by macrophages results in the activation of the inflammatory regulator nuclear factor kappa beta (NFκB). This leads to increased production of pro-inflammatory cytokines, as well as cyclooxygenase (COX) induced production of pro-inflammatory lipid mediators [436, 467]. Inflammatory signalling also delays the apoptosis of neutrophils, permitting migration to tissues, mediated by cell surface markers such as P-selectin glycoprotein ligand-1 (PSGL-1). This in turn contributes to exacerbated inflammation in tissues, potentially leading to organ failure and death [24]. Aside from the hyper-inflammatory response in sepsis, some patients enter a state of immunosuppression; triggered by the induced apoptosis of key adaptive immune response cells (B and T lymphocytes) [107].

The implementation of treatment “bundles” as directed by the guidelines from the Surviving Sepsis Campaign (SSC) have continued to be the standard of care in sepsis [2, 149]. Upon identification of sepsis, patients are provided multimodal therapy of supportive fluid resuscitation (intravenous fluids and vasopressors, with oxygen therapy
and mechanical ventilation) and empiric antibiotics [468]. Early goal directed therapy (EGDT) protocols were introduced in 2001, and therapeutic benefits were initially seen in sepsis [149], however recent debate has arisen over whether the improvements in mortality rate were specifically due to EGDT [11]. Further, none of the current therapies offered to patients target the inflammatory responses that contribute to sepsis severity. Although numerous animal studies and randomised control trials have been initiated into potential adjunct therapeutics (reviewed in [148]), none have shown to be successful in reducing sepsis mortality rates.

Evidence is accumulating that low-dose aspirin could be beneficial as an adjunct therapy for sepsis. A number of animal studies have shown that aspirin therapy in sepsis-related inflammatory conditions was associated with improved outcomes [16-18]. Further, two independent observational studies of patients in ICU with systemic inflammatory response syndrome (SIRS) and/or sepsis showed that patients already taking aspirin had reduced likelihood of mortality [61, 64, 220]. Taken together these data support the idea that low-dose aspirin therapy might be beneficial in patients with severe sepsis.

Aspirin is a cost-effective alternative to other pharmacological adjunct therapies, and at low doses has been shown to mediate resolution of inflammation, and to be associated with improved outcomes [469]. Unlike other non-steroidal anti-inflammatory drugs (NSAIDs), aspirin acetylates COX-2, triggering the production of specialized pro-resolving lipid mediators (SPM) such as 15-epi-LxA4; a synthetic analogue of the naturally occurring lipoxin A4 (LxA4). Both LxA4 and 15-epi-LxA4 have pro-resolution effects in local and systemic inflammation [20, 470]. Previous studies in healthy human volunteers have shown the use of low-dose aspirin (75 mg daily dose, for 10 days) resulted in a reduced local inflammatory response, presumably via increased production of 15-epi-LxA4 [19]. It has also been shown in vitro that aspirin in a dose equivalent to 1000 mg aspirin inhibits NFκB activation. The lower dose range required to inhibit NFκB activation has not yet been determined [18, 433, 471].

At present, three randomized clinical studies have been initiated to investigate the immunological impact of low-dose aspirin therapy in clinical sepsis (as described in section 1.4.2), including the Mechanism of Aspirin THERapy in Sepsis (MATHS) clinical trial. The results in this chapter are derived from preliminary analysis of the MATHS
trial, a randomised single centre trial initiated at the Victorian Infectious Disease Service (VIDS), Melbourne, investigating the effects of low-dose aspirin therapy at 100 mg and 300 mg doses in patients with sepsis.

Based on evidence from the literature and the reported mechanisms of aspirin therapy in inflammatory conditions described above, the following hypothesis was developed:

That low doses of aspirin will induce immuno-modulatory effects including production of 15-epi-LxA₄ and inhibition NFκB activation in patients with sepsis, consistent with a pro-resolving mechanism of action.

The MATHS clinical trial was designed to investigate the above hypothesis. This chapter describes the planned interim analysis of the first 29 patients.

In order to address the hypothesis, the specific aims of analyses in this chapter were:

- To collect and analyse blood from patients in a clinical trial to determine the effects of high (300 mg) and low (100 mg) doses of aspirin on the immune response in patients with sepsis

More specifically to:

- Quantitate levels of 15-epi-LxA₄ produced in plasma following aspirin therapy
- Determine whether aspirin therapy inhibits the activation of NFκB in peripheral blood mononuclear cells (PBMC)
- Determine the impact on cytokine levels in plasma as a measure of inflammatory activation
- Characterise changes in circulating immune cell populations to determine any effect of aspirin-therapy
3.2. Materials & Methods

3.2.1. MATHS clinical trial: patient recruitment and exclusion criteria

Patients in the Intensive Care Unit (ICU) and VIDS (Royal Melbourne Hospital, Melbourne Health, Australia) presenting with signs and symptoms of the SIRS and/or sepsis were asked for their voluntary participation. In the event of the patients being unable to respond, relatives gave informed consent. This study was performed in accordance with Human Research Ethics (application number ACTRN12611000649910. Protocol v1). Successful enrolment was based on the following inclusion and exclusion criteria:

**Inclusion criteria:** Critically ill adult patients over 18 years of age with sepsis (infected site plus SIRS) according to the criteria defined by Bone et al. (Table 1.1) [22].

**Exclusion criteria:** Patients with a contraindication to aspirin due to hypersensitivity to aspirin or NSAID drugs, platelet count <100,000×10⁹/l, coagulopathy with international normalised ratio (INR) >2 or active bleeding (e.g., trauma, gastrointestinal or intracranial bleeding) were excluded. Patients with pre-existing renal injury as per RIFLE guidelines [472] were also excluded. Patients had study medications ceased if there was evidence of hypersensitivity to aspirin (new onset of severe bronchospasm or urticarial rash), renal injury (serum creatinine doubling /GFR reducing by > 50%) or new onset of bleeding.

3.2.2. Protocol design for the MATHS clinical trial

All patients continued to receive optimal, multimodal therapy for their illness. The MATHS trial is an open label, randomised controlled trial. Upon enrolment into the MATHS trial, patients were randomised to either 100 mg, 300 mg daily aspirin doses or placebo (see Table 3.1). Patients regularly taking aspirin continued their regular dose of aspirin for the duration of sampling. These doses were selected as they have been shown to induce production of 15-epi-LxA₄, and a 300 mg dose was proposed to inhibit NFκB. Further, these doses are associated with an acceptably low risk of bleeding, based on a previous retrospective study that showed odds ratio (OR) of mortality of patients with high risk of bleeding was not affected in patients with low APACHE II scores (OR=1).
and significantly reduced in patients with APACHE II scores $\geq 15$ (OR<0.5) at admission who were regularly taking low-dose aspirin for prevention of cardiovascular events [63].

Two doses of aspirin were administered orally, or by nasogastric tube in ICU at time 0 (T0) and Day 1 (T24). Arterial blood sampling was performed immediately prior to first dose of aspirin/placebo (T0), and after 1 h (T1), 8 h (T8), 24 h (T24) and 48 h (T48). The above time-point intervals were determined on the basis of expected response times for 15-epi-LxA$_4$ production and NF$\kappa$B activation (within the first 8 h following aspirin) and analysis of immune cell populations over a range of time including before and after aspirin administration.

Patient demographics, background medical history (including history of cardiovascular disease, diabetes, renal disease, and/or cancer) were recorded, as well as Acute Physiology and Chronic Health Evaluation (APACHE) II score at enrolment (T0) and daily Sequential Organ Failure Assessment (SOFA) score (during sampling time). Once determined, site and source of sepsis were also recorded. Routine haematology values, including mean platelet volume (MPV) data were also recorded once daily, using the Coulter$^\circledR$ LH 700 haematology analyser (Beckman Coulter, USA) for the duration of sampling.

Table 3.1: Design of the Mechanisms of Aspirin Therapy in Sepsis (MATHS) clinical trial. Patients presenting with signs of SIRS/sepsis were asked for their voluntary participation. Once enrolled, patients were randomised to one of four trial groups, and a total of 180 participants are required to be enrolled for the completion of the trial.

<table>
<thead>
<tr>
<th>Group</th>
<th>Aspirin Dose</th>
<th>Exclusions</th>
<th>Number of subjects (planned)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>100 mg T0/100 mg T24</td>
<td>No aspirin/NSAID in the last 7 days</td>
<td>45</td>
</tr>
<tr>
<td>II</td>
<td>300 mg T0/300 mg T24</td>
<td>No aspirin/NSAID in the last 7 days</td>
<td>45</td>
</tr>
<tr>
<td>III</td>
<td>Previous dose T0/T24</td>
<td>-</td>
<td>45</td>
</tr>
<tr>
<td>IV</td>
<td>Untreated Controls</td>
<td>No aspirin/NSAID in the last 7 days</td>
<td>45</td>
</tr>
</tbody>
</table>
This study was powered to detect differences in baseline versus Day 2 15-epi-LxA4 concentrations in placebo, versus aspirin-treated groups. Baseline 15-epi-LxA4 concentrations and population standard deviations used to determine sample size were derived from published data from a randomised clinical trial of low-dose aspirin in healthy human subjects [20]. Baseline 15-epi-LxA4 was measured at 2.45 ng/mL (± 0.96), with no significant change over time in healthy subjects not taking aspirin [20]. From this it was assumed (regardless of changes occurring during the course of sepsis), that the combined aspirin groups would have 15-epi-LxA4 concentrations at least 0.6 ng/mL higher than untreated control group. With this in mind, it was calculated that a sample size of 45 participants per group (180 participants in total) was required to have 80% power to reject the null hypothesis (that change in 15-epi-LxA4 concentration of aspirin patients = change in 15-epi-LxA4 concentration of untreated controls, p<0.05), with a value of Sigma ± 0.96. This study was not powered to determine changes in clinical outcome. Clinical outcomes of patients will not be made available until the end of the trial.

3.2.3. Collection and processing of patient blood

3.2.3.1. Collection of blood

Blood specimens were collected into pre-treated ethylenediaminetetraacetic acid (EDTA) vacutainers (Beckton Dickinson) at time-points described in section 3.2.2, and stored at 4°C. Blood was processed within 4 h of the specimen being taken.

3.2.3.2. Processing

Human blood samples were centrifuged in the Heraeus Multifuge 1S-R centrifuge (Thermo Fisher Scientific) as described (section 2.5.1). Plasma fractions were dispensed into either cryovials (Sarstedt) or 1.7 mL micro-centrifuge tubes (Interpath Services) and stored at -80°C. Erythrocytes were lysed in TAC red cell lysis buffer (section 2.5.1) at 37°C for 5 min. Following centrifugation lysed erythrocytes were discarded. Cells were washed in FACS wash buffer (section 2.2.3) twice, then cells were resuspended in FACS wash buffer and 500 µL was dispensed into microcentrifuge tubes (Interpath Services), and centrifuged at maximum speed (16,000 × g) for 5 min at 4°C. Excess FACS wash was removed and pellets were stored at -80°C. Remaining cells were centrifuged, excess FACS wash buffer was discarded and aliquots (500 µL) were made in FACS freeze media.
(section 2.2.3) and slow frozen in the Nalgene® Mr Frosty freezing container (Sigma-Aldrich) at -80°C, prior to batch flow cytometry analysis.

3.2.4. Detection of 15-epi-LxA₄ in plasma of sepsis patients

3.2.4.1. Enrichment of plasma proteins and lipids

Plasma was enriched for proteins and lipid mediators including 15-epi-LxA₄, using the Bond Elute C₁₈ OH solid phase extraction column (Agilent technologies) prior to quantification using an immunoassay (described in section 2.5.4.1). Columns were pre-conditioned by washing with methanol and MQH₂O. Plasma samples were acidified to pH 3.5 with 1 mol/L HCl, then diluted 1:5 in MQH₂O and added to columns. Columns were centrifuged, and the eluate was discarded. Columns were washed with MQH₂O and petroleum ether, and plasma proteins and lipids were eluted with methyl formate. Solvents were evaporated under a stream of nitrogen gas, and dried pellets were resolubilised in 1 mL in 1× 15-epi-LxA₄-Extraction Buffer (supplied in the 15-epi-LxA₄ Enzyme Immunoassay (EIA) kit; Oxford Biomedical Research, Inc), prior to quantification.

3.2.4.2. Quantifying 15-epi-LxA₄ in plasma via enzyme linked immunosorbent assay (ELISA)

Quantification of plasma 15-epi-LxA₄ was performed using the 15-epi-LxA₄ EIA kit (Oxford Biomedical Research, Inc) according to the manufacturer’s specifications. Purified proteins and lipid mediators enriched from plasma were further diluted 1:10 in 1× 15-epi-LxA₄-Extraction Buffer, and 50 µL was added in duplicate to wells of a 96-well Costar® microplate (supplied in the kit), pre-coated with anti-15-epi-LxA₄ rabbit antibody. Next, 50 µL of diluted 15-epi-LxA₄-HRP conjugated antibody was added to each well, plates were mixed by shaking gently on a plate shaker, covered and incubated at RT for 1 h. Following incubation, plates were washed three times with 300 µL, 1× Wash Buffer and TMB substrate solution was added to each well, plates were mixed on a plate shaker (as described above) and incubated at RT for 30 min. Absorbance was measured at 650 nm using the POLARstar Optima multi-detection microplate reader (BMGLabtech). Concentrations of 15-epi-LxA₄ were determined using standard curves generated from 0 to 2 ng/mL, and adjusted for dilution factor and starting plasma volume.
3.2.5. Detection of NFκB p65 activation

3.2.5.1. Preparation of nuclear and cytoplasmic extracts

PBMC pellets were thawed, washed once in FACS wash buffer (section 2.2.3) and once in PBS. Nuclear and cytoplasmic extracts were prepared with the use of the NE-PER nuclear and cytoplasmic extraction reagents (Pierce) according to the manufacturers specifications. Cells were resuspended in ice-cold CER I buffer (supplemented with 1× cOmplete ULTRA protease inhibitor cocktail; Roche AG) and ice-cold CER II buffer, vortexed vigorously and incubated on ice. Cells were vortexed vigorously for 5 s and centrifuged as described and supernatants (cytoplasmic extracts) were transferred to a pre-chilled microcentrifuge tube and stored at -80°C. Remaining pellets (containing nuclei) were resuspended in ice-cold NER buffer (supplemented with 1× cOmplete ULTRA protease inhibitor cocktail; Roche AG), vortexed vigorously for and incubated on ice. This process was repeated every 10 min for a total of 40 min. Cells were centrifuged and supernatants (nuclear extracts) were transferred to a pre-chilled microcentrifuge tube and stored at -80°C.

3.2.5.2. Chemiluminescent detection of NFκB p65 activation

The activation of NFkB p65 in nuclear extracts (prepared as described) was detected using the NFkB p65 Transcription factor assay (Pierce) according to the manufacturers specifications. Working Binding Buffer was added to each well of the 96-well NFkB Assay Plate provided, prior to addition of nuclear extracts (added in duplicate). Plates were incubated at RT for 1 h (on a plate shaker), then washed three times with 1× NFkB Assay Wash Buffer. Plates were incubated with NFkB p65 primary antibody (1:1000; prepared in supplied Antibody Dilution Buffer) at RT for 1 h, under static conditions. Plates were washed three times as described then incubated with HRP-conjugated secondary antibody (1:10,000; prepared in Antibody Dilution Buffer) at RT for 1 h, under static conditions. Plates were washed four times as described then substrate working solution (prepared as described in section 2.5.5.2) was added. Chemiluminescence expressed as relative fluorescence units (RFU) was detected using the luminometer function on the POLARstar Optima multi-detection microplate reader (BMGlabtech).
3.2.6. **Determination of inflammatory cytokine profile by LEGENDplex™ multi-analyte flow assay kit**

Measurements of pro-inflammatory cytokines in plasma samples were made using the LEGENDplex™ Human Inflammation panel (13-plex) flow assay (BioLegend). All procedures were performed according to manufacturers instructions. Cytokine standards and samples were prepared in LEGENDplex™ Assay Buffer and were added to a 96-well U-bottom plate (Nunc A/S). Sonicated pre-mixed beads and detection antibodies were added to each well and then plates were incubated (in the dark) on a plate shaker at 2 h at RT. Following incubation, streptavidin–PE solution was added to each well and further incubated for 30 min at RT. Samples were analysed and data collected using the FACSCanto II (Beckton Dickinson). Data were analysed using the BioLegend LEGENDplex™ Data Analysis Software with standard curves generated from 0 to 50,000 pg/mL, and adjusted for sample dilution factor.

3.2.7. **Analysis of cell populations**

3.2.7.1. **Preparation of single cells from blood**

Leukocytes from patients were thawed at 4°C, then 500 µL DMEM complete media (prepared as described in section 2.2.3) containing 0.5 mmol/L EDTA was added drop wise. Specimens were then centrifuged as previously described and excess media was removed. The specimens were then washed twice with FACS wash buffer (section 2.2.3).

3.2.7.2. **Flow cytometry**

Approximately $10^6$ cells were stained in one of the two cocktails that follow, to characterize leukocyte and T lymphocyte populations:

**All Analyses:** Cells were incubated with Human TruStain FcX™ (BioLegend), according to manufacturer’s specifications (1:20) diluted in FACS wash buffer containing 5% Normal Goat Serum (NGS) for 30 min on ice. The antibodies used for staining are listed in Table 3.1. All antibodies used in this study were sourced from BioLegend. Staining was performed in 100 µL FACS wash buffer for 60 min on ice.
**Cocktail 1:** Following staining, cells were washed twice in FACS wash buffer and incubated with a fluorescently conjugated secondary antibody diluted in FACS wash buffer at 4°C overnight. The following day cells were washed twice in FACS wash buffer before being re-suspended in PBS for flow cytometry.

**Cocktail 2:** Following staining, cells were washed twice in FACS wash buffer and stored in FACS wash buffer at 4°C overnight. The following day cells were washed twice in FACS wash buffer before being re-suspended in PBS for flow cytometry.

**Batch 2:** The following day cells were washed in 1X Annexin Binding buffer and then stained with the Pacific Blue™ Annexin V Apoptosis Detection Kit with 7-AAD (BioLegend) at RT for 15 min according to manufacturers specifications. Cells were washed once in Annexin binding buffer before being re-suspended in PBS for data acquisition.

Compensation was performed using UltraComp eBeads (eBioscience) based on the single staining of each individual fluorochrome and correction for auto-fluorescence with unstained beads and cells. Additional control cells labelled with secondary antibody only were also tested.

Data was collected using the FACSCanto II flow cytometer (Beckton Dickinson). A five-decade logarithmic scale was used to process fluorescence signals and a linear scale was used to process light scattering signals. Data analysis was performed using FlowJo software (Version 9.7.1). Single cells were gated and then populations of cells were defined as being T lymphocytes (CD3⁺, CD4⁺, CD8⁺), monocytes/macrophages (CD11b⁺) and neutrophils (CD66b⁺) as well as other cell surface markers.
Table 3.2: Antibodies used in human flow cytometry analysis. Markers were selected based on evidence of an important role in sepsis, or in regulation/resolution of inflammation.

a) Primary Antibodies

<table>
<thead>
<tr>
<th>Marker</th>
<th>Clone</th>
<th>Conjugate</th>
<th>Dilution</th>
<th>Cocktail</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>HIT3a (mouse)</td>
<td>APC Cy7</td>
<td>1:20</td>
<td>1, 2</td>
</tr>
<tr>
<td>CD11b</td>
<td>ICR44 (mouse)</td>
<td>PE Cy7</td>
<td>1:20</td>
<td>1</td>
</tr>
<tr>
<td>CD66b</td>
<td>G10F5 (mouse)</td>
<td>PerCP Cy5.5</td>
<td>1:20</td>
<td>1</td>
</tr>
<tr>
<td>CD86</td>
<td>IT2.2 (mouse)</td>
<td>Alexa Fluor 647</td>
<td>1:20</td>
<td>1</td>
</tr>
<tr>
<td>CD162</td>
<td>KPL-1 (mouse)</td>
<td>-</td>
<td>1:100</td>
<td>1</td>
</tr>
<tr>
<td>CD4/CD25</td>
<td>RPA-T4/BC96 (mouse)</td>
<td>PE Cy5/PE</td>
<td>1:20</td>
<td>2 (Batch 1)</td>
</tr>
<tr>
<td>CD4</td>
<td>OKT4 (mouse)</td>
<td>Alexa Fluor 488</td>
<td>1:20</td>
<td>2 (Batch 2)</td>
</tr>
<tr>
<td>CD45</td>
<td>HI30 (mouse)</td>
<td>Brilliant Violet 510</td>
<td>1:20</td>
<td>2 (Batch 2)</td>
</tr>
<tr>
<td>CD8</td>
<td>SK1 (mouse)</td>
<td>PE Cy7</td>
<td>1:20</td>
<td>2</td>
</tr>
<tr>
<td>Annexin V</td>
<td>-</td>
<td>Pacific Blue</td>
<td>1:20</td>
<td>2 (Batch 2)</td>
</tr>
<tr>
<td>7-AAD</td>
<td>-</td>
<td>-</td>
<td>1:20</td>
<td>2 (Batch 2)</td>
</tr>
</tbody>
</table>

b) Secondary Antibodies

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Host</th>
<th>Conjugate</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat</td>
<td>Mouse</td>
<td>Brilliant Violet 421</td>
<td>1:1000</td>
</tr>
</tbody>
</table>
3.2.8. Statistical analysis

Statistical analyses were performed using GraphPad Prism version 6.0 for Macintosh (GraphPad Software). For baseline patient characteristics, continuous variables (age, physiological scores and length of hospital stay) were analysed using a two-tailed Mann-Whitney Test; categorical variables (gender, admission source, comorbidities and infection characteristics) were analysed using a chi square contingency table and statistical comparisons determined with Fisher’s exact test where p<0.05 was considered significant. For all other immunological analyses, unless stated, differences observed between two means were analysed using a two-tailed Mann-Whitney Test where p<0.05 was considered significant.
3.3. Results

3.3.1. Baseline patient characteristics

The MATHS clinical trial is continuing to recruit to a total of 180 participants with 45 patients per group to be enrolled. The data presented in this chapter represents the analysis of parameters measured from the first 29 patients enrolled into the study until June 2015. Given that a larger number of total participants were required for statistically significant differences to be observed, it should be noted that the analysis provided for this subset of patients might not be truly representative of the total cohort of patients in the trial.

Following enrolment into the study, patients were randomly assigned to 100 mg aspirin-treated, and 300 mg aspirin-treated and untreated control patients. Subjects regularly taking aspirin had blood samples taken as per the other trial patients (Table 3.3). Medical history data (including physiological scores, infectious site and source) were also collected. The median age (of all patients) was 43 years (IQR; 27-64). The age distribution between treatment groups was similar; with the exception of regular aspirin takers, who tended to be older and overall had a higher incidence of co-morbidities (67% vs 13% or 0 in the other groups; Table 3.3). Combined analysis of all aspirin-treated patient groups versus the untreated control group revealed there were no significant differences observed in baseline clinical characteristics analysed, with the exception of APACHE II score and viral source of infection (Table 3.3).

A number of possible sources of bias should be noted in the cohort of patients enrolled thus far. The untreated control group had the lowest mean APACHE II score of all treatment groups (8.25 ± 2.49), however this difference was not significant compared with 100 mg or 300 mg aspirin-treated groups. Regular aspirin-taking patients had higher APACHE II score (16.33 ± 2.5) compared with other participant groups. As mentioned above, these patients were also older and had one or more co-morbidities. It is noteworthy that viral infection was the cause of sepsis in 4 patients, all of who were randomised to the untreated control group. SOFA scores varied between patients in each group, however the mean SOFA score improved in all groups over the two days participants were in the trial, regardless of treatment. This is reflective of the absence of sepsis deaths in our trial participants as well as the small sample analysed to date.
Table 3.3: Patient characteristics for the MATHS trial cohort. At enrolment, general characteristics, physiological scores, comorbidities and infection characteristics were recorded for each patient. Significant differences were noted for APACHE scores and viral source of infection.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>100 mg aspirin (n=8)</th>
<th>300 mg aspirin (n=7)</th>
<th>Regular aspirin (n=6)</th>
<th>Untreated Control (n=8)</th>
<th>( P ) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>General Characteristics</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, median (IQR)</td>
<td>35 (25-44)</td>
<td>42 (35-50)</td>
<td>73 (64-82)</td>
<td>38 (22-54)</td>
<td>0.253</td>
</tr>
<tr>
<td>Male Gender, % (n)</td>
<td>87.5 (7)</td>
<td>57.1 (4)</td>
<td>83.3 (5)</td>
<td>50 (4)</td>
<td>0.209</td>
</tr>
<tr>
<td>Admission Source, % (n)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ICU</td>
<td>25 (2)</td>
<td>28.6 (2)</td>
<td>33.3 (2)</td>
<td>12.5 (1)</td>
<td>0.510</td>
</tr>
<tr>
<td>Ward</td>
<td>75 (6)</td>
<td>71.4 (5)</td>
<td>50 (3)</td>
<td>87.5 (7)</td>
<td>0.095</td>
</tr>
<tr>
<td>Other</td>
<td>0</td>
<td>0</td>
<td>16.7 (1)</td>
<td>0</td>
<td>0.128</td>
</tr>
<tr>
<td>Length of Hospital Stay, Days median (IQR)</td>
<td>4.5 (3-13.5)</td>
<td>9 (5-28)</td>
<td>7 (6-15)</td>
<td>4.5 (2-8.5)</td>
<td>0.128</td>
</tr>
<tr>
<td>Physiological Scores, % (n)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>APACHE* II, mean (SD)</td>
<td>11.25 (9.29)</td>
<td>11.43 (2.64)</td>
<td>16.33 (2.5)</td>
<td>8.25 (2.49)</td>
<td>0.011*</td>
</tr>
<tr>
<td>SOFA*, mean (SD)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>2.25 (2.19)</td>
<td>1.29 (1.11)</td>
<td>2.5 (2.43)</td>
<td>1.625 (1.19)</td>
<td>0.993</td>
</tr>
<tr>
<td>Day 1</td>
<td>1.14 (1.46)</td>
<td>1.14 (1.07)</td>
<td>2.17 (1.72)</td>
<td>1.25 (1.16)</td>
<td>0.821</td>
</tr>
<tr>
<td>Day 2</td>
<td>1.29 (1.80)</td>
<td>0.57 (1.13)</td>
<td>1.67 (1.37)</td>
<td>1 (1)</td>
<td>1.000</td>
</tr>
</tbody>
</table>

* APACHE, Acute Physiology and Chronic Health Evaluation; COPD, Chronic obstructive pulmonary disease; SOFA, Sequential Organ Failure Assessment.
<table>
<thead>
<tr>
<th>Treatment group</th>
<th>100 mg aspirin (n=8)</th>
<th>300 mg aspirin (n=7)</th>
<th>Regular aspirin (n=6)</th>
<th>Untreated Control (n=8)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Comorbidities, % (n)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Ischemic Heart Disease</td>
<td>12.5 (1)</td>
<td>0</td>
<td>66.7 (4)</td>
<td>0</td>
<td>0.647</td>
</tr>
<tr>
<td>Diabetes</td>
<td>12.5 (1)</td>
<td>0</td>
<td>33.3 (2)</td>
<td>12.5 (1)</td>
<td>1.000</td>
</tr>
<tr>
<td>COPD*</td>
<td>12.5 (1)</td>
<td>0</td>
<td>33.3 (2)</td>
<td>0</td>
<td>0.540</td>
</tr>
<tr>
<td>Renal Disease</td>
<td>25 (2)</td>
<td>0</td>
<td>0</td>
<td></td>
<td>1.000</td>
</tr>
<tr>
<td>Cancer</td>
<td>0</td>
<td>0</td>
<td>33.3 (2)</td>
<td>12.5 (1)</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>12.5 (1)</td>
<td>42.9 (5)</td>
<td>100 (6)</td>
<td>75 (6)</td>
<td>0.238</td>
</tr>
<tr>
<td>None</td>
<td>50 (4)</td>
<td>28.6 (2)</td>
<td>0</td>
<td>37.5 (3)</td>
<td>0.675</td>
</tr>
<tr>
<td>Infection Characteristics, % (n)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Site of infection</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Bloodstream</td>
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<td>28.6 (2)</td>
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<td>50 (4)</td>
<td>0.164</td>
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<tr>
<td>Respiratory</td>
<td>37.5 (3)</td>
<td>14.3 (1)</td>
<td>83.3 (5)</td>
<td>25 (2)</td>
<td>0.238</td>
</tr>
<tr>
<td>Urinary</td>
<td>0</td>
<td>28.6 (2)</td>
<td>0</td>
<td>12.5 (1)</td>
<td>1.000</td>
</tr>
<tr>
<td>Skin/Soft Tissue</td>
<td>25 (2)</td>
<td>0</td>
<td>0</td>
<td>12.5 (1)</td>
<td></td>
</tr>
<tr>
<td>Other</td>
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<td>42.9 (3)</td>
<td>16.7 (1)</td>
<td>25 (2)</td>
<td>1.000</td>
</tr>
<tr>
<td>Source of infection</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gram-positive bacteria</td>
<td>25 (2)</td>
<td>14.3 (1)</td>
<td>33.3 (2)</td>
<td>12.5 (1)</td>
<td>0.647</td>
</tr>
<tr>
<td>Gram-negative bacteria</td>
<td>12.5 (1)</td>
<td>57.1 (4)</td>
<td>0</td>
<td>25 (2)</td>
<td>1.000</td>
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<tr>
<td>Virus</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>50 (4)</td>
<td><strong>0.003</strong>*</td>
</tr>
<tr>
<td>Fungi</td>
<td>0</td>
<td>14.3 (1)</td>
<td>0</td>
<td>0</td>
<td>1.000</td>
</tr>
<tr>
<td>Specific organism not identified</td>
<td>62.5 (5)</td>
<td>14.3 (1)</td>
<td>66.7 (4)</td>
<td>12.5 (1)</td>
<td>0.110</td>
</tr>
</tbody>
</table>
3.3.2. Analysis of mean platelet volume (MPV) in patients with sepsis

MPV is a haematological marker of platelet size, function and activation, and is commonly used to indicate changes in platelet production rates or platelet stimulation [473, 474]. MPV has been used in conjunction with platelet count as predictor for mortality in ICU in other inflammatory conditions [475, 476], and recently increased MPV and decreased platelet count were shown to be associated with worse outcomes in patients with severe sepsis or septic shock [477, 478]. In this study, MPV data from diagnostic haematology test (using the Coulter® LH 700 haematology analyser at the Royal Melbourne Hospital) was analysed to investigate potential effects of aspirin therapy in trial participants (Figure 3.1). Individual patient MPV varied, however, the majority of patient values were within the normal MPV range between 7.4 and 10.4 femtolitres (fL). Combined analysis of all aspirin-treated groups versus the untreated control group revealed no significant differences in median values of MPV on any of the days sampled (Figure 3.1A). Comparisons of data from each treatment group showed that regular aspirin-taking patients had the highest baseline median MPV (10.5 fL), which may reflect more severe sepsis, consistent with the presence of co-morbidities and increased APACHE II score. Of note 300 mg aspirin-treated patients had the lowest baseline median value for MPV (7.9 fL) (Figure 3.1B).

While aspirin-treatment did not appear to significantly reduce MPV (consistent with limited published data) it is interesting to note that 300 mg aspirin-treated patients and regular aspirin-treated patients maintained consistent MPV for the duration of sampling (regardless of starting value). In contrast, untreated control patients showed a small trend in increased MPV over the three-day sampling period from a median value of 8.45 fL (day 1) to 10.5 fL (day 3). Likewise 100 mg aspirin-treated patients showed a non-discriminatory increase from a median value of 8.8 fL (day 1) to 9.5 fL (day 3). On day 3 statistically significant differences were observed between regular aspirin-taking patients and 300 mg aspirin-treated patients, however taking into consideration the baseline starting values for each group, this difference was more likely to be due to increased severity of condition in the regular aspirin-taking patients, rather than response to aspirin treatment.
Figure 3.1: Mean platelet volume (MPV) in blood is not significantly reduced with aspirin therapy in patients with sepsis, but is increased in regular aspirin-taking patients with sepsis. A) Combined analysis of all aspirin-treated groups versus untreated control group. B) Analysis of individual patient groups. Data are represented as circular symbols for individual patients within each group, the bar the mean ± SEM and expressed as femtolitre (fL) (n=3-7 per group/time-point, two-tailed Mann-Whitney test, *p<0.05).
3.3.3. Quantification of plasma 15-epi-LxA$_4$ in patients with sepsis

In both clinical studies and animal models of inflammation, 15-epi-LxA$_4$ was shown to contribute to resolution of inflammation; associated with improved outcomes [17, 19, 20, 434]. In this study, levels of 15-epi-LxA$_4$ were measured in plasma, as a surrogate measure of resolution of inflammation. Plasma extracted from whole blood of patients with sepsis were analysed using a quantitative ELISA. Concentrations derived from 15-epi-LxA$_4$ standard curves were expressed in ng/mL.

Figure 3.2 illustrates the levels of 15-epi-LxA$_4$ detected over the 48 h trial timeline in trial participants with sepsis. Combined analysis of all aspirin-treated groups versus the untreated control group revealed no significant differences in mean production of 15-epi-LxA$_4$ on any of the days sampled (Figure 3.2A). When analysing the differences between individual treatment groups, at 1 h following first aspirin dose, 100 mg aspirin-treated patients showed a 1.382 ng/mL aspirin-dependent increase in mean 15-epi-LxA$_4$ levels. While this was not significantly different from untreated control patients, significant differences were observed between 100 mg aspirin-treated patients and other aspirin-treated patient groups (Figure 3.2B; p<0.05, one-tailed Mann-Whitney). Also of note, initial baseline concentrations of 15-epi-LxA$_4$ recorded (prior to aspirin dosing) for 100 mg aspirin-treated patients and untreated control patients were higher than baseline 15-epi-LxA$_4$ levels observed in healthy volunteers in a previous study [20], suggesting that inflammation alone can trigger the increased production of plasma 15-epi-LxA$_4$. 
Figure 3.2: Plasma 15-epi-LxA₄ production is moderately increased with 100 mg aspirin therapy in patients with sepsis. A) Combined analysis of all aspirin-treated groups versus untreated control group. B) Analysis of individual patient groups. Data are represented as mean ± SEM, and expressed as ng/mL. Dotted line represents the baseline concentration of 15-epi-LxA₄ (2.45 ng/mL) in healthy patients determined in [20], (n=3-7 per group/time-point, one-tailed Mann-Whitney test, *p<0.05).
3.3.4. Detection of NFκB activation in patients with sepsis

Production of SPMs such as lipoxins was recently shown to inhibit the activation of transcription factors such as NFκB that contribute to exacerbation of inflammatory pathways [479, 480]. Nuclear extracts from PBMC pellets were analysed by sandwich ELISA. Chemiluminescent detection was used to measure levels of activation of the p65 subunit of NFκB, and mean values were expressed as the percentage NFκB p65 activation of normalised start value.

Combined analysis of all aspirin-treated patients versus untreated control group are shown in Figure 3.3A. Regardless of aspirin treatment, 8 h after first aspirin dose there was no increase observed in NFκB p65 activation compared with untreated control patients. Comparisons of treatment groups showed that while there was considerable variation between patients, the untreated control group had increased NFκB p65 activation peaking at 8 h following enrolment. In contrast, both 100 and 300 mg aspirin-treated patients showed no significant change in NFκB p65 activation compared with initial start value. Significant differences were seen between 100 mg aspirin-treated patients and untreated control patients (Figure 3.3B; p<0.05, one-tailed Mann-Whitney).

Regular aspirin-taking patients showed a small initial increase in mean NFκB p65 activation (1 h following aspirin-treatment), however this was not statistically significant. Following this NFκB p65 activation returned to a level comparable with other aspirin-treated groups (at 8 h post-aspirin dose 1), consistent with 15-epi-LxA4-induced resolution of inflammation, previously shown in vitro [479]. The high level of variation of NFκB p65 activation observed in untreated control and regular aspirin-taking groups is likely to be a consequence of the small sample size to date.
Figure 3.3: Decreased NFκB activation observed at T8 in 100 mg and 300 mg aspirin-treated patients with sepsis. A) Combined analysis of all aspirin-treated groups versus untreated control group. B) Analysis of individual patient groups. Data are represented as mean ± SEM, and expressed as percentage change from starting value, (n=5-8 per group/time-point, one-tailed Mann-Whitney test, *p<0.05).
3.3.5. Inflammatory cytokine production in patients with sepsis

A key process during the sepsis inflammatory cascade is the production of pro-inflammatory cytokines. As 100 mg aspirin treatment led to increased plasma 15-epi-LxA₄ levels, and all aspirin doses inhibited NFκB p65 activation in the initial analysis, it was hypothesised that pathways downstream of NFκB activation including the production of both inflammatory cytokines and chemokines would be impaired [446]. Levels of pro- and anti-inflammatory cytokines and chemokines produced in the plasma of patients with sepsis (24 h after the first aspirin dose) were detected using the Human Inflammation panel (13-plex) LEGENDplex™ flow assay. Concentrations of IL-1β, IL-6, IL-8, IL-10, IL-12p70, IL-17A, IL-18, IL-23, IL-33, IFN-α, IFN-γ, MCP-1 and TNF-α were assessed, calculated from standard curves with recombinant human cytokine standards, and expressed as pg/mL.

Compared with mean endogenous levels of pro-inflammatory cytokines described for normal human plasma by the multiplex assay manufacturer, in this study increases in plasma levels of IL-6, IL-8, IFN-γ, MCP-1 (Figure 3.4B-E), IL-17A, IL-18 and IL-23 (Figure 3.5B-D) were seen in all patients with sepsis. Aside from a few individual responses detected in patients of all treatment groups, the levels of IL-1β, TNF-α (Figure 3.4A, F), IL-12p70 (Figure 3.5A), IFN-α, IL-10 and IL-33 (Figure 3.6A-C) detected were however within the normal range of endogenous levels found in normal human plasma. No statistically significant change in the early response pro-inflammatory cytokines IL-1β and IFN-γ were observed with aspirin-treatment compared with the untreated control group. Significant differences in mean plasma TNF-α level were seen in the regular aspirin-taking group compared with untreated controls (Figure 3.4F; p<0.05, one-tailed Mann-Whitney test). Significant differences were also seen in the mean plasma levels of IL-18 in the 100 mg aspirin-treated group compared with the untreated control group (Figure 3.5C; p<0.05, one-tailed Mann-Whitney test).
Figure 3.4: Participants regularly taking aspirin have increased plasma levels of TNF-α. Plasma extracted from patients was analysed for levels of: A) IL-1β, B) IL-6, C) IL-8, D) IFN-γ, E) MCP-1 and F) TNF-α at 24 h post-aspirin dose 1. Data are represented as circular symbols for individual patients with the mean ± SEM and expressed as plasma concentration (pg/mL), (n=5-8 per group, one-tailed Mann-Whitney test, *p<0.05).
Figure 3.5: Individual responses in the levels of IL-1 and IL-12 related pro-inflammatory cytokines vary in plasma of patients with sepsis. Plasma extracted from patients was analysed for levels of: A) IL-12, B) IL-17A C) IL-18 and D) IL-23, at 24 h post-aspirin dose 1. Data are represented as circular symbols for individual patients with the mean ± SEM and expressed as pg/mL, (n=5-8 per group, one-tailed Mann-Whitney test, *p<0.05).
Figure 3.6: Individual responses in levels of other pro-inflammatory and anti-inflammatory cytokines detected vary in plasma of patients with sepsis. Plasma extracted from patients was analysed for levels of: A) IFN-α, B) IL-10 and C) IL-33, at 24 h post-aspirin dose 1. Data are represented as circular symbols for individual patients, with the mean ± SEM (n=5-8 per group) and expressed as plasma concentration (pg/mL).
3.3.6. Analysis of immune cell populations in patients with sepsis

In order to assess whether aspirin had an overall impact on immune responses in patients with sepsis, the proportions of circulating immune cell subsets in whole blood were analysed. Following preparation of single cell suspensions, cells were stained with the appropriate antibodies and immune cell subsets were quantified by flow cytometry. Patient samples were analysed in 2 batches in November 2014 and July 2015. Following the initial batch analysis, additional markers were added.

For all comparative flow cytometry analyses; the same antibodies, voltage settings on the flow cytometer (FACS Canto II) and gating strategies were used. The gating strategies for FACS experiments are shown in Figure 3.7.

3.3.6.1. Analysis of innate immune cell populations

Phagocytic cell types (monocytes, macrophages and neutrophils) represent the early response to systemic infection by mediating clearance of pathogens both directly, and by presenting antigens leading to indirect bacterial pathogen clearance by adaptive mechanisms. For this reason the main focus of the analysis was these cell populations. Sepsis interferes with phagocytic mechanisms depending on pro-inflammatory stimuli, and delayed neutrophil apoptosis in sepsis results in organ dysfunction [100]. In this study, the proportion of monocytes/macrophages (CD86⁺, CD11b⁺, CD66b⁻) and neutrophils (CD86⁻, CD11b⁻, CD66b⁻) were analysed over a 48 h period in patients with sepsis. Proportions of CD86⁺ monocytes/macrophages in all participant groups fell within the normal range (<15% of all circulating leukocytes), with regular aspirin-taking patients displaying lowest proportions (Figure 3.8A; p<0.05, one-way ANOVA). While there was a small increase in mean percentage of monocytes/macrophages was seen in 100 mg aspirin-treated patients 1-8 h following first aspirin dose, this was not significantly different from other participant groups. Proportions of circulating neutrophils overall were higher compared with monocytes/macrophages (Figure 3.7B). Similarly, there was no significant effect on proportions of neutrophils in blood for new aspirin-treated patients. Subjects who were regular taking aspirin had a significantly lower starting percentage of neutrophils and this remained lower than other groups (Figure 3.8B; p<0.005, p<0.0001, one-way ANOVA).
Figure 3.7: Gating strategy and definition of monocyte/macrophage, neutrophil and CD4⁺/CD8⁺ T lymphocyte populations for analysis of immune cell subsets by flow cytometry. Representative FACS plots of cell suspensions isolated from the whole blood of critically ill patients with sepsis. Cells were differentiated into a leukocyte/lymphocyte gate according to their side scatter (SSC-A) and forward scatter (FSC-A) profiles (top left panel). Forward height (FSC-H) and forward area (FSC-A) parameters were used to identify single cells (bottom left panel). Cells were then defined as being CD3⁻ or CD3⁺ (top middle panel). In the CD3⁺ population, cells were defined as CD4⁺ T lymphocytes, and CD8⁺ T lymphocytes (top right panel). In the CD3⁻ population, cells were defined as being CD86⁺ or CD86⁻ (bottom middle panel). In the CD86⁺ population, CD11b⁺ CD66b⁻ cells were defined as monocytes/macrophages, and the double positive population for CD11b and CD66b were defined as neutrophils (bottom right panel).
Figure 3.8: Proportions of monocytes/macrophages and neutrophils are altered following 100 mg aspirin in patients with sepsis. A) Overall, regular aspirin-taking patients had significantly less monocytes/macrophages than new 100 mg aspirin-treated patients. B) Regular aspirin-taking patients had significantly less neutrophils than all other treatment groups. Data are the combination of two independent batch analyses, represented as mean ± SEM, and expressed as a percentage of the total leukocyte gate (n=4-8 per group/time-point, one-way ANOVA, *p<0.05, ***p<0.005, ****p<0.0001).
3.3.6.1.1. Expression of cell surface activation and migration marker PSGL-1

PSGL-1 is expressed on surfaces of neutrophils, and has a high affinity for binding P-selectin on endothelial surfaces to facilitate rolling, adhesion and subsequent migration across vascular the endothelium [481]. It was of interest therefore to investigate PSGL-1 expression on the surface of CD86$^+$ neutrophils, in blood of aspirin-treated patients with sepsis as an indicator of possible effects on tissue inflammation.

Mean PSGL-1 expression on neutrophils appeared to be altered in aspirin-treated patients with sepsis, with the 300 mg aspirin-treated group and 100 mg aspirin-treated patients both showing increases in mean PSGL-1 at 8 h and 24 h following first aspirin dose, respectively (Figure 3.9A). However, further detailed analysis at each time-point showed that these mean increases were due to single outlier patients with extreme responses in the 300 mg (Figure 3.9Bi) and 100 mg aspirin-treated groups (Figure 3.9Bii). As a result, it was concluded that there was no significant difference in PSGL-1 expression on the surface of neutrophils in aspirin-treated groups compared with untreated control patients.
Figure 3.9: Mean changes in PSGL-1 expression on neutrophils are due to variability in individual patients. A) Changes in PSGL-1 surface expression on neutrophils. Data represent the mean ± SEM (n=3-8 per group/time-point), and expressed as % change from start value. B) Percentage change PSGL-1 surface expression at i) 8 h, and ii) 24 h post aspirin dose 1. Differences in means observed were due to single outliers in 300 mg and 100 mg aspirin-treated groups respectively, therefore no significant differences were observed between groups. Data represented as square symbols for individual patients and the bar, the mean ± SEM, (n= 3-6 per group).
3.3.6.2. Analysis of T lymphocyte populations

Several studies in both animal models [482, 483] and human sepsis [107] have shown that a decrease in circulating lymphocytes, accompanied by an increase in lymphocyte apoptosis, leads to immunosuppression and contributes to severity of this inflammatory condition. In this study, the proportions of CD4$^+$ and CD8$^+$ T lymphocytes circulating in whole blood of patients with sepsis were evaluated (over a 48 h period), to determine whether aspirin-treatment impacted these cells. Figure 3.10A represents the change in proportions of CD4$^+$ and CD8$^+$ T lymphocytes. While the detected proportions were lower (<2% and <3% total leukocytes respectively) compared with what is seen proportionally in healthy humans, this was consistent with published data from trauma patients [108]. Interestingly proportions of CD4$^+$ T lymphocytes remained similar for all aspirin-treated groups, but increases were observed in the untreated control patients by 48 h following first aspirin dose (Figure 3.10A; p<0.05, one-way ANOVA).

To investigate lymphocyte apoptosis, in a subset of 14 patients (Batch 2), the proportions of Annexin V$^+$, CD4$^+$ and CD8$^+$ T lymphocytes were also analysed (Figure 3.10B). Overall the proportion of apoptotic cells was higher than reported in previous studies. Small changes in levels of apoptosis in circulating CD4$^+$ T lymphocytes were observed, however in contrast with the study by Hotchkiss et al. [107], no significant increases in apoptosis were observed, and no apparent effect of aspirin was noted (Figure 3.10C, left panel). Likewise, analysis of apoptosis in CD8$^+$ T lymphocytes again showed similar proportions of apoptotic cells across all treatment groups. Again, no significant effect of aspirin-treatment was seen in the levels of CD8$^+$ T lymphocyte apoptosis.
Figure 3.10: Aspirin therapy reduces proportions of T lymphocyte populations, but does not impact lymphocyte apoptosis. A) Analysis of CD4⁺ and CD8⁺ T lymphocyte proportions in whole blood from patients with sepsis. Data are the combination of two independent batch analyses, represented as mean ± SEM and expressed as a percentage of total leukocyte gate (n=4-8 per group/time-point, one-way ANOVA, *p<0.05). B) T lymphocyte apoptosis was determined in batch 2 analysis, using Annexin V and 7-AAD. Apoptotic cells were defined as Annexin V⁺/7-AAD population. C) Proportion of apoptotic circulating CD4⁺ and CD8⁺ T lymphocytes. Data represent the mean ± SEM and expressed as percentage of apoptotic cells (n=1-5 per group/time-point).
3.4. Discussion

For a potential adjunct therapy of sepsis to be successful, it should be inexpensive to produce and easy to administer. It is accepted that adjunct therapies should also aim to resolve inflammation and promote a return to homeostasis, rather than inhibiting inflammation all together [484-486], as this can lead to immunosuppression. At low doses (equivalent with doses taken for cardio-protective effects), aspirin is the only NSAID shown to induce the production of SPMs including 15-epi-LxA₄ [14]. Increased production of 15-epi-LxA₄ not only interferes with inflammatory signaling (via inhibition of NFκB) [216, 479], but also acts to resolve inflammation by: restoring neutrophil apoptosis, inhibiting neutrophil chemotaxis to reduce tissue damage [101, 487, 488], and promoting increased phagocytosis of pathogens and apoptotic neutrophils from circulation [446].

Observational studies have demonstrated an association between long-term aspirin usage and reduced sepsis mortality [61, 64, 220], however none of these studies were able to investigate the inflammatory pathways that may have mediated reduced mortality in these patients. To address this, the MATHS clinical trial was initiated, to investigate the effects of aspirin-therapy on surrogate markers of inflammation (primarily 15-epi-LxA₄ and NFκB), and other immunological parameters in patients with sepsis.

The data presented here represents the preliminary analysis of all patients enrolled to date (n=29) in the MATHS clinical trial (single-centre study). It should be noted that not all patients had all time-points sampled (either due to early discharge, or voluntary withdrawal of participation), which may have impacted the degree of variation seen in some analyses. The main findings of this study showed that regular aspirin-taking patients were the most severely ill of all groups, as they were older and presented with one or more co-morbidities, and these patients had the highest APACHE II and SOFA scores on enrolment. MPV was not impacted by aspirin therapy. Analysis of primary read out parameters of surrogate markers revealed an increase in plasma 15-epi-LxA₄ in 100 mg aspirin-treated patients within the 1 h of first aspirin dose. Also, all aspirin-treated groups showed a degree of NFκB inhibition, compared with untreated controls. Increased levels of some pro-inflammatory cytokines were observed among all patient groups, however a significant additional increase in levels of TNF-α was seen in regular aspirin-taking
group, and increases in plasma IL-18 levels in 100 mg aspirin-treated group, compared with untreated control group. Circulating CD86+ monocyte/macrophage and neutrophil populations were altered soon after aspirin therapy, however the level of PSGL-1 expression on innate immune cell surfaces was not affected. Circulating proportions of T lymphocytes were decreased in all aspirin-treated groups, compared with the untreated control group, but no effect of aspirin on levels of lymphocyte apoptosis was observed.

Of the patients enrolled in the trial, the most severely ill (according to physiological scores and MPV values) were those regularly taking aspirin. These patients were also significantly older than patients in the other participant groups and presented with at least one comorbidity, which may have predisposed these individuals to develop sepsis [1]. While analysis of MPV data in all patients showed no clear effect of aspirin therapy on platelet volume, over the three days of sampling, untreated control patients showed a small increase in platelet volume. While these increases were moderate, larger increases in mean platelet volume are often associated with disease severity, suggesting the potential use of MPV as a prognostic biomarker for sepsis severity [473, 477, 478]. These results are consistent with the limited published data available, suggesting that aspirin has no role in actively decreasing MPV, however it should be noted that the both of these studies only analysed patients regularly taking aspirin as a cardio-protective therapy [489, 490]. Another published study in healthy volunteers receiving 7 days of aspirin treatment (81 mg daily)[491], also showed no decrease in MPV, consistent with the data presented in this study for 100 mg aspirin-treated patients.

This study was focused on the “pro-resolving” outcomes associated with aspirin therapy, and analysis of plasma 15-epi-LxA4 and inhibition of NFkB as surrogate markers were performed. Increases in plasma 15-epi-LxA4 levels were seen in 100 mg aspirin-treated individuals 1 h following aspirin treatment, however no significant differences seen in absolute concentration with that of the untreated control group. It has also been reported by others that detectable levels of 15-epi-LxA4 (2-3 ng/mL) can be seen in the absence of aspirin therapy [19, 20]; similarly confirmed in this study with quantifiable levels of 15-epi-LxA4 seen in untreated control patients. It was also noted that the baseline mean plasma 15-epi-LxA4 levels in two of the participant groups in this study were higher than what has previously been reported in healthy patients. This suggests that 15-epi-LxA4 production is increased under inflammatory conditions, and levels are further increased.
following aspirin therapy. While the source of this non-aspirin induced 15-epi-LxA₄ is unknown, it has been hypothesised that in the absence of aspirin, COX-2 may still become acetylated by endogenously produced acetylating agents, leading to the production of basal levels of 15-epi-LxA₄ [20].

Although aspirin was the first pharmacological agent shown to induce the production of 15-epi-LxA₄, it is not the only known inducer of 15-epi-LxA₄. Statins including atorvastatin and pioglitazone can also promote the production of 15-epi-LxA₄, via the S-nitrosylation of COX-2 in animal models of inflammation [443, 444]. One human study of statin therapy did show a similar response, however 4-week period yielded a less significant change in levels of 15-epi-LxA₄ (~0.83 ng/mL), compared with levels shown in the present study with low-dose aspirin (mean change ~1.48 ng/mL in 100 mg aspirin-treated patients) [445]. Clinical patient data regarding patient medication will not be made available until the trial is completed, and as such post-hoc analysis will need to be performed in order to evaluate the relative effect of statin therapy on 15-epi-LxA₄ production.

A recent study by Morris and colleagues also showed that the phenotypic variation in 15-epi-LxA₄ production seen in a skin blister model in healthy volunteers [492], could be due to acute-inflammatory response time. “Early resolving” participants exhibited low initial 15-epi-LxA₄ levels with levels increasing with increased inflammation, and “delayed resolvers” exhibiting initially increased 15-epi-LxA₄ levels, that decreased as inflammation progressed. While this may contribute to the variation in plasma 15-epi-LxA₄ levels seen in patients in this study, the MATHs trial was powered to determine differences in a larger sample size (45 patients per group) and so no final conclusions can be drawn until recruitment has been completed. Analysis of a larger cohort of patients would be required to determine whether increases in 15-epi-LxA₄ production were significantly different with low-dose aspirin therapy. While the preliminary results are promising, aspirin also triggers the production of a number of other SPMs. Resolution of inflammation is a complex process; proposed to be mediated by SPMs in combination [464]. As such, there is increased interest into the roles of other SPMs derived from polyunsaturated fatty acids (PUHA) in resolution of inflammation including: resolvins, protectins and maresins. Future studies, including the analysis in this
trial should investigate the role of resolvins, protectins and maresins following aspirin therapy, to determine their contribution to resolution of inflammation.

In the current study, aspirin-treatment prohibited the activation of NFκB by 8 h following first aspirin dose, regardless of the dose given, or prior therapy. This is the first study to show aspirin-induced inhibition of NFκB \textit{in vivo} in a human clinical trial of sepsis, and the results presented are consistent with that reported from \textit{in vitro} analyses of NFκB inhibition [216, 433, 493], as well as rodent models of inflammation [494]. While only 5-6 patients per group contributed to analysis at this time-point, these results are encouraging, and support the potential for aspirin-induced resolution of inflammation in sepsis acting via NFκB inhibition among other mechanisms. A recent study also showed that aspirin-triggered resolvin D1 inhibited NFκB activation, decreased neutrophil influx into kidney tissue, protecting against acute kidney injury in rats [13], further supporting a role for other SPMs with potent anti-inflammatory actions. NFκB is a key driver of inflammation by facilitating transcription of pro-inflammatory cytokines, therefore it was expected that inhibition of NFκB would impact the level of cytokines produced downstream. The novel information we aimed to provide is the lower range of aspirin required to produce this effect on NFκB.

This study is one of very few investigating a broad panel of cytokines in patients with sepsis or taking aspirin. Levels of pro-inflammatory and anti-inflammatory cytokines in plasma were consistent with early inflammation, however these were for the most part lower that what has been reported in patients with severe sepsis and septic shock [495]. This study did not include a healthy control group, but the plasma concentrations of pro-inflammatory cytokines and chemokines in this study were either moderately increased or unchanged compared with endogenous levels in healthy human plasma (shown in the LEGENDplex™ manufacturer information). The majority of the cytokine and chemokine panel investigated showed no effect of aspirin at the time-points assessed, similarly shown in exudates of blister fluid in healthy volunteers treated with low-doses of aspirin [19]. Significant increases were seen in plasma levels of TNF-α in the regular aspirin-taking group compared with untreated controls. While this suggested a more severe disease status, measured levels in this study were lower than seen in another study of severe sepsis and septic shock [495], and therefore no further conclusions can be drawn until the data from all samples in the trial (once completed) are available.
Levels of IL-18 were shown to increase in patients with sepsis, compared with trauma patients and healthy controls in a previous study, suggesting this marker could be used as an identifying marker of sepsis [496]. While all patient groups in the current study showed a high level of plasma IL-18 (>350 pg/mL) indicative of sepsis, mean plasma levels of IL-18 in the 100 mg aspirin-treated group were significantly higher than untreated control group. A number of biological roles have been described for IL-18, most notably in IFN-γ regulation [497], which was shown in one study to promote the resolution of bacterial infection in mice [498]. As plasma increases of IL-18 were seen with 100 mg aspirin-therapy, this might suggest that low-dose aspirin increases this cytokine in conjunction with 15-epi-LxA4, and may contribute to pro-resolving pathways in patients with sepsis.

Another key target of the sepsis inflammatory cascade are the host innate and adaptive immune responses. In this study, CD86 expression was analysed on the surface both monocytes/macrophages and neutrophils. For the duration of sampling the proportion of CD86⁺ monocytes/macrophages was lower in regular aspirin takers; keeping with the knowledge that sepsis decreases surface expression of CD86 resulting in macrophage dysfunction [66], which along with other data suggests more severe disease state. Further, a significant increase in the proportion of CD86⁺ monocytes/macrophages was seen with 100 mg aspirin-treatment (8 h) compared with regular aspirin takers (p<0.05, one-way ANOVA); suggestive of increased antigen-presenting capability, which may lead to enhanced phagocytosis of bacteria in circulation [446].

While not commonly expressed on neutrophil surfaces, pro-inflammatory stimuli can induce co-stimulatory molecule CD86 expression, which can induce signaling for T lymphocyte activation. Delayed neutrophil apoptosis is a known contributing factor to sepsis inflammation, organ dysfunction and increased mortality, demonstrated by increased CD86 surface expression following pro-inflammatory stimuli [499, 500]. This could be true for untreated control patients in this study (see Figure 3.8B), with increases in CD86 expression on neutrophils occurring at similar timeframe as increased NFκB activation (Figure 3.3 and 3.8B), however no significant correlations were found in the available datasets. Initial increases in proportions of CD86⁺ neutrophils were also seen early in 100 mg aspirin-treated patients (1 h following aspirin treatment), however this was not found to be statistically significant. As production of 15-epi-LxA4 has been
reported to override the signals for delayed apoptosis in neutrophils [101], it is possible that this mechanism contributes to the small proportional decrease of CD86+ neutrophils that was observed in this group of patients, occurring 8 h following aspirin-treatment and production of 15-epi-LxA₄ (at times consistent with inhibition of NFκB).

Cell surface expression of PSGL-1 and CD11b mediates adhesion of leukocytes to both platelets and vascular endothelium to promote rolling and migration. In a previous study however, 15-epi-LxA₄ was shown to interfere with neutrophil binding on vascular endothelium, preventing chemotaxis and transmigration and this contributed to resolving inflammation [487]. This inhibitory mechanism of 15-epi-LxA₄ could also explain why an initial increase in the proportion of CD86+ neutrophils was seen in this group of patients. Analysis of cell surface expression of PSGL-1 on CD86+ neutrophils showed an initial decrease in 100 mg aspirin-treated patients, however because no significant differences in PSGL-1 expression were determined, it is difficult to speculate whether aspirin impacted neutrophil rolling and migration. Further, soluble 15-epi-LxA₄ interaction with its surface receptor ALX on neutrophil surfaces have been suggested to trigger anti-inflammatory and pro-resolution effects during acute inflammation [501]. However, surface expression of ALX on neutrophils in this study appeared to be constitutive and did not change in all participant groups (data not shown). Analysis of cell surface expression PSGL-1 on circulating CD86+ monocytes/macrophages was also investigated, however no significant increases in proportion of these cells were seen in any of the treatment groups (data not shown).

The role of adaptive immune responses in the early development of sepsis have been debated, however one described effect of sepsis to the adaptive immune response is immunosuppression. In both experimental animal models and clinical studies alike, within 7 days of sepsis development a profound depletion of CD4+ T lymphocytes was observed [107, 483]. The proportions of both CD4+ and CD8+ T lymphocytes in this study appeared lower compared with proportions observed in healthy patients in a previous study [108], however no significant change was seen with any of the aspirin-treated patients over 48 h. Untreated control patients had significantly increased proportions of CD4+ T lymphocytes subsets at the 48 h sampling time, suggesting that the decrease in lymphocytes described by Hotchkiss et al. occurs following 48 h of identification of sepsis [107]. Also, the preliminary investigation into lymphocyte
apoptosis in the current study did not show any significant effect of aspirin therapy. Proportions of apoptotic CD4\(^+\) and CD8\(^+\) T lymphocytes did appear higher in patients in this study, compared with other published studies [107, 502]. This data suggests that our patients were entering immunosuppression phase of sepsis, however it should be noted that the number of patients per group in this batch analysis was very small (n=1-5). Further, levels of CD4\(^+\) T lymphocyte apoptosis measured at T0 did not correlate with APACHE II scores, suggesting that the apoptosis data presented here is not truly reflective of the total population of patients analysed.

As previously stated, the design of MATHS clinical trial was not powered to detect improvements in clinical outcomes. Complete clinical outcome data will not be released until the completion of the trial and as such it cannot be determined whether aspirin therapy improves outcomes at this time.

Other limitations of this study include a lack of samples from healthy patient controls for comparative analysis. This group was omitted from this initial trial due to difficulties in gaining human ethics approval to collect blood from healthy patients. Future studies should also include a healthy (non-septic) control group, for determination of baseline characteristics particularly for NF\(\kappa\)B activation and immunological parameters measured by flow cytometry (plasma cytokines and immune cell populations). Further, the contribution of other SPMs in resolution of inflammation should be considered in these patients, as these have recently been shown to reduce inflammation in animal models of systemic infection [13, 435, 503]. In this study, all analyses were performed the blood collected from patients with sepsis. While the results of this study to date are promising and support a role for low-dose aspirin induced resolution of inflammation, it is still unclear whether the effects seen in circulation extend to tissue inflammation, contributing to reduced organ-damage. With this in mind, an animal model of systemic bloodstream infection was established (described in Chapter 4 of this thesis). This model permits the investigation of tissue damage and potential organ failure seen in early development of sepsis.
3.5. Conclusion

Despite the number of adjunct therapies investigated for their potential in targeting excessive inflammation in sepsis, none have shown therapeutic benefit in clinical setting. Both animal models of infection, and observational studies in patients have shown increased evidence to support the use of low-dose aspirin in patients with sepsis; proposed to be mediated primarily by anti-inflammatory/pro-resolving actions [16-18, 61, 64, 220].

The results of this study to date are in keeping with the hypothesis and have shown that low-dose aspirin therapy (100 mg daily) induced the increased production of 15-epi-LxA₄ within 1 h of administration, with downstream effects including inhibiting NFκB activation (not seen previously with this dose). Further, low-dose aspirin moderately altered circulating leukocyte proportions, consistent with mechanisms of aspirin shown in other inflammatory conditions. While the results of this study are preliminary and extended analyses are required in a larger subset of patients to achieve statistical significance, the therapeutic benefits of aspirin therapy shown here are encouraging for the support of low-dose aspirin as an adjunct therapy of sepsis.
Chapter 4

Investigating the effects of aspirin treatment in a murine model of gram-positive induced sepsis
4.1. Introduction

Sepsis is a complex inflammatory condition that occurs in response to infection, and at its most severe, a hyper-inflammatory state leading to organ failure and death [24, 483]. Epidemiological studies have revealed that since 1987 there has been a shift, toward gram-positive organisms as the leading source of bloodstream infections [4]. Among these, *Staphylococcus aureus* is the most important pathogen that causes a range of diseases, from mild skin infections to septic shock. Currently, *S. aureus* is the most frequently isolated gram-positive organism in patients with sepsis. Animal models are widely used to study the immunology of infections and associated inflammatory conditions including sepsis, allowing the extended analysis of target organs commonly damaged.

Recently a number of published reports have prompted a debate as to whether mouse models of sepsis are a good representation of human disease (discussed in section 1.2.6). A meta-analysis of transcription profiles of inflammatory genes in PBMC, initially suggested a poor correlation between human systemic inflammation and the mouse models [222]. However, a later study analysed the same data sets using different methodology and concluded that the gene expression patterns were in fact similar [223]. Given the significant experimental differences between models that neither of these studies took into account, the issue remains controversial. For inter-species comparisons to be useful, considerations must be made in regards to time frames, doses used, and parameters measured in experimental protocols, as well as any acknowledging genetic variables that may exist [224].

Animal models are also commonly used in the development of novel adjunct therapies of sepsis. Of the many pharmacological agents showing promise in animal models of sepsis, none have shown considerable therapeutic benefit in human clinical trials; reflecting a lack of mouse models that accurately represent the human clinical condition, most likely due to the differences in responses to infection and inflammation (reviewed in [148]). One way to shed more light on the limitations of animal models is to carefully develop models to closely mimic that seen in patients, and to measure similar parameters as markers of outcome or severity.
The study described in the previous chapter suggests that low-dose aspirin therapy impacts immunological pathways as an adjunct therapeutic in clinical sepsis, more specifically by increasing the production of plasma 15-epi-LxA4 and inhibiting the activation of NFκB. Further, aspirin therapy via production of 15-epi-LxA4, can lead to increased activation of monocytes/macrophages while inhibiting activation and migration of neutrophils, and contribute to resolution of inflammation (reviewed in [469, 504]).

Aspirin and other NSAIDs have also been shown to induce L-selectin shedding on neutrophils, to reduced inflammation in tissues [505]. PSGL-1 is a well-described ligand of L-selectin [97], and an integral surface adhesion molecule for recruitment of neutrophils to tissues [98]. Decreased surface PSGL-1 on activated leukocytes has also been suggested to impact recruitment to other tissues. Therefore, it was of interest to investigate the expression of these two markers on leukocyte surfaces to determine whether sepsis or aspirin therapy had an effect.

A number of studies have shown a similar mechanism of action for aspirin therapy in other rodent models of inflammation (discussed in section 1.4.3). Of those showing similar beneficial effects of aspirin described above, aspirin doses administered in rodent model studies were 20-times higher than doses administered to patients in the clinical study in the previous chapter [18, 214]. In order to mirror the clinical study more closely, lower doses of aspirin were selected for this study.

The study described within this chapter aimed to establish a model of gram-positive bloodstream infection using *S. aureus*, in order to further explore the hypothesis that underlies the clinical trial described in the previous chapter. In order to facilitate comparison of this animal model with our human trial participants, where possible the same parameters measured in patients were studied in mice. In addition, the effect of aspirin treatment on the impact of sepsis on target organs was investigated.

The hypothesis was therefore; that low-doses of aspirin will induce immuno-modulatory effects in mice with *S. aureus*-induced sepsis.
In order to address the hypothesis, the specific aims of the experiments in this chapter were:

- To establish a model of gram-positive sepsis using *S. aureus* in wild-type C57BL/6 mice

- To determine the effects of two doses of aspirin (1 mg/kg and/or 10 mg/kg) on immune responses in wild-type C57BL/6 mice with gram-positive sepsis

More specifically to:

- Determine whether aspirin could improve clinical outcomes in mice with staphylococcal-induced sepsis

- Quantitate levels of 15-epi-LxA₄ produced in plasma of septic mice to determine an effect of aspirin therapy

- Determine whether aspirin therapy inhibits the activation of NFκB in PBMC

- Determine the impact on cytokine levels in plasma as a measure of inflammatory activation

- Characterise changes in immune cell sub-populations in blood and spleen as a representative organ in mice with sepsis to assess the effects of aspirin-therapy
4.2. Materials & Methods

4.2.1. Animal model of gram-positive sepsis

A bloodstream infection was induced by injecting $1 \times 10^7$ CFU of \textit{S. aureus} into the tail vein of female wild-type C57BL/6 mice. Mice were monitored for 2 days. At the end of the experiment, blood and target organs (spleen, kidney and liver) were sampled for bacteria and histology. Systemic inflammatory responses were also assessed.

Experiments involving animals were carried out with the approval of the RMIT Animal Ethics Committee (AEC Permit Number 1213). Six to eight-week-old female C57BL/6 mice were sourced from the Animal Resource Centre (ARC). Prior to experimentation, mice were acclimatised for a minimum of seven days within the RMIT Animal facility (RAF) (RMIT University, Australia). A maximum of four mice were housed in a single housing unit, were fed standard rodent chow and had access to sterilised-water \textit{ad libitum}.

4.2.1.1. Bacterial strain used in systemic infections

The methicillin-susceptible \textit{S. aureus} ATCC 25923 (SA25923) strain was used to establish a blood-stream infection in C57BL/6 mice. The selection of this strain (rather than an MRSA) was primarily for safety reasons to decrease risks to personnel. This strain was kindly provided by Senior Technical Officer, Helen Williams (RMIT University, Australia).

SA25923 was grown in broth culture at 37°C overnight, and the absorbance at 595 nm was measured. The broth culture was diluted to achieve an absorbance measurement of 1.0 (equivalent to approximately $1 \times 10^8$ CFU/mL) and then bacteria were harvested by centrifugation, washed and re-suspended in sterile PBS to achieve a final concentration of $1 \times 10^8$ CFU/mL.

4.2.1.2. Mouse model of systemic infection

Experimental groups of 14-15 mice received a single dose of $1 \times 10^7$ CFU of SA25923 (in PBS) in a total volume of 100 µL by intravenous tail-vein injection (see Figure 4.1). Viable counts were determined by plating serial dilutions of the inoculum.
Figure 4.1: **Timeline for *S. aureus* sepsis model.** Female C57BL/6 mice (n=44) received $10^7$ CFU of SA25923 by i.v injection in the tail-vein. Mice received oral low-dose aspirin by gavage (represented in red symbols) (1 mg/kg; n=14), or high dose aspirin (represented in blue symbols) (10 mg/kg; n=15). Aspirin was administered in two doses; 1 h following infection and 24 h following first aspirin dose. Naïve mice (represented in purple; n=10) were not infected and did not receive aspirin therapy, and infected only mice (represented in green symbols; n=15) did not receive aspirin. Mice were weighed prior to infection, monitored every 2-4 h and weighed every 6 h for signs and symptoms of systemic inflammation. Monitoring was performed for 2 days according to established criteria (Table 2.2).
Aspirin (1 mg/kg or 10 mg/kg; equivalent to a human dose of 100 mg (low-dose) or 1000 mg (high-dose) respectively, prepared as described in section 2.2.2, Sigma-Aldrich) was administered to groups of 14-15 mice by oral gavage using a s/steel blunt ended gavage needle (18G; Popper and Sons Inc, USA). Infected only control mice (n=15) did not receive aspirin. Aspirin treatment was administered on day 0 and on day 1 of the experiment (~24 h following the first treatment).

Mice were weighed every 6 h and monitored every 2-4 h following infection and aspirin treatment for signs and symptoms, indicating the progression/resolution of sepsis. Monitoring was performed for 2 days according to the criteria as outlined in Table 2.2 (Chapter 2). Humane end-point criteria for this study were: mice displaying a clinical score of 3 for any one criterion, or a total score from multiple criteria of ≥10 (note >9 was expected), or mice displaying ≥15% weight loss.

4.2.1.3. Termination of experiment and sample collection

On day 2, mice were euthanised via CO₂ inhalation; blood was collected immediately by cardiac puncture into syringes pre-loaded with 200 µL sterile Alsever’s solution (described in section 2.4.2). Mice were transferred to a biosafety level 2 cabinet to minimise contamination with air microorganisms, organs were removed and divided into sections for analysis of bacterial load, histology and other immunological parameters. Blood was processed by centrifugation as described in section 2.5.1, then plasma and PBMC pellets were aliquoted into microcentrifuge tubes and stored at -80°C, for batch analysis.

4.2.2. Determination of bacterial load in blood and organs

An aliquot of blood collected into Alsever’s solution was diluted 1:2 in BHIB, and a total volume of 100 µL was then spread plated onto BHIA and colony counts were performed after overnight incubation at 37°C.

Spleen, kidney and liver were collected into pre-weighed tubes containing 2 mL BHIB, filled tubes were weighed and the tissue was homogenised with the Bio-Gen™ PRO200 homogeniser (PRO Scientific). Homogenates (100 µL) were spread plated onto BHIA, and colony counts were performed as described above.
4.2.3. Histological assessment of organ damage

Tissues harvested from mice were embedded in Tissue-Tek® O.C.T™ medium (Sakura Finetek) and stored at -80°C. Sections were cut (as described in Section 2.4.3), mounted onto StarFrost® supereclean slides (ProSciTech) and stored at -80°C.

4.2.3.1. Haematoxylin and eosin staining and scoring of inflammation

Frozen 10 µm sections of spleen were fixed in 4% neutral buffered formalin (Thermo Fisher Scientific). Sections were washed in PBS once before being stained with haematoxylin and eosin. Stained sections were examined under light microscopy for assessment and scoring of tissue damage. Images were captured using a Leica DM2500 microscope and Leica Application Suite software (Leica).

H & E stained spleen sections were scored in a blinded manner for the presence of inflammation. A semi-quantitative scoring system was used, as described by Wiersinga et al. [506] where assessment of inflammation, apoptosis/necrosis leading to abscess formation and thrombus formation was made. Minor modifications were made to the score-range, with each parameter listed above being graded on a scale of 0 to 3: 0, none; 1, mild; 2, moderate; 3, severe. The total “splenic inflammation score” was expressed as the sum of the scores for each parameter, with the maximum total spleen inflammation score being 12.

4.2.3.2. Immunohistochemical detection of S. aureus in spleen

Frozen 10 µm sections of spleen were fixed in ice-cold acetone. Sections were air dried and rehydrated in PBS for 10 min then were permeabilised with PBS containing 0.2% Triton X-100. Fc Receptor blocking was performed by incubation with PBS/1% BSA and serum from the host species of the secondary antibody (described in section 2.4.3.2) for 1 h at RT. Slides were inverted to remove excess blocking solution, and the primary antibodies were added (rabbit anti-S. aureus, (1:200; Abcam), and rat anti-mouse CD11b, (1:100; BioLegend) in PBS/1% BSA) and sections were incubated overnight at 4°C. Sections were washed three times for 10 min in PBST and secondary antibodies were added (anti-rabbit Cy3 (1:1000; Abcam) and anti-rat Alexa Fluor® 488 (1:800; Life Technologies) in PBS/1% BSA), and slides were incubated for 1 h at RT. Following this, slides were washed 3 times in PBST then incubated with DAPI nuclear stain in PBS/1%
BSA (2 µg/mL) for 20 min at RT (in the dark). Slides were washed frequently in PBST over 30 min, mounted in Mowiol (described in section 2.4.3.2) and examined the following day. Images were captured as described above. Images were overlayed for display using Adobe Photoshop software (Adobe).

4.2.4. Detection of 15-epi-LxA₄ in plasma of mice with sepsis

4.2.4.1. Enrichment of plasma proteins and lipids

Plasma was enriched for proteins and lipid mediators including 15-epi-LxA₄, using the Bond Elute C₁₈ OH solid phase extraction column (Agilent technologies) prior to quantification using an immunoassay (described in section 2.5.4.1). Columns were pre-conditioned by washing with methanol and MQH₂O. Plasma samples (500 µL) were acidified to pH 3.5 with 1 mol/L HCl, then diluted 1:4 in MQH₂O and added to columns. Columns were centrifuged as described, and the eluate was discarded. Columns were washed with MQH₂O and petroleum ether, and plasma proteins and lipids were eluted with methyl formate. Solvents were evaporated under a stream of nitrogen gas, and dried pellets were resolubilised in 100 µL 1× 15-epi-LxA₄-Extraction Buffer, prior to quantification using the 15-epi-LxA₄ EIA kit (Oxford Biomedical Research Inc).

4.2.4.2. Quantification of 15-epi-LxA₄ in plasma by ELISA

Quantification of plasma 15-epi-LxA₄ was performed according to the manufacturers specifications. Purified proteins and lipid mediators enriched from plasma were further diluted 1:5 in 1× 15-epi-LxA₄-Extraction Buffer, and 50 µL was added in duplicate to wells of a 96-well Costar® microplate, pre-coated with anti-15-epi-LxA₄ rabbit antibody. Next, 50 µL of diluted 15-epi-LxA₄-HRP conjugated antibody was added to each well, plates were mixed briefly by shaking gently on a plate shaker, covered and incubated at RT for 1 h. Following incubation, plates were washed three times with 300 µL, 1× Wash Buffer and TMB substrate solution was added to each well, plates were mixed on a plate shaker (as described above) and incubated at RT for 30 min. Absorbance was measured at 650nm using the POLARstar Optima multi-detection microplate reader (BMGlabtech). Concentrations of 15-epi-LxA₄ were determined using standard curves generated from 0 to 2 ng/mL, and adjusted for dilution factor and starting volume of plasma.
4.2.5. Detection of NFκB activation

4.2.5.1. Preparation of nuclear and cytoplasmic extracts

Nuclear and cytoplasmic extracts were prepared with the use of the NE-PER nuclear and cytoplasmic extraction reagents (Pierce) according to the manufacturers specifications. PBMC pellets were washed once in FACS wash buffer and once in PBS. Then cells were resuspended in ice-cold CER I buffer (supplemented with 1× cOmplete ULTRA protease inhibitor cocktail; Roche AG) and ice-cold CER II buffer, vortexed vigorously and incubated on ice. Cells were vortexed vigorously for 5 s and centrifuged as described and supernatants (cytoplasmic extracts) were transferred to a pre-chilled microcentrifuge tube and stored at -80°C. Remaining pellets (containing nuclei) were resuspended in ice-cold NER buffer (supplemented with 1× cOmplete ULTRA protease inhibitor cocktail; Roche AG), vortexed vigorously for and incubated on ice. This process was repeated every 10 min for a total of 40 min. Cells were centrifuged and supernatants (nuclear extracts) were transferred to a pre-chilled microcentrifuge tube and stored at -80°C.

4.2.5.2. Chemiluminescent detection of NFκB p65 activation

The activation of the NFκB p65 in nuclear extracts (prepared as described) was detected using the NFκB p65 Transcription factor assay (Pierce) according to the manufacturers specifications. Working Binding Buffer was added to each well of the 96-well NFκB Assay Plate provided, prior to addition of nuclear extracts (added in duplicate). Plates were incubated at RT for 1 h (on a plate shaker), and washed three times with 1× NFκB Assay Wash Buffer. Plates were incubated with NFκB p65 primary antibody (1:1000; in supplied Antibody Dilution Buffer) at RT for 1 h, under static conditions. Plates were washed three times as described then incubated with HRP-conjugated secondary antibody (1:10,000) at RT for 1 h, under static conditions. Plates were washed four times as described then substrate working solution (prepared as described in section 2.5.5.2) was added. Chemiluminescence expressed as relative fluorescence units (RFU) was detected using the luminometer function on the POLARstar Optima multi-detection microplate reader (BMGlabtech).
4.2.6. Quantification of plasma cytokine levels using the LEGENDplex™ multi-analyte flow assay kit

Levels of pro-inflammatory cytokines in plasma samples were quantified using the LEGENDplex™ Mouse Inflammation panel (13-plex) flow assay (BioLegend). All procedures were performed according to manufacturers instructions (as described in section 2.5.6). Cytokine standards and samples were prepared in LEGENDplex™ Assay buffer and were added to a 96-well U-bottom plate (Nunc A/S). Sonicated pre-mixed beads and detection antibodies were added to each well and then plates were incubated (in the dark) on a plate shaker at 600 rpm for 2 h at RT. Following incubation, streptavidin–PE solution was added to each well and further incubated for 30 min at RT. Samples were analysed and data collected using the FACSCanto II (Beckton Dickinson). Data were analysed using the BioLegend LEGENDplex™ Data Analysis Software with standard curves generated from 0 to 50,000 pg/mL, and samples adjusted for dilution factor.

4.2.7. Analysis of cell populations

4.2.7.1. Preparation of single cell suspensions

4.2.7.1.1. Blood

Following centrifugation and removal of plasma, cells were re-suspended in TAC red cell lysis buffer (prepared as described in section 2.5.1) and incubated at 37°C for 5 min to lyse contaminating erythrocytes. Cells were then washed by centrifugation and re-suspended in FACS wash buffer (prepared as described in section 2.2.3).

4.2.7.1.2. Spleen

Spleens were collected into ice-cold DMEM complete media (prepared as described in section 2.2.3). Cells were placed in petri dishes and were gently teased through a 40 µm cell strainer (Biologix). Following a washing step cells were re-suspended in TAC red cell lysis buffer and incubated at 37°C for 5 min to lyse contaminating erythrocytes. Cells were then washed and re-suspended in FACS wash buffer.

4.2.7.2. Flow cytometry

For flow cytometry, 10^6 cells from blood or spleen were incubated with Fc receptor block (purified anti-mouse CD16/32 Antibody; BioLegend), at 4 µg/mL in 50 µL FACS wash buffer.
buffer for 30 min on ice. The antibodies used for staining are listed in Table 2.3 (Chapter 2). Staining was performed in 100 µL FACS wash buffer for at least 30 min on ice. Following this cells were washed twice in FACS wash buffer, before being re-suspended in PBS for data acquisition.

Compensation was performed using UltraComp eBeads (eBioscience) based on the single staining of each individual fluorochrome and correction for auto-fluorescence with unstained beads and cells. Data was collected using the FACSCanto II as described above. A five-decade logarithmic scale was used to process fluorescence signals and a linear scale was used to process light scattering signals. Single cells were gated and populations of cells were defined as T lymphocytes (CD3+, CD4+, CD8+), monocytes/macrophages (CD3-, CD11b+, Ly6G-) and neutrophils (CD3-, CD11b+, Ly6G+).

4.2.8. Statistical analysis

All statistical analyses were performed using GraphPad Prism version 6.0 for Macintosh (GraphPad Software). Unless stated, the differences observed between two means were analysed using a two-tailed Mann-Whitney test where p<0.05 was considered significant.
4.3. Results

4.3.1. Establishing a model of gram-positive sepsis in C57BL/6 wild-type mouse strain

Two dose-finding studies were performed in order to determine the number of *S. aureus* (SA25923) that would induce sepsis in wild-type C57BL/6 mice, and permit analysis over an approximately 48 h, without reaching the humane endpoint criteria. Mice were initially infected with either $10^6$ or $10^7$ CFU by tail vein injection and monitored every 2-4 h for a period of 48 h (to mirror human subject sampling times described in section 3.2.2) and weight and clinical parameters were recorded (see Table 2.2).

Although mice were monitored for changes over a range of criteria, the only criteria that represented a measurable difference were change in weight. Mice infected with $10^6$ CFU *S. aureus*, displayed few detectable symptoms and varying degrees of weight loss indicating this dose was not effective in inducing sepsis. When compared with naïve mice, mice that received the $10^7$ CFU dose of *S. aureus* displayed moderate loss of weight over time (10-15% weight lost compared with initial start value) (data not shown). A dosage of $10^7$ CFU was chosen for the following studies.

4.3.2. Effect of aspirin treatment in mice with gram-positive sepsis

4.3.2.1. Observations of clinical and physical parameters

Mice were infected with $10^7$ CFU of *S. aureus* by tail-vein injection at time 0 (T0). Control mice were not infected or treated with aspirin, and used to determine baseline values (naïve mice). Following this, two doses of aspirin (at 1 h and 24 h post-infection) were administered orally, to groups of 14-15 mice. A separate group of mice (n=15) were also infected but did not receive aspirin therapy (infected only control mice). For the duration of the experiment the mice were monitored every 2-4 h for changes in weight and clinical parameters for sepsis progression (according to criteria outlined in Table 2.2). None of the mice that were infected with *S. aureus* met the criteria for humane end-point intervention during the course of the study (described in section 4.2.1.2).
Naïve mice maintained a steady weight over time. In contrast, mice infected with *S. aureus* began to lose weight 12 h after infection (Figure 4.2A; p<0.05, one-way ANOVA). After a second dose of aspirin (24 h post-infection), low-dose aspirin-treated mice (1 mg/kg) began to re-gain weight, with mean weights returning to starting values equivalent with naïve mice 48 h post-infection (Figure 4.2A; p<0.05, one-way ANOVA).

Interestingly, mice treated with high-dose aspirin (10 mg/kg) and infected only control group displayed significant weight loss (p<0.05, one-way ANOVA) compared with naïve mice.

This trend was also shown in clinical scores. Although individual variation was seen in mice of all treatment groups, following a second dose, clinical scores of low-dose aspirin-treated group decreased, compared with infected only and high dose aspirin groups, which continued to increase (Figure 4.2B; p<0.05, one-way ANOVA).
**Figure 4.2**: Wild-type mice with *S. aureus* sepsis demonstrate improvements in A) weight and B) clinical score following low-dose aspirin therapy. C57BL/6 Mice were weighed and clinical scores determined at T0 and every 6 h until 48 h post-infection. Infected control mice and high-dose aspirin-treated mice exhibited significant weight loss; low-dose aspirin-treated mice regained weight and were equivalent to naïve mice by 48 h. Clinical scores of infected control mice and high-dose aspirin-treated mice increased significantly; clinical scores of low-dose aspirin-treated mice decreased following a second dose of aspirin. Weight data are expressed as percentage change from start weight; Clinical scores are expressed as range between 0-4 (according to criteria in Table 2.3), and all data represent mean ± SEM (n=3-15 per group/time-point, one-way ANOVA, *p<0.05).
4.3.2.2. Bacterial load of *S. aureus* in blood and organs

The bacterial burden was enumerated in mice to determine whether aspirin treatment improved clearance of bacteria from blood and organs. Evidence from a previous study suggests that wild-type C57BL/6 mice are more resistant to infection with *S. aureus*, than other strains [241], and this may be why a relatively high dose of $10^7$ was required to induce observable symptoms of sepsis.

Blood was diluted 1:2 and plated onto BHI agar plates. No viable bacteria were measured in the blood or organs of naïve mice (data not shown). Within 12 h of being infected with $10^7$ CFU *S. aureus*, all mice showed a minimum 3-fold drop in the number of viable bacteria in the bloodstream. Decreases in bacterial load continued, with lowest counts observed at 48 h post-infection (Figure 4.2A).

In order to determine the extent of the systemic spread of bacteria to organs, homogenates of spleen, kidney and livers were diluted and plated onto BHI agar plates. Initial counts at 12 h post-infection in organs showed the highest level of bacterial burden with colony-forming units (CFU) in spleen, comparable with the initial inoculum. Following this time-point there was a gradual decrease in bacterial counts retrieved (as seen in the blood), suggestive of bacterial clearance.

No significant differences were seen between treatment groups at either 12 or 24 h post-infection (Figure 4.3B-D). Remarkably, at 48 h post-infection low-dose aspirin-treated mice showed a 4-5 log reduction in bacterial count with complete clearance of viable bacteria observed in all target organs assessed, while infected only controls and high-dose aspirin-treated mice still had detectable bacterial counts (Figure 4.3; p<0.05).
Chapter 4

A

Blood

Infected Only (n=5)
Infected + 1mg/kg aspirin (n=4-5)
Infected + 10mg/kg aspirin (n=4-6)

B

Spleen

Infected Only (n=5)
Infected + 1mg/kg aspirin (n=4-5)
Infected + 10mg/kg aspirin (n=4-6)

C

Kidney

Infected Only (n=5)
Infected + 1mg/kg aspirin (n=4-5)
Infected + 10mg/kg aspirin (n=4-6)

D

Liver

Hours after infection with S. aureus

* Indicate significant difference
Figure 4.3: Effective clearance of *S. aureus* from organs in low-dose aspirin-treated mice by 48 h post-infection. **A** Blood was diluted two-fold and plated onto BHI agar plates determine colony-forming units (CFU) normalized for volume (mL). **B** Spleen, **C** kidney, and **D** liver homogenates were ten-fold diluted and plated on BHI agar plates to determine CFU/g tissue. At 48 h post-infection, low-dose aspirin-treated mice showed complete clearance of viable bacteria in the target organs sampled compared with infected control and high-dose aspirin-treated mice. Data represent mean ± SEM, (n=4-5 per group/time-point, two-tailed Mann-Whitney test *p*<0.05).
4.3.2.3. Assessment of organ damage in *S. aureus* infected and aspirin-treated C57BL/6 mice

4.3.2.3.1. Histological scoring

The extent of damage sustained by organs induced by systemic staphylococcal infection in all treatment groups was determined 48 h post-infection. Spleens of naïve, infected and aspirin-treated mice (n=3-5 per group) were stained with haematoxylin and eosin (H & E). When compared with the naïve mice (Figure 4.4A), no significant increase in inflammation or organ damage was seen in any of the treatment groups, with all spleen sections receiving scores of 0 for all parameters (Figures 4.4B-D). From bacterial load results (Figure 4.3), remaining bacteria in infected only mice and high-dose aspirin-treated mice at 48 h post-infection did not appear to induce extended tissue damage, and no differences were seen compared with low-dose aspirin-treated mice that demonstrated complete bacterial clearance at this time-point. Attempts to perform histological analyses of other organs were unsuccessful, as incorrect freezing technique caused tissue damage.

4.3.2.3.2. Immunolocalisation of *S. aureus* and inflammatory cells in spleen tissue

To confirm the presence of bacteria and immune cells in organs 48 h after systemic staphylococcal infection, spleen sections were analysed by immunofluorescence (IF) microscopy. Spleen sections from naïve (n=3), infected (n=5) and aspirin-treated mice (n=4), at 48 h post-infection were stained for the localisation of *S. aureus*, as well as monocyte/macrophage surface marker CD11b. Representative images are shown in Figure 4.5. Infected control and high-dose aspirin-treated mice had positively stained *S. aureus* at 48 h post-infection, while low-dose aspirin-treated mice did not have any detected *S. aureus* staining, comparable with naïve control mice. These data were reflective of bacterial loads retrieved from spleen of low-dose aspirin-treated mice (Figure 4.3). Staining of *S. aureus* within the spleen showed no specific localization patterns, with bacteria visible in all areas of the spleen. CD11b*+* cells were detectable in all treatment groups, and co-localisation of CD11b*+* cells and *S. aureus* was visible in infected and high-dose aspirin-treated groups, suggestive of leukocyte-mediated bacterial clearance. These leukocyte populations were also further assessed by flow cytometry analysis in both blood and spleen populations (see section 4.3.2.7).
Figure 4.4: No evidence of tissue damage in spleen of C57BL/6 mice with *S. aureus* sepsis. Haematoxylin and eosin (H & E) stained sections of spleen were observed under light microscopy. Representative sections from A) naïve, B) infected only, C) infected and low-dose aspirin and D) infected and high-dose aspirin. Scale bar: 50 µm. Original magnification: 10x.
Figure 4.5: *S. aureus* and CD11b⁺ cells are co-localised in spleen at 48 h post-infection. Representative images showing the detection of *S. aureus* (red) and CD11b (green), in mouse spleens. Infected only and high-dose aspirin-treated mice had detectable *S. aureus* in spleen. Low-dose aspirin-treated mice had no detectable *S. aureus* in the spleen. Scale bar: 50 µm. Original magnification: 40x. Arrows indicate immunoreactivity for *S. aureus*. 
4.3.2.4. Quantification of plasma 15-epi-LxA₄ in mice with sepsis

Plasma 15-epi-LxA₄ was used as a surrogate marker of resolving inflammation. In this study, proteins and lipids were enriched in plasma from naïve mice, infected only controls and infected mice treated with either low or high-dose aspirin, and levels of 15-epi-LxA₄ were analysed using a quantitative ELISA. Concentrations were analysed for individual mice and mean values were expressed in ng/mL derived from standard curves (as described in section 4.2.3). All infected mice tended to have increasing levels of 15-epi-LxA₄ compared with naïve mice, however no significant differences in 15-epi-LxA₄ levels were seen between infected only controls and aspirin-treated mice (Figure 4.6).

4.3.2.5. Determination of NFκB activation

In order to determine whether the inhibition of NFκB activation observed in aspirin-treated patients in clinical study (see Chapter 3) also occurs in the mouse model of systemic infection, nuclear extracts from PBMC pellets from whole blood of naïve, infected and aspirin-treated mice were analysed using a p65 specific sandwich ELISA. Chemiluminescent detection was used to measure levels of activation of the p65 subunit of NFκB, and mean values of luminescence were expressed as a percentage of normalised start value. When compared with naïve mice, all infected and aspirin-treated mice showed increase in NFκB p65 activation by 24 h post-infection (Figure 4.7). Of interest, high-dose aspirin-treated mice showed a significant increase in NFκB p65 activation, compared with naïve mice (Figure 4.7; p<0.05). At 12 h post-infection, no significant differences were observed between infected only mice and low-dose aspirin-treated mice.
Figure 4.6: Levels of 15-epi-LxA₄ detected in plasma of C57BL/6 mice with sepsis were not affected by aspirin treatment. Levels of plasma 15-epi-LxA₄ were measured by immunoassay at baseline (0 h), 12 h and 24 h post-infection. All data are represented as mean ± SEM, (n=3-5 mice per group/time-point).
Figure 4.7: Low-dose aspirin did not significantly affect activation of NFκB in nuclear extracts of C57BL/6 mice with sepsis. Nuclear extracts from naïve, infected and aspirin-treated mice at baseline (0 h), 12 h and 24 h post-infection were analysed for activation of the p65 subunit of NFκB. High dose aspirin-treated mice showed significantly increased NFκB activation, compared with naïve mice. All data are represented as mean ± SEM and expressed as percentage change from (0 h) time-point (n=3-5 per group/time-point, two-tailed Mann-Whitney test, *p<0.05).
4.3.2.6. Levels of inflammatory cytokine production

Analysis of 13 different cytokines in the plasma extracted from mice with systemic staphylococcal infection was performed using the bead-based sandwich LEGENDplex™ immunoassay. The concentrations of IL-1α, IL-1β, IL-6, IL-10, IL-12p70, IL-17A, IL-23, IL-27, MCP-1, IFN-β, IFN-γ, TNF-α, and GM-CSF were assessed and the mean values expressed as pg/mL, derived from standard curves performed with corresponding recombinant mouse cytokine standards (described in section 4.2.5).

While no significant differences were observed between infected or treatment groups for IL-1β production, individual mice showed some increased production; with a single mouse in the infected only group (366.33 pg/mL) at 24 h post-infection, and a single mouse in the low-dose aspirin-treated group (439.26 pg/mL) at 48 h post-infection (Figure 4.8A). A significant increase in the production of IL-6 was seen at 24 h post-infection, in the infected only (mean concentration of 721.87 pg/mL) and aspirin-treated mice (low-dose and high-dose aspirin-treated mice 508.87 pg/mL and 1,019.78 pg/mL, respectively), compared with the naïve mice (17.23 pg/mL) (Figure 4.8B). Mean concentrations of IFN-γ and MCP-1 were also elevated at the 24 h time-point, but there was considerable variation between individuals, only MCP-1 in infected only mice showed significant differences compared with naïve mice. Similar to IL-1β production, levels of GM-CSF or TNF-α did not differ. Interestingly, the same mice that showed increases in IL-1β levels also showed similar increases in detected levels of GM-CSF and TNF-α (Figure 4.8C and 4.8F).

No significant differences were observed in plasma levels of IL-1α, IL-10, IL-12p70, IL-17A, IL-23, IL-27, or IFN-β in infected or aspirin-treated mice compared with naïve mice at either 24 h or 48 h post-infection, however responses for cytokines varied with individual mice from each group exhibiting some increased production (Figure 4.9 and 4.10). One mouse in the low-dose aspirin-treated group showed increased levels of IL-1α (483.26 pg/mL), IL-10 (1183.51 pg/mL) and IFN-β (48.44 pg/mL) compared with plasma levels of naïve mice at 48 h post-infection.
Figure 4.8: Plasma IL-6 is significantly increased in wild-type mice with sepsis. Levels of (A) IL-1β, (B) IL-6, (C) GM-CSF, (D) IFN-γ, (E) MCP-1 and (F) TNF-α in the plasma of mice at 24 h and 48 h post-infection. Data are represented as circular symbols for individual mice with the bar mean ± SEM, (n=3-5 per group/time-point, two-tailed Mann-Whitney test, *p<0.05).
Figure 4.9: Individual mice with sepsis showed increases in IL-12 related cytokines in plasma. Levels of: A) IL-12p70, B) IL-17A, C) IL-23 and D) IL-27 at 24 h and 48 h in the plasma of mice at 24 h and 48 h post-infection. Data are represented as circular symbols for individual mice with the bar mean ± SEM, (n=3-5 per group/time-point).
Figure 4.10: Individual mice with sepsis showed increases of pro-inflammatory and anti-inflammatory cytokines in plasma. Levels of: A) IL-1α, B) IL-10 and C) IFN-β in the plasma of mice at 24 h and 48 h post-infection. Data are represented as circular symbols for individual mice with the bar mean ± SEM, (n=3-5 per group/time-point).
4.3.2.7. Analysis of leukocyte/lymphocyte populations in *S. aureus* infected and aspirin-treated C57BL/6 mice

In order to determine whether aspirin treatment has an effect on immune responses in mice with *S. aureus*-induced sepsis, flow cytometry analysis on whole blood and spleen was performed. Following preparation of single cell suspensions, cells were stained with the appropriate antibodies and immune cell subsets were quantified. For all comparative flow cytometry analyses; the same antibodies, voltage settings on the flow cytometer (FACS Canto II) and gating strategies were used. The gating strategies for the FACS experiments are shown in Figure 4.11. In the CD3⁻ population, monocytes/macrophages were defined as CD11b⁺, Ly6G⁻, and neutrophils as CD11b⁺, Ly6G⁺ populations. CD3⁺ T lymphocytes were defined as either CD4⁺ or CD8⁻ T lymphocytes (Figure 4.12).

Proportions of monocyte/macrophage and neutrophil populations in C57BL/6 mice were analysed in blood and spleen at 12, 24 and 48 h post-infection (see Figure 4.13). All mice showed moderate decreases in the proportion of monocytes/macrophages in blood by 48 h post-infection, however no significant differences were seen cell proportions in either blood or spleen, compared with naïve mice.

T lymphocytes are important for regulation of innate immune response [36], however there is debate regarding the role of T lymphocytes in early sepsis (within the first 48 h following onset of symptoms). Here, the proportions of CD4⁺ and CD8⁻ T lymphocytes in whole blood and spleen were analysed at 12, 24 and 48 h post-infection. No significant differences in the proportions of circulating CD4⁺ or CD8⁻ T lymphocytes was observed in infected and aspirin-treated mice, compared with naïve mice. In contrast, proportions of splenic CD4⁺ T lymphocytes decreased at times in low-dose aspirin-treated mice, compared with naïve mice (Figure 4.14; p<0.05, one-way ANOVA), and splenic CD8⁺ T lymphocytes decreased at times in infected control mice, compared with naïve mice (p<0.05, one-way ANOVA).
Figure 4.11: Gating strategy for analysis of immune cell subsets in blood and spleen of C57BL/6 mice. Representative FACS plots of cells isolated from the spleen or whole blood of naïve, infected and aspirin-treated mice over a period of 48 h. Approximately $10^5$ events were collected. The leukocyte gate was set using side scatter (SSC-A) and forward scatter (FSC-A) profiles (left panel). Forward height (FSC-H) and forward area (FSC-A) parameters were used to identify single cells (middle panel). Histograms were used to define CD3$^-$ or CD3$^+$ (right panel).
Figure 4.12: Definition of leukocyte sub-populations in blood and spleen of C57BL/6 mice. In the CD3- population, monocytes/macrophages were defined as CD11b+, Ly6G-, and neutrophils as CD11b+, Ly6G+ populations (Left panel). CD3+ T lymphocyte, CD4+ and CD8+ cell populations (right panel).
Figure 4.13: Proportions of circulating and splenic monocyte/macrophage and neutrophil populations were not significantly affected by infection or aspirin therapy. Neither infection nor aspirin treatment had an effect on the percentage of CD11b−Ly6G− monocytes/macrophages or CD11b+Ly6G+ neutrophils in either the blood or spleen of mice, compared with naïve mice. Data are represented as mean ± SEM, and expressed as a percentage of total events collected though the leukocyte gate (n=3-6 per group/time-point).
Figure 4.14: Proportions of circulating CD4⁺ or CD8⁺ T lymphocytes were not significantly affected by infection or aspirin treatment. In contrast, low-dose aspirin-treated mice had significantly reduced splenic CD4⁺ T lymphocytes, compared with naïve mice. Infected control mice showed a significant decrease in proportion of CD8⁺ T lymphocytes in spleen, compared with naïve mice. Data are represented as mean ± SEM, and expressed as a percentage of total events collected though the leukocyte gate (n=3-6 per group/time-point, one-way ANOVA, *p<0.05).
4.3.2.7.1. Markers of activation and migration markers

Cell surface activation and migration markers were analysed, to determine whether aspirin therapy might inhibit migration of lymphocytes to tissues limiting inflammatory damage. L-selectin is a well known cellular activation and migration marker expressed on the surfaces of all leukocytes. PSGL-1 (described in Chapter 3) is also expressed on the surface of leukocytes and facilitates migration into inflamed tissues [481, 507].

No significant differences in the proportion of circulating L-selectin\(^+\)CD11b\(^+\)Ly6G\(^-\) monocytes/macrophages were seen at 24 h post-infection, compared with the naïve group (Figure 4.15A, left panel). In contrast, all infected and aspirin-treated groups showed significant increases in proportions of L-selectin\(^+\) monocytes/macrophages, compared with the naïve group (Figure 4.15B, left panel; \(p<0.05\)), with a small non-significant decrease observed with low-dose aspirin. High-dose aspirin-treated mice showed significant increases in proportions of L-selectin\(^+\)CD11b\(^+\)Ly6G\(^+\) neutrophils in blood, compared with infected mice (Figure 4.15A, middle panel; \(p<0.05\)), however no significant differences were seen compared with naïve mice. In spleen, neither infection nor aspirin therapy affected proportions of L-selectin\(^+\) neutrophils compared with the naïve group (Figure 4.15B, middle panel). Proportions of L-selectin\(^+\)CD3\(^+\) T lymphocytes were low in all of the groups; however all infected mouse groups had increased proportions in blood compared with naïve mice (Figure 4.15A, right panel; \(p<0.05\)). Splenic proportions of L-selectin\(^+\)CD3\(^+\) T lymphocytes were significantly higher in high-dose aspirin-treated mice, compared with naïve group (Figure 4.15B, right panel; \(p<0.05\)).

Proportions of PSGL-1\(^+\)monocytes/macrophages in blood at 24 h post-infection were decreased in infected control and low-dose aspirin-treated groups, compared with the naïve mice (\(p<0.05\); Figure 4.16A, left panel). Splenic proportions of PSGL-1\(^+\) monocytes/macrophages were also decreased in infected only group compared with naïve mice (Figure 4.16B, left panel; \(p<0.05\)). No significant differences were observed in the proportion of PSGL-1\(^+\)neutrophils, consistent with what was seen in patients in the previous chapter study (Figure 4.16, middle panel). Similarly, PSGL-1 expression on CD3\(^+\) T lymphocytes was not affected by infection or aspirin therapy (Figure 4.16, right panel).
In summary, neither bloodstream infection with \textit{S. aureus}, nor aspirin treatment significantly impacted proportions of circulating or splenic monocyte/macrophage or neutrophil populations. In contrast, infection with \textit{S. aureus} decreased proportions of splenic CD8$^+$ T lymphocytes and low-dose aspirin decreased splenic CD4$^+$ T lymphocyte proportions.

Proportions of circulating L-selectin$^+$ monocytes/macrophages were not affected by bloodstream infection or aspirin therapy, however infection lead to increased L-selectin expression on splenic monocytes/macrophages. High-dose aspirin-treated mice had increased L-selectin$^+$ neutrophils, compared with infected mice. PGSL-1 expression was reduced on monocytes/macrophages in blood of infected mice and aspirin increased this. Taken together with the observation that low dose aspirin-treated mice had cleared bacteria by 48 h post-infection, these relatively small shifts in cell populations are reflections of the impact of aspirin in inflammatory response.
Figure 4.15: Proportions of L-selectin positive monocytes/macrophages, neutrophils and CD3+ T lymphocytes in A) blood and B) spleen of C57BL/6 mice, 24 h post-infection with S. aureus. Data are represented as circular symbols for individual mice with the mean ± SEM, and expressed as percentage of L-selectin positive cells, (n=3-6 per group, two-tailed Mann-Whitney test, *p<0.05).
Figure 4.16: Proportions of P-selectin Glycoprotein Ligand 1 (PSGL-1) positive monocytes/macrophages, neutrophils and CD3+ T lymphocytes in A) blood and B) spleen of C57BL/6 mice, 24 h post-infection with S. aureus. Data are represented as circular symbols for individual mice with the mean ± SEM, and expressed as percentage of positive cells (n=3-6 per group, two-tailed Mann-Whitney test, *p<0.05).
4.4. Discussion

The limited number of published studies investigating aspirin treatment in animal models of inflammation report encouraging results, with increased survival seen in a canine model of endotoxemia with 40-100 mg/kg aspirin for example [508]. Similarly, in mouse models of systemic inflammation both treatment with aspirin or epimeric lipoxins (like 15-epi-LxA4) resulted in attenuation of lung and kidney injury [214, 509] and had protective effects in a gram-negative induced peritonitis [17]. As *S. aureus* is a frequent cause of sepsis in a clinical setting [4], this study aimed to determine whether aspirin treatment (low-dose or high dose) contributes to resolution of inflammatory conditions in wild-type C57BL/6 mice with *S. aureus*-induced sepsis. An additional aim was to develop a mouse model that will closely mimic human sepsis and facilitate future detailed analysis in target organs. C57BL/6 mice were selected for use in this study, as they are less susceptible to systemic infection with *S. aureus* [241], therefore providing a robust model for the analysis of immunological responses in the early development of sepsis.

The primary read-outs from this study were CFU of *S. aureus* in organs and body weight (as a surrogate of overall condition). To dissect pathways that may be involved in reductions in inflammation, plasma 15-epi-LxA4 levels, NFκB activation in PBMCs, cytokine profiles and immune cell populations were all measured.

The results from the primary read-outs show that that low-dose aspirin therapy does improve outcomes in C57BL/6 mice with *S. aureus* sepsis. Low-dose aspirin-treated mice recovered more quickly from weight loss, had reduced disease severity (clinical scores) (Figure 4.2) and remarkably, a complete clearance of detectable bacteria from organs by 48 h post-infection (Figure 4.3 & 4.5). Interestingly, and unlike the clinical study neither 15-epi-LxA4 production nor NFκB inhibition were significantly impacted in infected mice, regardless of aspirin therapy (Figure 4.6 and Figure 4.7, respectively). Low-dose aspirin therapy appears to enhance bacterial clearance and inflammation by other means, presumably by affecting immune function. Flow cytometry analysis did reveal impacts of low dose aspirin on monocytes/macrophage and neutrophil populations, particularly on expression of markers of activation and migration (Figure 4.14 and 4.15).
One of the difficulties encountered in this study of the mouse model of sepsis was the difficulty in identifying the signs of sepsis. While monitoring was performed according to accepted criteria (see Table 2.2), and body temperature was also recorded, weight loss was the only consistent indicator that correlated with disease severity. This is likely to be in part due to the instinctive behavior or rodents to avoid showing any signs of weakness that might alert a predator. This clinical signs and inflammatory changes in this study were consistent with early development of sepsis following bloodstream infection. As this model represents early stages of sepsis, it is likely that symptomatic responses had not yet fully developed. Low-dose aspirin-treated mice did start regaining weight following the second aspirin dose, returning to start weight values comparable with naïve mice indicating improved overall clinical condition. At 48 h post-infection these mice also demonstrated complete clearance of *S. aureus* in all organs sampled (Figure; p<0.05).

C57BL/6 mice were shown in a previous study to have an enhanced capacity to limit bacterial growth in target organs following intravenous infection with *S. aureus*, compared with other more susceptible strains of mice [241]. While this could explain why in the present study histological analysis of the spleen revealed no obvious organ damage, despite high bacterial loads (Figure 4.3). It is more likely that this model represents early stages of sepsis development and 48 h post-infection may have been too early for damage to be apparent. These findings, along with decreases in bacterial load, raised the question of the role of innate immune responses in mediating enhanced bacterial clearance. Immunohistochemistry revealed monocytes/macrophages/neutrophils (CD11b+) co-localised with *S. aureus*, supporting their role in clearance. Consistent with the bacterial load data, no *S. aureus* was detected in spleens of low-dose aspirin-treated mice.

The 1 mg/kg dosage given to mice (equivalent to 81-100 mg daily doses administered in published clinical studies proposing that) is hypothesised to induce the increase 15-epi-LxA₄, leading to activation of monocyte/macrophage populations with the understanding being that this activation may enhance phagocytosis [446, 484], and similarly reduces influx of neutrophils associated with inflammation and damage [19]. While the 10 mg/kg dose is only 10 times greater, its equivalent dosage in humans would be ~1000 mg daily dose, closer to the dosage administered for pain relief. At this dose, it is not anticipated that the resolving pathways are activated (as this has only been shown
with low doses), and therefore not activate the proposed phagocytosis and clearance of bacteria.

The results of our human randomized controlled clinical study described in Chapter 3 showed moderate increases in plasma 15-epi-LxA₄ production and decreases in NFκB activation in blood of 100 mg aspirin-treated patients with sepsis. Although small increases in plasma 15-epi-LxA₄ production were seen in all infected groups of mice in this study, the overall levels were lower compared with levels seen in patients in the previous chapter, and no apparent effect of aspirin was noted. Post-treatment with LxA₄ and 15-epi-LxA₄ have also been linked to better outcomes in numerous animal sepsis models [17, 510]. Mice with gram-negative sepsis showed reduced bacterial load and improved survival when treated with a combination 15-epi-LxA₄ and ceftadizime therapies [17].

While this differed with results observed in our clinical study, it is comparable with data reported in studies investigating lipoxin production in other rodent models of infection, even with aspirin doses 10 times greater than in this study [214]. In a previous study of LPS treated mice, 15-epi-LxA₄ was detected in lung fluid but not blood after treatment with 10 mg/kg aspirin, the maximum dose used in the present study. Indeed, significant increases in plasma 15-epi-LxA₄ have been observed in mice when treated with doses of 200 mg/kg of aspirin or greater [18]. Similar to what has been shown in both trial participants in Chapter 3 and other published clinical studies, infected only mice in this study did produce detectable levels 15-epi-LxA₄ in the absence of aspirin, with 48 h levels comparable with levels seen in trial participants. While the source of this 15-epi-LxA₄ is unclear, these results confirm that inflammation alone can moderately increase 15-epi-LxA₄ production in the absence of aspirin [19, 214].

It is possible that the reason for the lack of aspirin-dependent responses observed in this study is due to the time frame over which the mice were sampled for analysis. This animal model of sepsis was designed following analysis of the first batch of patients from the MATHS trial, where interim results showed changes in primary parameters of 15-epi-LxA₄ and NFκB occurring later (8 h following aspirin). This provided the basis for the 12, 24 and 48 h time-points selected in the current study. However, these time-points
are no longer reflective of patient response times (~1-8 h aspirin-induced response) and as such, the peak increases for both of these parameters will likely have been missed.

While patients enrolled in the clinical study have sepsis on entry to the trial, mice in this study were infected at T0 and did not have pre-existing bacteremia at the time of the first aspirin dose. This will likely have affected the responses, with baseline concentrations of 15-epi-LxA4 lower in mice, compared with patients (Figures 4.6 and 3.2, respectively). Plasma 15-epi-LxA4 production was shown to moderately increase within 1 h of aspirin therapy in sepsis patients in Chapter 3. The kinetics of 15-epi-LxA4 synthesis in organs that are the primary site of inflammation may differ from that in plasma, and warrant further investigation in future studies. In a recent mouse study, levels of 15-epi-LxA4 were shown in lungs to increase as rapidly as 15-30 min following aspirin treatment, while plasma levels did not change [214]. Future studies should investigate the production of 15-epi-LxA4 in plasma at earlier time-points following aspirin administration, as well as evaluating 15-epi-LxA4 production in target organs; to determine whether the aspirin-specific response occurs rapidly as seen in patients (Chapter 3). Further, 15-epi-LxA4 production has been linked to the inhibition of NFκB [216, 480, 493].

Infected and aspirin-treated mice showed increased activation of NFκB, suggesting the progression of inflammatory signalling, but again no effect of aspirin was shown. Interestingly, high-dose aspirin-treated mice (equivalent to 1000 mg daily dose) showed the highest proportional increase in NFκB activation, contradicting what has been shown previously with similar doses in both in vitro and ex vivo models of inflammation [18, 432, 433].

During sepsis, inflammation is regulated by transcriptional activation of pro-inflammatory cytokines and mediators that contribute to prolonged inflammation and organ damage [124, 511]. The cytokines IL-1β, IL-6, IFN-γ and TNF-α have been studied in a number of other reports on mouse models of sepsis. In an attempt to identify new possible biomarkers, and markers of aspirin treatment, a wider panel of cytokines was chosen. A significant increase in plasma levels IL-6 was seen in all infected and aspirin-treated mice (p<0.05), however no effect of aspirin was shown. A number of biological roles have been described for IL-6 in sepsis, including controlling the induction of acute-
phase responses and temperature regulation [126]. Body temperatures were measured in all mice during the experiment using an infrared thermometer, but measurements were highly variable and this parameter was not a clear measure of sepsis progression in this model (data not shown), and no correlations could be made with levels of IL-6 measured. IL-6 production has also been used a prognostic marker for outcome in sepsis patients, and has been previously suggested as a diagnostic biomarker for bacteraemia (as discussed in section 1.2.4). IL-6 production in infected mice in this study remained raised even 48 h following infection, allowing for clear differentiation with naïve mice, and therefore in this model IL-6 could be used in the confirmation of bacterial sepsis in future animal studies. Small increases were also observed with chemokine MCP-1 (Figure 4.8) in infected mice at 24 h, further confirming systemic inflammation in these mice. Of the other cytokines analysed, individual responses varied, however mice in infected control group displaying increased levels of plasma cytokines at 24 h also had higher bacterial counts in organs, consistent with systemic inflammation. While IL-1β and TNF-α are two of the most documented cytokines involved in the sepsis inflammatory cascade [512, 513], no significant increase was observed for either cytokine in plasma of infected or aspirin-treated mice. The lack of differences observed in these cytokines could be due to the time frame of sampling for analysis, the production of different cytokines will occur at various stages of acute sepsis progression [514]. Additionally, the half-lives of cytokines are predominantly short in vivo and cytokines are often restricted to local site of production. It would be of interest in future studies to measure local cytokine production, in target organs following inflammation, to determine if there is any aspirin-related effect. Early response cytokines IL-1β and TNF-α are involved in the initiation of the sepsis inflammatory cascade, the results from 24 to 48 h time-points analysed in this study also suggest that infected mice were in between early and late phases of inflammation [515].

Aspirin only has documented antibacterial effects in high doses. Therefore, It is unlikely that the clearance of bacteria from organs was due to a direct antibacterial effect of aspirin therapy [17, 218, 516]. Our flow cytometry analysis focused on monocyte/macrophage and neutrophil populations as effector populations for bacterial clearance. Although there was no significant difference in the proportions of monocytes/macrophages (CD11b+ Ly6G−), or neutrophils (CD11b+ Ly6G+) in blood or spleen compared with naïve mice, a general trend in decreased monocyte/macrophage proportions in blood was noted over the course of the experiment. The most likely
explanation for this is that following activation monocyte/macrophage populations are migrating to target tissues in order to mediate bacterial clearance.

Activation and tissue migration markers L-selectin and PSGL-1 were analysed on leukocyte surfaces in blood and spleen of mice. L-selectin expression on circulating monocytes/macrophages in this study were not significantly impacted by infection or aspirin treatment, however there were increased proportions of monocytes/macrophages expressing L-selectin in spleen of all infected mice at 24 h post-infection. This suggests that these cells were activated and targeted to enter circulation and other target organs. In contrast, decreased proportions of PSGL-1+ monocytes/macrophages in blood of all infected mice, which was moderately increased with aspirin (Figure 4.15A left panel), also suggesting these cells were more likely to be activated for recruitment to tissues.

These relatively small shifts in the monocyte/macrophage populations at 24 h post-infection in low-dose aspirin-treated mice led to complete clearance in bacteria from organs by 48 h, indicating that the migration and activation of this population are critical for their effector function. Further, decreases in L-selectin expression observed in low-dose aspirin-treated groups could be due to the ability of aspirin to induce L-selectin shedding, preventing neutrophil-endothelial interactions, and facilitate resolving inflammation as shown in previous studies [505, 517]. While the effects of infection on leukocyte PSGL-1 expression are limited, decreases of PSGL-1 expression observed in this study could be due to a similar shedding event as described for L-selectin, with similar reductions in inflammation proposed [518]. To ascertain whether this is the case, it would be necessary in future to determine levels of soluble L-selectin and PSGL-1 in plasma and tissues. Future studies should also consider the transcriptional analysis of L-selectin and PSGL-1 ligands on endothelial surfaces to determine whether surface expression of these markers on leukocytes is indicative of adhesion and migration. As PSGL-1 is a major ligand for L-selectin, it was also suggested that PSGL-1 and L-selectin form complexes on cell surfaces for effective leukocyte transmigration and thus expression of each marker could be dependent on the other [519].

Analysis of lymphocyte populations was less revealing, however the proportion of splenic CD4+ T lymphocytes was significantly reduced in low-dose aspirin-treated mice, compared with naïve mice (Figure 4.13; p<0.05, one-way ANOVA), and splenic CD8+
T lymphocytes were significantly reduced in infected control mice compared with naïve mice (Figure 4.13; p<0.05, one-way ANOVA).

The role of the adaptive immune response in the early stages of sepsis has been widely debated. Given that the current model represents early stages of sepsis, the lack of significant changes in T lymphocyte populations is consistent with previous studies suggesting that T lymphocytes are not critical for early sepsis responses [110, 520]. While significant increases in L-selectin expression was observed in all infected and aspirin-treated mice, the proportion of CD3⁺ (inclusive of both CD4⁺ and CD8⁺) T lymphocytes expressing L-selectin were lower by comparison with monocyte/macrophage and neutrophil cells, further confirming these cells are not critically involved in early immune responses in sepsis. Interestingly and unlike L-selectin, expression of PSGL-1 was high on CD3⁺ T lymphocytes in both blood and spleen for all mice (including naïve group). One study also recently showed that unlike monocytes/macrophages and neutrophils, PSGL-1 is expressed on T lymphocytes, but the modifying enzymes required for functional activity are not constitutively expressed [521]. Further analysis would need to be performed to determine whether PSGL-1 on CD3⁺ T lymphocytes is functionally active in this model.

A number of differences in responses were seen between patients and mice in this sepsis model (due to time frame of infection, modes of infection, progression of infection prior to treatment with aspirin, age differences). The results of our analysis suggest that while some similarities were observed with regular aspirin taking patients, this model of infection was not an accurate representation of the overall responses seen in other patient groups in the clinical study. In order to address these differences, future studies will aim to investigate a number of additional variables including: further adaptation to timelines for sampling to more closely mirror the response timeline with that of the patients.

Patients in the MATHS clinical trial (described in the previous chapter) were already identified as having sepsis prior to commencement of aspirin-therapy (with the exception of regular aspirin-takers), thus future models of infection should consider inclusion of a group that are infected for a minimum of 12-24 h to induce inflammatory response prior to administration of aspirin therapy. Another major difference between this model and the standard patient care for sepsis is the absence of antibiotic therapy. Any impacts on
immunological or clinical parameters in the current study were the effect of low-dose aspirin in the absence of antibiotic therapy. As the role of aspirin in the treatment of sepsis is to serve as an adjunct therapy, future studies will consider the combined use of aspirin and antibiotic therapy. Also, a high proportion of participants in the MATHS clinical trial presented with at least one pre-existing health condition that can increase likelihood of infection and sepsis development. Given this, future studies should investigate the parameters measured in this study in a variety of mouse strains (including diabetic and other immuno-compromised mice) to ascertain whether aspirin improves recovery or outcomes.

While there is evidence to suggest aspirin at all doses affects the GI tract and can cause adverse bleeding, the doses used in this study (equivalent with 100 mg and 1000 mg daily dose, administered for two days) were chosen to represent low- and medium-doses administered in humans, and delivered in 2 doses over a 48 h period. As such, it was not anticipated that adverse bleeding effects would be seen (based on the study by Winning et al. (2010) [63]). Indeed, aspirin given to mice at a 5 mg/kg daily dose for up to 5 days did not result in any adverse bleeding, in a previous study [522]. Having said this, the limitations of not having an aspirin only control should be considered, with clinical measurements of weight loss being a primary parameter, if aspirin did affect the GI tract of mice in this study, it may have contributed to early weight loss. It would be useful to include an aspirin-only control group in future studies to investigate whether this is indeed the case, and evaluate any other potential adverse effects of low-dose aspirin (such as bleeding), in the absence of infection. Further, aspirin alone was shown in healthy volunteers to impact the neutrophil-endothelial interactions by inducing premature shedding of activation marker L-selectin [505], it would also be worthwhile to confirm this in our mouse model. And while it is unclear what effects aspirin alone has on the surface expression of PSGL-1, an aspirin-only control group to investigate this in this mouse model should be considered. An aspirin-only control group could also serve as an added comparison, providing baseline responses for the anti-inflammatory effects of low-dose aspirin (15-epi-LxA₄ production, NFκB inhibition), in the absence of infection.
4.5. Conclusion

In this study, the effects of aspirin therapy on our inflammatory pathways of prior interest, (plasma 15-epi-LxA₄ and NFκB activation) seen in patients, were not observed in mice. It is however clear, that low-dose aspirin therapy had a beneficial effect in mice, with complete clearance of bacteria from organs shown in the absence of supportive antibiotic therapy.

Taken together, these results support the potential for low-dose aspirin as an adjunct therapeutic within this model of sepsis. Future studies will investigate the mechanism in which aspirin-therapy mediates clearance of bacteria from organs and will include a group of mice also receiving anti-microbial therapy, to better model the current therapies offered to patients with sepsis.
Chapter 5

Investigating the effects of aspirin treatment in *in vitro* and *in vivo* models of device-related biofilm infection
5.1. Introduction

Biofilm forming bacteria are implicated in a number of serious infectious diseases, and the use of indwelling medical devices such as intravenous and urinary catheters, as well as pacemakers and orthopaedic devices, present opportunity for planktonic nosocomial bacteria such as Staphylococcus species to attach and “switch” to biofilm forming phenotypes [5-8]. Staphylococcus aureus and Staphylococcus epidermidis are well-studied human pathogens. A common constituent of the nasal microflora, S. aureus has the potential to establish opportunistic infections upon disruption of epithelial barriers [523, 524]. Many staphylococcal species have the ability to form biofilms. The increased incidence of anti-microbial resistance, coupled with advances in medical care for severe illness, necessitating the use of indwelling medical devices. This can lead to both an increased likelihood of developing device-related biofilm infection, and risk of severe outcomes [525, 526].

Several bacterial factors are involved in the formation of S. aureus biofilms. Previous studies have shown the significance of the intracellular adhesion (ica) locus in the attachment of staphylococcal biofilms and production of the polysaccharide intracellular adhesion (PIA), the main contributor to total biofilm [270, 527, 528]. Quorum-sensing molecules including the accessory gene regulator locus (agr) [337, 338], staphylococcal accessory regulator (SarA) [339, 340] and transcription factor Sigma B (σB) [529, 530] are involved in regulating the formation of staphylococcal biofilms. Cell death, lysis and subsequent release of genomic DNA during biofilm development in S. aureus is highly regulated by cidA (murein hydrolase regulator with other functions in reducing antibiotic tolerance) [531]. Analysis of biofilm structure has also showed that extracellular DNA (eDNA) is abundant in staphylococcal biofilms, similarly described in biofilms of Pseudomonas aeruginosa (P. aeruginosa). Extracellular DNA contributes to the integrity of the biofilm structure and also in resistance to conventional antibiotic therapy [532]. Exposure to DNases results in diminished eDNA in the biofilm matrix (in both S. aureus and other organisms) contributing to decreased biofilm stability [328, 533] and adherence [534] in vitro. This effect also translates to the clinical setting, where recombinant human DNAses have been successfully used in conjunction with antibiotic therapy to treat cystic fibrosis patients with P. aeruginosa infections [535]. Similarly, recombinant human
DNase therapy was shown to be effective in diminishing eDNA in biofilms isolated in young children with recurrent otitis media [536].

As discussed in section 1.3.5.3, biofilm infections are difficult to treat, particularly in the case of prostheses such as hip joints, and there is a need for new therapeutic options [537-539].

Salicylic acid (SA), a major metabolite of aspirin inhibits NFκB and other intracellular signalling pathways in mammalian cells [61, 432]. Salicylic acid has anti-biofilm effects against other biofilm forming organisms [540, 541]. Treatment with SA in vitro was shown to inhibit biofilms of *P. aeruginosa* and species of *Candida, Salmonella* and *Streptococcus* [542][35-37]. The effects of SA inhibition on *P. aeruginosa* have been well studied, with flagella-mediated motility [319, 543] and quorum-sensing in biofilm regulation impacted [544]. Similarly, therapeutic doses of aspirin were shown to impact levels of secreted proteases in biofilms and planktonic cultures of *Candida spp* [541, 545, 546]. Recent evidence supporting the potential for SA inhibition of staphylococcal biofilms was shown in an *in vitro* model of infective endocarditis by Kupferwasser et al. [221]. Staphylococcal cultures treated with SA at therapeutic concentrations were significantly impacted via global regulatory pathways mediated by sarA, contributing to reduced bacterial adhesion and virulence [221]. The same group also reported that aspirin therapy impacted the ability of *S. aureus* to cause chronic bacterial endocarditis in an *in vivo* rabbit model [384].

The experimental approach of this chapter addresses the hypothesis that treatment with SA will impact staphylococcal biofilm formation *in vitro* by interfering with PIA production and eDNA release. Further, the effect of aspirin therapy on biofilm formation and inflammation *in vivo* was tested in a mouse model of staphylococcal device-related biofilm infection.
Chapter 5

The specific aims of experiments outlined in this chapter were:

- To determine whether SA treatment impacts \textit{in vitro} staphylococcal biofilm formation

More specifically:

  - The effect of SA treatment on polysaccharide production and bacterial viability
  - The effect of SA treatment on extracellular DNA release
  - The effect of SA treatment on the transcriptional regulation of biofilm-related target genes

- To investigate staphylococcal biofilms in a model of device-related infection in C57BL/6 mice

More specifically:

  - The effect of low-dose aspirin treatment on staphylococcal biofilm polysaccharide production on stainless steel and catheter implants
  - The effects of low-dose aspirin treatment on bacterial growth in infected sites surrounding stainless steel and catheter implants
  - The effects of low-dose aspirin treatment on both local and systemic inflammation induced by stainless steel and catheter implants
5.2. Materials & Methods

5.2.1. *In vitro* study

5.2.1.1. Bacterial strains

Strains of *S. aureus* and *S. epidermidis* used in this study are outlined in Table 2.1.

5.2.1.2. Culturing of bacterial biofilms with or without SA

Stock solutions of SA (1 mol/L) were prepared by dissolving lyophilised SA (Sigma-Aldrich) in 70% ethanol (Merck KGaA), and diluted 1:50 in Brain Heart Infusion broth (BHIB: Oxoid) to a final concentration of 20 mmol/L SA. Vehicle controls were prepared by diluting 70% ethanol 1:50 in BHIB to a final concentration of 1.5% (vehicle control). Bacterial cultures grown overnight at 37°C were diluted in BHIB containing 20 mmol/L SA or vehicle in 200 μL in a suspension of $10^5$ CFU. Negative control wells contained only BHI broth and either 20 mmol/L SA or vehicle. Plates were covered and incubated at 37°C for either 24 or 48 h.

5.2.1.3. Quantitative biofilm assay (crystal violet method)

Biofilm assays were performed using published methods [547] with minor modifications. Following incubation of the biofilm culture, the contents of each well were aspirated and washed three times with 200 μL tap water. Plates were stained with 200 μL aqueous crystal violet solution (2.3% (v/v): Sigma-Aldrich) for 15 min at RT. Excess stain was removed and wells were washed briefly with tap water twice. Plates were left to air dry at RT. Once dried, biofilms were resolubilised in 300 μL of 33% (v/v) glacial acetic acid (Merck KGaA). Crystal violet staining was quantitated by measuring absorbance at a wavelength of 595 nm using an iMark™ microplate reader (Bio-Rad, USA).

5.2.1.4. Bacterial viability analysis

Following incubation of biofilms, planktonic cells were removed and wells were washed twice with tap water as above. The adherent material in each well was re-suspended in 100 μL BHI broth after scraping and vigorous pipetting. A 20 μL aliquot of this suspension was 10-fold serially diluted in BHI broth and plated on BHI agar medium. Viable bacterial counts were performed following incubation 37°C for 24 h.
5.2.1.5. Confocal microscopy

Sterilised glass coverslips (13 mm round: ProSciTech) were placed in individual wells of a sterile 24-well plate. Following this 1 mL bacterial culture (diluted as described above) in BHIB containing 20 mmol/L SA (prepared as described in section 5.2.1.2) or an equal concentration of 70% ethanol (vehicle) was added to each well. Biofilms were incubated at 37°C as described in Table 5.1.

Table 5.1: Treatment groups for the qualitative analysis of 48 h bacterial biofilms by immunofluorescent confocal microscopy.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Media incubation (first 24 h)</th>
<th>Media Incubation (second 24 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>SA1698 or SE35984 biofilm in BHIB/vehicle&lt;sup&gt;a&lt;/sup&gt;</td>
<td>BHIB/vehicle replaced with fresh BHIB/vehicle</td>
</tr>
<tr>
<td>Group 2</td>
<td>SA1698 or SE35984 biofilm in BHIB/vehicle</td>
<td>BHIB/vehicle replaced with BHIB/SA&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group 3</td>
<td>SA1698 or SE35984 biofilm in BHIB/SA</td>
<td>BHIB/SA replaced with BHIB/vehicle</td>
</tr>
<tr>
<td>Group 4</td>
<td>SA1698 or SE35984 biofilm in BHIB/SA</td>
<td>BHIB/SA replaced with fresh BHIB/SA</td>
</tr>
</tbody>
</table>

<sup>a</sup> BHIB with vehicle control media equal concentrations as SA containing media

<sup>b</sup> SA in BHIB

Following incubation, excess culture media was aspirated and wells were washed twice in PBS (Bioline), then fixed in 4% neutral buffered formalin (Thermo Fisher Scientific) for 10 min. Biofilms were then permeabilised in 0.2% Triton X-100 (Sigma-Aldrich) for 10 min, and washed three times in PBS. Coverslips were then incubated on 25 µL of rabbit anti-<i>S. aureus</i> antibody (1:200; Abcam) in PBS/1% BSA for 60 min. Following incubation, coverslips were returned to the wells and washed three times in PBS/0.1% BSA, before being incubated on 25 µL of goat anti-rabbit Cy3 (1:1000; Abcam), and SYTOX Green nucleic acid direct stain (2 µmol/L) (Life Technologies) in PBS/1% BSA for 45 min. Following incubation coverslips were returned to the wells, washed three times in PBS/0.1% BSA, and once in sterile MQH2O. Coverslips were then mounted in Mowiol (prepared as described in section 2.4.3.2). Imaging was performed using a Nikon
A1 inverted confocal microscope and NIS Elements C control software (Nikon, Japan) using lasers at 405, 488, 561 nm. Images were overlayed for display using the Adobe Photoshop program (Adobe).

5.2.1.6. Quantitation of extracellular DNA (eDNA) release in biofilms

Biofilms of SA1698 and SE35984 were grown at 37°C for 24 h in 96-well plates in either BHIB containing 1, 5, 10 or 20 mmol/L SA or vehicle (prepared as described in section 5.2.1.2). Following incubation, the contents of each plate were aspirated and washed twice in PBS. Biofilms were then fixed in 4% neutral buffered formalin (Thermo Fisher Scientific) for 10 min and stained with SYTOX Green (2 µmol/L) in PBS (Life Technologies) for 45 min. Following incubation; biofilms were washed three times with PBS and the contents of each well were resuspended in 100 µL of PBS. Fluorescence absorbance was measured at a wavelength of 488 nm using the POLARstar Optima mult-detection microplate reader (BMGlabtech).

5.2.1.7. Gene expression analysis

5.2.1.7.1. Isolation of RNA from staphylococcal biofilms

Following incubation of bacterial biofilms, excess media was removed and RNA was isolated using the Isolate II RNA mini kit (Bioline) according to the manufacturers protocol with some minor adaptations. 100 µL TE buffer (10 mmol/L Tris-HCl (Merck KGaA), 1 mmol/L EDTA (Calbiochem) pH 8.0) containing 50 µg/mL lysostaphin (Sigma-Aldrich) and 3 mg/mL lysozyme (Sigma-Aldrich) was added to each well. Biofilms were scraped into the buffer to form a lysate and contents were transferred to a microcentrifuge tube and incubated at 37°C for 1 h. Next, 350 µL of Buffer RLY (Bioline) containing 1% β-mercaptoethanol (Bio-Rad) was added to each tube and vortexed vigorously for 5 s. The combined contents of each tube were transferred to an ISOLATE II filter (violet) in a 2 mL collection tube and centrifuged at 11,000 × g for 1 min at RT to reduce viscosity. Next, 350 µL of 70% ethanol was added to each lysate and mixed by pipetting, then contents were transferred to an ISOLATE II RNA Mini column (blue) in a 2 mL collection tube, which was centrifuged again at 11,000 × g for 30 s. The flow through was discarded and 350 µL Membrane Desalting Buffer (MEM) was added to the column, which was centrifuged again at 11,000 × g for 1 min. Samples were then washed by addition of 200 µL Buffer RW1, and centrifuged at 11,000 × g for
30 s. Next, 600 µL Buffer RW2 was added and centrifugation was performed as described above, followed by an additional wash in 250 µL Buffer RW2, and centrifuged at 11,000 × g for 2 min to dry the membrane completely. Columns were then placed into a new microcentrifuge tube and RNA was eluted in a total volume of 80 µL in nuclease-free water (elution was performed in two steps of 40 µL nuclease-free water). Contaminating DNases were removed using the TURBO DNA-free™ kit (Life Technologies) according to the manufacturers specifications. Concentration and purity of the total RNA were determined using the NanoDrop 2000™ (Thermo Fisher Scientific).

5.2.1.7.2. Reverse transcription of RNA

cDNA was synthesised from ~1 µg in vitro RNA using the Omniscript RT Kit (Qiagen, Australia) with 0.8 µg/µL Random Hexamers (Qiagen) according to the manufacturer's instructions. The cDNA was then precipitated by ethanol precipitation (as a clean-up step), and the resulting cDNA was then used as the template in a semi-quantitative SYBR green assay (qRT-PCR).

5.2.1.7.3. Ethanol precipitation of DNA

To purify and concentrate cDNA 2.5 volumes of ice-cold 96% (v/v) ethanol and 0.1 volume of sodium acetate were added to DNA preparations. The DNA was precipitated at -20°C for 1 h and pelleted by centrifugation (13,000 × g, 15 min, 4°C). After washing the pellet with 70% ethanol the reaction was centrifuged as above. The DNA pellet was dried in a laminar flow hood and resuspended in 10 µL nuclease-free water (Qiagen).

5.2.1.7.4. Semi-quantitative real-time PCR (qRT-PCR)

Real-Time semi-quantitative PCR analysis for the genes listed in Table 5.2, were performed using the Rotor-Gene Q real-time cycler and software (Qiagen) and 2× SensiFAST™ SYBR® Green master mix (Bioline), with cycling conditions according to the manufacturer’s specifications. PCR reactions contained 10 ng template DNA, 10 pmol oligonucleotides, and were performed in duplicate in a total volume of 20 µL. Each series contained a non-template control (NTC). All oligonucleotides were purchased from GeneWorks (GeneWorks, Australia) according to publications listed in Table 5.2, or were designed using Primer 3 plus software [548], on the basis of nucleotide sequences for
S. aureus genes of interest. RT-PCR conditions were as follows: denaturation 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, 60°C for 15 s and 72°C for 30 s. A final extension phase of 72°C for 7 min was used. The \textit{gyrB} (staphylococcal DNA gyrase subunit B) gene was used as reference gene for quantifications. Relative gene expression was calculated using the method previously described by Pfaffl [549] and expressed as fold change.
Table 5.2: Oligonucleotides used in quantitative real-time PCR in this study.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Type</th>
<th>Sequence</th>
<th>(T_m) (°C)</th>
<th>Amplicon Length</th>
<th>Function</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>gyrB</td>
<td>F</td>
<td>5’- GGTGGCGACTTTGATCTAGC-3’</td>
<td>54</td>
<td>170 bp</td>
<td>DNA gyrase subunit B (Reference gene)</td>
<td>[296]</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>5’- TTATACAACGGTGCTGTGC-3’</td>
<td>52</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>agr</td>
<td>F</td>
<td>5’-TCACAGACTCATTCATTGCCATT-3’</td>
<td>50</td>
<td>193 bp</td>
<td>Global gene regulator Virulence determinant (Pathogenesis)</td>
<td>[296]</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>5’-CAACGATGCATAGCAGTGTT-3’</td>
<td>52</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sarA</td>
<td>F</td>
<td>5’-TTTCGTTTGTTCAGTG-3’</td>
<td>48</td>
<td>398 bp</td>
<td>Global regulator of multiple virulence and biofilm related genes</td>
<td>[530]</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>5’-TCGAGCAAGATGCATCAA-3’</td>
<td>47</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sigB</td>
<td>F</td>
<td>5’-TTGATTCTTTGCCCCAGTTTC-3’</td>
<td>48</td>
<td>355 bp</td>
<td>Transcription factor affecting RNA Polymerase</td>
<td>[530]</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>5’-GCGAAAGAGTCGAAATCAGC-3’</td>
<td>52</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spa</td>
<td>F</td>
<td>5’-TATGCGCTTTTAAATGCTG-3’</td>
<td>46</td>
<td>119 bp</td>
<td>Cell surface associated virulence factor</td>
<td>[296]</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>5’-TTGGAGCTTGGAGTCATTTA-3’</td>
<td>48</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cidA</td>
<td>F</td>
<td>5’-ATGGATGTTGCTTCGAAAT-3’</td>
<td>48</td>
<td>166 bp</td>
<td>Membrane-associated murein hydrolase regulator</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>5’-GGCTTGACGTCAATCATCA-3’</td>
<td>50</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CcpA</td>
<td>F</td>
<td>5’-GTGTCGCGTTGTGTTAATGG-3’</td>
<td>52</td>
<td>190 bp</td>
<td>Catabolite control protein regulates polysaccharide production</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>5’-GTCCACGAGCAAGTGTGCA-3’</td>
<td>52</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
5.2.2. In vivo biofilm infection model

A mouse model of contaminated medical devices was established by implanting contaminated stainless steel (s/s) or catheter section implants in a subcutaneous pocket in C57BL/6 mice. Mice were monitored for 4 days. At the end of the experiment the implant site was sampled for bacteria and histology. Systemic inflammatory responses were also assessed.

Experiments involving animals were carried out with the approval of the RMIT Animal Ethics Committee (AEC Permit Number 1426). Six to eight-week-old female C57BL/6 mice were sourced from the Animal Resource Centre (ARC, Australia). Prior to experimentation, mice were acclimatised for a minimum of seven days within the RMIT Animal facility (RAF) (RMIT University). A maximum of four mice were housed in a single housing unit, mice were fed standard rodent chow and had access to sterilised-water *ad libitum*.

5.2.2.1. Contaminated implants

SA1698 was grown in BHIB at 37°C overnight. In this study, sterile 4 mm diameter surgical grade s/s balls (Bearings Australia, Australia) as a model of a contaminated metal prosthesis, or ~5mm sections of urinary catheter (Bard, Australia) were contaminated with SA1698. Implants were incubated at 37°C for 3 h in a 100 µL suspension of approximately $3 \times 10^5$ CFU SA1698, prior to implantation. Non-implanted s/s and catheter implants incubated with SA1698 were briefly sonicated and cultured to confirm the infective dose upon implantation.

5.2.2.2. Mouse implant model

Six mice per experimental group received either s/s ball to represent a prosthetic joint, or latex-coated catheter implants to represent an indwelling catheter, contaminated with SA1698 (see Figure 5.1). Mice were anaesthetised via intra-peritoneal injection of ketamine (100 mg/kg - Ketamav: Mavlab, Australia)/xylazine (10 mg/kg - Rompun: Bayer, Germany). Once mice were deeply anaesthetised, fur on the back of the mouse was shaved to expose the skin and washed with 80% ethanol. A 10 mm incision was made with a sterile scalpel and a sub-cutaneous pocket was introduced under the surface of the skin by inserting blunt end scissors to a depth of 10 mm.
SA1698 contaminated s/s or catheter implants inserted in skin pockets of mice

All mice received 10 mm incision on their back

Day 0 Day 1 Day 2 Day 3 Day 4

- Sham surgery
- S/s + S. aureus (+ aspirin)
- S/s + S. aureus (No aspirin)
- Catheter + S. aureus (+ aspirin)
- Catheter + S. aureus (No aspirin)

Figure 5.1: Timeline for mouse implant infection model. Female C57BL/6 mice were anaesthetized and had either s/s (n=6) or catheter implants (n=6) inserted into subcutaneous pockets of skin. Following surgery, half of the mice in each implant group (n=3 represented in red and blue symbols) received an oral dose of aspirin by gavage (1 mg/kg). Aspirin was administered once daily for a period of 4 days. Green and grey symbols represent mice with s/s and catheter implants (respectively) not receiving aspirin therapy. Sham surgery mice (represented in purple symbol) underwent surgery but did not receive an implant or aspirin therapy. Mice were weighed prior to surgery, and mice were weighed and monitored for signs and symptoms of inflammation twice daily. Monitoring was performed according to established criteria as outlined in the previous study (Table 2.2).
Contaminated implants were inserted into the subcutaneous pockets, incisions were closed with 2 sutures (Dysilk 12 mm black braided: Dynek Pty Ltd., Australia) and wounds were covered with standard adhesive dressing (Leukoplast®: Smith & Nephew, Australia). Aspirin (1 mg/kg, prepared as described in section 4.2.1.5, Sigma-Aldrich) was administered by oral gavage to half of the mice in experimental implant groups (n=3; see Figure 5.1). A separate group of mice underwent mock-surgery (n=3), whereby a 10 mm incision was made and closed with 2 sutures as described above, without an implant being inserted (sham surgery group).

5.2.2.3. Termination of experiment and sample collection

Mice were euthanised via CO₂ inhalation; blood was collected immediately by cardiac puncture into syringes pre-loaded with 200 µL sterile Alsever’s solution (prepared as described in section 2.4.2). Mice were transferred to the biosafety level 2 cabinet to minimise contamination with air microorganisms, swabs of contaminated exudate sites were taken and stored in BHIB for quantification of bacterial load and implants were removed for quantification of surface biofilm. Skin surrounding contaminated implants was removed for analysis of inflammatory infiltrates by histology and immunohistochemistry.

5.2.2.4. Determination of bacterial load

Swabs collected into 1 mL BHIB were serially diluted and 100 µL was spread plated on the surface of BHIA plates and incubated at 37°C overnight, following which viable bacteria were quantified.

Blood collected by cardiac puncture was diluted 1:2 in BHIB and a volume of 100 µL was then spread plated onto BHIA and incubated as described above.

5.2.2.5. Determination of bacterial-induced damage in organs

Tissues harvested following euthanasia of mice were embedded in Tissue-Tek® O.C.T™ medium (Sakura Finetek) immediately following harvest and were snap frozen over liquid nitrogen. Sections were cut at 10 µm thickness, mounted onto StarFrost® superclean slides (ProSciTech Pty Ltd) and stored at -80°C.
5.2.2.5.1. Haematoxylin and eosin staining and scoring of inflammation

Frozen 10 µm sections of skin were fixed in 4% neutral buffered formalin (Thermo Fisher Scientific). Sections were washed in PBS once before being stained with haematoxylin and eosin. Histological sections were observed under light microscopy to determine the extent of damage and inflammation to tissues. Images were captured using a Leica DM2500 microscope and Leica Application Suite software (Leica). Epidermal thickness was measured (µm) to determine effects on wound healing, using the Leica Application Suite software (Leica).

H & E stained skin sections were scored in a blinded manner for the presence of inflammation. A semi-quantitative scoring system was used, as described in the study by Kugelberg et al. [550]. The parameters used to describe the inflammatory response included inflammation in the dermis and subcutaneous tissues (inclusive of influx of leukocytes, and epidermal thickening observed in these tissues). The parameters listed were scored from 0-3, as follows: 0, no inflammation (no evidence of leukocyte influx); 1, minimal inflammation (a few leukocytes present); 2, moderate inflammation (moderate presence of leukocytes); 3, severe inflammation (abundance of leukocytes present).

5.2.2.5.2. Immunohistochemical detection of S. aureus in skin

Frozen 10 µm sections of skin were fixed in ice-cold acetone. Sections were air dried and rehydrated in PBS for 10 min, then permeabilised with PBS containing 0.2% Triton X-100. Fc Receptor blocking was performed as described in section 2.4.3.2, for 1 h at RT. Slides were inverted to remove excess blocking solution and primary antibodies (rabbit anti-S. aureus, 1:200; Abcam, and rat anti-mouse CD11b, 1:100; BioLegend) in PBS/1% BSA were added, and sections were incubated overnight at 4°C. Sections were washed three times for 10 min in PBST and an anti-rabbit Cy3® (1:1000; Abcam) and anti-rat Alexa Fluor® 488 (1:800; Life Technologies) conjugated secondary antibodies in PBS/1% BSA were added. Slides were incubated for 1 h at RT. Sections were washed three times for 10 min in PBST then slides were incubated with DAPI nuclear stain in PBS/1% BSA (2 µg/mL) for 20 min at RT (in the dark). Slides were washed frequently in PBST over 30 min, mounted in Mowiol (section 2.4.3.2) and examined the following day. Images were captured as described above. Images were overlayed for display using Adobe Photoshop software (Adobe).
5.2.2.6. Analysis of cell populations

5.2.2.6.1. Preparation of single cell suspensions for flow cytometry

5.2.2.6.1.1. Blood

Following centrifugation and removal of plasma, cells were re-suspended in TAC red cell lysis buffer (prepared as described in section 2.5.1) and incubated at 37°C for 5 min to lyse contaminating erythrocytes. Cells were then washed by centrifugation and resuspended in FACS wash buffer (prepared as described in section 2.2.3).

5.2.2.6.1.2. Spleen

Spleens were collected into ice-cold DMEM complete media (prepared as described in section 2.2.3). Cells were placed in petri dishes and were gently teased through a 40 µm cell strainer (Biologix). Following a washing step cells were re-suspended in TAC red cell lysis buffer (prepared as described in section 2.5.1) and incubated at 37°C for 5 min to lyse contaminating erythrocytes. Cells were then washed and resuspended in FACS wash buffer.

5.2.2.6.2. Flow cytometry

For flow cytometry, 10^6 cells from blood or spleen were incubated with Fc receptor block (Purified anti-mouse CD16/32 Antibody, BioLegend), at 4 µg/mL in 50 µL FACS wash buffer for 30 min on ice. The antibodies used for staining are listed in Table 2.3 (Chapter 2). Staining was performed in 100 µL FACS wash buffer for at least 30 min on ice. Following this cells were washed twice in FACS wash buffer before being re-suspended in PBS for data acquisition.

Data was collected using the FACSCanto II (Beckton Dickinson). A five-decade logarithmic scale was used to process fluorescence signals and a linear scale was used to process light scattering signals. Data analysis was performed using FlowJo software (Version 9.7.1). Single cells were gated and then populations of cells were defined as being T lymphocytes (CD3+, CD4+, CD8+), monocytes/macrophages (CD11b+) and neutrophils (Ly6G+) as well as other cell surface markers.
5.2.3. Statistical analysis

Statistical analyses were performed using GraphPad Prism version 6.0 for Macintosh (GraphPad Software). Unless stated, differences observed between two means were analysed using a two-tailed Mann-Whitney Test, where $p<0.05$ was considered significant.
5.3. Results

5.3.1. In vitro study

5.3.1.1. The effects of SA on growing staphylococcal biofilms

Staphylococcal biofilms were grown in 24-well plates in the presence or absence of 20 mmol/L SA over 24 h or 48 h and the ability to form biofilms was assessed quantitatively using the crystal violet method.

Figure 5.2A shows the biomass of biofilms after 24 h incubation. There was a significant 2.4-fold decrease in biofilm formation in the SA1698 (reference-MRSA) strain treated with SA when compared to the vehicle (p<0.0001). Likewise, SE35984 (a known biofilm former) showed a 12.4-fold decrease in biofilm formation when treated with SA, compared with vehicle-treated (p<0.0001). Weak biofilm forming S. aureus clinical isolate 6 also showed a 1.2-fold decrease in biofilm formation following 24 h incubation (p<0.0001). Figure 5.2B shows total biofilms formed after 48 h incubation. A significant (2.8-fold) decrease in biofilm formation was also seen in the SA1698 treated with SA compared to the vehicle (p<0.0001). SE35984 showed a significant (17.2-fold) decrease in biofilm formation when treated with SA, compared to the vehicle (p<0.0001). Following 48 h incubation, weak biofilm forming S. aureus clinical isolates 5 and 6 also showed small decreases (1.3-fold and 1.5-fold, respectively) in biofilm formation (p<0.0001).

5.3.1.2. The effect of SA on viability of staphylococcal species

Biofilms of staphylococcal isolates were grown over a 48 h period in the presence or absence of SA as previously described and bacterial viability was determined. Figure 5.3 illustrates the differences seen between treated and untreated biofilms. All of the biofilm isolates grown in the absence of 20 mmol/L SA displayed a significant increase in CFU of at least 3.5 log_{10} on BHIA compared with SA-treated biofilms; where bacterial counts comparable with initial inoculum (p<0.05, p<0.005).
Figure 5.2: SA treatment significantly reduces staphylococcal biofilm formation. Biofilms of clinical isolates and reference strains SA1698 and SE35984 were grown in the presence or absence of 20 mmol/L SA at 37°C. Biofilm formation was measured by crystal violet absorbance at 595nm. Graphs represent; A) 24 h incubation, B) 48 h incubation. Data represent mean ± SEM of three independent experiments (n=15 per group, two-tailed Mann-Whitney test, ****p<0.0001 vs. vehicle).
Figure 5.3: SA treatment impacts staphylococcal post-biofilm growth. Biofilms of clinical isolates and reference strains SA1680, SA1698 and SE35984 were grown in the presence or absence of 20 mmol/L SA at 37°C for 48 h. Planktonic cells were removed and samples were serially diluted and plated for viable CFU. Data represent mean ± SEM of three independent experiments (n=6 per group, two-tailed Mann-Whitney test, * p<0.05, ** p<0.005 vs. vehicle) and expressed as (Log$_{10}$ CFU/mL).
5.3.1.3. Confocal microscopy analysis of staphylococcal biofilms

Formation of bacterial biofilms of SA1698 and SE35984 were also assessed by immunofluorescence confocal microscopy. Biofilms were grown on glass coverslips over a 48 h period in the presence or absence of 20 mmol/L SA to assess the impact of SA on biofilm formation (see Table 5.1). Coverslips were then stained with *Staphylococcus* antiserum and detected with a Cy3-conjugated secondary antibody and SYTOX green (showing eDNA). Images taken on the Nikon A1 confocal microscope are shown in Figure 5.4.

Biofilms formed in absence of SA for 48 h demonstrated a uniform 3-dimensional mature biofilm with an abundance of extracellular DNA as shown by the SYTOX green staining (80-100% biofilm surface coverage). In contrast biofilms formed in the presence of SA for 48 h showed minimal detection of attached of bacteria but no uniform 3-dimensional biofilm, and no visual detection of extracellular DNA (0-15% biofilm surface coverage).

Biofilms formed in the presence of SA in the first 24 h then replaced with vehicle medium demonstrated some bacterial attachment and maturation, however there were visible gaps in biofilm structure and less detectable extracellular DNA compared with biofilms formed in vehicle media for 48 h. Interestingly, biofilms formed in vehicle media for 24 h then replaced with SA containing media showed differences between SA1698 and SE35984; with greater impact seen in weaker biofilm forming strain SA1698.
**S. aureus ATCC-BAA 1698**

1. 
2. 
3. 
4. 

**S. epidermidis ATCC-35984**

1. 
2. 
3. 
4. 

Figure 5.4: SA treatment impacts staphylococcal biofilm formation. Biofilms of reference strains SA1698 and SE35984 were formed in the presence or absence of 20 mmol/L SA at 37°C. Over 48 h, biofilms were grown on glass coverslips at 37°C as follows: 1) Vehicle only (48 h); 2) Vehicle (24 h), then SA (24 h); 3) SA (24 h), then vehicle (24 h); or 4) SA (48 h). Coverslips were fixed and stained with SYTOX green and anti-*Staphylococcus* (Cy3, red). Staining was visualised by confocal microscopy. Original magnification: 10x
5.3.1.4. Quantification of eDNA in staphylococcal biofilms

Confocal microscopy analysis demonstrated the impact of 20 mmol/L SA treatment on biofilms of grown over 48 h. In order to further determine the extent to which SA treatment impacted biofilm formation, staphylococcal biofilms (SA1698 and SE35984) were grown in the presence of increasing concentrations of SA for 48 h. Determination of eDNA release was performed by SYTOX green nucleic staining and relative fluorescence intensity units (RFU) was measured.

Figure 5.5 represents the fluorescence output for both SA1698 and SE35984. For both strains, SA treatment as low as 1 mmol/L significantly impaired eDNA release in biofilms grown over 48 h (Figure 5.5A; p<0.005). Weaker biofilms formed by SA1698 were more significantly impacted, with SA concentrations of 1 mmol/L reducing the total amount of eDNA by half (p<0.0001), compared with vehicle. Stronger biofilms formed by SE35984 showed increased resistance to SA treatment, with the same level of eDNA decrease seen with SA concentrations of 5 mmol/L or greater (Figure 5.5B). The strongest effect of SA treatment was shown in both strains with 10 mmol/L and 20 mmol/L SA with decrease in eDNA release below $0.5 \times 10^5$ RFU (p<0.0001).
Figure 5.5: SA impairs extracellular DNA release in staphylococcal biofilms. Biofilms of A) SA1698 and B) SE35984 were grown in 96-well plates in BHIB/vehicle or BHIB containing, 1, 5, 10 or 20 mmol/L SA at 37°C for 48 h. Release of eDNA was semi-quantitatively measured by SYTOX green fluorescence (2 μmol/L in PBS). Data are expressed as relative fluorescence units (RFU) and represent the mean ± SEM of three independent experiments (n=19 per group, two-tailed Mann-Whitney test, ** p<0.005, **** p<0.0001 vs. vehicle).
5.3.1.5. The effect of SA on biofilm-related gene expression in \textit{S. aureus}

Many genes have been implicated in the formation and maturation of staphylococcal biofilms. Of note, the genes involved in the regulation of the biofilm process such as the accessory gene regulator (\textit{agr}), staphylococcal accessory regulator (\textit{sarA}), alternative sigma factor B (\textit{sigB}) and other virulence factors such as staphylococcal protein A (\textit{spa}). Changes in gene transcription were assessed in biofilms of SA1698, in the presence or absence of 5 mmol/L SA. Treatment with 5 mmol/L SA showed a significant decrease in the gene expression of \textit{sarA} when compared to both an untreated and vehicle treated biofilms. A trend toward decreased gene expression of \textit{sigB} was also seen, however the differences between SA treated and vehicle treated biofilms were not statistically significant (Figure 5.6).

Other genes previously reported as contributing to regulation of the biofilm were analysed to determine whether SA treatment interfered with their gene expression. Membrane-spanning protein CidA forms part of a two-component regulatory system to facilitate bacterial lysis and cell death, contributing to the release of eDNA [328]. In the above analysis eDNA levels were impacted by SA treatment, thus gene expression of \textit{(cidA)} was assessed. Catabolite control protein (\textit{CcpA}) is activated in the presence of glucose or sucrose, and was shown to up-regulate the genes responsible for synthesis of polysaccharide for the biofilm [551]. As a result, gene expression of \textit{CcpA} was evaluated for a potential impact of SA treatment. No significant differences were observed between vehicle and SA treated biofilms for either of the above genes of interest (Figure 5.6).
Figure 5.6: SA treatment decreases gene expression of key biofilm regulatory gene sarA in mature staphylococcal biofilms. Biofilms of reference MRSA strain SA1698 were grown in the presence or absence of 5 mmol/L SA at 37°C for 48 h. Relative transcript levels were compared against gyrB for a panel of biofilm related genes including agr, sarA, sigB, cidA and CcpA as well as a virulence gene spA. Data expressed as fold-change relative to gyrB and represents the mean ± SEM of three independent experiments (n= 3-5 per group, two-tailed Mann-Whitney test, *p<0.05 vs. vehicle).
5.3.2. The effects of low-dose aspirin in an in vivo model of device-related biofilm infection

5.3.2.1. Analysis of clinical parameters associated with inflammation

Mice had either 4mm s/s balls or catheter implants contaminated with reference MRSA strain SA1698 surgically implanted into subcutaneous pockets of skin to induce a localised biofilm infection. Approximately $3 \times 10^5$ CFU per s/s implant (Standard Deviation [SD] = $1.3 \times 10^5$) and approximately $2.7 \times 10^5$ CFU per catheter ([SD] = $5.9 \times 10^4$) was delivered to subcutaneous pockets for infection. For comparison, a separate group of mice underwent mock-surgery (but did not receive an implant or aspirin treatment), to determine baseline values (sham surgery mice). Following this, low-dose aspirin (1 mg/kg: equivalent to a dose of 100 mg/day in a human) was administered to mice from each experimental group; the first dose administered 1 h following surgery. Aspirin treatment was given once daily for the following three days. For the duration of the experiment the mice were monitored for changes in weight as a primary parameter for change in condition. None of the mice with S. aureus contaminated implants displayed dramatic weight loss meeting criteria for humane end-point intervention (described in section 4.2.1.2).

As shown in Figure 5.7, all mice displayed initial weight loss (within 24 h of surgery), no significant differences in weight loss were observed among experimental groups with all mice maintaining weight or regaining weight by termination of the experiment (96 h post-implantation). Aspirin treatment did not have any significant effect on weight change in implanted mice.
Figure 5.7: No significant differences were observed in weight loss of mice with subcutaneous implants, regardless of aspirin therapy. Mice were weighed prior to surgery and aspirin treatment, then were monitored and weighed twice daily (morning and evening) for a total of 4 days. Data represents the mean ± SEM and expressed as percentage of start weight (n=3 per group).
5.3.2.2. Analysis of the effects of low-dose aspirin treatment on biofilm formation

Following termination of the experiment, implants were removed and the surface of each implant was assessed for measurable biofilm formation using the crystal violet absorbance method [547]. Quantitative detection of biofilms formed on implants retrieved from mice after 4 days incubation is shown in Figure 5.8A. All implanted mice had detectable biofilms formed on the surface of implants; catheters appeared to provide better surface for bacterial attachment and biofilm formation compared with s/s implants. Small differences were observed between aspirin and non aspirin-treated mice with s/s implants with a two-fold decrease in biofilm formation in aspirin-treated mice (Figure 5.8A; p<0.05, one-tailed Mann-Whitney). No statistically significant differences were observed in catheter-implanted mice.

Bacterial load in exudates surrounding skin were quantified to determine whether aspirin treatment improved mediated clearance of bacteria. Following removal of implants, exudates in surrounding areas were swabbed and incubated in BHI broth. In order to determine bacterial load, swabs were spread plated onto BHIA plates. CFU were counted following incubation at 37°C for 24 h. SA1698 growth was not seen in exudates from sham surgery mice (data not shown). Following 4 days incubation, all exudates from infected sites yielded viable bacterial growth (Figure 5.8B). No significant differences observed between treatment groups and there was no apparent effect of aspirin noted. No bacteria were detected in the blood of any of the mice (regardless of implant and aspirin therapy) confirming that this infection was localised to the site of implantation (data not shown).
Figure 5.8: A) Aspirin treatment results in decreased biofilm formation on contaminated s/s implants removed from C57BL/6 mice. Contaminated implants were removed and analysed for surface biofilm formation. Data represents mean ± SEM (n=3 per group, one-tailed Mann-Whitney test, *p<0.05 vs. s/s non-aspirin treated), B) Bacterial load of exudates surrounding contaminated implant sites were not significantly reduced with aspirin treatment. Swabs were taken from contaminated implant sites and incubated in BHIB. Following this samples were plated on BHIA, colonies were counted and data was log transformed. Data represents mean ± SEM (n=3 per group).
5.3.2.3. Analysis of inflammation and damage in skin surrounding infected sites

5.3.2.3.1. Histological scoring

To assess whether aspirin treatment had a beneficial effect on inflammation, sections of skin surrounding the surgical site of implant were analysed. Sections of skin from sham surgery, s/s-implanted mice and catheter-implanted mice were stained with haematoxylin and eosin. Assessment of inflammation in dermis and subcutaneous tissues were scored as described in section 5.2.3.5.1, and epidermal thickness was measured. Skin from infected mice showed signs of inflammation with varying degrees of distortion of subcutaneous tissues, and increased infiltration of leukocytes seen in dermis and subcutaneous tissue compared with sham surgery mice (shown in Figure 5.9). Aspirin-treated mice had slightly lower mean inflammation scores compared with non aspirin-treated mice, however as individual variation was seen in each group, this was not statistically significant (Figure 5.10). Interestingly in implanted mice, inflammation due to contaminated implants also resulted in significantly increased thickness observed in the epidermal layer of the skin (p<0.005, one-way ANOVA) possibly due to scar tissue development, and this was reduced with aspirin therapy (p<0.05, one-way ANOVA).

5.3.2.3.2. Immunofluorescence microscopy analysis

As S. aureus was detected in exudates surrounding tissue, immunofluorescence (IF) microscopy was performed to determine localisation of S. aureus and presence of leukocytes (monocytes/macrophages and neutrophils) in skin surrounding contaminated implants. Skin sections from sham surgery, aspirin and non aspirin-treated s/s and catheter-implanted mice (n=3 group) were stained for S. aureus and monocyte/macrophage/neutrophil surface marker CD11b. No detectable staining for S. aureus was seen in sham surgery mice (Figure 5.11 top left panel). In contrast, all implanted groups (both aspirin and non aspirin-treated) showed immunoreactivity for S. aureus, (indicated by arrows in Figure 5.11, left panel) and CD11b. Likewise weak immunoreactivity was seen for CD11b in sham surgery skin sections compared with implanted mice groups. S. aureus appeared to localise toward the edge of epidermal skin surfaces. CD11b+ cells appeared to be co-localised with S. aureus indicating leukocyte mediated clearance of bacteria. Aspirin treatment appeared to moderately reduce the amount of CD11b positive cells infiltrating into the skin of catheter-implanted mice.
Figure 5.9: Aspirin therapy results in modest reductions in inflammation and epithelial thickness in skin of implanted mice. Haematoxylin and eosin (H & E) stained sections of skin were assessed for levels of inflammation. Representative sections from: A) sham surgery, B) s/s implant with aspirin, C) s/s implant without aspirin, D) catheter implant with aspirin, and E) catheter implant without aspirin groups are shown (n=3 per group). Arrows indicate increasing leukocyte inflammation in dermis and subcutaneous tissues, as well as increased epithelial thickness. Ep, Epidermis; D, Dermis; and SC, Subcutaneous tissue. Scale bar: 50 µm. Original magnification 10x.
Figure 5.10: Inflammation scores for skin sections of s/s and catheter-implanted mice show a trend towards reduced inflammation and reduced epidermal thickness with low-dose aspirin treatment. H & E stained skins sections were scored according to levels of inflammation in dermis and subcutaneous tissues, and relative epidermal thickness. Data expressed inflammation (score) or thickness (µm) with symbols representing individual mice, and the bar the mean ± SEM (n=3 per group, one-way ANOVA *p<0.05, **p<0.005).
Figure 5.11: *S. aureus* and CD11b\(^+\) cells are co-localised in infected skin surrounding s/s and catheter implants 4 days post-implantation. Representative images showing the detection of *S. aureus* (Cy3, red) and CD11b (Alexa Fluor\(^\circledR\) 488, green) in mouse skin. No significant effect of aspirin therapy was seen in the skin of s/s-implanted mice, small reduction of CD11b\(^+\) cells in skin of catheter-implanted mice treated with aspirin. Scale bar: 50 µm. Original magnification: 10x. Arrows indicate immunoreactivity for *S. aureus*. 
5.3.2.4. Analysis of leukocyte populations

In order to determine whether aspirin treatment has an effect on immune responses in mice with *S. aureus* contaminated s/s or catheter implants, the proportions of circulating immune cell subsets in blood and spleen were analysed. Following preparation of single cell suspension, cells were stained with the appropriate antibodies and immune cell subsets were quantified by flow cytometry. For all comparative flow cytometry analyses; the same antibodies, voltage settings on the flow cytometer (FACS Canto II) and gating strategies were used. The gating strategies for the FACS experiments are shown in Figure 4.10 and 4.11 (Chapter 4).

5.3.2.4.1. Monocyte/macrophage and neutrophil populations

The role of innate immune cells (monocytes/macrophages and neutrophils) has previously reported as early responders of infection and inflammation. The proportions of monocyte/macrophage and neutrophil populations in C57BL/6 mice were analysed in both blood and spleen 96 h after surgical implantation of contaminated s/s and catheter implants. There was a small increase in blood monocyte/macrophage and neutrophil populations in catheter-implanted mice compared with sham surgery mice and s/s-implanted mice, however this was not significant (Figure 5.12; p=0.05). Aspirin-treated mice with s/s implants showed a trend to a decrease in circulating neutrophils compared with non aspirin-treated mice and sham surgery mice, although again this was not significant (Figure 5.12; p=0.05). Analysis of populations of monocytes/macrophages and neutrophils in spleen showed the highest proportion of these cells were in sham surgery mice and aspirin-treated s/s-implanted mice.
Monocytes/Macrophages

Blood

Cell Count ($x10^5$)

Spleen

Cell Count ($x10^6$)

Neutrophils

Cell Count ($x10^6$)

Figure 5.12: Proportions of monocytes/macrophages and neutrophils were not significantly altered in blood in blood of catheter-implanted mice and in spleen of all implanted mice. Leukocytes were extracted from the blood and spleen of female C57BL/6 mice, stained for surface epitopes and analysed by flow cytometry. Data represents the mean ± SEM of cell number in leukocyte gate (n=3 per group), differences between means were analysed by one-tailed Mann-Whitney test.
5.3.2.4.1.1. Cell surface expression of L-selectin and PSGL-1

Cell surface expression of L-selectin and PSGL-1 on monocytes/macrophages and neutrophils in blood and spleen were assessed as markers of activation and migration. Expression levels of L-selectin and PSGL-1 on monocyte/macrophage surfaces were low in blood and spleen of all mice (Figure 5.13).

No significant differences were seen in blood or spleen for L-selectin expression on monocytes/macrophages in either s/s or catheter-implanted mice compared with sham surgery mice. Catheter-implanted mice displayed the lowest proportion of L-selectin expression on monocytes/macrophages (Figure 5.13Ai and iii). L-selectin expression on neutrophils in blood remained unchanged in all mice, regardless of aspirin treatment (Figure 5.13Aii). In spleen, a 2-fold reduction in the expression of L-selectin on neutrophils was seen in both s/s and catheter-implanted mice compared with sham surgery mice (Figure 5.13Aiv). Aspirin treatment however did not affect L-selectin expression on neutrophils in spleen. Mean PSGL-1 expression on circulating monocytes/macrophages was lower in all implanted mice compared with sham surgery mice. Mice with s/s implants had a small reduction in PSGL-1 expression on the surface of both monocyte/macrophage and neutrophils in spleen with aspirin treatment (Figure 5.11Biii and iv). PSGL-1 on neutrophils in blood (Figure 5.13Bii) showed a small decrease in s/s-implanted mice compared with sham surgery mice, however no significant effect of aspirin was seen.
Figure 5.13: A) L-selectin and B) PSGL-1 expression on monocytes/macrophages and neutrophils were not significantly altered in blood and spleen of mice with contaminated implants. Small decreases in blood and splenic expression of PSGL-1 in monocytes/macrophages and neutrophils in aspirin-treated s/s-implanted mice. Data are expressed as percentage of total monocytes/macrophages or neutrophils. Data represents mean ± SEM (n=3 per group).
5.3.2.4.2. Analysis of T lymphocyte populations

To evaluate the extent of contribution of the adaptive immune response in this model of device-related biofilm infection, the proportions of CD4$^+$ and CD8$^+$ T lymphocytes in C57BL/6 mice were determined in both blood and spleen, 96 h following surgical implantation of contaminated s/s and catheter implants (Figure 5.14). Similar to proportions observed in innate immune cell populations, catheter-implanted mice had the highest counts of CD4$^+$ T lymphocytes in blood, with counts 34-fold higher (aspirin-treated) and 25.8-fold higher (non aspirin-treated) than sham surgery mice, however this was not found to be statistically significant (Figure 5.14; p=0.05). In contrast, splenic CD4$^+$ T lymphocyte analysis revealed that implanted mice had a minimum of 6-fold reduction in CD3$^+$CD4$^+$ cells, compared with sham surgery mice. Proportions of CD8$^+$ T lymphocytes in blood were low (<100 CD3$^+$CD8$^+$ cells), however the highest counts were seen in catheter-implanted mice. The trends observed for CD4$^+$ T lymphocytes in the spleen were also seen in CD8$^+$ T lymphocytes proportions, however none of these differences were statistically significant. Sham surgery mice had the highest splenic CD8$^+$ T lymphocyte response compared with implanted mice.
Figure 5.14: Proportion of CD4$^+$ and CD8$^+$ T lymphocytes were not significantly altered in blood in blood of catheter-implanted mice and reduced in spleen of all implanted mice. Lymphocytes were extracted from the blood and spleen of female C57BL/6 mice, stained for surface epitopes and analysed by flow cytometry. Data are represented as mean ± SEM of cell number in leukocyte gate (n=3 per group), differences between means were analysed by one-tailed Mann-Whitney test.
5.4. Discussion

*S. aureus*, *P. aeruginosa* and *Candida spp* are three of the most common sources of biofilm infections. Unsurprisingly, these pathogens are also frequent sources of bloodstream infections contributing to development of sepsis [552, 553]. Biofilms formed by these pathogens on devices have been shown to be resistant to both phagocytosis by macrophages and neutrophils [312, 554, 555] and antibiotic therapy [556, 557]. Failure to effectively clear infection contributes to persistence of biofilm. Anti-biofilm activities of aspirin and its major metabolite SA against models of *P. aeruginosa* and *Candida spp* infections have been shown, promoting investigation into the potential as an adjunct therapy of staphylococcal biofilm infections.

5.4.1. *In vitro* study

The main findings of this study are that formation of staphylococcal biofilms was impaired with the sub-inhibitory concentration of 20 mmol/L SA. Viability of the bacteria was not impacted, however growth rate was impaired. Concentrations of as low as 1 mmol/L SA reduced levels of eDNA released in staphylococcal biofilms, with maximum decrease seen with 20 mmol/L SA. At the transcriptional level, out of a panel of known biofilm regulating genes, only the transcription of *sarA* was significantly impacted in mature biofilms with 5 mmol/L SA under the conditions tested.

Inhibitory effects of aspirin and its major metabolite SA have been shown *in vitro* in biofilms of *P. aeruginosa* [540, 558] and *Candida spp* [541, 546], and to reduce growth rates of *P. aeruginosa* [558]. In this study, growth rates of SA1698 and SE35984 treated with 20 mmol/L SA were affected, with reduced numbers of growing bacteria in logarithmic phase (data not shown). SA-treated cultures also demonstrated an approximate 8 h delay in growth time to reach stationary phase. The bacteriostatic effect of SA was also shown in the viable number of bacteria recovered, following biofilm formation, with SA-treated biofilms comparable with initial inoculum.

Extracellular DNA is an integral part of staphylococcal biofilms; crosslinking of eDNA within the extracellular polymeric matrix contributes to increased resistance to antimicrobial therapy and immune attack. In a previous study, staphylococcal biofilms treated with increasing concentrations of DNAse I showed a significant reduction in detectable levels of eDNA [345]. Likewise in this study, concentrations of SA as low as
1 mmol/L lead to a significant reduction in eDNA in the biofilm. Biofilms treated with 20 mmol/L SA showed maximum reduction of eDNA release and this was confirmed by both confocal microscopy (Figure 5.3) and semi-quantitative analysis (Figure 5.4). To my knowledge, this is the first study to address the impacts of SA-treatment on the release of eDNA as a mechanism of inhibition in staphylococcal biofilms. SA-treatment was shown in this study to impact two key components of staphylococcal biofilm-formation; biomass (detected by crystal violet absorption) and eDNA release (semi-quantitative fluorescent detection), which lead to reduced structural integrity of the biofilm.

Resistance of staphylococcal biofilms to host immune responses was shown in a previous study, with macrophages failing to successfully phagocytose staphylococcal biofilms in a co-culture model [357]. Similar results were seen in this study, confirmed in a single experiment, where RAW 264.7 cells (mouse macrophage cell line) failed to phagocytose mature biofilms of SA1698 or SE35984 (data not shown). Polymorphonuclear neutrophils (PMN) were shown in a previous study to successfully mediate phagocytosis of staphylococcal biofilms [345]. Similarly, it has been shown that low-dose aspirin therapy leads to increased leukocyte phagocytosis of bacteria in vivo [446]. It was of interest therefore to investigate whether pre-treatment of leukocytes with aspirin enhanced phagocytic ability. In a single experiment performed in this study, leukocytes purified from whole blood of mice were able to phagocytose staphylococcal biofilms, however there was no improvement in phagocytic ability when leukocytes were pre-treated with 1 mmol/L aspirin (data not shown). SA-treatment does appear to impair mechanisms of staphylococcal biofilms regulation, however it is still unclear whether this impacts susceptibility to immune attack. It was also of interest to determine whether the effects of SA treatment on S. aureus biofilms were impacted at a transcriptional level.

In other published studies on SA-treated biofilms of P. aeruginosa, quorum-sensing pathways that contribute to regulation of biofilm formation were impacted [559]. Similar mechanisms have been described in the regulation of staphylococcal biofilm formation [325]; with transcription of genes involved quorum sensing significantly decreased following treatment with SA [221]. Here, expression of the global regulator sarA was significantly reduced in SA-treated mature biofilms of SA1698. Other genes involved in regulating biofilm formation appeared to be unaffected: including genes associated with regulation of autolysis (contributing to eDNA release) and PIA production (contributing
to biomass) (Figure 5.5). This result was unexpected as the production both of these components were both impaired in biofilms treated with SA. It may be that regulation of these processes is occurring at an earlier stage of biofilm formation, and therefore not detected at the times investigated. Given this, future studies should investigate the impact of SA treatment on these genes at earlier stages of biofilm formation. While the exact regulatory mechanisms of SA-mediated inhibition are still poorly understood, this study has demonstrated two key components of staphylococcal biofilm-formation (biomass production and eDNA release) are physically impaired with SA treatment. These results support the potential of salicylates in the adjunct therapy of device-related biofilm infections; and raise the question of whether aspirin therapy has similar impacts on biofilm formation in an in vivo model of device-related infection? To address this, the effects of aspirin therapy on biofilm development in vivo, and host immune responses were investigated in a wild-type mouse model of device-related biofilm infection.

5.4.2. In vivo study

Implantation of contaminated s/s balls or catheter sections into subcutaneous pockets of C57BL/6 mice resulted in the development of an acute biofilm infection, which remained localised. Low-dose aspirin therapy (1 mg/kg/day for 5 days) impacted biofilm formation (2-fold decrease) on the surface of s/s implants, however the number of CFU determined in exudates surrounding s/s implants was however not affected. Aspirin therapy had no effect on biofilm or CFU in mice catheter implants. Modest reductions in skin inflammation scores were seen with aspirin therapy in both s/s and catheter-implant models, with reduced leukocyte influx into subcutaneous tissues and dermal layers of skin. Immunohistochemistry of skin surrounding implants showed the co-localising of *S. aureus* and CD11b⁺ cells. A decrease in inflammation in the vicinity of catheter implants was evident by the number of CD11b⁺ cells present. Flow cytometry analysis showed systemic inflammation and a general increase in the proportion of inflammatory cells in catheter-implanted mice (regardless of aspirin therapy), with significantly higher proportions of circulating monocytes/macrophages (CD11b⁺ Ly6G⁻), neutrophils (CD11b⁺ Ly6G⁺) and T lymphocytes (CD4⁺ and CD8⁺ T lymphocytes) in blood, compared with s/s-implanted mice.

Of the published studies of device-related biofilm infection models in vivo, none have investigated the potential therapeutic benefit of low-dose aspirin. The results of this
in vivo study have confirmed that low-dose aspirin therapy reduces staphylococcal biofilms formed on s/s-implanted mice, however this effect was not similarly observed in catheter-implanted mice. This difference is likely to relate to the differences in surface characteristics for promoting biofilm formation as catheters were found to support development of greater biofilm biomass than s/s implants. Stainless steel implants in previous animal-implant studies more readily allowed for the formation of a fibrous capsule in vivo, providing bacteria an optimal surface for attachment and biofilm formation, when compared to titanium surfaces [560, 561]. In contrast, hydrogel-coated latex catheters have been associated with reduced adhesion of bacteria [562], however in the sectioning of catheters in this study, inner latex surfaces of catheters were exposed to bacteria. As a result, there was an increase in total surface area, and surface roughness (compared with hydrogel coated outer sections), which may have contributed to increased bacterial attachment. To address whether the differences between implant surfaces truly impact biofilm formation and resistance, future studies should further characterise the differences in surface characteristics between s/s and catheter implants, and the impact on bacterial attachment and maturation of biofilms in vivo.

Aspirin therapy has been shown to improve outcomes in the rabbit model of endocarditis where disease is contributed to by biofilm formation. Reduced vegetation weight and bacterial load were found in rabbits treated with 4-50 mg/kg dose range of aspirin [384, 516, 563]. In the mouse model described here, similar decreases in biofilm formation were seen in s/s-implanted mice treated with lower doses of aspirin than the rabbit studies (Figure 5.8A). While bacterial loads at the implant site were not affected by low-dose aspirin therapy (Figure 5.8B), it should be noted that mice in our study had not received antibiotics as would be routinely done in a clinical setting. Evidence from in vitro studies have shown that aspirin and vancomycin combined had a greater inhibitory effect against staphylococcal biofilm formation, than either treatment did individually [564].

There were limitations associated with the “swab and count” technique used to enumerate bacterial load of exudates in this model. The technique was reliant on careful swabbing of the exudate area, in order to avoid contamination from other commensal flora of the skin. Further, the number of planktonic bacteria in the exudate solution may have been present in fewer numbers, and swabbing may not have given a true representative level of bacterial burden in the skin and surrounding areas. The use of more regulated sampling
techniques, for example; sampling of standard section of skin surrounding wound, then homogenising and plating on appropriate selective media (as described for other systemic organ CFU counts), or sampling a restricted volume of exudate (as similarly described for blood CFU counts) may have yielded more reliable measurements and should be considered in future experiments.

Clinically relevant outcomes flow from aspirin use in preventing other biofilm associated diseases. Embolic complications are reduced in patients with infective endocarditis who are on long term aspirin [565]. Also, in an observational study of haemodialysis patients with tunnelled catheters, patients previously taking aspirin had decreased risk of developing of staphylococcal bacteraemia [218].

A scenario can be constructed where pre and post-operative aspirin could be used to prevent device-related infections. Various strategies are currently used to prevent venous thromboembolism. Heparinoids are most commonly used but these are associated with bleeding including into prosthetic joints. Many orthopaedic surgeons prefer alternatives including aspirin. Large retrospective studies have shown that aspirin is equally effective as DVT prophylaxis with no increase in bleeding risk [566]. As mentioned above, significant reductions were shown in S. epidermidis biofilm formation (up to 99%), when biofilms were treated with aspirin combined with vancomycin [564]. In the absence of aspirin, this effect was not seen. Aspirin could be used as prophylaxis against device-related biofilm infection, then to potentiate killing with in combination with post-operative antibiotics.

In this study, although systemic inflammation was detected, bacteria were not found in the blood of any of the mice (data not shown), suggesting that in this model the infection remained localised to the vicinity of the implant in subcutaneous pockets of the skin, regardless of aspirin therapy.

Aspirin therapy was recently shown in a previous study to delay wound healing in mice, by reducing the production of 12-hydroxyheptadecatrienoic acid (a derivative of arachidonic acid); resulting in impaired re-epithelialization and delayed wound closure [567]. This detrimental effect only occurs at high-doses (180 mg/kg; equivalent to 14g dose for a human of ~80 kg). In contrast, the production of specialised pro-resolving mediators (SPMs) such as lipoxins and resolvins triggered by low-dose aspirin were
shown to enhance wound healing [19, 568]. In the current study, histological analysis showed complete re-epithelialization and wound healing, with increased epidermal thickness seen in all implanted mice (Figure 5.9). Epidermal thickness was reduced in aspirin-treated mice, suggesting enhanced wound healing. Future studies will investigate a range of time-points to determine if the time frame for wound healing is reduced in the presence of aspirin, and to further characterise the contribution of SPMs produced in local sites surrounding implants in potentially accelerating wound healing and reducing inflammation. Immunohistochemistry of skin surrounding the implant showed infiltrates of CD11b+ cells co-localised with *S. aureus*, suggesting interactions with monocytes/macrophages/neutrophils to initiate bacterial clearance from tissues, however no significant effect of aspirin therapy could be determined at the time-points analysed for s/s-implanted mice (Figure 5.11). Direct analysis of interaction of monocytes/macrophages with biofilm on the implant surface *in vivo* were not possible in this study, but will be addressed in future studies.

Complications of intravascular device biofilm infections *in vivo* include dissemination leading to bacteraemia and development of sepsis [569, 570]. With this in mind, the focus of the immunological analysis performed in this study was detecting bacteraemia and measuring the systemic inflammatory response. Regardless of aspirin therapy, mice with catheter implants had a tendency to greater weight loss, increased bacteria in exudates, and increased proportions of all immune cell populations (monocytes/macrophages, neutrophils and T lymphocytes) in blood. Although bacteria were not detected in the blood of these mice, significant increases in systemic inflammatory responses suggest that infection with catheter-implant may lead to the development of the systemic inflammatory response syndrome (SIRS). In contrast, s/s-implanted mice did not appear to have altered proportions of circulating monocytes/macrophages, neutrophils or T lymphocytes and no effect of aspirin was noted.

Although proportions of circulating neutrophils were not impacted by aspirin therapy, analysis of cell surface markers of activation and migration (L-selectin and PSGL-1) were performed (mirroring the analyses performed in patients with sepsis (Chapter 3) and mice with systemic staphylococcal infection (Chapter 4)). Levels of migration marker PSGL-1 expression was reduced 2-fold in monocytes/macrophages and neutrophils of aspirin-treated mice with s/s implants, consistent with the possibility that chemotaxis is being
inhibited. However, as this was not statistically different a more detailed analysis of infiltrates at the local implant site as well as other target organs (such as kidney and liver) would be required to assess this in future.

The main difference between this model and the standard patient care for device-related infections is the absence of antibiotic therapy. This study is the first to my knowledge to demonstrate effects of aspirin therapy in an in vivo model of device-related biofilm infection. Reductions on biofilm formation were seen in s/s-implanted mice in the absence of antibiotics. Indeed, clinical studies of low-dose aspirin therapy (equivalent to 80-100 mg) have not shown any direct anti-bacterial action [218, 384]. Future studies will investigate the effects of low-dose aspirin in conjunction with antibiotic therapy, to replicate therapeutic approaches currently given in patients with similar device-related infections.

The sub-cutaneous models of device-related biofilm infection described in this preliminary in vivo study, used contaminated urinary catheter sections and s/s balls and foreign body implants due to the ease of use and standardised implant sizes. The study allowed the observation of the effects of low-dose aspirin on biofilm formation on the implant and bacterial burden in surrounding tissues, as well as histological damage and to some degree the host inflammatory responses. However, the design for these experimental models was limited to only mimic sub-cutaneous local infection, and may not truly represent prosthetic-joint infection or catheter-related infection in a clinical setting.

Developments to the experimental design of these models could potentially allow for greater understanding of the biofilm formation in the context of the respective infection, and may show a clearer effect of aspirin therapy. For example, the use of intravascular catheters inserted in the sub-cutaneous pockets might be a better material to better replicate the localised infection, and the potential spread to the bloodstream, and development of sepsis [360]. This is on the basis of a previous clinical study showing that prophylactic aspirin therapy reduced risk of bloodstream infection in patients with intravascular catheters inserted [218]. Alternatively, the use of urinary catheter with adjustments made the experimental design should be considered. Insertion the catheter in closer proximity to, or directly into the bladder (as described in the studies by Guiton et al. [571, 572]) would broaden the scope of this study, allowing the investigation aspirin-
related effects on catheter-associated urinary tract biofilm-infections.

Similarly, adjustments could be made to the s/s-implant model to better replicate clinical prosthetic-joint infections. Clinical prosthetic-joint infection are difficult to treat as interstitial spaces near joints lack suitable blood flow, which in turn limits access to both critical immune responses and effective delivery of antimicrobial therapy [355]. The 2011 study by Prabhakara et al. used contaminated s/s insect pins that were inserted in the tibia of C57BL/6 mice to study the characteristics of biofilm development and host immune responses, closely mimicking clinical prosthetic-implant infection [356]. Similarly, antimicrobial coated orthopaedic-grade s/s Kirschner (K)-wires were inserted in mouse femur prior to infection with S. aureus, to evaluate the efficacy of antimicrobial coatings as a preventative approach of biofilm formation [573]. Developments made to the type of implant used and the location of implant insertion (closer to a joint) in the present model would similarly permit the investigation of aspirin therapy in a more representative model of prosthetic joint infection.

Further, while effects seen with low-dose aspirin seen in this study are encouraging, higher doses of aspirin therapy (5-10 mg/kg) in endocarditis models of infection were shown to be more effective in reducing bacterial vegetations and should be considered in future studies in these models of device-related biofilm infection. Further development of this model could also include an experimental group with aspirin therapy administered 24 h after contaminated implants are inserted, to determine whether responses to aspirin treatment are clearer following onset of inflammation. This group could more accurately represent a subset of patients with established device-related infections [218, 385]. Also, future studies should investigate in more detail the local immune response; particularly the effect of inflammation induced by NFκB, as this was shown to be reduced with low-dose aspirin therapy in patients with systemic inflammation (Chapter 3). Detailed analysis of measures in local infection site will improve our understanding of the mechanism of low-dose aspirin therapy in these implant models of device-related infection.

As previously discussed in section 1.4.2, aspirin at all doses can potentially cause damage to the gastrointestinal (GI) tract. The extent of the damage however is dependent on several factors including dose and term of administration [422]. As previously mentioned in section 4.4, doses used in this mouse study were aimed at mimicking low-dose aspirin therapy (100 mg) in humans, which in short-term administration did not significantly
increase GI injury [63]. Similarly, aspirin given to mice at a 5 mg/kg daily dose for up to 5 days in a previous study, did not result in any adverse bleeding [522]. Given that the dosage of aspirin administered to mice in this study was at a lower dose (1 mg/kg) over a 4 day period, it was not anticipated that there would be any significant damage to the GI tract. However, the inclusion of an aspirin only control group should be considered, to confirm there is no evidence of GI damage.

In summary, this study has shown that SA affects staphylococcal biofilm formation by decreasing total biomass and impacting extracellular DNA release; supporting the hypothesis that SA-treatment results in weakened staphylococcal biofilms formed in vitro. Future studies will investigate the impact of phagocytosis on these weakened biofilms, as well as examining the response of SA-weakened staphylococcal biofilms to traditional antibiotic therapy. The in vivo study of device-related MRSA infection showed a moderate reduction of biofilm formation on the surface of s/s balls with low-dose aspirin. Catheter-implanted mice were less impacted by aspirin therapy, and displayed a systemic inflammatory response characteristic of SIRS. This suggests that infection on this type of implant can lead to a more severe inflammatory state. To determine what contributes to these systemic inflammatory responses; future studies will first investigate local sites of inflammation, to improve our understanding of interactions between host immune response and bacteria on the surface on the implant. This will also clarify the potential impact of low-dose aspirin therapy on local inflammatory processes.
5.5. Conclusion

Infectious complications associated with indwelling medical devices are a major healthcare problem. Current approaches in treating complex device-related biofilm infections are invasive and do not always effectively clear infection, leading to re-establishment of biofilm [574]. Further, replacement of contaminated devices can increase the risk of bacterial dissemination resulting in bloodstream infection and development of sepsis. Results of studies described within this chapter have shown that the aspirin’s major metabolite SA impacts regulatory processes in vitro resulting in impaired staphylococcal biofilm formation. Low-dose aspirin also reduced biofilm formed on s/s implant surfaces in vivo. These results, along with its cost-effectiveness and ease of use, highlight a potential role for low-dose aspirin as an adjunct therapeutic of device-related biofilm infections.
Chapter 6

General Discussion
As discussed in section 1.2.5, current therapies for severe sepsis do not directly target the hyper-inflammatory/suppressed immune responses that characterise the sepsis syndrome, and indeed careful analysis over eight years of EGDT have not shown any significant benefit in reducing sepsis mortality rates [11]. Although a number of pharmacological agents have been investigated for their potential as adjunct therapies, none have shown significant therapeutic benefit when reaching clinical trials in humans (reviewed in [148]). For a novel adjunct therapy of sepsis to be successful, it should be inexpensive, be effective against all sources of sepsis, and aim to mediate resolution of inflammation rather than trying to inhibit inflammation completely.

Evidence is accumulating to support the potential of aspirin in the adjunct therapy of sepsis, with the prediction that the mechanisms of action previously described in other inflammatory conditions (via production of pro-resolving lipid mediators including 15-epi-LxA₄) [19, 20], will also be beneficial in sepsis patients. In October 2015, in a review was published by Toner et al., also proposing a role for aspirin in the adjunct therapy of sepsis, with proposed mechanisms of action similarly described as above [575]. In addition, improved outcomes have been reported in animal and observational studies of septic patients [18, 61, 214, 220]. With this in mind, the MATHS clinical trial and contributing studies of this thesis were initiated, to investigate any effect of aspirin-therapy in clinical sepsis, as well as experimentally in mouse models of gram-positive sepsis and device-related biofilm infection.

In this discussion, the key findings of this work will be discussed, with a particular focus on how the results contribute to the greater understanding of mechanisms of aspirin therapy in affecting systemic inflammatory responses. The limitations and strengths of these studies and in particular the differences between sepsis in humans and the mouse model will be discussed. Furthermore, the potential implications of these findings will be discussed along with suggestions for future experiments stemming from this work.

6.1. Key findings of this study

Overall, the findings of the interim analysis on the first patients in the MATHS clinical trial are that short-term administration of low-dose aspirin therapy (100 mg/day) does in fact impact immunological pathways in patients with sepsis, and these could potentially
lead to improved outcomes. In addition, aspirin therapy at equivalent doses showed therapeutic benefit in mice with *S. aureus*-induced sepsis, and impacted biofilm formation in mice with contaminated s/s implants.

### 6.2. Low-dose aspirin as a potential adjunct therapy of systemic inflammation

#### 6.2.1. The role of pro-resolving lipid mediators

Lipoxins and aspirin-triggered epimers are well-studied lipid mediators with functions in resolving inflammation. These mediators were first described by Charles Serhan and colleagues in 1984, and are produced via the metabolism of AA in human leukocytes [576]. In this study, 15-epi-LxA4 was used as a primary read-out parameter for determining an effect of aspirin therapy in both patients with sepsis and mice with *S. aureus*-induced sepsis.

Previous studies have shown that lipoxin production occurs rapidly following onset of inflammation [12, 439]. Similarly, plasma levels of 15-epi-LxA4 increase rapidly following aspirin administration [19, 214]. Peak responses of 15-epi-LxA4 in plasma of patients in this study were seen within 1 hour of receiving low-dose aspirin therapy; consistent with reports from studies investigating localised 15-epi-LxA4 production following inflammation, leading to resolution of inflammation [19]. Interestingly, detectable levels of 15-epi-LxA4 were also observed in the absence of aspirin therapy in both the human and animal studies, as was seen in previous studies [19, 214]. A number of sources have been suggested including the endogenous production of 15R-HETE, leading to 15-epi-LxA4 production by cytochrome p450 [577], and in the case of the participants in the trial it is also possible that statin therapy could also result in moderate increases in 15-epi-LxA4 production [443]. In future studies, the source of this endogenously produced 15-epi-LxA4 should be investigated further. A number of downstream effects of 15-epi-LxA4 production have been described contributing to resolution of inflammation, however very few of these have been studied within the context of sepsis.
The production of 15-epi-LxA4 leads to the inhibition of NFκB, and this has been shown in vitro in both human and mouse cell types [480, 493], and in ex vivo stimulation of human leukocytes [216]. To my knowledge, this study is one of only a few that has investigated the in vivo activation of NFκB in PBMCs in both patients and mice with sepsis. Development of sepsis increased activation of NFκB in both untreated control patients and infected mice, indicating increases in inflammation, and an inhibitory effect of aspirin therapy was shown in patients within 8 h of receiving aspirin therapy, regardless of dose administered. These results are promising considering the sample size, and support the potential for NFκB as indicative marker for aspirin-induced anti-inflammatory/pro-resolution effects in sepsis.

A consequence of NFκB activation in sepsis is the production of pro-inflammatory cytokines and chemokines. Lipoxin A4 and 15-epi-LxA4 have been shown to impact transcription and subsequent production of pro-inflammatory cytokines [128, 216]. Similarly, post-treatment with LxA4 and 15-epi-LxA4 lead to attenuated production of pro-inflammatory cytokines and chemokines, with improved outcomes shown in animal models of sepsis [17, 510].

The levels of pro-inflammatory cytokines and chemokines produced in both patients and mice in this study were indicative of early inflammation, plasma cytokine levels in a lower range when compared with levels reported from patients with severe sepsis and septic shock [495]. The only significant aspirin-related effect seen was an increase in levels of plasma IL-18 in patients treated with 100 mg aspirin. In a previous study, an increase in IL-18 contributed to resolution of bacterial infection in mice [498]. It is possible that the same mechanism of action is responsible in the current study; however further investigation would be required to confirm any correlation.

Levels of TNF-α were also significantly increased in regular aspirin-taking patients, although as levels were within normal range, the only conclusions that can be made are that these patients were more severely ill compared with other groups. This was also evident from other clinical characteristics including increased age, presence of co-morbidities, and highest MPV recorded. Further, this effect on TNF-α was not seen in mice, possibly because mice did not have existing bacteraemia prior to receiving aspirin therapy. Other well-known pro-inflammatory cytokines and chemokines did not appear to
be affected by aspirin therapy, keeping with results seen in both exudates from localised infection in humans, and mice with induced peritonitis following low-dose aspirin therapy in a previous study [19]. Future studies will investigate whether the lack of differences seen in other plasma cytokine levels were due the time frame for analysis, by performing similar analysis on plasma sampled at earlier time-points following aspirin administration. The short half-life of cytokines in circulation could also account for why significant differences were not seen for commonly reported cytokines. Moreover, it is possible that detected levels of cytokines in plasma are dilute and do not adequately reflect the expected level of inflammation in affected tissues. Future studies should consider the measurement of cytokines either directly from affected tissues for a clearer understanding of more localised inflammatory responses, or measurement from in vitro stimulated PBMC to determine the predominant leukocyte type for cytokine production in sepsis.

Both LxA₄ and 15-epi-LxA₄ are produced as a result of interactions between cells of the host immune response following inflammation, and both mediate impact innate immune responses in inflammation, most notably by interfering with the activation and functions of neutrophils [14]. During sepsis, activation of neutrophils is prolonged, delaying apoptosis; leading to exacerbated inflammation, damage and organ failure contributing to increased mortality rates [578]. Pre-treatment with 15-epi-LxA₄ was shown to reverse the delayed apoptosis of isolated human neutrophils in a previous study [101]. Further, LxA₄ and 15-epi-LxA₄ were both shown to attenuate activation and migration of neutrophils, which lead to reduced neutrophil-mediated tissue damage [487].

In this study, CD86 expression was analysed on the surface of circulating monocytes/macrophages as a marker of antigen presenting activity, and in the case of neutrophils, delayed apoptosis [499]. Increases in the proportions of circulating monocytes/macrophages and neutrophils expressing CD86 were seen shortly after the first aspirin dose in 100 mg aspirin-treated patients, however these were not significantly different from other treatment groups. Further, no significant difference was seen in surface expression of the known activation and migration marker PSGL-1 on circulating neutrophils in patients with sepsis, or in blood or spleen of mice with S. aureus-induced sepsis or device-related infection, regardless of aspirin therapy. Interestingly, differences were seen in mice with sepsis following low-dose aspirin therapy in the proportion of
splenic and circulating PSGL-1⁺ monocytes/macrophages and splenic L-selectin⁺ monocytes/macrophages, suggesting that in the early stages of sepsis, aspirin therapy activates monocytes/macrophages, for enhanced phagocytic-mediated clearance of bacteria [446]. Complete clearance of viable bacteria was seen in target organs of low-dose aspirin-treated mice with *S. aureus*-induced sepsis following second dose, in the absence of antibiotic therapy. As low-dose aspirin is does not appear to be directly antibacterial, further investigation is required to confirm whether this is a downstream effect of aspirin. Additionally, immunohistochemistry analysis of spleen as a representative organ also revealed the co-localisation of *S. aureus* and CD11b⁺ cells (including monocytes/macrophages and neutrophils). The combination of these results supports the idea that at least in the early stage sepsis shown in this study aspirin does not significantly impact neutrophils, however activates monocytes/macrophages, leading to the enhanced clearance of bacteria from organs often damaged in sepsis.

All of the studies described in this thesis showed a benefit of aspirin therapy, current knowledge suggesting that 15-epi-LxA₄-mediated actions contribute to this, as these actions are both anti-inflammatory and contribute to the resolution of inflammation. It is known however, that 15-epi-LxA₄ is not the only SPM synthesised following aspirin therapy. As outlined in section 1.4.6.2, a number of recent studies have shown that aspirin also triggers the synthesis a number of other epimeric SPMs including resolvins and protectins. As similarly described for 15-epi-LxA₄, aspirin-triggered resolvin D1 (AT-RvD1) is a more potent anti-inflammatory mediator than naturally produced RvD1, with a greater capacity to inhibit of leukocyte infiltration, and increased resistance to inactivation by enzymes [503]. Post-treatment with AT-RvD1 also protected mice from both inflammation induced acute lung injury and endotoxin-induced acute kidney injury by inhibiting NFκB-mediated activation [13, 503]. Taking this into consideration, it is unlikely resolution of inflammation is due to any of these SPMs individually, but more likely the contribution of many, with a switch from traditional pro-inflammatory lipid mediators to the production of anti-inflammatory mediators, which combined trigger resolution of inflammation [464]. With this in mind, future studies in all models should consider what role other naturally occurring and aspirin-triggered SPMs play in resolution of inflammation following sepsis.
The design of the MATHS clinical trial could be improved by adding a time-point approximately 1 h after the second dose, and the inclusion of a healthy control group (or access to one). This is currently restricted by the limitations of the human ethics aspects of the study and number of samples that could be taken from seriously ill patients. These additional samples would make the analysis easier to interpret.

The results from patients enrolled in the MATHS clinical trial will also contribute to understanding the immunological basis for effects of aspirin therapy in an ongoing primary prevention trial; the AspiriN To Inhibit SEPSIS (ANTI SEPSIS) study, which is a sub-study of ASPirin in Reducing Events in the Elderly (ASPREE) trial. The ANTI SEPSIS trial will determine whether the prophylactic administration of low-dose aspirin can reduce mortality in the elderly population as a result of sepsis, as well as reduce the development of severe infections requiring hospitalisation and minimising ICU admission following identification of severe sepsis in hospitals.

As mentioned, differences were observed in aspirin-dependent responses between trial participants and mice with sepsis. These factors lead to the conclusion that the current mouse model used was not an accurate comparison with human systemic inflammatory disease. The significance and implications of these differences as well as suggestions for future studies are discussed below.

6.2.2. Differences observed between patients with sepsis and the mouse model

Although aspirin therapy did have beneficial effects in the mouse model of *S. aureus*-induced sepsis described in this study, the predicted changes in surrogate markers of increased plasma 15-epi-LxA₄ and NFκB inhibition were not seen.

The major difference between these two studies is the time frame and model development. Patients in the clinical study described in Chapter 3 were enrolled into the clinical trial following identification of SIRS/sepsis, and then aspirin therapy was administered. In contrast, mice were infected with *S. aureus*, and aspirin therapy was administered after 1 h. Therefore, in mice bacteraemia had not yet developed and induced septic changes prior to aspirin administration. Moreover, patients had different infectious sources, and initial sites of infection contributing to the development and severity sepsis,
whereas mice were infected via the same route with the same strain of \textit{S. aureus}. While the animal model is a more defined system for investigation of the immunological responses of sepsis in target organs as well as the bloodstream, it cannot represent all of the responses that can arise from different sources and sites of infection that contribute to sepsis in humans [1, 30]. Future studies should also include a number of pathogenic sources (gram-positive and gram-negative bacteria, as well as fungal sources) so that the impact of aspirin can be tested in a range of infections, to better model the spectrum of infections seen in humans.

The factors that can be addressed to enhance the mouse model include allowing bacteraemia and sepsis symptoms to develop before administration of aspirin, and sampling at earlier and additional time-points to permit clarification of the question as to whether timing aspirin does induce changes in the key surrogate markers in mice.

There were two significant considerations that directed the current design of the mouse model. In order to allow mice to develop sepsis for 24 h prior to administering aspirin the experiment duration would have had to be extended to 72 h, given that sepsis can lead to rapid mortality, and there would likely be a high mortality in the untreated control group. Animal welfare and issues are not insignificant in this type of study, and animal ethics committees do not consider death an acceptable endpoint. For this reason the decision was taken to perform experiments using a wild-type strain of mice with some relative resistance to \textit{S. aureus}, and administer aspirin immediately following infection, in the initial 48 h study to give the best chance of detecting an effect of aspirin. Based on the data that has now been obtained, an improved model would include additional parameters, including a group where sepsis is allowed to develop for 24 h before aspirin treatment, and groups where mice are given supportive antibiotic therapy as well as aspirin as adjunct therapy.

The sampling time-points used in the current mouse study were initially selected based on data from a preliminary analysis of the first 15 patients in the trial (Batch 1), which indicated that the peak responses for all parameters were expected to be in the 8-24 h time frame. Later, the combined analysis of the 29 septic patients enrolled to date now suggest that the response is more rapid, however it may not be possible to clearly predict this until the trial is complete. Also, the prohibitive cost of analysis of multiple parameters meant
that a limited number of time-points had to be selected, as a result of the selection of the 12, 24 and 48 h time-points it is likely that aspirin-induced changes in the surrogate markers were missed.

Similar differences were also noted for NFκB inhibition. Although increases in NFκB activation were seen, the observed change in mice were of a lower magnitude compared to patients, with largest differences measured at 24 h post-infection; consistent with early development of inflammation. Given that maximum inflammation driven by NFκB had not fully developed, it is therefore not surprising that an effect of aspirin was not seen. In future developments of the model described above; inducing sepsis prior to aspirin therapy will allow for the detection of 15-epi-LxA₄ within time frames previously published as well as the inhibition of established inflammation driven by NFκB, as seen in patients and other previously published studies [19, 214, 216, 480].

There are number of other differences that should be taken into consideration in the future development animal models of sepsis; epidemiological evidence suggests that in Australia, men are more likely to develop sepsis than women [29, 30]. Also, age and presence of comorbidities contribute to increases the risk of patients developing sepsis [4, 579-581]. A high proportion of the patients enrolled into the clinical study had at least one pre-existing condition. In fact, patients regularly taking aspirin were older, and all presented with at least one co-existing disease that contributed to their overall increased severity of inflammation. In contrast, mice used in sepsis studies were all healthy female, wild-type C57BL/6 strain of mice, age-matched at 6-8 weeks old. A previous study reported that C57BL/6 demonstrate increased resistance to bloodstream infections with *S. aureus* due to their genetic background [241], and this strain was chosen for initial studies to reduce the risk of rapid mortality after bloodstream infection. The laboratory mice in this study were also specific pathogen free (SPF), meaning that they would also have a reduced spectrum of pre-existing immune memory to bacterial pathogens.

Future development of this model to reflect a more accurate representation of the spectrum of human clinical sepsis will need to consider all of the previously described variables including: inducing sepsis in mice of both genders, in a variety of ages, and strains of mice with increased susceptibility (such as BALB/c or A/J mice), or strains of mice representing patients with co-morbidities like Type 2 diabetes (e.g. B6.Cg-m +/+)
Lepr^{db/J (db/db) obese type 2 diabetic mice). Further, considerations should also include a group of mice that also receive antibiotic therapy, mirroring the multi-modal therapies offered to patients with sepsis and in a previous study, was shown to decrease systemic inflammation and improve survival in a mouse model of gram-negative sepsis [17]. Additionally, the inclusion of an aspirin-only group should be considered, in order to determine any effect of aspirin on the gastro-intestinal tract. This group of mice would also facilitate determination of baseline 15-epi-LxA₄ and NFκB responses to aspirin, in the absence of infection.

Regardless of the differences in time lines, protocols and surrogate measures compared with patients, it remains that aspirin therapy did have beneficial effects in mice with S. aureus-induced sepsis. Clinical outcomes including changes in weight and clinical scores given on the basis of parameters outlined in Table 2.2 were significantly improved in mice treated with low-dose aspirin (equivalent to 100 mg dose in patients with sepsis). Additionally, low-dose aspirin-treated mice showed complete clearance of S. aureus from target organs by 48 h post-infection, in the absence of traditional antibiotic therapy.

### 6.3. Low-dose aspirin as a potential adjunct therapy of device-related biofilm infection

A series of studies were performed to investigate the additional direct effects of aspirin on pathogenic bacteria. Aspirin and its major metabolite SA have previously been shown by others to inhibit formation of biofilms of P. aeruginosa [540, 558] and Candida spp [541, 546]. In a manner that extends these observations and broadens the range of pathogens, this study has shown that SA inhibits staphylococcal biofilm formation in vitro. The mechanism by which this occurs is by impacting major regulatory processes including, production of polysaccharide biomass and extracellular DNA release. Given that these two processes are integral to resistance of the biofilm [312, 556], the combined effect of SA leads to formation of a weaker biofilm that could potentially be more susceptible to both immune attack and traditional antimicrobial therapies. Further, this study confirmed that transcription of global regulator gene sarA was significantly decreased in biofilm forming strains even up to 24 h following treatment with SA [221]. These results raised the question of whether aspirin therapy similarly impacts biofilm formation in vivo.
Beneficial effects have been shown following aspirin use in biofilm-associated diseases. Reductions in bacterial vegetation size and weight have been shown in aspirin-treated rabbits with biofilm-related endocarditis [384, 563]. Further, aspirin therapy is associated with reduced embolic complications in patients with infective endocarditis, and was considered a suitable prophylactic therapy in preventing thromboembolism [385, 565, 566]. Aspirin therapy also contributed to reduced incidence of staphylococcal bacteraemia in haemodialysis patients with tunnelled catheters [218]. These studies provided further evidence supporting the potential benefit of aspirin therapy in device-related biofilm infections.

This study is the first to demonstrate an effect of low-dose aspirin therapy in an in vivo model of subcutaneous device-related biofilm infection. Mice with S. aureus contaminated s/s implants had reduced biofilm formation, following 4 days of low-dose aspirin therapy. In contrast, mice with catheter implants were not affected by aspirin therapy; this was also reflected in the different systemic responses observed between the different implant types. Aspirin therapy was also shown to reduce epidermal thickness, in skin surrounding infected wounds which in other localised infection studies was suggestive of enhanced wound healing through rapid resolution of inflammation, presumably facilitated by the local production of pro-resolving mediators including 15-epi-LxA_4  [19, 568]. Future investigations in this model should include the quantification of local 15-epi-LxA_4 production and NFκB inhibition, and subsequent impact on local inflammation following aspirin therapy in device-related biofilm infections.

### 6.3.1. Differences observed between patients with device-related infections and mouse models in this study

As similarly noted in section 6.2.2, there were a number of differences noted between the mice in the experimental models of device-related biofilm infections study and their clinical counterparts, which need be considered.

Although the models investigated in this study did provide useful information on the effects of aspirin as an adjunct therapy for sub-cutaneous device-related biofilm infections, they were not ideal representative models of clinical intravascular catheter
infections, or prosthetic joint infections. Adaptations to the experimental models (as discussed in section 5.4.2), such as use of intravascular catheter inserted into the bloodstream, would better facilitate investigations into the systemic spread of localised infections, mimicking the predisposition of patients with intravascular catheters to bloodstream infection, endocarditis and sepsis development [582, 583]. Similarly, the continued use of urinary catheters with the adjustment of inserting closer to or directly into the bladder would allow the investigation of catheter-associated urinary tract infection, and determining the potential for aspirin as an adjunct therapeutic in this model [366, 376, 584]. Further, modifications to both the shape and size of s/s implants, as well as inserting implants in closer proximity to joints would permit the evaluation of aspirin therapy in a more representative model of clinical prosthetic-joint infections [573].

Aside from modifications to experimental implant designs, there are other considerations to be made when comparing the mice in the present study, to humans with clinical device-related infections. As similarly noted in section 6.2.2, age and presence of other pre-existing conditions were two clear points of difference. Patients with clinical device-related infections are older by comparison and have numerous pre-disposing health issues (patients requiring joint replacement, due to deterioration of natural joints, or patients requiring catheterisation; either for replenishment of fluids, or to assist urinary excretions) and in some cases these patients had deteriorating immune responses [353]. In contrast, mice used in the device-related biofilm models were all healthy female C57BL/6 strain of mice, age-matched at 6-8 weeks old. Mice in this study were also specific-pathogen free (SPF), and thus would not have pre-existing memory response to infectious pathogens. In order to more reliably reflect the patients commonly presenting with these types of infections, future studies should consider the use of aged mice of both genders, as well as mice from a variety of different genetic backgrounds (as described in section 6.2.2). In this way, the study would be able to determine the potential therapeutic range of low-dose aspirin, in these device-related infection models.

The beneficial effects of aspirin therapy in this model were similar to that observed in mice with sepsis in the absence of antibiotic therapy. Indeed, in vitro studies have demonstrated increased efficiency in diminishing biofilm formation of staphylococci, following incubation with salicylate and vancomycin in combination [564]. Further, combination therapy including administration of 15-epi-LxA₄ and ceftadizime improved
clinical outcomes in mice with systemic inflammation [17], supporting a more significant impact of aspirin as an adjunct therapy. In order to replicate therapies given to patients and determine whether outcomes are more significantly impacted, future studies of this model should also include antibiotic therapy. Furthermore, while therapeutic benefit was shown in this study, only one low dose of aspirin was investigated. Significant reduction of bacterial vegetations was observed in rabbits with infective endocarditis when treated with higher doses of aspirin (8-12 mg/kg) [384, 516, 563]. Indeed, high doses of aspirin (100 mg/kg and above, 10 fold greater than the highest dose used in the current study) are associated with bleeding and other negative side effects in both humans and rodents [585, 586]. In future studies however, it would be worthwhile investigating doses of aspirin comparative with the endocarditis studies (3-10 mg/kg); to determine whether a moderately increased aspirin dose would be more or less beneficial in adjunct therapy of device-related biofilm infection models. As similarly described in section 6.2.2, the inclusion of an aspirin-only control group should be considered in future studies, to determine any effects on the gastro-intestinal tract, and provide baseline responses to aspirin in the absence of infection.
6.4. Conclusion

In summary, this thesis has contributed to the understanding of the therapeutic benefits of low-dose aspirin therapy in systemic and localised inflammation following infection. Low-dose aspirin (100 mg) increases plasma 15-epi-LxA4 production, inhibited NFkB activation and changes in circulating innate immune cell proportions in patients with sepsis. While effects on these parameters were not shown in mice with S. aureus-induced sepsis, overall clinical condition was improved and complete clearance of bacteria from target organs was seen with equivalent aspirin doses, in the absence of antibiotic therapy. Future improvements to the design of the model will be required to better model the human spectrum of disease. Further, this study showed for the first time that low-dose aspirin therapy decreased biofilm formation on the surface s/s implants in a mouse model of device-related biofilm infection.

The results from this study provide support for a role for low-dose aspirin in the adjunct therapy of both sepsis and device-related biofilm infection. Completion of the MATHs clinical trial will be required to provide definitive evidence to support advancing to a prospective trial in septic patients. Further research is required in the development of both mouse models, in order to dissect the mechanisms of aspirin-induced responses and determine whether adapted models are more representative of patients of sepsis and device-related infections, respectively.
References
References


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References


References


512. Kurt ANC, Aygun AD, Godekmerdan A, Kurt A, Dogan Y, Yilmaz E: Serum IL-1β, IL-6, IL-8, and TNF-α levels in early diagnosis and management of neonatal sepsis. Mediators of Inflammation 2007, 2007:31397.


References


544. Bacalso M, Xu T, Yeung K, Zheng D: Biofilm formation of *Pseudomonas aeruginosa PA14* required lasI and was stimulated by the *Pseudomonas* quinolone signal although Salicylic acid inhibition is independent of the pqs pathway. *Journal of Experimental Microbiology and Immunology* 2011, 15:7.


Appendices
3 September 2013

Dr Anna Walduck
School of Applied Sciences
RMIT University

Dear Anna,

AEC 1213: Could aspirin improve outcomes in severe staphylococcal infections.

I am pleased to advise that this project has been approved by the RMIT University Animal Ethics Committee (AEC) for the period from 1 August 2013 until 1 August 2016.

Animals

Your application has been approved to use the following animals, over the duration of the project.

<table>
<thead>
<tr>
<th>Species (and common name)</th>
<th>Strain [Indicate (*) if Genetically Modified]</th>
<th>Sex</th>
<th>Age</th>
<th>Total Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>BALB/c</td>
<td>M/F</td>
<td>5-8 weeks</td>
<td>282</td>
</tr>
<tr>
<td>Mouse</td>
<td>C57BL/6</td>
<td>M/F</td>
<td>6-8 weeks</td>
<td>282</td>
</tr>
</tbody>
</table>

The use of animals in scientific procedures is strictly regulated by the Australian code of practice for the care and use of animals for scientific purposes. The above project is conducted under a Scientific Procedures and Premises License issued by the Bureau of Animal Welfare.

Responsibilities of Investigators

1. Dr. Anna Walduck
2. Ms Jacinta Ortega

Responsibilities of investigators are described in the Australian code of practice for the care and use of animals for scientific purposes (section 3). Investigators have a ‘personal responsibility for all matters related to the welfare of animals they use and must act in accordance with all requirements of the code. This responsibility begins when an animal is allocated to a project and ends with its fate at the completion of the project’ (6.3.1.1).

Amendments and extensions
Appendix 1

If you find reason to amend your research method you should advise the AEC and prepare a request for minor amendment form. Please note that the AEC may only deal with ‘minor’ amendment requests. Major amendments to projects normally require a new project application.

Adverse events or unexpected outcomes
As the primary investigator you have a significant responsibility to monitor the research and to take prompt steps to deal with any unexpected outcomes. You must notify the AEC immediately of any serious or unexpected adverse effects on animals, or unforeseen events, which may affect the ethical acceptability of your project.

Unwell animals must be immediately reported via the care forms available at the RMIT Animal Facility. In the case of any emergency, the Animal Welfare Officer, Dr Rebecca Wilcox, may be contacted at any time. In case of any unexpected animal death, the researcher has a responsibility to organise an autopsy so as to determine the cause of death.

Investigator guidelines for record keeping
Investigators are required to adhere to the strict guidelines regarding record keeping for their project. Note that records associated with a project ‘should be available for audit by the institution and authorised external reviewers’. Failure to maintain proper records may result in a compliance breach of the Code and place at risk the researcher’s capacity to carry out research with animals.

Conditions of approval
The AEC may apply conditions of approval beyond the submission of annual/final reports. There are no specific conditions attached to this project, except that described elsewhere in this letter.

Reports
Approval to continue a project is conditional on the submission of annual and final reports. Annual reports are requested in December each year, and must be submitted whether or not the project has commenced or is inactive. Report forms are available at http://www.rmit.edu.au/governance/committees/aec.

Failure to submit reports will mean that a project is no longer approved, and/or that approval will be withheld from future projects.

All reports or communication regarding this project are to be forwarded to the secretary.

On behalf of the AEC I wish you well with your research.

Nupur Nag, PhD
Acting Coordinator, Animal Ethics and Gene Modification
On behalf of
RMIT Animal Ethics Committee
22 January 2015

Dr Anna Walduck
School of Applied Sciences
RMIT University

Dear Anna,

AEC 1426: A model of surgical wound infection to test the of effect two novel treatments

I am pleased to advise that this project has been approved by the RMIT University Animal Ethics Committee (AEC) for the period from 22 January 2015 until 21 January 2018. An approved version of the application is attached.

Animals
Your application has been approved to use $n=100$ mice (C57BL/6J mice) over the duration of the project.

The use of animals in scientific procedures is strictly regulated by the Australian code of practice for the care and use of animals for scientific purposes. The above project is conducted under a Scientific Procedures and Premises License issued by the Bureau of Animal Welfare.

Responsibilities of Investigators
1. Dr Anna Walduck
2. Ms Jacinta Ortega
3. Associate Professor Vipul Bansal

Responsibilities of investigators are described in the Australian code of practice for the care and use of animals for scientific purposes (section 3). Investigators have a ‘personal responsibility for all matters related to the welfare of animals they use and must act in accordance with all requirements of the code. This responsibility begins when an animal is allocated to a project and ends with its fate at the completion of the project’ (s.3.1.1).

Amendments and extensions
If you find reason to amend your research method you should advise the AEC and prepare a request for minor amendment form. Please note that the AEC may only deal with ‘minor’ amendment requests. Major amendments to projects normally require a new project application.

Adverse events or unexpected outcomes
As the primary investigator you have a significant responsibility to monitor the research and to take prompt steps to deal with any unexpected outcomes. You must notify the AEC immediately of any serious or unexpected adverse effects on animals, or unforeseen events, which may affect the ethical acceptability of your project.

Unwell animals must be immediately reported via the care forms available at the RMIT Animal Facility. In the case of any emergency, the Animal Welfare Officer, Dr Rebecca Wilcox, may be contacted on
at any time. In case of any unexpected animal death, the researcher has a responsibility to organise an autopsy so as to determine the cause of death.

**Investigator guidelines for record keeping**
Investigators are required to adhere to the strict guidelines regarding record keeping for their project. Note that records associated with a project should be available for audit by the institution and authorised external reviewers. Failure to maintain proper records may result in a compliance breach of the Code and place at risk the researcher’s capacity to carry out research with animals.

**Conditions of approval**
The AEC may apply conditions of approval beyond the submission of annual/final reports. There are no specific conditions attached to this project, except that described elsewhere in this letter.

**Reports**
Approval to continue a project is conditional on the submission of annual and final reports. Annual reports are requested in December each year, and must be submitted whether or not the project has commenced or is inactive. Report forms are available at [http://www.rmit.edu.au/governance/committees/aec](http://www.rmit.edu.au/governance/committees/aec).

Failure to submit reports will mean that a project is no longer approved, and/or that approval will be withheld from future projects.

All reports or communication regarding this project are to be forwarded to the secretary.

On behalf of the AEC I wish you well with your research.

Dr Brad Hayward  
Research Ethics Coordinator  
*On behalf of*  
RMIT Animal Ethics Committee

cc: Ms Tricia Murphy, RAF manager
Appendix 3

OFFICE FOR RESEARCH

MELBOURNE HEALTH HUMAN RESEARCH ETHICS COMMITTEE
ETHICAL APPROVAL OF A RESEARCH PROJECT

A/Prof Damon Eisen
Victorian Infectious Diseases Service (VIDS)

8th August 2011

Dear A/Prof Damon Eisen,

MH Project Number: 2011.143

Project Title: Randomised, open label, phase II study of aspirin in sepsis patients in ICU to determine the PK/PD of aspirin.

HREC Approval Date: 8th August 2011

I am pleased to advise that the above project has received ethical approval.

Participating Sites:

• The Royal Melbourne Hospital

Approved Documents:

• Study Protocol Version 2 dated 18th July 2011
• The Royal Melbourne Hospital Person Responsible Information and Consent Form Version 2 dated 18th July 2011

Site Specific Assessment:

Please note: You cannot commence this study until you have completed all the requirements of the Site Specific Assessment and have received the “Approval to Conduct a Research Project at Melbourne Health” certificate.

Conditions of Ethics Approval:

In order to comply with the National Statement on Ethical Conduct in Human Research 2007, Guidelines for Good Clinical Research Practice and Melbourne Health Research Policies and Guidelines you are required to:

• Submit a copy of this letter to the Radiation Safety Officer (RSO) at Melbourne Health, for addition of the project to the Licence for Research Involving Human Volunteers held by the Department of Human Services Radiation Safety Section Radiation Safety Licence (if your project involves exposure to ionising radiation). Note: You cannot commence the project...
until you have received notification from the RSO that the project has been added to the Licence;

- Notify the HREC of the actual start date of the project;
- Submit to the HREC for approval any proposed amendments to the project including any proposed changes to the Protocol, Participant Information and Consent Form/s and the Investigator Brochure;
- Notify the HREC of any adverse events in accordance with the Melbourne Health Guidelines for Monitoring and Reporting of Safety in Clinical Trials Involving Therapeutic Products and Other Clinical Research, July 2009;
- Notify the HREC of any unforeseen events;
- Notify the HREC of your inability to continue as Principal Investigator or any other change in research personnel involved in the project;
- Notify the HREC if a decision is taken to end the study prior to the expected date of completion or failure to commence the study within 12 months of the HREC approval date;
- Notify the HREC of any other matters which may impact the conduct of the project.

**Reporting**

You are required to submit to the HREC:

- An Annual Progress Report every 12 months (or more frequently as requested by the reviewing HREC) for the duration of the project. This report is due on the anniversary of HREC approval. Continuation of ethics approval is contingent on submission of an annual report in a timely manner; and
- A comprehensive Final Report upon completion of the project.

The HREC may conduct an audit of the project at any time.

Please refer to the Office for Research website to access forms such as the Amendment Form, Annual Report/Final Report Form, Guidelines for Monitoring and Reporting of Safety in Clinical Trials Guidelines and Adverse Event Report Forms, and other information and news concerning research at Melbourne Health: [http://www.mh.org.au/www/342/1001127/displayarticle/1001352.html](http://www.mh.org.au/www/342/1001127/displayarticle/1001352.html)

A list of those HREC members present at the review of this project can be obtained from the above website.

Yours sincerely,

Ms. Angela Gray  
Manager, Melbourne Health Human Research Ethics Committee