Declaration

I hereby declare that the work herein, now submitted as a thesis for the degree of Doctor of Philosophy at Charles Darwin University is the result of my own investigations, and all references to ideas and work of other researchers have been specifically acknowledged. I hereby certify that the work embodied in this thesis has not already been accepted in substance for any degree, and is not currently being submitted in candidature for any other degree.
Abstract

Despite substantial advances in its management, severe sepsis remains the most common cause of death in intensive care units, and has a mortality of 30-50%. Within Australia, there are an estimated 15,000 cases of severe sepsis each year. However the epidemiology of sepsis in the tropical Northern Territory, a region with a high incidence of acute infections and chronic diseases, and a high proportion of Indigenous residents, has not been described. The pathophysiology of sepsis is complex and incompletely understood. A pivotal factor in sepsis pathophysiology is dysfunction of the endothelium, a metabolically active organ which lines the entire vascular system.

Following the literature reviews in section A, this thesis is divided into three parts. In section B, I present a 12-month prospective cohort study based at Royal Darwin Hospital describing the epidemiology of sepsis in the Top End of the Northern Territory. This study found that the incidence of sepsis in this region is five-fold higher than that in temperate Australia and elsewhere, and that most of this burden occurs in Indigenous people.

In section C, I undertook an observational study examining endothelial function in patients with sepsis and healthy controls, using a novel technique called peripheral arterial tonometry. This study found that peripheral arterial tonometry is a feasible technique for monitoring endothelial function in sepsis and that endothelial function is impaired in proportion to disease severity. It also identified several potential targets for development of therapeutic intervention to improve the endothelial and microvascular dysfunction of sepsis.

One of the most promising potential adjunctive therapies for sepsis is the statins, a class of lipid-lowering drugs. Section D describes the rationale and protocol for a currently recruiting randomised trial of atorvastatin to improve endothelial function in patients with severe sepsis.
Plain language summary of this thesis

The term “sepsis” means an infection which has triggered a widespread response by the infected person’s body. This response may be manifested by fever, increased heart rate and breathing rate, or a raised white blood cell count. Sepsis does not mean local infection (e.g. an abscess of the foot, without fever): it refers to the case where inflammation has spread beyond the original focus of infection to involve the whole body. The term “severe sepsis” means sepsis that results in damage to vital organs, such as the kidneys, liver and heart. Commonly used terms that roughly correspond to sepsis include “blood poisoning” and “septicaemia”.

Sepsis is a common and devastating problem, and according to a recent large US study, it causes more deaths each year than heart attacks. Approximately 15,000 cases of severe sepsis occur in Australia and New Zealand every year and around one third of these patients die as a result. However, it is not known how common sepsis is in tropical Northern Australia. This region is different from Australia as a whole, because a high proportion (around 25%) of its residents are Indigenous; many of its residents have chronic diseases such as diabetes or kidney failure; and a different range of bacteria cause infections in this region compared to elsewhere.

Following reviews of the relevant literature in Part A, in part B of this thesis I describe a study which aimed to find out how common sepsis is in the Top End of the Northern Territory, who gets it, what are the common responsible germs, and what are the risk factors for dying of sepsis. This study found that sepsis is five times more common in this region than in the rest of Australia, Europe or North America. Most of this difference comes from the figures in Indigenous people, in whom sepsis is twenty times more common than in other regions. However, among patients hospitalised with sepsis, the risk of dying appears to be lower at Royal Darwin Hospital (RDH) than it is in most other studies. For example, of those with severe sepsis who went to the intensive care unit, 21% died, compared with 38% in a study from the rest of Australia and 36% in a large European study. This is true despite the sepsis being just as severe at RDH as in other studies. The reasons for this are unclear, but one possible explanation is that patients here are, on average, younger than those reported from elsewhere. Even though a low proportion of patients die from sepsis in the Top End, a high number of patients die, because sepsis is so much more common here than elsewhere. Therefore, sepsis is an important problem in the Top End. Most of the burden is shouldered by Indigenous Australians, particularly those with poor
nutrition and kidney disease. The results of this study can be used to design strategies to prevent sepsis, and to decrease the number of people dying from sepsis.

Chapters 6 and 7 of part B both evaluate scoring systems to estimate the severity of sepsis, and help to predict who will die or develop more severe illness. Both of these chapters describe new uses for these scoring systems to help in developing local treatment guidelines to improve outcomes from sepsis.

Part C of the thesis concerns “endothelial function” in patients with sepsis. The “endothelium” is a group of cells which form the lining of all the blood vessels in the human body, ranging from the largest (the aorta for example) to the smallest (the capillaries, which need a microscope to be seen). There are more than 100 trillion endothelial cells in the human body, and if they were all laid out, they would cover an area of approximately twenty-seven tennis courts, or 27,000 Monopoly boards. The endothelium was once thought to be simply a passive cell layer which kept the blood vessels smooth and prevented blood clotting – akin to a layer of Teflon. However it is now known that endothelial cells are very active; they sense changes in their environment, and can respond to these changes in multiple ways (usually by releasing messenger chemicals into the blood stream). The function of endothelial cells is to keep the blood flowing (i.e. to prevent it from clotting); to stop the blood cells and fluid from leaking out into the tissues; to regulate how big or small individual blood vessels are; and to act as part of the immune system, responding to foreign material such as bacteria. Together these jobs are known as “endothelial function”.

So what does this have to do with sepsis? During sepsis, the endothelium becomes activated and damaged, and starts not to do its job properly. It stops being able to control the tightness and size of blood vessels (causing blood pressure to become too low, and meaning that not enough blood flows to vital organs); It allows cells and fluid to leak out into the tissues (causing the patient to swell up); it allows the blood to clot in small blood vessels (also depriving some organs of proper blood flow); it amplifies the body’s response to infection, making sepsis worse (instead of damping it down, as it is meant to do). Many of these effects have only been measured in laboratory animals with sepsis.

It is difficult to measure endothelial function in people with sepsis. A few previous studies have done so using complicated or invasive techniques which require highly trained staff and a very co-operative patient. We used a relatively new technology, peripheral arterial
tonometry (or PAT) to measure endothelial function in patients with sepsis. PAT is easy to perform, requires no needles, and the results are generated by a computer. It uses a thimble-like device to record the pulse in the fingertips. PAT has been used in many studies to measure endothelial function in people who have or are at risk of heart disease, but it has never been used in sepsis before. Because people with sepsis often have poor blood supply to their fingertips, it was unclear if PAT would work in this setting. The aim of the study described in Chapter 9 was to determine the feasibility of PAT to measure endothelial function in sepsis, to determine if endothelial function is impaired in sepsis patients compared with healthy patients, and to explore the correlations of the PAT results.

This study found that PAT was indeed a feasible technique in sepsis patients (i.e. it worked in the majority of patients). Endothelial function was impaired in sepsis patients compared with healthy people, and this impairment correlated with disease severity; in other words the sicker a patient was, the worse their endothelial function. Chapters 11-13 present further results from this study, identifying several biochemical targets for potential treatments aiming to improve endothelial function in people with sepsis.

Finally, in part D of the thesis, I present the detailed plans for a clinical trial of a drug called atorvastatin (Lipitor – a member of a family of cholesterol medications called the “statin” family) to try to improve endothelial function and thus decrease organ failure and death in patients with severe sepsis. Atorvastatin is the most commonly prescribed medication in Australia, and is used to treat high cholesterol. However, atorvastatin also happens to have some other beneficial side effects, including improving endothelial function. Several studies, including a study performed as part of this thesis (presented in Chapter 16) have suggested that people with sepsis are less likely to die if they are taking a drug from the statin family. However, these studies were not clinical trials – they were observations of groups of people who happened to be taking a statin. Therefore it has not been proven that statins are of benefit in sepsis; the only way to definitively do so is a clinical trial, where patients with sepsis are randomly allocated to take either a statin or an identical placebo pill (which contains no active drug). The protocol for such a study is presented in Chapter 17. This particular trial is testing whether statins improve endothelial function in sepsis. It is currently in progress, and has enrolled 36 of a planned 76 patients.
Dedication

This work is dedicated to my daughters Zoë, Bella, Poppy and Amélie, the last two of whom were born during my PhD candidature - may you grow up to a world where Indigenous health inequality is a thing of the past, and where clinical and translational research continues to make incremental improvements in outcomes from sepsis; I also dedicate it to the memory of my cousin Lucy Lieberman, who died tragically at the age of 15 during the course of this work.
Acknowledgements

Issac Newton said that if he had seen further than other men, it was because he had stood on the shoulders of giants. Of course, such great leaps forward seldom occur. Science is a collaborative and incremental process. In the work described in this thesis, I have stood on the shoulders of giants, but many people helped me climb up to those shoulders, held me steady while I was there and passed me binoculars to help me see into the distance.

It is traditional in theses to acknowledge one’s supervisors and collaborators first and one’s family last. I think this an example of positivist thinking distorting reality. I owe a great debt of gratitude to my wife, my love and my best friend, Joanne Walsh. Despite many obstacles, she has steadfastly supported and encouraged me through this process, while simultaneously looking after our small children, earning a real salary to supplement my student’s income, and generally holding our lives together. Joey, thank you for believing in me – I am a very lucky man.

Sepsis research is a crowded and competitive field. This has helped by giving me a wealth of research to draw upon and learn from; it has also hindered by creating a culture that may be suspicious of novelty. There are several international researchers I wish to thank for their advice – often in reply to unsolicited emails from a complete stranger (me) – these are Derek Angus, Jacques Creteur and Mark Astiz. I wish also to thank Jean-Louis Vincent, who allowed me to take a tour of his ICU and research laboratories at the Hôpital Erasme of the Université Libre de Bruxelles. Peter Kruger, from the Princess Alexandra Hospital in Brisbane deserves special thanks. When he could have seen me as a competitor, he made me a collaborator and has been very generous with his time and knowledge. Professor David Celermajer has also been very helpful with his encyclopaedic knowledge of endothelial function and his useful input to several manuscripts.

Closer to home, there are many people I wish to thank at the Menzies School of Health Research. Mark McMillan and Alex Humphrey both worked with me as research assistants; they both excelled at their jobs and without their hard work collecting, entering and checking data, my PhD would have taken six years rather than three. I also would like to thank Bart Currie, who was always a wealth of wise advice when asked; Ross Andrews and John Condon, who both lent me their epidemiology expertise; Joseph McDonnell for his advice on advanced statistical questions and his clever Stata tricks; Robin Liddle for helping
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For most of my candidature, I sat in the “Beach Club”, and my fellow beach-clubbers were an endless source of useful advice and conversation and were also great sounding boards; thanks to Steven Tong, Paul Burgess, Christabelle Darcy, Tom Snelling, Naor Bar Ze’ev, Jacqui Boyle and Annette Dougal. Towards the end I shared an office with Peter Morris and then Nick Douglas – I also thank them their advice and forbearance. The Menzies School of Health Research is truly a dynamic and intellectually enriching environment for any health researcher.

Much of the research reported in this thesis was conducted in the Intensive Care Unit of Royal Darwin Hospital (RDH). I am grateful to the director of the unit Dianne Stephens, who was a collaborator on all of the major projects in this thesis, and is strongly supportive of research despite running a very busy ICU. Jane Thomas, the research co-ordinator at RDH ICU was a pivotal part of the FRESH study and the STREAMS trial, screening and recruiting patients and collecting data. Her obsessive attention to detail, dedication, and great rapport with and concern for patients make her the perfect research co-ordinator. Without Jane, clinical research in a stressful environment like the ICU would be impossible. I would also like to thank the other intensive care specialists, Paul Goldrick and Sarah Collins who were always helpful and ready for a friendly chat, and the nursing staff of the ICU, for help with study procedures. Some of the control patients for the FRESH study were recruited and had measurements taken in the RDH Hospital in the Home unit; thus I would like to thank Paulene Kittler and the other nursing staff of HITH. I am also grateful to all of the patients and their families who so selflessly agreed to take part in the studies reported here, not for tangible benefit to themselves, but to help future patients.
Thanks to Ric Price for all his helpful advice and support, particularly in the field of databases and statistics. Tsin Yeo, who completed a PhD on endothelial function and L-arginine supplementation in malaria during my candidature was an indispensable resource and was basically an extra supervisor. Much of the work in this thesis builds upon work Tsin and colleagues had pioneered in malaria studies in West Papua. Tsin was always generous with his time and ideas, and taught me much of what I know about endothelial pathophysiology. Allen Cheng was a fantastic associate supervisor. Whether he was in Darwin or Melbourne, he always gave me a useful and definitive answer to any query in a timely fashion – usually within minutes. I particularly benefited from his expertise on epidemiology and biostatistics. I also learnt much from Allen’s seemingly effortless way of churning out manuscripts and of expressing difficult concepts in apt phrases.

Throughout my candidature, I received a PhD scholarship from the National Health and Medical Research Council, for which I am grateful, and which took the pressure off having to do too much clinical work. Thanks are also due to my clinical colleagues in the Royal Darwin Hospital department of infectious diseases and the division of medicine for sharing the clinical load and never complaining that I was only working 0.2 of a full-time position at the hospital.

I have the kind of tangential and distractible mind which would go crazy if concentrating only on science for three years. Thus I would like to thank the following people for keeping me sane: during the first year, Simone de Beauvoir, Irène Nemirovsky and Guy de Maupassant; during the second year, Marcus Tullius Cicero, Gaius Julius Caesar and Gaius Marius; and during the third year Zuben el Genubi, John Dobson and Omega Centauri. I also would like to thank the boys from my book/beer club and cycling group for further fruitful and fun distractions.

Many of my friends and family suffered the fate of having to listen to me explain a concept from or bemoan a setback in my research. These discussions often helped me straighten out my ideas. Thanks to Jo Walsh, Richard Davis, Lawrence Dadd, Geneviève Campbell, Justine Davis, Anna Davis, David Steinberg and Denise Davis for listening. Thanks also to Denise Davis, Richard Davis, John Jablonka and David Steinberg for help with proof reading, an unenviable task if ever there was one. I also wish to thank my children, Zoë, Bella, Poppy and Amélie: for putting up with the mental and physical absences induced in me by the process of doing my PhD; for always making me laugh; and for helping me keep perspective
on what is important. Without lots of help with childcare, I would not have been able to complete this thesis; of course Jo Walsh bore the brunt of this, but thanks also to David Steinberg; John Jablonka, Yvonne Walsh; and Denise, Richard, Justine and Anna Davis for this support.

Finally, I wish to thank the best PhD supervisor there ever was: Professor Nicholas Anstey. Nick’s enthusiasm, optimism, diplomacy, diligence, friendliness, sense of humour and unwavering support were the ocean upon which my little PhD boat has sailed for the last three years. Nick has been very generous and trusting in providing all the funding and resources needed for my research. Despite this, he has allowed me a lot of independence, and has let me develop ideas even when he didn’t agree with them (he usually turned out to be right, of course). Nick took a gamble on me by allowing me to commence a PhD in what was a relatively new field for Menzies, for which I am eternally grateful. Nick’s careful (and sometimes, it must be said, excessive!) attention to detail was the perfect foil for my tendency to cut corners. His dedication to his students, both myself and others, is unparalleled. On one occasion, I emailed Nick when he was in the UK, forgetting about the time difference, saying I needed to speak to him. Minutes later he was talking to me via Skype. When I realised he looked a bit sleepy, I asked him what time it was. He said it was 3am, but this was the only time that day he could give some attention to his research students back in Australia! Nick, thank you for teaching me the trade, and I hope this thesis is a fitting testament to the quality of my principal supervisor.
Publications arising from this work

Published manuscripts


Manuscripts submitted to peer-reviewed journals, undergoing review


2. Davis JS, Yeo TW, Thomas JT, Stephens D, Kruger P, Anstey NM. Dynamic near-infrared spectroscopy compared with peripheral arterial tonometry to measure microvascular reactivity in severe sepsis.

Manuscripts planned for submission to peer reviewed journals

1. Davis JS, Cheng AC, Stephens DP, Anstey NM. Comparison of APACHE II scores for predicting mortality in patients with sepsis admitted to the intensive care unit or hospital wards.

2. Davis JS, Yeo TW, Anstey NM. Single versus repeated use of near-infrared spectroscopy sensors.

Abstracts published and/or presented at national or international meetings

1. Davis JS*, Thomas JH, Yeo TS, Blenk KH, Boutlis CS, Celermajer DS, Stephens DP, Anstey NM. *Finger Reactive hyperaemia to measure Endothelial function in Sepsis and Health (the FRESH study).* Presented at the Annual Scientific Meeting of the Australasian Society for Infectious Diseases, Hobart, Tasmania, 2007.

2. Davis JS*. *Statins – have we finally found an adjuvant therapy for sepsis that works?* Presented at the Annual Scientific Meeting of the Australasian Society for Infectious Diseases, Maroochydore, Queensland, 2008.


Declaration of the author’s contribution

This thesis is substantially my own work and was written under the supervision of Professor Nicholas Anstey. All of the chapters of this thesis which are not multi-author papers were written by me. All of the chapters which are multi-author papers were originally drafted and predominantly written by me, apart from Chapter 13, of which I am the second author. All of the statistical analyses were performed predominantly by me. I also made up all of the acronyms in this thesis!

The PRESTO study, reported in Chapters 5-7 was planned and designed by me. Much of the data collection for this study was performed by Mark McMillan and Alex Humphrey, my research assistants. My co-authors in Chapters 5-7 contributed to the final draft of the manuscripts, helped with study procedures and commented on data analysis.

The FRESH study, reported in Chapters 8-13 was planned and designed by Nick Anstey, Tsin Yeo, Craig Boutlis and me. Patient recruitment, study procedures and data collection were primarily performed by Jane Thomas and Mark McMillan. I recruited and performed endothelial function measurements on approximately 15% of the subjects. The following people also helped recruit patients and take measurements: Karl Blenk, Anthony van Asche, Steven Tong and Paulene Kittler. The HPLC assays for amino acids were designed and conducted by Yvette McNeil, Christabelle Darcy and Catherine Jones. ELISA assays were performed by Kim Piera. Cytokine bead array assays were performed by Tonia Woodberry, with some clumsy help from me. I performed all data management and analysis. The first draft of Chapter 13 was written by Christabelle Darcy. I contributed to the analytical plan, and the subsequent drafts of the paper. Although I wrote the first draft of Chapter 11, Christabelle Darcy is listed as equal first author, for her important intellectual contribution to the analysis and manuscript.

The STOPWATCH study, reported in Chapter 8, was planned and designed by Kim Piera, Tonia Woodberry, Christabelle Darcy and me. Others who helped with blood collection or gave their blood are acknowledged in the manuscript. The plasma amino acid HPLC was done by Yvette McNeil. I performed integration of the HPLC trace of approximately half of the samples. I performed the data analysis and wrote the first draft of the manuscript.
The study reported in Chapter 14 was planned and carried out by me and Tsin Yeo, who were also the volunteers. I performed the analysis and wrote the manuscript.

The protocol for the STREAMS trial (Chapter 17) was written by me, but much of the study design was, by necessity, taken from that of the national STATInS study.
List of Abbreviations

ACh – Acetylcholine
ADMA – Asymmetric dimethyl arginine
ANZICS – Australia and New Zealand Intensive Care Society
APACHE – Acute Physiology And Chronic Health Evaluation
CAT – Cationic Amino acid Transporter
DDAH - Dimethylarginine dimethylaminohydrolase
dNIRS – Dynamic near-infrared spectroscopy
ED – Emergency department
EDRF – Endothelial-derived relaxing factor
eNOS – Endothelial nitric oxide synthase
FMD – Flow mediated dilatation of the brachial artery
FRESH – Finger Reactive hyperaemia to measure Endothelial function in Sepsis and Health
GTN – Gliceryl Trinitrate
ICAM-1 – Intercellular adhesion molecule-1
ICU – Intensive Care Unit
IDO - Indoleamine 2,3-dioxygenase
IFNγ – Interferon gamma
IL – Interleukin
IQR – Interquartile range
iNOS – Inducible nitric oxide synthase
LPS – Lipopolysaccharide
NIRS – Near-infrared spectroscopy
NO – Nitric oxide
NOS – Nitric oxide synthase
PAT – Peripheral arterial tonometry
PRESTO – Prospective Epidemiology of Sepsis in the TOp end.
PSI – Pneumonia Severity Index
PWA – Pulse wave amplitude
RDH – Royal Darwin Hospital
RH-PAT – Reactive hyperaemia peripheral arterial tonometry
SDMA – Symmetric dimethyl arginine
SIRS – Systemic inflammatory response syndrome
STATInS – STudy of Atorvastatin In Sepsis
STREAMS – STatins to Reduce Endothelial dysfunction; Adjunctive Management in Sepsis
SD – Standard deviation
SEM – Standard error of the mean
SOFA – Sequential organ failure assessment
StO₂ – Tissue oxygen saturation
TLR – TOLL-like receptors
TNFα – Tumor necrosis factor alpha
VEGF – Vascular endothelial growth factor
VOT – Vascular occlusion test
vWF – von Willebrand Factor
WPB – Weibel-Palade body
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Section A - Introduction and Literature Reviews.

“The summer’s flower is to the summer sweet
Though to itself it only live and die,
But if that flower with base infection meet,
The basest weed outbraves his dignity:
For sweetest things turn sourest by their deeds;
Lilies that fester smell far worse than weeds”

William Shakespeare (1564-1616)
Chapter 1. Background, scope and aims of the thesis
1.1 Background

Severe sepsis is responsible for over 13,000 hospitalisations in Australasia [1] and 215,000 deaths in North America [2] every year, and is increasing in incidence [3, 4]. The hospital mortality from severe sepsis remains at 25-50% [5, 6], even in the presence of broad-spectrum antibiotic treatment, source control and modern advances in supportive care.

Despite the fact that sepsis is a syndrome seen every day in every large hospital, the precise mechanisms leading to organ dysfunction and death in patients with sepsis are not well understood. As a corollary of this, few effective adjunctive sepsis therapies have been discovered. The importance of the endothelium and microvasculature in the pathophysiology of sepsis has been increasingly recognised in recent years, and this field of study holds promise for improving the understanding, prevention and treatment of sepsis-related organ dysfunction.

1.2 Scope and structure of the thesis

The broad questions addressed in this thesis fall into three areas, whose unifying feature is a desire to improve outcomes from sepsis through improved understanding. The first area is the epidemiology of sepsis, and attempts to define the scope of the problem in the tropical Northern Territory of Australia. The second focuses on the pathophysiology of sepsis, and explores several aspects of endothelial function in patients with sepsis, including some potential therapeutic targets. The third area concerns adjunctive treatment of sepsis with statins, a therapy which improves endothelial function.

This thesis is divided into five sections. Section A contains literature reviews providing the bedrock upon which rests the remainder of the thesis. In Chapter Two, the current understanding of the epidemiology of sepsis around the world and in Australia, as well as methodological issues important for studies of sepsis epidemiology are reviewed. Chapter Three is a literature review summarising background, methodological and pathophysiological issues relevant to the study of endothelial function in sepsis. Chapter Four is a systematic review aiming to determine if the plasma concentration of arginine (the precursor of nitric oxide, essential for endothelial and immunological function) is decreased in patients with sepsis.

Section B (Chapters 5-7) concerns the epidemiology and risk stratification of sepsis and reports results derived from a prospective cohort study of sepsis epidemiology conducted
at Royal Darwin Hospital (RDH). Section C (Chapters 8-15) presents the methodology and results of an observational study of endothelial function in patients with sepsis at RDH (Chapters 8-13), as well as investigations into the use of near-infrared spectroscopy as a tool to measure microvascular function in sepsis (Chapters 14-15). Section D (Chapters 16-17) addresses statins as an adjunctive therapy in sepsis, and contains a cohort study examining their effect on mortality, as well as a protocol for a randomised controlled trial of atorvastatin in severe sepsis which is currently underway.

In summary, Section B defines the scope of the problem, Section C investigates and develops tools for the assessment of endothelial function in sepsis and Section D uses this information and these tools to commence a clinical trial aiming to improve sepsis outcomes by targeting the endothelium.

The majority of the chapters are manuscripts which have been submitted to peer-reviewed journals for publication. Chapters seven, eight and ten have been published, and are presented as published papers, with no alteration from the published format. This is to avoid dismantling their peer-review status. The unpublished but submitted papers have been reformatted for the thesis, but their content is unaltered from the submitted manuscripts. References are listed at the end of each chapter rather than at the end of the thesis.
1.3 Aims and hypotheses

The detailed aims and hypotheses for each of the three strands comprising the rope of this thesis are as follows:

1.3.1 The epidemiology of sepsis in the Top End of the Northern Territory

1.3.1.1 Aims

1) To define the incidence of sepsis, severe sepsis and septic shock in the primary drainage area of Royal Darwin Hospital (RDH).
2) To describe the microbiology, clinical features, demographics and severity of sepsis at RDH over a one year period.
3) To determine predictors of Intensive Care Unit (ICU) admission, length of stay and mortality in RDH patients with sepsis.

1.3.1.2 Hypotheses

1) The incidence of sepsis in the drainage area of RDH is higher than that of Australia as a whole, as well as that of North America and Europe.
2) Patients with sepsis at RDH have a younger age, a higher severity of sepsis (as estimated by APACHE II scores and SOFA scores) and a different distribution of causative organisms than Australia as a whole, and than other regions with published data.
3) Severe sepsis which does not require admission to ICU is a common condition and has a similar mortality to that requiring ICU admission.

1.3.2 Endothelial function in sepsis

1.3.2.1 Aims

1) To evaluate the use of peripheral arterial tonometry (PAT) for the assessment of endothelial function in patients with sepsis.
2) To compare endothelial function in patients with and without sepsis, both at baseline, and longitudinally over the first 7 days of illness.
3) To examine the relationship between endothelial function, disease severity, and the following parameters
   i) The severity and outcome of sepsis
   ii) The degree of endothelial activation (as measured by serum levels of soluble adhesion molecules) and
   iii) Plasma concentration of L-arginine and dimethylarginines.
1.3.2.2 Hypotheses

1) PAT will be a feasible method for measuring reactive hyperaemia (and thus estimating endothelial function) in patients with sepsis.

2) Baseline endothelial function is impaired in patients with sepsis compared with non-septic controls.

2) The change in endothelial function over the first 48-72 hours of illness will correlate with disease severity and outcome.

3) Serum ICAM-1, e-Selectin and Angiopoietin-2 will be elevated in patients with sepsis compared with non-septic controls, with the degree of their elevation correlating with the degree of endothelial dysfunction.

4) The degree of endothelial dysfunction (as measured by PAT) and its change over the first 48-72 hours will correlate with the severity of illness and with the development of organ failure or death.

5) Plasma L-arginine levels and arginine/ADMA (asymmetric dimethylarginine) ratio will be low in patients with sepsis, and will correlate with the degree of endothelial dysfunction.

1.3.3 Adjunctive therapy with atorvastatin in sepsis

1.3.3.1 Aims

1) To determine if pre-existing statin use is protective against death in patients admitted to RDH with sepsis.

2) To develop the tools and protocol for a randomised controlled trial assessing the effect of adjunctive atorvastatin on endothelial function and outcomes in patients with severe sepsis.

1.3.3.2 Hypotheses

1) Patients admitted to hospital with sepsis who are taking a statin will be less likely to have died 28 days and 1 year after admission than those not taking a statin.

2) In adult ICU patients with severe sepsis, adjunctive therapy with atorvastatin will lead to improved endothelial function (as measured by PAT, near infrared spectroscopy and plasma markers of endothelial activation) over the first 48 hours of treatment.
1.4 References


Chapter 2. The Epidemiology of Sepsis
2.1 Background and definitions

2.1.1 What is sepsis?

The word “sepsis” has several meanings, but in this thesis, I have used the term as defined by Bone et al. in 1992, meaning an infection with a systemic response [1]. Sepsis is an excessive or dysregulated host response to infection; organ dysfunction resulting from severe sepsis is the “collateral damage” in the war between the host and pathogen. As Sir William Osler presciently noted in 1904,

“Except on few occasions, the patient appears to die from the body’s response to infection rather than from it.” [2]

This thesis does not deal with sepsis in infants and neonates, which has different pathophysiology, definitions and manifestations than it does in adults [3-7].

2.1.2 Historical aspects

Severe and overwhelming infection has been a major problem for human societies since earliest recorded history, but it is only in the last one hundred years that the pathophysiology of sepsis has begun to be understood [8]. The origins of the term “sepsis” lie in ancient Greece [9], where two forms of biological decomposition were described: “sepsis” and “pepsis”. “Sepsis” referred to putrefaction and “pepsis” to digestion or chemical decomposition. The word sepsis was used in this context by Hippocrates (460-370 BC), Aristotle (384-322 BC) and Galen (129-199 AD) [10]. However, it was not until the 1680s that the first descriptions of bacteria were made (Leeuwenhoek’s “animalcules”), and the 19th century that the link between bacteria and infection finally began to be elucidated, when the French chemist Louis Pasteur (1822-1895) discovered that microscopic single-celled organisms caused putrefaction [11]. The Hungarian surgeon Ignaz Semmelwiess hypothesised in 1863 that childbed fever was caused by “decomposed animal matter that entered the bloodstream” [12]. In (1867-1936) recognised that systemic signs of inflammation could be triggered by the release of bacteria into the bloodstream. Thus, for the first time, the source of infection as a cause of sepsis was clearly recognised [13].

The early to mid 20th century discovery of antibiotics and development of intensive care medicine transformed severe sepsis from an affliction which was not understood and...
usually fatal to a treatable albeit complicated condition. However in the late 20th and early 21st centuries, the incidence of sepsis is increasing and the mortality remains unacceptably high [14, 15].

2.1.3 Definitions and classification

Research regarding sepsis and sepsis epidemiology has been hampered in the past by a lack of consensus definitions. Multiple poorly-defined terms referring to sepsis are found in the literature prior to 1992. These include “septicaemia” [16, 17], “sepsis syndrome” [18-20], “septic conditions” [21], and the lay term “blood poisoning” [22].

In 1991, the USA Society of Critical Care Medicine (SCCM) and American College of Chest Physicians (ACCP) held a consensus conference on sepsis definitions [1]. The result was a set of tools facilitating the conduct, communication and application of sepsis research which are still used today (Table 3-1). The concept of a “systemic inflammatory response syndrome” (SIRS) was introduced, which may be triggered either by an infection or by non-infectious insults, such as trauma or pancreatitis (Table 3-2). Sepsis was defined as a suspected or proven infection with a systemic response, as manifested by at least two SIRS criteria, severe sepsis as sepsis with consequent organ dysfunction, and septic shock as sepsis with recalcitrant hypotension (Table 3-1). Most scientific publications after 1991 used this definition of sepsis, but the term continues to be used by both medical professionals and lay people with a variety of meanings, including referring to local infection such as abscesses and cellulitis. This is one of the reasons why public awareness of sepsis as a major problem facing our health systems is lacking; most people simply do not understand what sepsis is [23].
Table 2-1. Sepsis Definitions from the ACCP/SCCM consensus conference, 1991 [1].

<table>
<thead>
<tr>
<th>Definition</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Infection</strong></td>
<td>Microbial phenomenon characterised by an inflammatory response to the presence of microorganisms or the invasion of normally sterile host tissue by those organisms</td>
</tr>
<tr>
<td><strong>Systemic Inflammatory Response Syndrome (SIRS)</strong></td>
<td>The systemic inflammatory response to a variety of clinical insults. The response is manifested by two or more of the criteria in table 3-2.</td>
</tr>
<tr>
<td><strong>Sepsis</strong></td>
<td>The systemic response to infection; An infection plus two or more SIRS criteria</td>
</tr>
<tr>
<td><strong>Severe Sepsis</strong></td>
<td>Sepsis associated with organ dysfunction, hypoperfusion or hypotension. Hypoperfusion may include lactic acidosis, oliguria or acutely altered mental status.</td>
</tr>
<tr>
<td><strong>Septic Shock</strong></td>
<td>Sepsis-induced hypotension (systolic BP&lt;90mmHg or a reduction of ≥40mmHg from baseline) despite adequate fluid resuscitation PLUS Evidence of perfusion abnormalities</td>
</tr>
</tbody>
</table>

The 1991 definitions began to be criticized in the late 1990s as lacking specificity, and as not being useful to the clinician at the bedside [24-26]. Hence a second consensus conference, which included European as well as north American participants was held in 2001 [5]. They concluded that the 1991 definitions of sepsis, severe sepsis and septic shock were robust and should continue to be used. However, they proposed that the 1991 definition of SIRS was too narrow and defined SIRS as “some of” a list of twenty-four criteria, including the original four, but adding blood markers of inflammation (C-reactive protein, procalcitonin), hyperglycemia, oedema and various organ dysfunctions. Presumably “some of” means two or more but this is not specified. Most clinical sepsis research, including that reported in this thesis, continues to use the 1991 definitions of SIRS [27-38], as the 2001 definition is impractical (a long list of criteria to remember or to put on data forms), vague, and not likely to be any more specific than the 1991 definitions. The second major proposal made by the 2001 conference was a staging system for sepsis, called PIRO – predisposition, infection, response and organ dysfunction. Unlike the 2001 SIRS definitions, the PIRO
concept has gained currency [39-42] and appears to be a useful framework for describing an individual patient’s condition, or for developing prognostic scoring systems [43]. Finally, the 2001 conference suggested that sepsis be defined as “proven or suspected infection” with SIRS criteria, rather than just confirmed infection as in the 1991 definitions.

Table 2-2. SIRS criteria from the ACCP/SCCM consensus conference, 1991 [1].

<table>
<thead>
<tr>
<th>Abnormal temperature</th>
<th>Temperature &gt;38°C or &lt;36°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tachycardia</td>
<td>Heart rate&gt;90 beats/minute</td>
</tr>
<tr>
<td>Tachypnoea</td>
<td>Respiratory rate&gt;20 breaths/minute OR PaCO₂&lt;32 mm Hg</td>
</tr>
<tr>
<td>Abnormal White blood cell count</td>
<td>White blood cell count&gt;12,000/mm³ or &lt;4,000/mm³ OR Band forms &gt;10% of white blood cells</td>
</tr>
</tbody>
</table>

An alternative method of improving the specificity of the 1991 sepsis definitions is to require three rather than two SIRS criteria. This method is being increasingly used in clinical drug trials, where specificity is more important than sensitivity, and where more severely ill patients are sought (e.g. the PROWESS trial of activated protein C for severe sepsis [44]). However, as a consequence of requiring three rather than two SIRS criteria, sensitivity suffers; thus most prospective studies attempting to describe sepsis epidemiology use two SIRS criteria in their definition of sepsis [20, 29, 32, 45-48].

In addition to finessing the criteria for SIRS, improving the accuracy of the diagnosis of infection would greatly improve the specificity and clinical utility of sepsis diagnostic criteria. Distinguishing infectious SIRS (i.e. sepsis) from non-infectious SIRS is a holy grail of critical care and infectious diseases research, which unfortunately continues to elude us. A rapid and reliable means of making this distinction would have many important ramifications, including limiting unnecessary antibiotic use, improving accuracy of clinical trials, and allowing early and appropriate specific therapy. Many candidate markers have been proposed to distinguish sepsis from non-infectious SIRS. These include rapid techniques to identify the presence of microorganisms directly in clinical samples and markers of host inflammatory and immune responses. Examples of the microbiological approaches include nucleic acid detection using multiplex PCR on DNA extracted directly from blood [49-52], and quantitative plasma bacterial endotoxin assays [53-55]. Many aspects of host responses to infection have been studied in this regard, including C-reactive protein [56, 57], procalcitonin [58, 59], biphasic aPTT [60], triggering receptor expressed on
myeloid cells-1 (TREM-1) [61, 62], eosinopenia [63] and multiplex proteomics [64] or genomics [65]. Unfortunately, none of these techniques has proved to be sufficiently sensitive, specific and practical to be in common use.

Although the term sepsis is often taken to imply bacterial infection, neither the 1991 nor 2001 definitions require the infection to be of bacterial origin. Indeed, fungal infection is an increasingly common cause of sepsis in ICUs, accounting for 19% of infections in ICU patients in an international study including data from 1,265 ICUs in seventy-five countries in 2007 [66]. Viral and parasitic infections, such as severe influenza or malaria due to Plasmodium spp. can also cause syndromes clinically indistinguishable from bacterial sepsis.

Throughout this thesis, except where noted, I have used the 1991 consensus conference sepsis definitions of Bone et al [1], including the requirement of two rather than three SIRS criteria in the definition of sepsis.

2.2 Methodologies used to conduct studies of sepsis epidemiology

Even though most studies of sepsis epidemiology have used more consistent definitions of sepsis in the last twenty years than previously, there continues to be great heterogeneity in the methodology used in such studies. These differences must be borne in mind when comparing data reported by different studies. The major points of variation in methodology are summarised below.

2.2.1 – Retrospective, point-prevalence or prospective study design

2.2.1.1 Retrospective database analysis.

Most large studies of sepsis epidemiology are conducted by retrospective analysis of databases [14, 15, 67-76] (Table 2-3; where studies report incidence and mortality over large periods of time, only the latest data were included in this table). This approach allows for large numbers of patients to be included (e.g. Martin et al analysed data from 750 million admissions to USA hospitals [15]), and is suited to analysing trends over long periods of time [14, 15, 68]. However, the most significant problem with retrospective studies is lack of detailed clinical data and thus a likely under-estimation of sepsis incidence. Most such studies use International Classification of Disease (ICD-9) coding, and include combinations of codes for various kinds of infection, organ failure and non-traumatic shock. These studies rely heavily on the accuracy of coding which was generally performed for hospital billing purposes rather than with research in mind. A US study of hospital billing
concluded that use of discharge ICD coding underestimates the occurrence of sepsis by up to 25% [77]. Such studies may also lack specificity; for example, a patient with discharge diagnoses of a urinary tract infection and heart failure due to a recent acute myocardial infarction would be counted as having severe sepsis (codes for infection plus organ failure).
Table 2-3. Retrospective studies of sepsis epidemiology
(adapted and expanded from [78])

<table>
<thead>
<tr>
<th>Region (first author, year of publication)</th>
<th>Period data collected from</th>
<th>Sampling frame</th>
<th>Number of admissions screened</th>
<th>ICU incidence of sepsis/severe sepsis</th>
<th>Mortality of Sepsis/Severe sepsis</th>
<th>Population incidence of sepsis</th>
<th>Population incidence of severe sepsis</th>
</tr>
</thead>
<tbody>
<tr>
<td>USA [67] (Angus, 2001)</td>
<td>Jan-Dec 1995</td>
<td>Whole hospital</td>
<td>6,621,559</td>
<td>NA/11.2%</td>
<td>NA/28.6%</td>
<td>NA</td>
<td>300 per 100,000</td>
</tr>
<tr>
<td>USA [69] (Teres, 2002)</td>
<td>1998-1999 ICU</td>
<td>21,480</td>
<td>NA/11.3%</td>
<td>NA/36%</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>USA [15] (Martin, 2003)</td>
<td>1979-2000 Whole hospital</td>
<td>750 million</td>
<td>NA</td>
<td>17.9%/NA</td>
<td>240 per 100,000</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>France [70] (Annane, 2003)</td>
<td>1993-2000 ICU</td>
<td>100,554</td>
<td>NA/8.2%</td>
<td>NA/61.2%</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>UK [71] (Padkin, 2003)</td>
<td>1995-2000 ICU</td>
<td>56,673</td>
<td>NA/27.1%</td>
<td>NA/47.3%</td>
<td>NA</td>
<td>NA</td>
<td>51 per 100,000</td>
</tr>
<tr>
<td>Location</td>
<td>Year(s)</td>
<td>Unit</td>
<td>Population</td>
<td>Increase</td>
<td>Men/Women</td>
<td>Rate/Hospital</td>
<td>Rate/Population</td>
</tr>
<tr>
<td>-------------------</td>
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<td>------------</td>
<td>----------</td>
<td>------------</td>
<td>---------------</td>
<td>----------------</td>
</tr>
<tr>
<td>Norway [73]</td>
<td>1999</td>
<td>Whole hospital</td>
<td>700,107</td>
<td>NA</td>
<td>7.1%/27%</td>
<td>149 per 100,000</td>
<td>47 per 100,000</td>
</tr>
<tr>
<td>(Flaaten, 1999)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Victoria, Australia [68] (Sundarajan, 2005)</td>
<td>Jul 1999 – Jun 2003</td>
<td>Whole hospital</td>
<td>3,122,515</td>
<td>NA</td>
<td>10.2%/31.1%</td>
<td>194 per 100,000</td>
<td>76 per 100,000</td>
</tr>
<tr>
<td>Madrid, Spain [76] (Inigo, 2006)</td>
<td>2001</td>
<td>Whole hospital</td>
<td>537,223</td>
<td>NA</td>
<td>NA/33%</td>
<td>NA</td>
<td>141 per 100,000</td>
</tr>
<tr>
<td>USA [14] (Dombrovskiy, 2007)</td>
<td>1993-2003</td>
<td>Whole hospital</td>
<td>391,571,824</td>
<td>NA</td>
<td>NA/37.7%</td>
<td>NA</td>
<td>134.6 per 100,000</td>
</tr>
<tr>
<td>Valencia, Spain [74] (Ballester, 2008)</td>
<td>1995-2004</td>
<td>Whole hospital</td>
<td>NA</td>
<td>NA</td>
<td>42.5%/NA</td>
<td>114 per 100,000</td>
<td>NA</td>
</tr>
<tr>
<td>Singapore [75] (Yang, 2009)</td>
<td>2004-2007</td>
<td>Whole hospital</td>
<td>305,637</td>
<td>NA</td>
<td>19.9%</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>
2.2.1.2 Point prevalence and cross-sectional studies

Point-prevalence studies record the number of patients with sepsis in an ICU at a given time (Table 2-4) [79-81], and may extrapolate from this to estimate population incidence [80]. This study design is logistically easier than prospective cohort studies but also has significant limitations, including failure to capture seasonal or stochastic variation, and inability to determine true disease burden because of a lack of data on illness duration.
<table>
<thead>
<tr>
<th>Region (first author, year of publication)</th>
<th>Period data collected from</th>
<th>Sampling frame</th>
<th>Number of admissions screened</th>
<th>ICU incidence of sepsis/severe sepsis</th>
<th>Mortality of Sepsis/Severe sepsis</th>
<th>Population incidence of sepsis</th>
<th>Population incidence of severe sepsis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mexico [79] (Leon-Rosales, 2000)</td>
<td>March 1995</td>
<td>ICU</td>
<td>895</td>
<td>49.4% / 16.5%</td>
<td>17.8% / 45.7%</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Netherlands [80] (van Gestel 2004)</td>
<td>December 2001</td>
<td>ICU</td>
<td>455</td>
<td>NA / 29.5%</td>
<td>NA</td>
<td>60 per 100,000</td>
<td>54 per 100,000</td>
</tr>
<tr>
<td>Germany [81] (Engel, 2007)</td>
<td>2003</td>
<td>ICU</td>
<td>3,877</td>
<td>12.4% / 11.0%</td>
<td>NA / 55.2%</td>
<td>85 to 116 per 100,000</td>
<td>76 to 110 per 100,000</td>
</tr>
</tbody>
</table>
2.2.1.3 Prospective cohort studies

The most powerful and commonly used study design to determine sepsis epidemiology is the prospective cohort study (Table 2-5) [20, 29, 32, 45-48, 82-90]. This has the advantage of being able to use strict and consistent definitions of sepsis and severe sepsis and of being able to access real-time clinical information to confirm diagnoses and to attribute organ dysfunction to infection. Published studies using this methodology vary in their recruitment period from 2 weeks [32] (which could almost be called a cross-sectional study) to 2 years [90].
Table 2-5. Prospective cohort studies of sepsis epidemiology
(adapted and expanded from [78])

<table>
<thead>
<tr>
<th>Region (first author, year of publication)</th>
<th>Period data collected from</th>
<th>Sampling frame</th>
<th>Number of admissions screened</th>
<th>ICU incidence of sepsis/ severe sepsis</th>
<th>Mortality of Sepsis/Severe sepsis</th>
<th>Population incidence of sepsis</th>
<th>Population incidence of severe sepsis</th>
</tr>
</thead>
<tbody>
<tr>
<td>USA [46] (Pittet, 1995)</td>
<td>April 1992</td>
<td>Surgical ICU</td>
<td>170</td>
<td>49%/16%</td>
<td>NA/35%</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>France [45] (Brun-Buisson, 1995)</td>
<td>Jan-Feb 1993</td>
<td>ICU</td>
<td>11,828</td>
<td>NA/9%</td>
<td>NA/56%</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Italy [83] (Salvo, 1995)</td>
<td>Apr 1993– Mar 1994</td>
<td>ICU</td>
<td>1,101</td>
<td>9.6%/5.1%</td>
<td>36%/52.2%</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>USA [20] (Sands, 1997)</td>
<td>Jan 1993– Apr 1994</td>
<td>Whole hospital</td>
<td>9,763</td>
<td>20%/10%</td>
<td>NA/34%</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Europe, Canada, Israel [48] (Alberti, 2002)</td>
<td>May 1997– May 1998</td>
<td>ICU</td>
<td>14,364</td>
<td>37.7%/25.4%</td>
<td>38.7%/47.8%</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Australia and New Zealand [29] (Finfer, 2004)</td>
<td>May 1999 – July 1999</td>
<td>ICU</td>
<td>5,878</td>
<td>NA/11.8%</td>
<td>NA/37.5%</td>
<td>NA</td>
<td>77 per 100,000</td>
</tr>
<tr>
<td>France [84] (Brun-Buisson, 2004)</td>
<td>Nov-Dec 2001</td>
<td>ICU</td>
<td>3,738</td>
<td>16.6%</td>
<td>NA/41.9%</td>
<td>NA</td>
<td>95 per 100,000</td>
</tr>
<tr>
<td>Brazil [47] (Silva, 2004)</td>
<td>May 2001– Jan 2002</td>
<td>ICU</td>
<td>1,383</td>
<td>46.9%/27.3%</td>
<td>33.9%/46.9%</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Slovakia [85] (Zahorec, 2005)</td>
<td>July-Dec 2002</td>
<td>ICU</td>
<td>121</td>
<td>NA/7.9%</td>
<td>NA/51.2%</td>
<td>NA</td>
<td>80-90 per 100,000</td>
</tr>
<tr>
<td>Country</td>
<td>Study [Year] (Reference)</td>
<td>Dates</td>
<td>Setting</td>
<td>Cases</td>
<td>Infection Rate</td>
<td>C. septicum Isolation Rate</td>
<td>Other</td>
</tr>
<tr>
<td>-----------------</td>
<td>--------------------------</td>
<td>------------------</td>
<td>------------------</td>
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<td>----------------</td>
<td>---------------------------</td>
<td>-------</td>
</tr>
<tr>
<td>24 European</td>
<td></td>
<td>May 2002 (2 weeks)</td>
<td>ICU</td>
<td>3,147</td>
<td>37%/30%</td>
<td>27%/32.2%</td>
<td>NA</td>
</tr>
<tr>
<td>countries [32]</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Vincent, 2006)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>[88]</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Cheng, 2007)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spain</td>
<td></td>
<td>April-June 2002 and Oct-Dec 2002</td>
<td>ICU</td>
<td>4,317</td>
<td>NA/11.9%</td>
<td>NA/54.3%</td>
<td>NA</td>
</tr>
<tr>
<td>[87]</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Blanco, 2008)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Canada</td>
<td></td>
<td>March 2003 to Nov 2004</td>
<td>ICU</td>
<td>6,298</td>
<td>NA/19.0%</td>
<td>NA/38.1%</td>
<td>NA</td>
</tr>
<tr>
<td>[86]</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Martin, 2009)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uganda</td>
<td></td>
<td>July 2006-Nov 2006</td>
<td>Whole hospital</td>
<td>NA</td>
<td>NA</td>
<td>23.7%/NA</td>
<td>NA</td>
</tr>
<tr>
<td>[89]</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thailand</td>
<td></td>
<td>July 2004-June 2006</td>
<td>ICU</td>
<td>2,057</td>
<td>NA/21.6%</td>
<td>NA/49.7%</td>
<td>NA</td>
</tr>
<tr>
<td>[90]</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.2.2 Intensive care-based vs. whole hospital-based studies

Most studies of sepsis epidemiology include only patients admitted to an intensive care unit. For example, of the thirty-one studies listed in tables 3-3 to 3-5, only eleven included patients admitted to hospital beds outside the ICU. When such data are used to extrapolate population-based incidences (for example in [29, 71, 80]), sepsis incidence is underestimated as the presumption is made that all sepsis hospital admissions receive care in an ICU. In fact both sepsis and severe sepsis are common among patients in general hospital wards, with only 32% [91], and 52% [31] of severe sepsis patients receiving care in an ICU in two recent studies. Furthermore, ICU bed availability and ICU admission policies vary widely between countries and over time, which will artefactually skew estimates of sepsis incidence and outcomes if data are included from the ICU but not from the hospital as a whole.

2.2.3 Hospital vs. community-based studies

Essentially all published studies which quantify sepsis epidemiology are hospital-based. They only include patients who are admitted to a hospital with sepsis, making the assumption that no one who meets sepsis criteria would be cared for outside the hospital setting. This is unlikely to be the case, particularly in regions with poor access to health care or geographical isolation. A proportion of patients with severe sepsis are likely to die before reaching hospital; while this proportion is probably small in the developed world, it is likely to be substantial in resource poor countries. A larger proportion with mild sepsis probably never require hospital admission and are cared for in the community or do not seek medical advice. A large retrospective study of nearly 200,000 presentations to US emergency departments found that 13% of those who met sepsis criteria and 2.3% with severe sepsis were sent home without hospital admission [92]. Thus hospital-based studies of sepsis epidemiology are likely to underestimate the population incidence of sepsis.

2.2.4 The ideal study of sepsis epidemiology

The ideal study of sepsis epidemiology would be a prospective cohort study which captures every hospital admission to all of the hospitals in the region or country of interest. It would also include visits to emergency departments and community clinics for sepsis which did not require hospital admission. The standard 1991 definitions would be used [1], and data would be collected on both sepsis and severe sepsis. The recruitment period would cover at least one complete seasonal cycle and preferably several. Such a study has never been
published, and would be logistically extremely difficult to perform. Thus all studies of sepsis epidemiology make compromises of one kind or another, and they should be interpreted with these issues in mind.

2.3 Sepsis epidemiology internationally

2.3.1 Overview

It has been estimated that eighteen million cases of sepsis occur per year worldwide [93] and if the mortality rate observed in multiple studies of approximately 30% is applied to this, then sepsis is likely to be a leading global cause of mortality [93], exceeding deaths from HIV, tuberculosis and malaria. Sepsis is also the leading cause of death in non-coronary ICUs in developed countries [24]. Despite these startling figures, sepsis receives considerably less media attention, public awareness and research and health-care funding than many other more visible problems such as breast cancer, HIV infection and heart disease.

2.3.2 North America

The two largest studies of sepsis epidemiology in North America were published in 2001 [67] and 2003 [15]. In the 2001 paper, Angus et al analysed discharge data from over six million hospital episodes from all non-federal hospitals in seven US states in 1995 [67]. They estimated that there were 751,000 cases of severe sepsis each year in the USA, with a mortality of 28.6% and a total annual cost of US$16.7 billion. Martin examined discharge data from 1979-2000 from more than 750 million hospital admissions across the USA [15], and found a progressive increase in sepsis incidence over time of 8.7%, but a decrease in the hospital mortality rate from 27.8% in 1979-1984 to 17.9% in 1995-2000. Despite this drop in mortality rate, the total number of deaths increased over this period because of the increasing incidence. Other studies from the USA [69],[14, 20, 82] and Canada [86] reported data consistent with the findings of these two large studies: sepsis and severe sepsis are common and costly conditions in the USA and their incidence is increasing.

2.3.3 Europe

There are more published studies of sepsis epidemiology from Europe than from any other region [32, 45, 70-74, 76, 80, 81, 83-85, 87]. These studies show a remarkable variation between countries in incidence and outcomes of sepsis (Figure 2-1). For example, in the SOAP study (Sepsis Occurrence in Acutely ill Patients), the hospital mortality of sepsis requiring ICU admission ranged from 14% in Switzerland to 41% in Portugal. However the
population-based incidence of sepsis and severe sepsis is reasonably consistent in these studies, and is similar to that reported from other regions of the world. Of eight European studies which reported population-based incidence of severe sepsis, six found incidences within the range 45-95 per 100,000 population per year [71, 73, 80, 81, 84, 85], with the two outliers both being from Spain [76, 87]. Furthermore, the incidence of severe sepsis has been shown to be increasing over time in studies from France [70], Spain [74] and the UK [72], a similar finding to studies from the USA [14, 15] and Australia [68, 94].

**Figure 2-1. ICU mortality and ICU sepsis incidence in 13 European countries from the SOAP study.**

This figure was directly reproduced from [32]

![Graph showing ICU mortality and ICU sepsis incidence in 13 European countries](image)

\[ Y = 0.51x + 0.11 \]
\[ R^2 = 0.80 \]

### 2.3.4 Low or Middle Income and Tropical Countries

There are few published data about the epidemiology of sepsis in resource-poor or tropical countries. Despite this, according to data from the World Health Organisation, it is likely that the majority of the global burden of morbidity and mortality from severe infections occurs in the developing world [95]. Within the last six years, data have been published from Turkey [96], Brazil [47], Uganda [89] and Thailand [90, 97]. All of these apart from the Uganda study are from middle-income countries and based in tertiary referral hospitals with the availability of sophisticated intensive care services. However, the Turkish study found a very high hospital mortality rate of 87.3% which they attributed to an insufficient number of ICU beds [96]. The Brazilian and Thai studies found hospital mortality rates for severe sepsis of 46.9% [47] and 49.7% [90] to 52.6% [97] respectively, which are similar to
rates reported from Europe and the USA. The Ugandan study differed from all others in that 84.9% of the patients were HIV infected, and there was very limited availability of supportive care such as oxygen therapy and vasopressors. Despite this, they reported a hospital mortality rate for sepsis of only 23.7%. None of these studies determined population-based incidence rates. Further studies are needed before one can even begin to quantify the burden of sepsis in developing countries.

2.4 Sepsis epidemiology in Australia

2.4.1 Studies before Finfer et al (2004)

In 2004, the first Australian study of the epidemiology of sepsis was published [29]. Prior to this time, multiple studies had addressed the epidemiology of particular infections in Australia, but none had specifically addressed the syndrome of sepsis. These include, for example, studies on bacteraemia in general [98], invasive pneumococcal disease [99], melioidosis [100, 101] and infections due to *Haemophilus influenzae* [102].

2.4.2 Finfer et al (2004)

In 1999, The Australia and New Zealand Intensive Care Society (ANZICS) clinical trials group performed the first and only nationwide study of sepsis epidemiology in Australia and New Zealand [29]. They prospectively collected data on all ICU admissions meeting the 1991 SCCM severe sepsis criteria over a 3-month period in twenty-three ICUs. They found an adult population-based incidence of severe sepsis of 77 per 100,000 population per year, and a hospital mortality of 37.5%.

This study is likely to have underestimated the population-based incidence of severe sepsis for several reasons. Firstly, the study was restricted to patients admitted to ICUs, and thus would have missed a significant number of patients who required hospital but not ICU admission [31, 91]. Secondly, the denominator of ICU admissions used for the estimation of severe sepsis incidence included only 180 of 195 ICUs in Australia and New Zealand, but the method of calculation assumed that this included every ICU admission in Australia and New Zealand. Finally, this study only included hospitals in temperate Australia, potentially missing an increased incidence of bacterial infection and severe sepsis in tropical northern Australia.
2.4.3 Sundararajan et al (2005)

The only other study specifically addressing the epidemiology of sepsis in Australia was conducted in Victoria, the second most populous state in Australia [68]. Sundararajan and colleagues used a large administrative database to retrospectively analyse data from over three million hospital admissions from between July 1999 and June 2003. They included all hospital admissions (not only ICU admissions) and examined both sepsis and severe sepsis. They found that the incidence of sepsis increased over the 4-year study period, from 166 to 194 per 100,000, and the severe sepsis incidence also increased from 65 to 76 per 100,000. Although this study examined a large number of hospital admissions, it was retrospective, relying on ICD coding and thus is likely both to have underestimated the incidence of sepsis [77] and falsely attributed some cases of organ dysfunction to infection.

While the incidence of severe sepsis in Victoria found in this study (76 per 100,000 per year) appears remarkably similar to that found in Australasia as a whole (77 per 100,000 per year [29]), it is actually substantially less. This is because Finfer et al only included patients admitted to ICUs, whereas Sundararajan et al included all hospital admissions, of whom only 50% received care in an ICU; thus the rate of severe sepsis requiring ICU admission in the Victorian study would be approximately 33 per 100,000 per year, compared with 77 per 100,000 per year in the Australasian study.

2.4.4 The Australian Resuscitation of Sepsis Evaluation Study (ARISE)

While not specifically aiming to describe sepsis epidemiology in Australia, the ARISE study did report some data relevant to it [31, 94]. Prior to the ARISE study, the authors performed a retrospective analysis of ICU admissions for sepsis originating from hospital emergency departments (EDs) [94]. These data had been routinely collected from the majority of Australian and New Zealand ICUs as part of the ANZICS adult patient database. They found that over the period 1997 to 2005, the ICU-based incidence of sepsis increased and the hospital mortality steadily decreased from 35.6% to 21.2%. The ARISE study was a prospective cohort study of severe sepsis presenting to Australian hospital EDs over a 3-month period in 2006. They found that only 52.4% of severe sepsis patients were admitted to an ICU, and that those not admitted to an ICU had a similar hospital mortality (21.4%) to those who were (24.7%), although it is possible that the high mortality in the non-ICU patients may have been due to treatment limitation orders.
2.4.5 Comparisons of Australian and international data

Allowing for the differences in methodology, the epidemiology of sepsis in temperate Australia appears to be broadly similar to that reported from the USA and Europe [103] (Tables 2-1 to 2-3). The population-based incidence of severe sepsis in Australia of 77 per 100,000 [29] population per year is consistent with those reported from France (95 per 100,000) [84], Germany (76 per 100,000) [81], the Netherlands (54 per 100,000) [80] and the UK (51 per 100,000) [71]. The only population-based severe sepsis incidence reported from the USA appears substantially higher than this at 300 per 100,000 [67], but this may be partially explained by the USA study including all hospital admissions whereas all of the abovementioned comparator studies only included patients admitted to an ICU. However, the reported incidence of severe sepsis as a proportion of all ICU admissions (11.8%) appears to be lower in Australia than in most international studies, which range from 10%-18% in studies from the USA [15, 46, 67, 82], 27.1%-28.7% in the UK [71, 72], 30% in Europe as a whole [32] and 25.4% in an international study including Europe, Canada and Israel [48].

The hospital mortality of severe sepsis in Australia of 31.1% [68] and 37.5% [29] is similar to that reported from the USA (28.6% [67], 36% [69], 37.7% [14], 35% [46]) and Europe as whole (32.2%) [32]. The observation that sepsis incidence is increasing and the mortality of sepsis is decreasing over time is a common theme linking Australian studies [94, 104] with those from the USA [14, 15] and Europe [70, 72]. Furthermore, the focus of infection and causative organisms are similar in Australian studies [29, 104] to those from other countries [32, 66], with pneumonia being the most common focus, and with a shift to a predominance of gram positive infections over the last two decades [103].

2.5 Epidemiology of sepsis in indigenous populations.

There are no published studies specifically addressing the epidemiology of sepsis in indigenous populations, either in general or in specific countries. However, many studies have demonstrated that the burden of infectious diseases in general and of several specific infections is significantly greater in indigenous people than in non-indigenous people from the same country [105-113]. Moreover, US studies have shown racial disparities in outcomes from sepsis, with African Americans having higher rates of hospitalisation for and mortality from sepsis [15, 114] than whites, but these studies did not report any data about Native Americans.
General mortality rates in Australian Indigenous people greatly exceed those not only of non-Indigenous Australians, but also of indigenous people in New Zealand and North America (Figure 3-2 [115]). While the epidemiology of sepsis has not been described in Australian Indigenous people, two small ICU-based studies suggest that sepsis is significantly more common in Indigenous than non-indigenous Australians [115, 116]. Ho et al retrospectively examined data on 11 years of admissions to a tertiary ICU in Western Australia [116]. They found that Indigenous people represented 3.2% of Western Australia’s population, but accounted for 9.5% of emergency ICU admissions; furthermore the primary diagnosis was sepsis other than pneumonia in 14.5% of Indigenous admissions, compared with 4.0% of non-Indigenous (p=0.001). Despite this fact, when adjusted for comorbidity and illness severity, mortality was similar in Indigenous and non-Indigenous people. Stephens retrospectively analysed data from the Royal Darwin Hospital ICU [115]. She found that despite comprising 28% of the hospital’s drainage population, Indigenous people accounted for 45% of admissions to RDH ICU. The proportion of ICU admissions due to sepsis or septic shock was 24% in Indigenous people compared with 15% in non-Indigenous (p<0.001). Finally, hospital mortality rates were not significantly different in Indigenous than non-Indigenous people.

Figure 2-2. Overall mortality rates for Indigenous Australians compared with non-Indigenous Australians and with other indigenous populations.

(Directly reproduced from [115])

2.6 Microbial epidemiology of sepsis

The microbial epidemiology of sepsis has changed significantly over the last two decades, with a widespread shift from predominantly Gram negative to predominantly Gram positive
bacterial pathogens [15, 117] (Figure 2-3). More recently, fungi (particularly Candida spp.) have emerged as important causative organisms in sepsis [15, 32]. For example in two recent large international studies, fungi were the primary causative organisms in 21.7% of severe sepsis patients [48] and 19.4% of infected ICU patients [66]. While these changes are fairly consistent in large international studies, there is significant variation between and within countries in specific factors such as the prevalence of MRSA and multi-resistant gram negative organisms [117]. For example, the microbial ecology and epidemiology of infection appears to be different in tropical northern Australia than in temperate Australia [118], with more Gram negative bacteria [119], and the presence of tropical pathogens such as *Burkholderia pseudomallei* [120] and community-acquired *Acinetobacter baumanii* [121].

Determining the local microbial epidemiology of sepsis is obviously a vital step in improving outcomes by ensuring early appropriate antibiotic selection [122, 123].

**Figure 2-3. Change in microbial epidemiology of sepsis in the USA, 1979-2001**  
(Directly reproduced from [15])
2.7 Conclusions

Sepsis is a syndrome of global importance and probably accounts for more deaths annually than any other infectious disease. Furthermore, the incidence of sepsis is progressively increasing in North America, Europe and Australia. Despite this, awareness of sepsis as a public health problem is significantly less than for higher profile diseases such as breast cancer, malaria and HIV infection. The epidemiology of sepsis has been extensively described in the USA and Europe, but there remains a significant knowledge gap about sepsis epidemiology in the developing world and in indigenous populations globally.
2.8 References


112. Kermode-Scott B: Canada has world's highest rate of confirmed cases of A/H1N1, with Aboriginal people hardest hit. *Bmj* 2009, 339:b2746.


Chapter 3. Endothelial and microvascular function in sepsis
3.1 What is the endothelium?

3.1.1 Historical perspective

The endothelium is an active layer of cells which lines the entire cardiovascular system. It is increasingly being recognised as an organ in its own right [1, 2] and is now known to be of pivotal importance in human health and disease. The concept of the endothelium was first proposed by the Swiss anatomist Wilhelm His in 1865, in a monograph entitled “The membranes and cavities of the body” [3]. Prior to this time, it was not recognised that there was any significant difference between epithelium and endothelium. Once this concept was generally accepted, the endothelium was initially thought of as a passive layer of cells which functioned simply as a semi-permeable membrane [4]. Over the last half of the 20th century, it was increasingly recognised that the endothelium is a highly complex and active organ. Its roles and functions span the gamut of human physiology, ranging from the regulation of vascular growth, tone and permeability through interaction with the coagulation system to antigen presentation and control of inflammation.

3.1.2 Anatomy of the endothelium and microcirculation

Endothelial cells lie at the interface between the blood stream, and every tissue and organ of the human body. All blood vessels and lymphatics, from the aorta to the smallest capillaries, are lined by a single continuous layer of endothelial cells. Together these cells form an organ composed of approximately $10^{13}$ cells and covering an area of 4000-7000 m$^2$ [1], or approximately five Olympic-sized swimming pools. Endothelial cells vary greatly over space and time in their phenotypic characteristics [5]. Examples of this heterogeneity include the fact that when activated by proinflammatory cytokines, endothelial cells change from having anticoagulant to procoagulant properties [6], and that endothelial cells in the central nervous system differ in size and shape from those in the peripheral circulation [5]. Endothelial cells are joined by tight junctions, preventing the egress of cells and fluid into tissues when in their resting state. The microcirculation is comprised of arterioles, capillaries and venules, and is defined as blood vessels which are less than 100 μM in diameter, where oxygen delivery to the tissues occurs [7].

3.1.3 Physiology of the endothelium in healthy subjects

Although endothelial cells have myriad roles in normal human physiology, their physiological functions can be broken into five main groups – the regulation of vascular tone; the regulation of the coagulation system; leukocyte trafficking and barrier function;
immunological functions; and vascular growth and remodelling. Following a brief précis of
the arginine/nitric oxide system, each of these functions will be briefly described in turn.

3.1.3.1 The Arginine/Nitric Oxide system
Nitric oxide (NO), until recently believed to be simply an atmospheric pollutant, is probably
the most versatile gas in mammalian physiology, acting as a direct vasodilator [8], cell-to
cell signalling molecule [9, 10], neurotransmitter [11], anti-apoptotic agent [12, 13], free
radical [14], and antibacterial effector molecule of immune cells [15, 16]. The initial steps in
recognising the importance of NO in human physiology were made by Furchgott and
Zawadski, who discovered that endothelial cells express a substance which relaxes vascular
smooth muscle in response to acetyl choline (ACh), which they dubbed endothelial derived
relaxing factor (EDRF) [17]. EDRF was identified as NO seven years later by two research
teams in parallel [8, 18].

NO is produced from its precursor L-arginine by the action of nitric oxide synthases (NOS)
[19]. There are three isoforms of NOS: NOS1 (neuronal NOS [nNOS]), NOS2 (inducible NOS
[iNOS]), and NOS3 (endothelial NOS [eNOS]) [20]. Endothelial NOS is constitutively
expressed in endothelial cells, platelets and myocytes, generating constant but low
concentrations of NO which are essential for maintaining the vasculature in a healthy and
quiescent state [21]. Endothelial NOS expression is calcium-dependent and is stimulated by
intrinsic signals including ACh and shear stress of the blood vessel wall. iNOS is calcium-
independent and expressed in a wider variety of cell types, including monocytes and
epithelial cells. iNOS can cause massive and sustained upregulation of NO production in
response to stimuli such as pro-inflammatory cytokines or pathogen associated molecular
patterns.

L-arginine is a conditionally essential amino acid [22], meaning that mammals can
endogenously produce sufficient amounts during health, but are unable to do so during
severe physiological stress. L-arginine is primarily synthesised from citrulline in the proximal
renal tubule and intestinal epithelium [23]. In health, endogenous production of L-arginine
(15-20 g/day) is a more important source than dietary intake (5-6g/day) [24, 25]. The
primary routes by which L-arginine is metabolised are by arginase to urea and ornithine, or
by NOS to NO and citrulline [26, 27]. L-arginine is transported into endothelial cells via a γ-
cationic amino acid transporter (CAT) system [28]. Endothelial NOS is concentrated inside
endothelial cells in small pockets of the cell membrane called caveolae, which are co-
located with CAT transporters, so that L-arginine is brought from extracellular fluid directly into contact with eNOS [29]. Several other amino acids can compete with L-arginine for transport into endothelial cells via the γ–CAT transporter system. These include lysine, ornithine and methylated arginines (asymmetric dimethyl arginine [ADMA] and symmetric dimethyl arginine [SDMA]) [28, 30]. As well as competing with L-arginine for transport into endothelial cells, a more important role of ADMA is as a competitive inhibitor of NOS [31]. This may explain why chronically raised plasma ADMA concentrations have been associated with endothelial dysfunction and increased cardiovascular risk in multiple cohorts [32-35]. The role of ADMA in endothelial physiology has been extensively reviewed elsewhere [36, 37], and will be addressed further in chapter 11.

3.1.3.2 Regulation of vascular tone
Arguably the most important role of the endothelium is the regulation of vascular tone, and thus of the blood supply to organs and tissues. The most important stimuli for endothelial NO release are shear stress from increased blood flow [38], ACh, thrombin, and bradykinin [39]. The endothelium expresses several other substances in addition to NO which influence vascular tone. These include vasodilator substances (endothelium derived hyperpolarising factor [EDHF] [40], prostacyclin [41]) and vasoconstrictors (endothelin [42], angiotensin II [43] and thromboxane A2 [44]). Under normal conditions, resting vascular tone is determined by the balance between these opposing influences. Endothelial cells act as a network of co-ordinated input-output devices, regulating local blood flow in response to changes in oxygen demand, metabolic stress and other local conditions [1].

3.1.3.3 Regulation of coagulation
Under normal circumstances, the endothelium has an antithrombotic and fibrinolytic effect, without which blood would not remain as a liquid. This is achieved through three main mechanisms: i) the secretion of anticoagulant proteins such as thrombomodulin, tissue plasminogen activator (tPA), and heparan sulphate; ii) inhibition of platelet activation by the secretion of prostacyclin and NO; and iii) physical protection of the underlying collagen matrix from the blood stream [45]. However, the endothelium is like a resting cat ready to pounce – when activated by inflammation or trauma, endothelial cells can rapidly release pre-formed procoagulant factors such as Von Willebrand factor and tissue factor, setting off a cascade of local thrombogenesis [46].
3.1.3.4 Leukocyte trafficking and barrier function.

The healthy endothelium controls the egress of cells, proteins and fluid from the bloodstream into the interstitium. Endothelial cells are joined by tight junctions, forming a protective barrier which can allow selective passage of ions, water and other molecules [47]. Quiescent endothelial cells do not physically interact with leucocytes and platelets. However, when activated, endothelial cells upregulate surface expression of adhesion molecules such as intracellular adhesion molecule 1 (ICAM 1), E-selectin, P-selectin and vascular cell adhesion molecule (VCAM) [48-50]. These allow leucocytes to roll along the endothelial surface, adhere, and migrate through the now-compromised tight junctions into the tissues.

3.1.3.5 Immunological function of endothelial cells.

As well as regulating macrophage and neutrophil migration into tissues, endothelial cells play an important role in both the innate and the adaptive immune systems [51]. Endothelial cells express toll-like receptors (TLRs), and endothelial TLR4 is an important mechanism for sensing bacterial lipopolysachharide (LPS) in blood [52]. Endothelial cells are also antigen presenting cells [53], and act both as targets for and sources of inflammatory cytokines [54].

3.1.3.6 Vascular growth and remodelling.

Endothelial cells secrete vascular endothelial growth factor (VEGF) and angiopoietins, which are important in the embryonic formation of blood vessels, adaptive vascular remodelling, and in pathological neovascularisation [55].

3.2 Clinical assessment of endothelial function

3.2.1 Overview

What do clinical researchers and clinicians mean when they discuss “measuring endothelial function”? Given all of the physiological roles discussed above, this could have a broad range of meanings. However, the use of this term in the clinical literature generally refers to the regulation of vascular tone, or more specifically, to the ability of blood vessels to vasodilate in response to either ACh or shear stress induced by increased blood flow [4, 56]. The term “endothelial activation” refers to a change in endothelial cell phenotype from thromboresistant to prothrombotic, from anti-inflammatory to pro-inflammatory, and from an impenetrable barrier to a conduit for cells and fluid into the tissues. Endothelial cells are normally kept in a quiescent state by constant low-level production of NO by eNOS, which
keeps the cell deactivated by several mechanisms, including direct down-regulation of the activity of the transcription factor nuclear factor kappa beta [21]. Endothelial cells can be activated by a relative deficiency of endothelial NO, proinflammatory cytokines, hypoxaemia or direct trauma.

The terms “endothelial activation” and “endothelial dysfunction” are sometimes used interchangeably. However, some authors argue that endothelial activation should only be considered dysfunctional if it is a “net liability to the host” [1]. Endothelial cell activation has probably evolved as an adaptive local response to bacterial invasion or trauma. The local inflammatory response and microthrombosis could serve to “wall off” an infection from the rest of the host organism. However, when a local infection leads to widespread endothelial activation, upregulation of inflammation and consequent organ dysfunction, this is known as severe sepsis, and is clearly an example of endothelial dysfunction.

3.2.2 Association of endothelial dysfunction with vascular disease, and influences on endothelial function.

Unless otherwise stated, the term “endothelial function” from here on will be used as defined above (the ability of blood vessels to vasodilate in response to either ACh or shear stress induced by increased blood flow). The majority of research about endothelial function concerns not sepsis, but the role of endothelial dysfunction in cardiovascular pathophysiology. Endothelial dysfunction has been shown to be an important proximate step in the chain of events leading from normal blood vessels (such as coronary arteries), to fatty streak, then atheromatous plaque and eventually stenosis-induced ischaemia or infarction [57]. Endothelial dysfunction is now emerging as a putative novel cardiovascular risk factor, alongside more traditional factors such as hypertension, smoking and diabetes mellitus [58].

There are many factors influencing endothelial function which need to be considered when attempting to clinically measure it. In healthy people, there is both diurnal variation, and variability from one day to the next of up to 20-30% [59]. Behaviours such as eating a high fat meal [60], and both active [61, 62] and passive [63] cigarette smoking can also deleteriously affect endothelial function. Even mood and psychological stress has been shown to influence endothelial function [64, 65].
3.2.3 Estimation of endothelial activation and damage using circulating markers

The majority of clinical studies concerning the question of endothelial dysfunction in sepsis use circulating markers of endothelial activation as surrogate measures for endothelial dysfunction. According to Professor John Deanfield, a leading expert on endothelial function, these measures can “complement physiological tests of endothelial vascular control” [56], but “as a result of biological and assay availability and variability, these factors currently have only a very limited role in the assessment of individual patients” [56].

These factors include (but are not limited to) the following: i) adhesion molecules such as ICAM-1 and VCAM [66]; ii) selectins such as E-selectin and P-selectin [67]; iii) coagulation proteins such as von Willebrand Factor and thrombomodulin [68, 69]; iv) vascular growth factors such as VEGF and angiopoietin-2 [70]; and v) Circulating endothelial progenitor cells and endothelial cell microparticles [71, 72]. All of these factors can be found in the bloodstream in soluble circulating forms and are detectable soon after an acute insult to the endothelium [73]. Most studies have found an association of chronically raised plasma markers of endothelial activation with established vascular disease and cardiovascular risk factors [66, 71]. However the degree to which they correlate with actual clinical measurements of endothelial function and their value in predicting vascular events have not been definitively determined.

3.2.4 Clinical methods for estimating endothelial function

Clinical methods for estimating endothelial function use endothelial-dependent vasomotion. There are several different methods of determining vessel responses, and various stimuli can be employed, and the most commonly used of these will be discussed below. Reactive hyperaemia is a normal physiological response following a period of ischaemia, and is dependent upon the ability of the endothelium to upregulate NO production in response to increased vessel wall shear stress. Endothelial-dependent vasomotion can also be tested using responses to ACh. Endothelial-dependent vasomotion is an estimate of local endothelial nitric oxide bioavailability. Thus it reflects not only vasoregulatory aspects of endothelial function, but other important aspects of NO activity as well, including thromboresistance, endothelial cell quiescence and cell adhesion [56].

3.2.4.1 Coronary angiography

The first clinical studies assessing endothelial function used quantitative coronary angiography to observe coronary artery responses to increased blood flow [74] or locally
delivered ACh [75]. These endothelium-dependent stimuli were compared with responses to glycercyl trinitrate (GTN), which directly relaxes vascular smooth muscle and is thus an endothelium-independent stimulus. They found impaired endothelial-dependent responses or paradoxical vasoconstriction in those with atherosclerosis. Subsequently, impaired endothelium-dependent responses were found in patients with cardiovascular risk factors but angiographically smooth arteries [76, 77]. However, the need for less invasive tests soon became clear, and thus the use of peripheral blood vessel responses began to be explored.

3.2.4.2 Venous plethysmography
Venous plethysmography tests vascular responses in forearm blood vessels but is complex to perform and requires arterial cannulation. Venous, but not arterial, blood flow is occluded by a pressure cuff on the forearm, and changes in forearm blood flow are measured by strain-gauge plethysmography in response to intra-arterial agents such as ACh and GTN. This technique has limited use in clinical research laboratories and is rarely applied outside this setting.

3.2.4.3 Flow mediated dilatation of the brachial artery measured by ultrasound
Portable ultrasound machines can be used to estimate the change in diameter of the brachial artery following a period of forearm ischaemia. This technique was first described by Celermajer in 1992 [78], and has become by far the most commonly used and widely validated method for assessing endothelial function in the clinical setting. It is generally referred to as simply flow mediated dilatation, or FMD. In this technique, the subject has an arterial pressure cuff placed on either the forearm or upper arm, and blood flow is completely occluded for 3-5 minutes. An ultrasound probe is placed over the brachial artery in the upper arm, and two-dimensional ultrasound is used to measure the brachial artery diameter at baseline and at maximal flow, following cuff release [79].

Although the brachial artery is being imaged by this technique, the vascular beds of interest (coronary, cerebral and renal arteries for example) are elsewhere. Thus FMD has been compared with invasive measurement of coronary artery endothelial function and been found to correlate well, with an abnormal FMD (<3%) having a 95% positive predictive value for coronary artery endothelial dysfunction [80]. FMD responses have also been shown to be nearly entirely dependent on endothelial cell NO release [81], making it a true test of endothelial function. FMD has become the standard clinical test of endothelial function because of its convenience and non-invasive nature; however it is operator dependent and
difficult to learn. The measured diameter of the brachial artery depends on the position and angle of ultrasound probe placement, as well as the amount of pressure applied by the operator. FMD also requires a very co-operative subject who can lie very still for at least 10-15 minutes.

### 3.2.4.4 Cutaneous Laser Doppler flowmetry

Laser Doppler flowmetry uses reflected laser light to measure the speed and volume of red blood cells flowing through the skin microcirculation. Various stimuli have been used along with laser Doppler flowmetry to induce cutaneous endothelium-dependent vasodilatation, including ACh delivered by iontophoresis, local heat, and post-ischaemic reactive hyperaemia [82]. While measuring cutaneous vascular responses might be an attractive idea for those interested in the microcirculation, laser Doppler responses to reactive hyperaemia do not correlate with FMD responses [83], and furthermore appear not to be NO-dependent [84]. Thus it is unclear if impaired cutaneous vascular responses as measured by laser Doppler have any significant relationship to coronary artery endothelial function or its clinical correlates.

### 3.2.4.5 Reactive hyperaemia peripheral arterial tonometry

Reactive hyperaemia peripheral arterial tonometry (RH-PAT) is a recently developed technique for measuring endothelial function [85, 86]. FMD moved from invasive coronary testing to non-invasive testing of a large peripheral artery, the brachial. RH-PAT extends this concept further by assessing reactive hyperaemia in the fingertip. Thimble-like probes are placed over a fingertip on each hand, and a sensitive pressure transducer (tonometer) records the digital pulse wave amplitude, averaging changes over time to estimate pulsatile blood volume in the finger tip (figure 3-1; see also figures 9-3 and 9-4 for a picture of an RH-PAT monitor in use). An arterial pressure cuff is placed on the upper arm or forearm to render the hand ischaemic for 5 minutes, and then released. The average digital pulse wave amplitude 60-120 seconds after cuff release is compared with the resting value to derive a ratio, the RH-PAT index, which quantifies reactive hyperaemia (see figure 1 in chapter 10). This ratio also accounts for systemic changes in vascular tone by adjusting for the signal from the hand that was not occluded. RH-PAT correlates well with both directly measured coronary artery endothelial function [87], and with brachial artery FMD [83, 88]. An Impaired RH-PAT index has recently been shown to be predictive of cardiovascular adverse events after seven years of follow up [89]. Responses have also been shown to be at least 50-60% dependent on endothelial cell NO release [90].
Like FMD, RH-PAT responses have been shown to be impaired in patients with established vascular disease [87], and with cardiovascular risk factors [91-94]. RH-PAT has also been used in intervention studies to assess changes in endothelial function [95]. RH-PAT is currently a patented technology (Endopat 2000; Itamar Medical; Caeserea, Israel) which is FDA-approved and marketed in the USA for routine clinical use for outpatient assessment of endothelial function [96].

RH-PAT has several potential advantages over other methods for the assessment of endothelial function in patients with sepsis. First, unlike venous plethysmography it is non-invasive and relatively simple to use. Second, unlike laser Doppler, it assesses a vascular bed which is more than skin-deep, and has been shown to correlate with other measures of endothelial function and to be NO-dependent. Third, unlike FMD, it does not require a highly skilled operator, is user independent, and measures responses in a mixture of microcirculatory vessels and larger vessels.

However, RH-PAT also has some limitations and disadvantages. Since most studies have not assessed PAT responses to direct vasodilators (such as nitrates), the relative importance of endothelium dependent versus independent vasodilatation in PAT responses are unclear. The digital vasculature is very sensitive to changes in sympathetic tone and thus one might expect that PAT responses would be difficult to measure in patients with severe peripheral vasoconstriction, such as those receiving high doses of vasopressor drugs for septic shock. The finger probes used for RH-PAT measurements are expensive, at approximately $US40 per pair, and are single use only. Finally, since the monitor used for RH-PAT is a commercial
patented device, not all details of the computer algorithm for calculating RH-PAT index are freely available, and many of the published studies using RH-PAT were sponsored by the manufacturer of the device.

3.2.4.6 Dynamic near infrared spectroscopy

Near-infrared spectroscopy (NIRS) uses reflected radiation to estimate the amount of oxygenated and deoxygenated haemoglobin in tissue microvasculature [97]. It was originally used to estimate cerebral oxygenation and function [98-100], but more recently, NIRS-derived skeletal muscle tissue oxygen saturation (StO₂) has emerged as a tool for evaluating resuscitation [101, 102], tissue oxygen consumption [103] and microvascular reactivity [104] in critical illness.

In these contexts, NIRS may be used to continuously estimate thenar muscle StO₂ at rest [105, 106], but the addition of a vascular occlusion test (as in FMD or RH-PAT) provides more information and is known as dynamic NIRS (dNIRS). Dynamic NIRS can be used to estimate tissue oxygen consumption (the rate of decrease in StO₂ following cuff inflation), as well as reactive hyperaemia (the rate of increase of StO₂ following cuff release) [104, 107-109]. Dynamic NIRS has not been compared with any other method of estimating endothelial function, and indeed none of the studies using dNIRS use the term “endothelial function”. NIRS has been included in this discussion of clinical methods for estimating endothelial function because it is an increasingly accepted means of assessing microcirculatory responses in critical illness, and appears to quantify at least an aspect of reactive hyperaemia. However, it is unclear how much of dNIRS responses are due to factors other than NO-dependent vasodilatation, such as recruitment of small blood vessels or interactions between haemoglobin and hypoxic cells.

3.3 Endothelial function in sepsis

Endothelial and microvascular dysfunction are increasingly recognised to be of critical importance in the pathophysiology of sepsis [7, 110, 111]. Traditional approaches to the treatment of patients with severe sepsis focus on normalising macro-haemodynamic variables, such as central venous pressure, mean arterial pressure, cardiac output and systemic vascular resistance. However, ongoing metabolic distress, evolving organ dysfunction and death often occur in sepsis despite the achievement of these macro-haemodynamic endpoints [112, 113], and this is likely to be largely due to dysfunction of the endothelium and abnormalities of the microcirculation. Although the study of microcirculatory abnormalities in sepsis is a rapidly growing field, endothelial function is
more relevant to this thesis, and thus I have touched on the closely related problem of the microcirculation only where it is relevant.

3.3.1 In-vitro and animal studies assessing endothelial function in sepsis

Experiments in animal models, as well as those in cells and tissues [114-116], conclusively demonstrate that sepsis is a state of profound endothelial activation, damage and dysfunction. One of the earliest studies to investigate this area found marked impairment of reactive hyperaemic responses of single arterioles in a rat model of sepsis [117]. Impaired endothelial vasomotor and barrier function, as well as evidence of pathological endothelial activation has been subsequently found in multiple animal models of sepsis ranging from rats [118-122], to pigs [123-125] to primates [126-128]. Elevated circulating concentrations of endothelial adhesion molecules correlated with immunohistochemical changes in skin endothelial cells in a mouse model of sepsis [129], supporting the extrapolation of circulating damage markers with changes in endothelial structure and function. Endothelial bioavailability of NO has been shown to be decreased in animal models of sepsis [130], and this decrease is at least in part due to decreased expression of eNOS [121].

3.3.2 Human studies assessing circulating markers of endothelial activation and damage in sepsis

3.3.2.1 Overview

Exposure of endothelial cells to pro-inflammatory cytokines (such as IL-1, IL-6, TNFα, and IFNγ) leads to increased surface expression and subsequent shedding of adhesion molecules and release of preformed angiopoietin-2 and von Willebrand factor (vWF) [73]. Endothelial cells also secrete pro-inflammatory cytokines such as IL-6 [54], but these are not useful as markers of endothelial activation, as they are also produced by several other cell types including monocytes and lymphocytes. It is important to note that none of these markers of endothelial activation are specific for sepsis; thus their potential value lies more in prognostication and understanding endothelial pathophysiology than as diagnostic tools.

3.3.2.2 Adhesion molecules

Plasma concentrations of ICAM-1, VCAM-1 and E-selectin (also known as ELAM-1) have all been shown to be raised in patients with sepsis and to correlate with severity and outcome [131-133], but E-selectin appears to correlate the most closely with disease severity [134]. E-selectin is expressed only by endothelial cells, as opposed to ICAM and VCAM which are also expressed on leucocytes. Furthermore, E-selectin has a shorter half-life and more
closely parallels the time course of endothelial inflammation than does ICAM-1 [135]. Endothelial cell specific molecule-1, or endocan, is a recently described circulating marker which is only produced by endothelial cells and is raised in proportion to sepsis severity [136, 137]. However, its relative merits compared with E-selectin and ICAM-1 have not been established.

3.3.2.3 Coagulation proteins
Plasma concentrations of thrombomodulin [138, 139], vWF [132, 140] and tissue factor [141] are all raised in inflammation and sepsis, but in general their correlation with disease severity and outcome is inferior to that of adhesion molecules. Furthermore their degree of elevation is often less marked than that of adhesion molecules: for example, plasma thrombomodulin is 1.5 to 3 times higher in sepsis patients than controls [138, 139], whereas E-selectin is 5 to 10 times higher [131, 132]. vWF is stored in endothelial cell Weibel Palade bodies, along with angiopoietin-2, and is rapidly released upon endothelial cell activation. However vWF is also released by other cell types, in particular platelets. In contrast to ICAM-1 and E-selectin, vWF has not been consistently shown to be correlated with sepsis severity and outcome [142].

3.3.2.4 Vascular growth factors
Vascular endothelial growth factor (VEGF) [143-146] and angiopoietin-2 [147-152] are also elevated in patients with sepsis, and correlate with disease severity. Unlike VEGF, angiopoietin-2 is markedly elevated in sepsis, and has been shown in several studies to predict mortality [153, 154]. As well as being released by endothelial cells, angiopoietin-2 acts upon them to increase activation, inflammation and capillary leak, and thus is likely to be a mediator as well as a marker of organ dysfunction in sepsis [151, 155-157].

3.3.2.5 Circulating endothelial cells and microparticles
In addition to a change in phenotype towards thrombogenesis and adhesiveness, activated endothelial cells may swell, detach from the underlying matrix and enter the circulation [158, 159]. Circulating endothelial cells are rarely found in healthy individuals, but have been described in a variety of pathological states including cardiovascular disease, cancer and inflammation [71, 158]. Part of the physiological response to the detachment of endothelial cells is the recruitment of immature endothelial progenitor cells from the bone marrow to replace them [160, 161]. The number of circulating endothelial progenitor cells increases in patients with sepsis within six hours of diagnosis [162] and correlates with mortality [163]. Endothelial damage also results in the release of microparticles into the
circulation [164]. Endothelial microparticles are also increased in patients with sepsis, but may have a negative correlation with mortality [165]. Circulating endothelial progenitor cells and microparticles are both present in small numbers, are difficult to measure and have not been precisely defined, and thus are currently not appropriate for use in a clinical setting.

3.3.3 Human studies measuring reactive hyperaemia in inflammation and sepsis

The sensitivity of endothelial function to inflammatory stimuli has been demonstrated in human volunteers. Following exposure to either intravenous lipopolysaccharide (LPS) [166, 167] or typhoid vaccination [168], reactive hyperaemia becomes acutely impaired within 3 to 8 hours of an inflammatory insult. Reactive hyperaemia also becomes impaired in previously healthy children at the time of common childhood infections, and remains so for at least 2 weeks. Furthermore, chronic infections such as HIV [169] and periodontitis [170] are also associated with substantial endothelial dysfunction. Hence there is ample evidence from human subjects to suggest that reactive hyperaemia might be impaired in sepsis, the pinnacle of all inflammatory insults.

Unlike the estimation of endothelial dysfunction using circulating markers, relatively few studies have measured reactive hyperaemia in humans with sepsis. Table 3-1 lists all published studies which have reported reactive hyperaemia in patients with sepsis. Two studies focussing on other syndromes (trauma and multiple organ failure) are not included in this table but do report some data from patients with sepsis [171, 172]. Of the nine studies reporting reactive hyperaemic responses in adults with sepsis, eight reported significantly decreased reactive hyperaemia compared with controls. Both of the two studies enrolling neonates rather than adults [173, 174] found an increase rather than a decrease in reactive hyperaemic responses in septic patients compared with controls, suggesting that neonates may have different pathophysiological responses than adults and should be considered separately.

Among the adults, the reported degree of reactive hyperaemia varies substantially with the methodology used, but the common theme which emerges is that reactive hyperaemic responses in sepsis patients are diminished by approximately half compared with those in healthy controls. Only one of these studies reported longitudinal data [175] and most did not compare the degree of observed impairment in vascular reactivity with disease severity or plasma markers of inflammation. Thus several important questions remain, including:
what is the best method for measuring endothelial dependent vascular reactivity in sepsis? Does the degree of impairment of reactive hyperaemia correlate with disease severity and outcomes? Does the degree of impairment of reactive hyperaemia correlate with circulating markers of endothelial activation? How long does sepsis-related endothelial dysfunction persist and what is its relationship to clinical recovery? The study reported in Chapters 9-13 will attempt to answer these questions.
### Table 3-1. Studies reporting reactive hyperaemic responses in patients with sepsis

<table>
<thead>
<tr>
<th>Author (Year)</th>
<th>Method</th>
<th>Stimulus for eliciting RH</th>
<th>N sepsis</th>
<th>N controls</th>
<th>Summary of Results</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hartl (1988) [176]</td>
<td>Venous plethysmography</td>
<td>3 minute arterial occlusion</td>
<td>12</td>
<td>10c</td>
<td>Absence of RH in 9 of 12 subjects</td>
<td>NA</td>
</tr>
<tr>
<td>Astiz (1991) [177]</td>
<td>Venous plethysmography</td>
<td>5 minute arterial occlusion</td>
<td>23</td>
<td>10</td>
<td>Relative RH in forearm blood flow: Controls 342%; Sepsis 247%; Septic shock 150%</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Astiz (1995) [178]</td>
<td>Venous plethysmography</td>
<td>3 minute arterial occlusion</td>
<td>8</td>
<td>9</td>
<td>Relative RH in forearm blood flow: Controls 300%; Sepsis 152%</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Poschl (1994) [174]</td>
<td>Laser Doppler</td>
<td>1 minute arterial occlusion</td>
<td>12d</td>
<td>15</td>
<td>Relative RH in thigh skin: Controls 208%; Septic full-term 243%; Septic preterm 250%;</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Young (1995) [179]</td>
<td>Laser Doppler</td>
<td>2 minute arterial occlusion</td>
<td>11</td>
<td>19</td>
<td>Relative RH in arm skin: Healthy young 281%; Healthy old 269%; Sepsis 140%</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Nevière (1996) [180]</td>
<td>Invasive Laser Doppler</td>
<td>3 minute arterial occlusion</td>
<td>16</td>
<td>10c</td>
<td>Relative RH in leg muscle: Control 515%; Sepsis 163%</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Martin (2001) [173]</td>
<td>Laser Doppler</td>
<td>1 minute arterial occlusion</td>
<td>12d</td>
<td>20</td>
<td>Relative RH in hand skin: Control 137%; Sepsis 270%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Sair (2001) [181]</td>
<td>Invasive Laser Doppler and Venous Plethysmography</td>
<td>20 minute arterial occlusion</td>
<td>6</td>
<td>7</td>
<td>Whole limb reperfusion: (ml/100ml/min): Controls 10.6; Sepsis 4.3</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Kubli (2003) [182]</td>
<td>Laser Doppler</td>
<td>ACh iontophoresis</td>
<td>8</td>
<td>16</td>
<td>Relative RH in arm skin: Healthy 600%; ICU 510%; Septic 269%</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Vaudo (2007) [175]</td>
<td>FMD</td>
<td>4 minute arterial occlusion</td>
<td>45</td>
<td>25</td>
<td>Brachial artery FMD: Sepsis 8.7%; Controls 9.9%</td>
<td>0.05</td>
</tr>
<tr>
<td>Kienbaum (2008) [183]</td>
<td>Venous Plethysmography</td>
<td>Intra-arterial ACh</td>
<td>8</td>
<td>11</td>
<td>Change in forearm vascular resistance (mmHg/min/ml): Sepsis -3.5; Control -5.7</td>
<td>NS</td>
</tr>
</tbody>
</table>

a. RH- Reactive hyperaemia  
b. p value comparing sepsis group with controls  
c. All controls are healthy volunteers except in Hartl (post-operative) and Nevière (non-septic ICU patients)  
d. All patients are adults except in Poschl and Martin, which enrolled neonate
3.3.4 Human studies using near-infrared spectroscopy in sepsis

For reasons discussed in 3.2.4.6, studies using NIRS have not been included in table 3-1. Although dNIRS may not exactly be measuring endothelial function, it does measure one aspect of microvascular reactivity and thus studies of dNIRS in sepsis will be briefly described here. The two main relevant parameters estimated by dNIRS are the downslope in StO$_2$ with cuff inflation (deoxygenation rate), and the upslope of StO$_2$ immediately following cuff release (StO$_2$ recovery rate).

Because of in-vitro and animal evidence suggesting impaired cellular oxygen utilisation in sepsis [7, 184], one would expect the deoxygenation rate to be slower in sepsis compared with controls. This has been found to be the case in one study [185], but not in several others [103, 107, 108]. In contrast, the StO$_2$ recovery rate has been consistently found to be slower in patients with sepsis than controls, and to correlate with disease severity [103, 104, 107, 108]. More research is needed to inform the interpretation of these studies, including the comparison of dNIRS with other methods of estimating endothelial function.

3.4 The endothelium as a therapeutic target in sepsis

Given that activation and subsequent dysfunction of the endothelium and microcirculation are pivotal events early in the progression of sepsis, there is emerging interest in adjunctive sepsis therapies which target the endothelium [112, 186]. The majority of adjunctive therapies tested in sepsis to date have either been harmful or have failed to show benefit [187, 188]. The likely reason for this is that many of these studies have targeted a single nodal point in the overlapping and redundant network of inflammatory activation that characterises sepsis, such as tumour necrosis factor [189, 190], interleukin 1 [191], bacterial endotoxin [192], and platelet activating factor [193, 194]. The pathophysiology of sepsis is a complex system, exhibiting chaotic behaviour and non-linear dynamics [195, 196]. Hence such linear single-mediator approaches are bound to fail, and targets which have multiple interacting roles, such as endothelial cells, or agents with multiple actions, such as statins, are more attractive candidates.

3.4.1 Proven therapies for sepsis which incidentally target the endothelium

The international evidence-based surviving sepsis guidelines recommend the use of activated protein C [197], early goal directed therapy [198], lung-protective ventilation strategies [199] and intensive insulin therapy [200] in selected patients with severe sepsis (although it should be noted that there is controversy about the process and
recommendations of these guidelines [201]). Professor William Aird suggests that a unifying theme of these four strategies may be a protective effect on the endothelium [186]. For example, activated protein C protects endothelial cells from inflammatory mediators and apoptosis [202, 203]; goal directed therapy avoids hypoxaemia and acidosis, both of which can cause endothelial dysfunction by oxidative stress; lung-protective ventilation avoids stretch-injury and thus activation of pulmonary endothelium [204], and intensive insulin therapy avoids hyperglycaemia-induced endothelial damage [205, 206].

3.4.2 Statins to improve endothelial function in sepsis

HMG CoA reductase inhibitors, or statins, have multiple effects apart from lipid lowering, and these are known as pleiotropic effects [207]. Statins are of benefit in animal models of sepsis [208-210], and decrease the inflammatory response to LPS in human volunteers [211]. Multiple cohort studies have suggested that statin use is protective against the development of and mortality from sepsis [212, 213]. The mechanisms and impact of the pleiotropic effects of statins on sepsis pathophysiology has been extensively reviewed [214, 215].

In addition to anti-inflammatory, immunomodulatory and anti-oxidant effects, statins also improve endothelial function and nitric oxide bioavailability. In animal models, statins increase eNOS activity, decrease iNOS activity, and improve endothelial function [130, 216, 217]. In healthy human volunteers, simvastatin pre-treatment prevents LPS-induced vascular hyporeactivity [218]. Statins improve endothelial function in patients with chronic vascular disease [219, 220], both with chronic use, and within four hours of a dose [221]. Thus statins are an attractive candidate for targeting the endothelium in sepsis, but to date there have been no published clinical trials assessing this strategy.

3.5 Conclusions

There is overwhelming evidence that endothelial and microvascular dysfunction are of central importance in sepsis pathophysiology and contribute to organ failure and death. The prevailing concepts of therapeutic and resuscitation targets in severe sepsis are evolving to reflect this. However, many issues remain to be resolved. The two most important of these are determining the best method for monitoring endothelial function and its response to therapy in sepsis, and finding one or more adjunctive therapies which will target the endothelium and hopefully decrease mortality in patients with sepsis.
3.6 References


Chapter 4. Is plasma arginine concentration decreased in patients with sepsis? A systematic review and meta-analysis
4.1 Preamble

In 2008, I attended the 28th symposium of the International Society for Intensive Care and Emergency Medicine, in Brussels, Belgium. Whilst there, I attended a talk by one of the world authorities on the role of arginine and nitric oxide in sepsis and trauma, Dr Juan Ochoa. Dr Ochoa pointedly stated on several occasions that plasma concentrations of arginine were decreased in patients with trauma, but not those with sepsis. In reply to an audience comment that there were several studies reporting decreased plasma arginine concentrations in patients with sepsis, he opined that these findings were artefactual, due to the enrolled patients having undergone trauma or surgery prior to developing sepsis.

My reading of the literature until this point had been that sepsis was a hypoargininaemic state, which may be an important piece of the puzzle of endothelial and immune dysfunction in sepsis. However, following this talk, further literature review suggested that it was not clear whether plasma arginine concentrations are decreased in patients with sepsis or not. Hence I undertook the systematic review reported in this chapter. This manuscript has been accepted for publication to Critical Care Medicine.
4.2 Abstract

Introduction

L-arginine is a conditionally essential amino acid and plays an important role in immune and vascular function in sepsis. Plasma concentrations of L-arginine are decreased following trauma or surgery, but have been variably reported to be normal or decreased in patients with sepsis.

Methods

We searched Medline and Embase from database inception until January 2010 for the MESH terms “arginine”, “amino acids” and “sepsis” and reviewed all studies which reported plasma arginine concentrations in humans with sepsis. Studies were grouped according to the presence or absence of trauma and surgery. We performed a pooled quantitative analysis on the subset of studies which reported appropriate data.

Results

We identified 285 citations, of which 16 met inclusion criteria, and 9 were included in the quantitative analysis. Plasma arginine concentration was lower in sepsis compared with concurrent or historical controls in three of four studies of surgical sepsis, one of four of sepsis following trauma, and all eight studies of predominantly medical sepsis. In the quantitative analysis, mean plasma L-arginine concentration was 33.7 µmol/L (95% CI 25.0 to 42.3) lower in sepsis patients than in concurrent healthy controls (p<0.001).

Conclusion

Plasma concentrations of plasma L-arginine are substantially decreased in patients with sepsis, in the absence of trauma or surgery. There are not enough studies of sufficient quality to determine if this is also the case for trauma or surgery-associated sepsis.
4.3 Introduction

L-arginine, a non-essential amino acid in baseline physiological states, becomes essential at times of physiological stress such as sepsis, trauma and surgery, and is thus a “conditionally essential” amino acid [1]. L-arginine plays several vital roles in patients with sepsis: it is the substrate for nitric oxide synthase (NOS) [2], and sole source of nitric oxide, which is necessary for maintaining the microcirculation and for directly fighting bacterial infection. Furthermore, L-arginine is important for cell-mediated immune function [3, 4], protein synthesis [5], and wound healing [6, 7]. Nutritional supplementation with L-arginine is controversial in sepsis [8], with various studies showing either beneficial or harmful effects [9].

Plasma L-arginine levels are generally [8, 10, 11], but not always [12, 13], described in review articles as being low in humans with sepsis However, these articles selectively cite either other commentaries [10, 14, 15], or the same few clinical studies [16, 17, Chiarla, 2006 #340, 18], and none of them critically assess the available published evidence.

Moreover, several studies and commentaries suggest that plasma L-arginine concentrations are not decreased in sepsis [19, 20], or that they are more markedly decreased in trauma than in sepsis [8, 12, 13, 18]. By far the two most highly cited studies of plasma L-arginine concentrations in sepsis both reported that there was no significant difference between plasma L-arginine concentrations in sepsis patients compared with healthy controls [19, 20] (citations as estimated by Google Scholar, 2nd January 2010 for these two articles exceeded those for all other studies included in this review combined).

Plasma L-arginine has also been shown to be low in humans with severe trauma [18, 20], and other severe states of physiological stress [21]. Arginine consumption by macrophage and myeloid cell production of arginase is increased following trauma [22] and surgery [23, 24]. Since many patients in clinical studies of sepsis also have experienced recent trauma or surgery, it is unclear if sepsis alone is an arginine-deficient state.

Since it has important implications for understanding sepsis pathophysiology, and for clarifying the potential role of arginine supplementation in sepsis, we conducted a systematic review of the published literature to address the question: is plasma L-arginine concentration decreased in patients with sepsis compared with healthy controls?
4.4 Methods

We searched Medline and Embase, using the MESH headings “arginine”, “amino acids” and “sepsis”, and the limits “human”, from database inception until 2nd January 2010. We also hand searched the references of retrieved articles and of review articles. Studies were included if they met all of the following criteria: i) Included humans with a diagnosis of “sepsis”; ii) provided a definition of “sepsis”; iii) reported plasma L-arginine concentrations; iv) patients were receiving no specific amino acid supplements at the time of measurements (apart from standard enteral or parenteral nutrition); and vi) were reported in English. Data were extracted and recorded on a predefined checklist.

To be included in the quantitative analysis, studies also had to: i) Report mean L-arginine concentrations (rather than expressing it in graphical format alone, as a proportion of normal or a median); ii) include a control group (rather than historical controls or normal ranges); and iii) Report the standard deviation (SD) or standard error of the mean (SEM) for L-arginine concentrations in both the sepsis and control groups.

For the purposes of comparing plasma arginine concentrations in sepsis with controls, we calculated a quality score ranging from zero to seven points for each study by allocating one point for each of the following: i) A currently accepted definition of sepsis was used ii) Sepsis patients were enrolled within the first 48 hours of diagnosis or admission (because plasma arginine recovers over time in patients with sepsis) [25] iii) Plasma arginine concentrations were reported numerically rather than in graphical form only iv) Concurrent rather than historical controls were included; v) Included healthy controls rather than those with non-sepsis critical illness or infection; vi) At least twenty patients with sepsis were included and vii) blood was placed on ice or plasma was frozen within 30 minutes of blood collection (because arginine concentrations drop rapidly in ex-vivo whole blood at room temperature) [26]. If these features were not described in the paper, they were assumed to be absent. This score was designed prior to analysis, and based on specific considerations for studies reporting plasma arginine concentrations in sepsis.

A random-effects model was used for pooling of plasma L-arginine concentrations. Where studies did not provide SD, but reported SEM, SD was calculated as SEM multiplied by the square root of the sample number (SEM x √n). Quantitative analysis was performed using Revman version 5 (Cochrane collaboration, Oxford, UK)
4.5 Results

Results of the search

287 citations were identified, of which 16 were included for analysis, and 9 were included in the quantitative analysis (figure 4-1)

Figure 4-1. Flow Chart of Study selection

287 citations found from initial searches
  Medline 268, Embase 11, Hand-searches 8

251 excluded after review of abstract
  (Did not meet inclusion criteria)

36 Papers retrieved for detailed evaluation

20 excluded
  12 plasma arginine not reported, 3 review articles, 2 not in English, 2 in malaria not sepsis, 1 patients receiving IV amino acids

16 articles included in review
  9 included in quantitative analysis
<table>
<thead>
<tr>
<th>Author (year)</th>
<th>Subjects</th>
<th>Sepsis Durationa</th>
<th>Sepsis definition</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freund (1978)</td>
<td>15 adults with sepsis (9 surgical)</td>
<td>Not stated</td>
<td>Infection plus fever and leukocytosis</td>
<td>None</td>
</tr>
<tr>
<td>Freund (1979)</td>
<td>25 adults with sepsis (Mostly surgical, numbers not stated)</td>
<td>Not stated</td>
<td>Infection plus fever and leukocytosis</td>
<td>None</td>
</tr>
<tr>
<td>Askanazi (1980)</td>
<td>10 adults with trauma (5 of whom also had sepsis)</td>
<td>Not stated</td>
<td>Fever plus infection</td>
<td>15 “normal”</td>
</tr>
<tr>
<td>Vente (1989)</td>
<td>27 adults with sepsis (38 adults with “stress” (trauma, pancreatitis or ruptured aneurysm))</td>
<td>Not stated</td>
<td>Bacteraemia plus all of fever, tachycardia and leukocytosis</td>
<td>None</td>
</tr>
<tr>
<td>Ochoa (1991)</td>
<td>17 surgical sepsis 14 trauma 8 trauma and sepsis (all adults)</td>
<td>Not stated</td>
<td>Roughly corresponds to 1992 SCCM criteria for severe sepsis [27]</td>
<td>14 healthy volunteers</td>
</tr>
<tr>
<td>Sprung (1991)</td>
<td>15 adults septic shock (12 with pneumonia)</td>
<td>Not stated</td>
<td>Roughly corresponds to 1992 SCCM criteria for septic shock, PLUS acute confusion [27]</td>
<td>17 infection (But with no confusion or shock)</td>
</tr>
<tr>
<td>Chiarla (2000)</td>
<td>16 adults with trauma and sepsis</td>
<td>Not stated</td>
<td>Infection plus fever and leukocytosis</td>
<td>None</td>
</tr>
<tr>
<td>Druml (2001)</td>
<td>9 adults with severe sepsis (4 surgical)</td>
<td>Not stated</td>
<td>1992 SCCM criteria PLUS bacteraemia</td>
<td>8 healthy controls</td>
</tr>
<tr>
<td>Study</td>
<td>Patients Description</td>
<td>Time to Enrolment</td>
<td>Inclusion Criteria</td>
<td>Exclusion Criteria</td>
</tr>
<tr>
<td>------------------</td>
<td>----------------------</td>
<td>-------------------</td>
<td>--------------------</td>
<td>-------------------</td>
</tr>
<tr>
<td>Luiking (2003)</td>
<td>8 adults severe sepsis (focus not stated)</td>
<td>Within 48h of sepsis diagnosis</td>
<td>Not stated</td>
<td>5 critically ill without sepsis</td>
</tr>
<tr>
<td>Villalpando (2006)</td>
<td>6 adults septic shock (all medical)</td>
<td>Within 12h of septic shock criteria</td>
<td>1992 SCCM criteria</td>
<td>10 healthy volunteers</td>
</tr>
<tr>
<td>Chiarla (2006)</td>
<td>9 adults with trauma and sepsis</td>
<td>Not stated</td>
<td>Infection plus fever and leukocytosis</td>
<td>None</td>
</tr>
<tr>
<td>Engel (2006)</td>
<td>32 adults with trauma and sepsis</td>
<td>Not stated</td>
<td>1992 SCCM criteria</td>
<td>None</td>
</tr>
<tr>
<td>Van Waardenburg (2007)</td>
<td>19 children with trauma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Davis (2009)</td>
<td>56 sepsis patients (50 community-acquired sepsis)</td>
<td>Within 36h of admission</td>
<td>1992 SCCM criteria</td>
<td>27 healthy volunteers</td>
</tr>
</tbody>
</table>

a. Time elapsed between onset/diagnosis of sepsis and enrolment in the study
b. “Surgical” sepsis refers to patients managed exclusively in surgical ICUs, or who have undergone an operation.
c. 8 excluded from quantitative analysis as were receiving non-standard TPN with branched chain amino acids
Characteristics of included studies

The 16 included studies (table 4-1) were divided into three groups (table 4-2), based on the presence or absence of trauma or surgery in addition to sepsis. Group 1 included those studies whose patients had predominantly surgical (post-operative) sepsis. Group 2 included those which enrolled patients in whom trauma and sepsis co-existed. Group 3 comprised those studies enrolling patients with no recent trauma, and with predominantly non-surgical sepsis (such as pneumonia or urosepsis). The studies employed varying definitions of sepsis, and most did not report the time elapsed between the diagnosis of sepsis and the collection of blood for analysis (table 4-3). The quality scores were lower for the studies in groups 1 and 2 (mean quality score=1.8), than for those in group 3 (mean quality score=4.9), p=0.003 (Table 4-2).

Group 1 (surgical sepsis) included four studies, three of which reported decreased L-arginine concentrations in sepsis compared with historical controls [16, 21, 29], and one of which reported no statistically significant difference in plasma L-arginine concentrations in sepsis patients compared with healthy controls [20]. Three of four studies in group 2 (sepsis with trauma) reported no difference in plasma L-arginine in septic patients compared with historical [18, 30] or concurrent [19] controls. All eight studies which enrolled predominantly patients with sepsis unrelated to surgery or trauma found decreased plasma L-arginine in sepsis patients compared with concurrent controls [25, 31-37].
Table 4-2. Mean Plasma L-arginine Concentrations (µmol/L) and Quality Scores of Included Studies

<table>
<thead>
<tr>
<th>Author (year)</th>
<th>Quality score</th>
<th>Sepsis</th>
<th>Controls</th>
<th>p sepsis v. controls</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group 1 – Post-surgical/abdominal sepsis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Freund (1978)</td>
<td>0</td>
<td>19.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Freund (1979)</td>
<td>1</td>
<td>20&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vente (1989)</td>
<td>1</td>
<td>45&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ochoa (1991)</td>
<td>4</td>
<td>84.7</td>
<td>80.7</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Group 2 – Sepsis with trauma</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Askanazi (1980)</td>
<td>3</td>
<td>110</td>
<td>132</td>
<td>NS</td>
</tr>
<tr>
<td>Chiarla (2000)</td>
<td>1</td>
<td>114</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chiarla (2006)</td>
<td>1</td>
<td>87</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Engel (2006)</td>
<td>3</td>
<td>51</td>
<td>84</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Group 3 – Predominantly non-surgical sepsis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sprung (1991)</td>
<td>3</td>
<td>41</td>
<td>78</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Druml (2001)</td>
<td>4</td>
<td>46</td>
<td>84</td>
<td></td>
</tr>
<tr>
<td>Luiking (2003)</td>
<td>3</td>
<td>47.7</td>
<td>68.2</td>
<td></td>
</tr>
<tr>
<td>Villalpando (2006)</td>
<td>5</td>
<td>40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>75&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Van Waardenburg (2007)</td>
<td>5</td>
<td>38</td>
<td>68</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Kao (2008)</td>
<td>6</td>
<td>40.2</td>
<td>85.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Luiking (2009)</td>
<td>6</td>
<td>49</td>
<td>92</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Davis (2009)</td>
<td>7</td>
<td>38.6</td>
<td>80.3</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

<sup>a</sup> Actual figure not reported in paper - estimated from graph or figure in manuscript.

Excluded studies

Twenty of the thirty-six retrieved papers were excluded, of which twelve did not report plasma L-arginine concentrations, three were review articles, two included patients with severe malaria but did not describe sepsis criteria [38, 39], and one included patients receiving variable rates of intravenous amino acid infusions. Two studies were excluded because of being written in a language other than English (1 Russian, 1 Chinese), but they otherwise met inclusion criteria. The abstract of the first reports that plasma L-arginine was “increased” in thirty-seven patients with severe sepsis but does not say with what they are being compared, and does not give any numerical data [40]. The abstract of the second reports that plasma L-arginine was decreased in twelve burns patients with sepsis compared with nineteen non-septic burns patients (p<0.05), but also provides no actual L-arginine concentrations [41].
Quantitative data synthesis

Of the sixteen included studies, only nine reported sufficient information to be included in a pooled quantitative analysis. These nine studies included 160 patients with sepsis and 120 controls. With the exception of one study, all of these reported lower plasma L-arginine concentrations in sepsis patients than in controls. The pooled mean difference in L-arginine concentrations between sepsis and control groups was -33.7 µmol/L (95% CI -42.3 to -25.0). In other words, plasma L-arginine was, on average, 33.7 µmol/L lower in sepsis patients than in controls, and this difference was statistically significant (p=0.0001).

Sensitivity analysis

Only four of the included studies had quality scores of five or more out of a possible seven points, and reported sufficient data for quantitative analysis [25, 35-37]. When a quantitative analysis was performed combining only these studies, a similar but stronger result was obtained to the overall analysis: that plasma L-arginine is significantly lower in patients with sepsis than in healthy controls (with a mean [95% CI] difference of -42.5 µmol/L [-47.9 to -37.2]) (Figure 2b). Furthermore, the large and statistically significant degree of heterogeneity observed in the overall analysis ($I^2=70\%$, $p=0.001$) disappears ($I^2=0\%$, $p=0.83$), suggesting that this is a more robust analysis. Including the two small studies excluded on the basis of language of publication is unlikely to have made any difference to the overall results, as one of them described L-arginine concentrations as being higher in sepsis and one lower.

4.6 Discussion

This review demonstrates that plasma concentrations of L-arginine are acutely decreased in patients with sepsis, independent of trauma or surgery. Furthermore, the degree of hypoargininaemia in sepsis is considerable, with a mean difference of at least 33 µmol/L compared with controls. Given that healthy plasma L-arginine concentrations are approximately 70 to 80 µmol/L [42-44], this difference is large enough to potentially contribute to significant impairment of endothelial and immune function in patients with sepsis.

The signal from the studies of sepsis associated with trauma or surgery was more mixed, with four out of these eight studies showing decreased L-arginine concentrations in sepsis, and four showing no difference from controls or normal ranges. However, since this group of studies were of significantly lower quality than those concerning non-surgical sepsis, one
should interpret them with care. Since plasma arginine has been shown to increase towards normal over the first 2-4 days in patients with sepsis [45], but to remain low for at least 7 days in patients with trauma [22], an important explanation for a lack of difference in these studies may be a prolonged time between sepsis onset and blood collection for plasma amino acid analysis. Of the four studies which did not find hypoargininaemia in sepsis patients, three provided no information about the time of specimen collection in relation to sepsis onset [20] and in the remaining study, at least 3 to 4 days had elapsed prior to blood collection [19].

The findings of this systematic review provides an evidence base that supports the majority of review articles and editorials concerning arginine in sepsis [1, 8, 10, 11, 46]. Moreover, our findings are consistent with observations from both animal [47] and human [48] experimental models of sepsis, which both showed an acute decrease in plasma L-arginine concentration following intravenous administration of bacterial endotoxin. There are several probable underlying mechanisms for the observed hypoargininaemia of sepsis. These include decreased gastrointestinal absorption of arginine [49], increased arginase activity [50], and increased utilisation of arginine for protein synthesis [10].

This study has several limitations. The overall quantitative analysis has significant heterogeneity ($I^2$ of 70%), and most of the studies including patients after surgery or trauma were of poor quality for the purposes of comparing plasma L-arginine concentrations in sepsis subjects with controls. Since some studies report plasma L-arginine concentrations as secondary outcomes, the search strategy may have missed some relevant studies. However, a funnel plot (not shown) of the mean difference versus its standard error for each study was symmetrical, which suggests that there are unlikely to be missing or unpublished studies which would alter the conclusions of this meta-analysis.
Figure 4-2. Pooled quantitative analysis of studies reporting plasma arginine concentration in sepsis compared with healthy controls

4-2a. Pooled quantitative analysis of studies reporting plasma arginine concentration in sepsis compared with healthy controls

<table>
<thead>
<tr>
<th>Study or Subgroup</th>
<th>Sepsis Mean</th>
<th>Sepsis SD</th>
<th>Sepsis Total</th>
<th>Control Mean</th>
<th>Control SD</th>
<th>Control Total</th>
<th>Mean Difference</th>
<th>IV, Random, 95% CI</th>
<th>Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>Askanazi 1980</td>
<td>110</td>
<td>44.7</td>
<td>5</td>
<td>132</td>
<td>31</td>
<td>5</td>
<td>-22.00 [-69.68, 25.68]</td>
<td>1980</td>
<td></td>
</tr>
<tr>
<td>Ochoa 1991</td>
<td>84.7</td>
<td>48</td>
<td>25</td>
<td>80.7</td>
<td>17.2</td>
<td>14</td>
<td>4.00 [-16.86, 24.86]</td>
<td>1991</td>
<td></td>
</tr>
<tr>
<td>Sprung 1991</td>
<td>41</td>
<td>15.5</td>
<td>15</td>
<td>78</td>
<td>28.9</td>
<td>17</td>
<td>-37.00 [-52.82, -21.18]</td>
<td>1991</td>
<td></td>
</tr>
<tr>
<td>Druml 2001</td>
<td>46</td>
<td>18</td>
<td>9</td>
<td>84</td>
<td>14</td>
<td>8</td>
<td>-38.00 [-53.24, -22.76]</td>
<td>2001</td>
<td></td>
</tr>
<tr>
<td>Luiking 2003</td>
<td>47.7</td>
<td>12.4</td>
<td>8</td>
<td>68.2</td>
<td>11.4</td>
<td>5</td>
<td>-20.50 [-33.68, -7.32]</td>
<td>2003</td>
<td></td>
</tr>
<tr>
<td>Kao 2008</td>
<td>40.2</td>
<td>13.7</td>
<td>13</td>
<td>85.5</td>
<td>8.7</td>
<td>7</td>
<td>-45.30 [-55.15, -35.45]</td>
<td>2008</td>
<td></td>
</tr>
<tr>
<td>Davis 2009</td>
<td>38.6</td>
<td>16.6</td>
<td>56</td>
<td>80.3</td>
<td>19.8</td>
<td>27</td>
<td>-41.70 [-50.34, -33.06]</td>
<td>2009</td>
<td></td>
</tr>
<tr>
<td>Luiking 2009</td>
<td>49</td>
<td>12</td>
<td>10</td>
<td>92</td>
<td>17</td>
<td>16</td>
<td>-43.00 [-54.17, -31.83]</td>
<td>2009</td>
<td></td>
</tr>
</tbody>
</table>

Total (95% CI): 160, 120 [-33.67 [-42.31, -25.03]]

Heterogeneity: \( \tau^2 = 109.51; \chi^2 = 26.24, \text{df} = 8 (P = 0.0010); I^2 = 70\%

Test for overall effect: \( Z = 7.64 (P < 0.00001) \)

Notes: SD=Standard Deviation. IV=Inverse variance method. Random=Random effects model. CI=Confidence Interval. df=degrees of freedom.

4-2b. Restricted pooled quantitative analysis of higher quality studies reporting plasma arginine concentration in sepsis compared with healthy controls

<table>
<thead>
<tr>
<th>Study or Subgroup</th>
<th>Sepsis Mean</th>
<th>Sepsis SD</th>
<th>Sepsis Total</th>
<th>Control Mean</th>
<th>Control SD</th>
<th>Control Total</th>
<th>Mean Difference</th>
<th>IV, Random, 95% CI</th>
<th>Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kao 2008</td>
<td>40.2</td>
<td>13.7</td>
<td>13</td>
<td>85.5</td>
<td>8.7</td>
<td>7</td>
<td>-45.30 [-55.15, -35.45]</td>
<td>2008</td>
<td></td>
</tr>
<tr>
<td>Davis 2009</td>
<td>38.6</td>
<td>16.6</td>
<td>56</td>
<td>80.3</td>
<td>19.8</td>
<td>27</td>
<td>-41.70 [-50.34, -33.06]</td>
<td>2009</td>
<td></td>
</tr>
<tr>
<td>Luiking 2009</td>
<td>49</td>
<td>12</td>
<td>10</td>
<td>92</td>
<td>17</td>
<td>16</td>
<td>-43.00 [-54.17, -31.83]</td>
<td>2009</td>
<td></td>
</tr>
</tbody>
</table>

Total (95% CI): 98, 71 [100.0% [-42.53 [-47.88, -37.18]]

Heterogeneity: \( \tau^2 = 0.00; \chi^2 = 0.87, \text{df} = 3 (P = 0.83); I^2 = 0\%

Test for overall effect: \( Z = 15.58 (P < 0.00001) \)
In conclusion, sepsis which is not associated with trauma or surgery is a profoundly hypoargininaemic state, but there are not enough high quality studies to determine with certainty if this is also the case for sepsis which is associated with trauma or surgery. Whilst studies of arginine supplementation in human [9] and animal [51, 52] sepsis have had conflicting results, few studies have assessed the role of arginine alone as a therapeutic agent in human sepsis. Those which have done so suggest that intravenous arginine is safe [53, 54]. Given that plasma L-arginine concentrations are clearly decreased in humans with sepsis, the issue of exogenous arginine administration in sepsis should be revisited.
4.7 References


Section B – Epidemiology and Risk Stratification of Sepsis in the Top End of the Northern Territory

“O Rose, thou art sick!
The invisible worm
That flies in the night,
In the howling storm,

Has found out thy bed
Of crimson joy:
And his dark secret love
Does thy life destroy”

William Blake (1757-1827)
Chapter 5 . Sepsis in Tropical Australia: high disease burden with disproportionate impact on Indigenous populations
5.1 Preamble

5.1.1 The study site

The “Top End” of the Northern Territory (NT) includes the Darwin, Katherine and Arnhem regions, and is a tropical area of Northern Australia with a widely dispersed population (figure 5-1).

Figure 5-1. The Health Districts of the Northern Territory of Australia

Twenty-seven percent of the population of the Top End are Indigenous Australians, many of whom live in remote communities scattered across the Top End. Figure 5-2 shows a typical remote Aboriginal community. Darwin is the capital city of the Northern Territory, and contains the only tertiary referral hospital, and the only intensive care unit in the Top End. There are district hospitals in Gove and Katherine, and multiple community health clinics throughout the Top End. The Royal Darwin Hospital Intensive Care Unit (RDH ICU) has eight ventilated beds and ten co-located high-dependency unit beds, and receives approximately 1000 admissions per year. It caters to both children and adults, and to both surgical and medical patients. RDH ICU serves the Top End of the NT, but also frequently admits patients from the Kimberly region of Western Australia, and from East Timor and Indonesia. It is capable of providing advanced supportive care including ventilation, continuous renal replacement therapy, vasopressor support and invasive cardiovascular monitoring, but
does not have the facilities to perform Extra-Corporeal Membrane Oxygenation, or elective cardiac or neurosurgery.

Figure 5-2. A typical Top End Aboriginal Community (with thanks to Peter Farkas, former photographer at Royal Darwin Hospital)

5.1.2 Background to the PRESTO study (Prospective Epidemiology of Sepsis in the Top end)

Prior to the commencement of my PhD studies, the epidemiology of several important infections had been described in the Top End of the Northern Territory (such as melioidosis, rotavirus, trachoma, rheumatic fever, and invasive pneumococcal disease). All of these specific infections, were known to be more common in the NT (particularly in Indigenous people) than elsewhere in Australia. However, the epidemiology of sepsis was unknown in the Top End. While it was presumed that the incidence of sepsis was high in the Top End, there were no data available on incidence rates, causative organisms or outcomes and their predictors. Such data are crucial for the planning of sepsis-related research, to inform public health policy and planning, and to improve the local management of sepsis. Hence, the study described in this chapter was designed and commenced within the first 6 months of my PhD studies.
The PRESTO study (Prospective Epidemiology of Sepsis in the Top End) is a prospective cohort study aiming to comprehensively describe the local epidemiology of sepsis which was conducted at Royal Darwin Hospital between May 2007 and May 2008. Chapters 5-7 and Chapter 16 are manuscripts reporting results from the PRESTO study. Appendix 1 contains the clinical research forms used for data collection in the PRESTO study, and thus provides more detail about entry criteria and definitions. The current chapter describes the epidemiology of sepsis in the Top End of the NT.
5.2 Abstract

Background

Sepsis is an important cause of mortality worldwide, and is increasing in incidence. Sepsis epidemiology has been described in North America, Europe and temperate Australia, but there are few data from tropical regions, or those with a high-proportion of indigenous people.

Methods

Prospective cohort study in the major hospital for the tropical Northern Territory of Australia, a region where 27% of the population are Indigenous. We screened all adult (≥15 years) acute hospital admissions over a 12-month period for sepsis by standard criteria, and collected standardised clinical data. Principal outcome measures were the population-based incidence of community-onset sepsis and severe sepsis requiring intensive care (ICU) admission; 28-day mortality, direct costs and microbial epidemiology.

Results

There were 1,191 hospital admissions for sepsis in 1,090 patients, of which 604 (50.7%) were Indigenous; the average age was 46.7 years. The age-adjusted annual population-based incidence of sepsis was 11.79 episodes/1000 (mortality 5.5%), but for Indigenous people it was 40.78/1000 (mortality 5.7%). For severe sepsis requiring ICU, the incidence was 1.30/1000/year (mortality 21.5%), with an Indigenous rate of 4.74/1000 (19.3%). The median (IQR) direct cost per hospital episode of sepsis was US$6,257 ($3,129-$13,220) and for severe sepsis requiring ICU admission, US$17,810 ($10,100-$33,810).

Conclusions

The incidence of sepsis in tropical northern Australia is substantially higher than that for temperate Australia, as well as for other countries. Sepsis has a disproportionate impact on Indigenous people. The burden of sepsis in indigenous populations worldwide requires further study to guide appropriate resourcing of health care and preventative strategies.
5.3 Introduction

Sepsis (an acute infection with a systemic response) and severe sepsis (sepsis resulting in organ dysfunction) have high mortality and health-care costs [1, 2], and are increasing in incidence [3, 4]. Severe sepsis has been estimated to cause as many deaths annually in the United States as acute myocardial infarction [1]. The epidemiology of sepsis has been well described in North America [1], [3, 5] and Europe [6, 7], but there are few published data describing the epidemiology of sepsis in tropical regions [8, 9], or indigenous populations. Only a single national study has described the epidemiology of sepsis in Australia [10], and this study did not include any data from tropical Australia, or on indigenous status. Finfer and colleagues found that the population-based incidence of severe sepsis requiring intensive care unit (ICU) admission in Australia was 0.77 cases per 1000 per year, which is comparable to figures reported from Europe and North America [11].

The Northern Territory (NT) of Australia is a tropical area with a high proportion of Indigenous people. The population of the NT has a high prevalence of both infectious diseases [12] and chronic diseases [13], but the epidemiology of sepsis in the NT is unknown. Similar to many other indigenous peoples living worldwide, Australian Indigenous people have a lower life expectancy and a higher burden of chronic and infectious diseases than do non-Indigenous Australians [14, 15]. A study from Western Australia found that sepsis accounted for 14.5% of ICU admissions in Indigenous people, compared with 4.0% in non-Indigenous [16]. In a retrospective central Australian study, 60% of deaths in Indigenous people were attributable to infection, compared with 25% for non-Indigenous people [17].

Most large studies of sepsis epidemiology are retrospective database analyses, based on discharge coding [1, 3, 18], which is likely to significantly underestimate sepsis incidence [19]. Most prospective studies are limited to patients requiring ICU admission [6, 10], and are thus not representative of the true community burden of sepsis requiring hospitalisation. Sepsis which requires hospital but not ICU admission is common and has a high mortality [1], but is under-represented in the literature.

In this prospective study, we describe the clinical and epidemiological features of sepsis and severe sepsis in tropical northern Australia, including the population-based incidence in Indigenous and non-Indigenous populations, microbiology, outcomes and costs, and
compare these with published estimates for temperate Australia, North America and Europe.

5.4 Methods

Setting
Royal Darwin Hospital (RDH) is a 350-bed teaching hospital in tropical Australia (latitude 12.5°S), which serves as the only hospital for a population of 130,000 over an area of 115,000 km² (primary drainage area; Figure 5-1), and as a referral hospital (including for ICU care) for a total population of 170,000 over an area of 500,000 km². Indigenous Australians comprise 27% of the catchment population, and 30.3% of the population live in “remote” or “very remote” areas [20, 21].

Recruitment and data collection
We undertook a prospective cohort study comprising every adult (≥15 years) acute admission to RDH for a 365-day period. The study was approved by the Human Research Ethics Committee of the Northern Territory Department of Health and Families and Menzies School of Health Research. From 6th May 2007 through 5th May 2008, we evaluated daily every admission to RDH by admission diagnosis. All patients with an admission diagnosis which could possibly represent an infection, or whose admission diagnosis was missing, underwent screening for study inclusion, by examination of the medical record, observation chart and pathology results, and, where necessary, discussion with the patient’s treating clinician. In addition, daily screening rounds were conducted of the ICU and emergency department. Finally, all positive blood culture results for the period of the study were examined, and those from episodes not already enrolled in the study were evaluated. All data were collected by one of three trained study staff.

All patients who met pre-defined criteria for probable or definite infection, in addition to at least two criteria for the systemic inflammatory response syndrome (SIRS) [22] were enrolled. SIRS criteria were required to be present concurrently within a 24-hour period, within the first 48 hours of hospital admission. Patients’ discharge summaries and pathology results were assessed at the time of hospital discharge, and those with a non-infectious cause of SIRS were subsequently excluded.
Definitions

An acute admission was defined as any admission to the acute hospital, excluding day procedures and attendance for routine haemodialysis. More than one admission for sepsis could be counted for the same patient, but readmission within 14 days of discharge was not counted as a separate episode. Criteria for probable or definite infection were identical to those used in the PROWESS trial [23].

Severe sepsis was defined as sepsis plus at least one sepsis-related organ dysfunction or perfusion abnormality within the first 48 hours post admission, as defined in the PROWESS study [23]. An organism was considered to be the primary causative organism if it was a pathogen consistent with the clinical presentation, which was isolated from an appropriate specimen collected within a period from 24 hours before to 48 hours after presentation to hospital. Non-sterile site isolates were only included if they were cultured from deep pus specimens or from purulent sputum with a predominant growth of an organism seen on Gram stain. For each episode, the single most important causative organism was selected based on the clinical presentation.
Figure 5-3. Map of the Northern Territory of Australia, showing Darwin, primary drainage area of Royal Darwin Hospital, and referral area for Royal Darwin Hospital Intensive Care Unit.

Data management and statistical analysis
All decisions about focus of infection and causative organisms were considered by an infectious diseases physician. Following hand-checking of all case record forms, and data entry (Epidata 3.1, Epidata Association), 10% of entries were checked for errors, with a resulting error rate of <0.1% of fields. Denominators for population-based incidence calculations were taken from the Australian Bureau of Statistics (ABS) estimated population figures for June 2007 [24]. Sepsis incidence was only calculated for patients whose current residence was within the primary drainage area of RDH; the incidence of severe sepsis requiring ICU admission was calculated using the primary drainage area for the ICU, a significantly larger area (Figure 5-1). Age-adjusted rates were calculated using the direct method, against the 2001 Standard Australian Population [25]. Comparator studies of sepsis epidemiology were included if they used similar methodology and reported
comparable data to the present study. Factors associated with mortality and readmission for sepsis were assessed using logistic regression models with backwards stepwise selection. All single variables with a Wald $p$-value of $\leq 0.10$ were included in the initial model. APACHE and SOFA were not included, since their components were part of the model. Patients with active orders limiting life-sustaining treatment were excluded from the mortality risk factor analysis. Indigenous population estimates were taken from ABS data from the 2006 census [20]. Confidence intervals for age-adjusted rates were calculated using the Poisson distribution [26]; $p$ values of $<0.05$ were considered significant. All statistical calculations were performed using Stata v10 (Statacorp).

**Direct cost estimation**

Direct costs were considered from a health system payer perspective and conservatively estimated for each sepsis admission by: total cost = (length of stay outside ICU x ward bed-day cost [US$1043/day]) + (ICU length of stay x ICU bed-day cost [US$1758/day]) + (cost of retrieval and repatriation, if relevant). Bed-day costs and estimated cost per nautical mile of retrieval or repatriation were taken from NT Government estimates for RDH for 2009. All costs were expressed as US dollars using the mean exchange rate for the study period ($A1=$US0.877).
5.5 Results

Recruitment and baseline characteristics

There were 1,191 hospital admissions for community-onset sepsis in 1,090 patients over a 365-day period (Figure 5-2). Mean age was 46.7 years, 52.4% were male, and 50.7% were Indigenous (Table 1). Indigenous differed substantially from non-Indigenous in both demographics and comorbidities (Table 5-1).

Figure 5-4. Recruitment flowchart

- Acute hospital admissions: 20,969
- Eligible admissions: 15,963
- Identified for screening: 3,882
- Met infection criteria: 2,193
- Met ≥2 SIRS criteria: 1,291
- Subsequently excluded: 100
  (Not infection: 93, Duplication of enrolment: 7)
- Included in final analysis: 1,191 episodes, in 1090 patients
Table 5-1. Baseline characteristics of study subjects by Indigenous status

<table>
<thead>
<tr>
<th></th>
<th>Total (n=1191)</th>
<th>Indigenous (n=604)</th>
<th>Non-Indigenous (n=587)</th>
<th>P valuea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age mean (sd)</td>
<td>46.7 (17.4)</td>
<td>43.2 (14.4)</td>
<td>50.2 (19.2)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Male</td>
<td>624 (52.4)</td>
<td>261 (44.5)</td>
<td>363 (60.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Remote-dwelling</td>
<td>288 (25.5)</td>
<td>251 (43.2)</td>
<td>37 (6.8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hazardous alcohol useb</td>
<td>339 (46.3)</td>
<td>246 (62)</td>
<td>92 (28)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Current smokingc</td>
<td>413 (52.1)</td>
<td>266 (66.5)</td>
<td>147 (37.4)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Chronic renal disease</td>
<td>140 (11.8)</td>
<td>114 (19.4)</td>
<td>26 (4.3)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Chronic liver disease</td>
<td>111 (9.3)</td>
<td>80 (13.6)</td>
<td>31 (5.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diabetes</td>
<td>285 (23.9)</td>
<td>188 (32)</td>
<td>97 (16)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>COPD</td>
<td>159 (13.4)</td>
<td>98 (16.7)</td>
<td>61 (10.1)</td>
<td>0.001</td>
</tr>
<tr>
<td>Immunosuppression</td>
<td>50 (4.2)</td>
<td>13 (2.2)</td>
<td>37 (6.1)</td>
<td>0.001</td>
</tr>
<tr>
<td>Malignancy</td>
<td>58 (4.9)</td>
<td>17 (2.9)</td>
<td>41 (6.8)</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Note: All figures are n (%) unless stated otherwise. Remote-dwelling defined as per Accessibility and Remoteness Index of Australia [21]. Hazardous ETOH: >40 g ethanol ingestion/day for a male or >20 g/day for a female [27]. COPD: Chronic Obstructive Pulmonary Disease.

a. p-value comparing Indigenous with non-Indigenous subjects.
b. Denominator for hazardous alcohol use is 733 due to missing data
c. Denominator for current smoking is 793 due to missing data
**Sepsis incidence**

The population-based, age-adjusted incidence (95% CI) of sepsis was 11.8 (11.0-12.5) admissions/1000/year overall and 40.8 (37.1-44.5) in Indigenous people (Figure 5-5). The rates for severe sepsis requiring admission to ICU were 1.30 (1.08-1.52) per 1000 per year overall, and 4.74 (3.78-5.70) for Indigenous people.

There were a total of 15,963 adult acute hospital admissions during the study period, of which the 1,191 with sepsis comprised 7.46%. There were 835 ICU admissions during the same period, of which community-onset sepsis accounted for 190 (22.8%) and community-onset severe sepsis for 150 (18.0%).

**Details of infection.**

A causative organism was identified in 541 (45.4%) episodes of sepsis; *Staphylococcus aureus* was the most common causative organism and *Escherichia coli* was the most common blood isolate (Table 5-3). The most common focus of infection was skin and soft tissue overall, and pneumonia among those with severe sepsis (Table 5-4).

Nosocomial infections were not included in the study design, however 292 (24.5%) of community-onset sepsis had indicators of health care associated infection [28] (Table 5-3). There were only minor differences in the causative organisms in those with or without health-care associated infection (Table 5-3), and thus these groups were considered together for all analyses. A higher proportion of Indigenous (24.2%) than non-Indigenous (18.1%) patients had indicators of health care-associated infection (p=0.01), with this discrepancy being mainly due to higher rates of outpatient haemodialysis (6.5% Indigenous vs 0.3% non-Indigenous). There was no significant difference in the distribution of causative organisms in Indigenous compared with non-Indigenous patients.
Figure 5.5 – Population-based incidence of sepsis requiring hospital admission (a) and severe sepsis requiring intensive care admission (b) in tropical Australia compared with other regions.

a.  

b.  

Cited data were taken from the following publications: Victoria, Australia [4]; USA [3]; Norway [18]; Temperate Australia [10]; France [29]; and the UK [7]. Figures are age-adjusted number of incident cases per 1000 population per year. Vertical lines at the top of each bar represent 95% confidence intervals, where available.
<table>
<thead>
<tr>
<th></th>
<th>Total (n=1191)</th>
<th>Severe sepsis (n=272)</th>
<th>Non-severe sepsis (n=919)</th>
<th>P value&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Met 2 SIRS criteria – n (%)</td>
<td>438 (36.8)</td>
<td>55 (20.2)</td>
<td>383 (41.7)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Met≥3 SIRS criteria – n (%)</td>
<td>753 (63.2)</td>
<td>217 (78.8)</td>
<td>536 (58.3)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Required ICU admission</td>
<td>190 (15.6)</td>
<td>150 (55.2)</td>
<td>40 (4.4)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>APACHE II score&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8 (4-13)</td>
<td>16 (9-22)</td>
<td>6 (3-10)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>SOFA score&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1 (0-3)</td>
<td>4 (2-7)</td>
<td>1 (0-2)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hospital Length of Stay&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5 (3-11)</td>
<td>8 (4-18)</td>
<td>4 (3-9)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ICU Length of Stay&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4 (2-8)</td>
<td>4 (2-9)</td>
<td>3 (1.5-6)</td>
<td>NS</td>
</tr>
<tr>
<td>Estimated direct costs ($US/episode)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7,134</td>
<td>12,758</td>
<td>5,945</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>(5,945 – 7,134)</td>
<td>(11,511 – 15,357)</td>
<td>(4,756-5,945)</td>
<td></td>
</tr>
<tr>
<td>Hospital mortality&lt;sup&gt;d&lt;/sup&gt;</td>
<td>55 (5.0%)</td>
<td>42 (17.1%)</td>
<td>13 (1.5%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>28-day mortality&lt;sup&gt;e&lt;/sup&gt;</td>
<td>56 (5.5%)</td>
<td>39 (17.1%)</td>
<td>17 (2.1%)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Note: Severe sepsis=sepsis with consequent organ dysfunction within the first 48 hours of hospital admission. SIRS=Systemic inflammatory response syndrome, criteria as defined by Bone et al in 1992 [22]. ICU=Intensive Care Unit. APACHE =Acute Physiology and Chronic Health Evaluation. SOFA=Sequential Organ Function Assessment.

a. p-value comparing severe sepsis with non-severe sepsis. b. Median (interquartile range). c. Median (IQR), in days d. Denominator for hospital mortality=1190 (number of individual subjects [245 severe sepsis and 845 non-severe sepsis]), not 1191 (number of admissions). e. Reliable follow-up beyond hospital discharge is only available for NT residents, thus denominator=1028 subjects (228 severe sepsis and 800 non-severe sepsis).
**Predictors of mortality**

Mortality rates were low by Australian and international standards, with 28-day mortality for sepsis, severe sepsis and severe sepsis requiring ICU of 5.5%, 17.1% and 21.5% respectively (Table 5-2). They were not significantly different between Indigenous and non-Indigenous people, either for sepsis (5.7% Indigenous vs 5.2% non-Indigenous) or severe sepsis (15.9% Indigenous vs 18.9% non-Indigenous).

They were also no different for remote-dwelling subjects (6.7%) than for urban dwelling subjects (5.0%), p=NS. On multivariate analysis, the strongest independent predictors of 28-day mortality were: age, living in residential care, the number of SIRS criteria met during the first 48 hours of hospitalisation, and admission serum albumin of <35g/L (3.5g/dL) (Table 5-5). The crude and age-adjusted population-based sepsis mortality were 44.57 deaths/100,000 per year and 80.33 deaths/100,000 per year respectively.

**Severe sepsis admitted to hospital wards**

Of 272 admissions for severe sepsis, 122 (44.9%) were not admitted to ICU. Of these 122, 110 (90%) had none of the following factors that might modify the probability of ICU admission: active orders limiting life-sustaining treatment; metastatic cancer; or residence in a nursing home. Median (IQR) APACHE II scores and 28-day mortality rates for severe sepsis admitted only to the ward were 11 (9-13) and 10.7% compared to 20 (18-22) and 21.5% for those admitted to ICU.
### Table 5-3. Causative organisms in subjects with sepsis and an identified pathogen

<table>
<thead>
<tr>
<th>Causative Organism</th>
<th>Overall (n=541)</th>
<th>Blood culture positive (n=193)</th>
<th>Community-acquired (n=404)</th>
<th>Health care-associated indicators (n=137)</th>
<th>p-value&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Staphylococcus aureus</strong></td>
<td>136 (25.1)</td>
<td>33 (17.1)</td>
<td>99 (24.5)</td>
<td>37 (27.0)</td>
<td>NS</td>
</tr>
<tr>
<td>MSSA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>106 (19.6)</td>
<td>28 (14.5)</td>
<td>77 (19.1)</td>
<td>29 (21.2)</td>
<td>NS</td>
</tr>
<tr>
<td>nmMRSA&lt;sup&gt;c&lt;/sup&gt;</td>
<td>28 (5.2)</td>
<td>4 (2.1)</td>
<td>22 (5.5)</td>
<td>6 (4.4)</td>
<td>NS</td>
</tr>
<tr>
<td>mMRSA&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2 (0.4)</td>
<td>1 (0.5)</td>
<td>0</td>
<td>2 (1.5)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Escherichia coli</strong></td>
<td>96 (17.7)</td>
<td>56 (29.0)</td>
<td>74 (18.3)</td>
<td>22 (16.1)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Group A Streptococcus</strong></td>
<td>48 (8.9)</td>
<td>12 (6.2)</td>
<td>35 (8.7)</td>
<td>13 (9.5)</td>
<td>NS</td>
</tr>
<tr>
<td>Mixed G A Strep and S. aureus&lt;sup&gt;e&lt;/sup&gt;</td>
<td>29 (5.4)</td>
<td>-</td>
<td>28 (6.9)</td>
<td>1 (0.7)</td>
<td>0.005</td>
</tr>
<tr>
<td><strong>Streptococcus pneumoniae</strong></td>
<td>27 (5.0)</td>
<td>18 (9.3)</td>
<td>23 (5.7)</td>
<td>4 (2.9)</td>
<td>NS</td>
</tr>
<tr>
<td>Pseudomonas spp.</td>
<td>19 (3.5)</td>
<td>2 (1)</td>
<td>10 (2.5)</td>
<td>9 (6.6)</td>
<td>0.02</td>
</tr>
<tr>
<td>Mixed anaerobes</td>
<td>19 (3.5)</td>
<td>1 (0.5)</td>
<td>16 (4.0)</td>
<td>3 (2.2)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Burkholderia pseudomallei</strong></td>
<td>17 (3.1)</td>
<td>10 (5.2)</td>
<td>13 (3.2)</td>
<td>4 (2.9)</td>
<td>NS</td>
</tr>
<tr>
<td>Other beta-haemolytic streptococci</td>
<td>14 (2.6)</td>
<td>9 (4.7)</td>
<td>8 (2.0)</td>
<td>6 (4.4)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Haemophilus spp.</strong></td>
<td>13 (2.4)</td>
<td>2 (1)</td>
<td>11 (2.7)</td>
<td>2 (1.5)</td>
<td>NS</td>
</tr>
<tr>
<td>Acinetobacter spp.</td>
<td>11 (2.0)</td>
<td>7 (3.6)</td>
<td>9 (2.2)</td>
<td>2 (1.5)</td>
<td>NS</td>
</tr>
<tr>
<td>Klebsiella spp.</td>
<td>10 (1.9)</td>
<td>5 (2.6)</td>
<td>9 (2.2)</td>
<td>1 (0.7)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Viridans group streptococci</strong></td>
<td>7 (0.6)</td>
<td>7 (3.6)</td>
<td>3 (0.7)</td>
<td>4 (2.9)</td>
<td>NS</td>
</tr>
<tr>
<td>Neisseria gonorrhoea</td>
<td>7 (0.6)</td>
<td>2 (1)</td>
<td>6 (1.5)</td>
<td>1 (0.7)</td>
<td>NS</td>
</tr>
<tr>
<td>Proteus spp.</td>
<td>7 (0.6)</td>
<td>2 (1)</td>
<td>3 (0.7)</td>
<td>4 (2.9)</td>
<td>NS</td>
</tr>
<tr>
<td>Influenza virus</td>
<td>6 (1.1)</td>
<td>-</td>
<td>6 (1.5)</td>
<td>0</td>
<td>NS</td>
</tr>
<tr>
<td>Nocardia spp.</td>
<td>5 (0.9)</td>
<td>1 (0.5)</td>
<td>4 (1.0)</td>
<td>1 (0.7)</td>
<td>NS</td>
</tr>
<tr>
<td>Milleri group streptococci</td>
<td>5 (0.9)</td>
<td>2 (1)</td>
<td>4 (1.0)</td>
<td>1 (0.7)</td>
<td>NS</td>
</tr>
<tr>
<td>Shigella spp.</td>
<td>5 (0.9)</td>
<td>-</td>
<td>4 (1.0)</td>
<td>1 (0.7)</td>
<td>NS</td>
</tr>
<tr>
<td>Other&lt;sup&gt;f&lt;/sup&gt;</td>
<td>60 (11.1)</td>
<td>24 (12.4)</td>
<td>39 (9.7)</td>
<td>21 (15.3)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Gram positive bacterium</strong></td>
<td>285 (52.7)</td>
<td>90 (46.6)</td>
<td>212 (52.5)</td>
<td>73 (53.3)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Gram negative bacterium</strong></td>
<td>236 (43.6)</td>
<td>101 (52.3)</td>
<td>176 (43.6)</td>
<td>60 (43.8)</td>
<td>NS</td>
</tr>
</tbody>
</table>

<sup>a</sup> P value comparing community-acquired with health-care associated infections.

<sup>b</sup> MSSA=Methicillin susceptible Staphylococcus aureus.

<sup>c</sup> nmMRSA=Non multi-resistant methicillin-resistant Staphylococcus aureus.

<sup>d</sup> mMRSA=Multi-resistant methicillin-resistant Staphylococcus aureus.

<sup>e</sup> Where Group A streptococci and S.aureus were both isolated from pus specimens, a decision on principal causative organism was not made, and both were included together.

<sup>f</sup> “Other” includes Enterococcus spp., Enterobacter spp., Neisseria meningitidis, Candida spp., Bacteroides spp., Dengue virus, Plasmodium falciparum and Anaplasma phagocytophilum.
**Risk factors for readmission**

Of the 1090 individuals enrolled in the study, 81 were readmitted at least once for sepsis during the 12-month study period. In the context of the first admission for sepsis, the following were independent predictors of readmission within the study period (Odds ratio [95% CI]): end-stage renal disease (2.91 [1.35-6.23]), chronic liver disease (2.73 [1.44-5.21]) and being Indigenous (1.82 [1.11-2.99]).

**Estimated Costs**

The median (IQR) direct cost per episode of sepsis was $6,257 ($3,129 – $13,220), and for severe sepsis requiring ICU admission was $17,810 ($10,100 - $33,810). The total direct cost for all sepsis admissions over a one year period was conservatively estimated at $17,810,000. Including only patients living in the primary drainage area of RDH, the total cost was $14,375,000 or $140 per capita per year. Median costs were not significantly different for gram positive infections ($6,260) compared with gram negative infections ($7,300), but were higher for osteoarticular infection ($33,890) compared with all other foci of infection combined ($5,440). There were insufficient data to estimate indirect costs such as loss of productivity and life-years lost.

**Comparisons with other sepsis studies**

Compared with temperate Australia [10], the UK [7] and France [29], the tropical northern NT has a significantly higher population-based incidence of severe sepsis requiring ICU admission, but this difference was entirely accounted for by the extremely high incidence in Indigenous people (Figure 5-3b). The rates of sepsis were approximately six times higher in the present study than those reported from Victoria, in temperate Australia [4], and the USA [3] (Figure 5-3a), and much of this difference also derives from the rates in Indigenous people.

Patients in the tropical NT with severe sepsis requiring ICU were younger than those in temperate Australia and had a lower 28-day mortality despite similar APACHEII and SOFA scores (Table 5-6). Among those with severe sepsis, the two most common causative organisms (S. aureus and E. coli) were the same as those from temperate Australia [10], Canada [5] and Europe [6], but there were significantly more Gram negative bacteria and less fungi in the present study than in each of the others (table 5-6). Table 5-6 includes microbial epidemiology data only for community-acquired sepsis, with the exception of the Finfer Australian study, where these data were not able to be extracted from the published
paper. Since the data from the Finfer paper includes both community-acquired and nosocomial sepsis, the comparisons of causative organisms between the current study and temperate Australia should be interpreted with caution.

Table 5-4 Primary focus of infection according to sepsis severity.

<table>
<thead>
<tr>
<th>Focus of Infection</th>
<th>All sepsis (n=1191)</th>
<th>Severe sepsis (n=272)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin and soft tissue</td>
<td>389 (32.8)</td>
<td>45 (16.5)</td>
</tr>
<tr>
<td>Pleuropulmonary</td>
<td>365 (30.8)</td>
<td>122 (44.8)</td>
</tr>
<tr>
<td>Urinary sepsis</td>
<td>146 (12.3)</td>
<td>35 (12.9)</td>
</tr>
<tr>
<td>Intra-abdominal</td>
<td>126 (10.6)</td>
<td>30 (11.0)</td>
</tr>
<tr>
<td>Primary blood stream infection</td>
<td>39 (3.3)</td>
<td>11 (4.0)</td>
</tr>
<tr>
<td>Osteoarticular</td>
<td>28 (2.4)</td>
<td>5 (1.8)</td>
</tr>
<tr>
<td>ENT</td>
<td>28 (2.4)</td>
<td>1 (0.4)</td>
</tr>
<tr>
<td>Gynaecological</td>
<td>20 (1.7)</td>
<td>7 (2.6)</td>
</tr>
<tr>
<td>CNS</td>
<td>11 (0.9)</td>
<td>7 (2.6)</td>
</tr>
<tr>
<td>Cardiac</td>
<td>7 (0.6)</td>
<td>2 (0.7)</td>
</tr>
<tr>
<td>IV line-related</td>
<td>5 (0.4)</td>
<td>2 (0.7)</td>
</tr>
<tr>
<td>Other identified focus</td>
<td>10 (0.8)</td>
<td>1 (0.4)</td>
</tr>
<tr>
<td>No identified focus</td>
<td>13 (1.9)</td>
<td>4 (1.5)</td>
</tr>
</tbody>
</table>

Note: all figures are n(%). ENT=Ear nose and throat. CNS=Central Nervous System. IV=Intravenous.
Table 5-5. Risk factors for 28-day mortality on univariate and multivariate analysis, grouped according to the PIRO system.

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>Univariate OR (95% CI)</th>
<th>Multivariate OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Predisposing factors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age ≥45&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.7 (1.4-5.3)</td>
<td>2.3 (1.1-4.7)</td>
</tr>
<tr>
<td>Age ≥65&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.1 (3.1-12.1)</td>
<td>5.6 (2.7-11.6)</td>
</tr>
<tr>
<td>Female sex</td>
<td>0.6 (0.4-0.9)</td>
<td>-</td>
</tr>
<tr>
<td>Residential care</td>
<td>5.7 (2.2-14)</td>
<td>5.7 (1.7-18)</td>
</tr>
<tr>
<td>Chronic lung disease</td>
<td>3.5 (2.1-6.1)</td>
<td>-</td>
</tr>
<tr>
<td>Chronic Renal Disease&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.5 (1.4-4.6)</td>
<td>2.1 (1.0-4.4)</td>
</tr>
<tr>
<td><strong>Infection characteristics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skin/soft tissue focus&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.12 (0.04-0.34)</td>
<td>0.30 (0.10-0.88)</td>
</tr>
<tr>
<td>Pneumonia</td>
<td>3.0 (1.8-4.9)</td>
<td>-</td>
</tr>
<tr>
<td>Bacteraemia</td>
<td>2.9 (1.7-5.0)</td>
<td>-</td>
</tr>
<tr>
<td><strong>Response to infection</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Met 3 SIRS criteria&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.7 (2.18-14.85)</td>
<td>4.1 (1.4-12)</td>
</tr>
<tr>
<td>Met 4 SIRS criteria&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.4 (4.4-30)</td>
<td>5.2 (1.8-15)</td>
</tr>
<tr>
<td>Albumin&lt;35 mg/dL&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.6 (3.8-11)</td>
<td>4.9 (2.3-10)</td>
</tr>
<tr>
<td>Bilirubin</td>
<td>1.01 (1.01-1.02)</td>
<td>-</td>
</tr>
<tr>
<td>Acute confusion</td>
<td>5.9 (2.9-12.3)</td>
<td>1.3 (1.1-1.6)</td>
</tr>
<tr>
<td>Platelet count</td>
<td>0.996 (0.994-0.999)</td>
<td>-</td>
</tr>
<tr>
<td>Mean arterial pressure</td>
<td>0.966 (0.944-0.988)</td>
<td>-</td>
</tr>
<tr>
<td>Oxygen saturation</td>
<td>0.859 (0.787-0.936)</td>
<td>-</td>
</tr>
<tr>
<td><strong>Organ dysfunction</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Septic shock</td>
<td>7.1 (4.3-11.8)</td>
<td>2.3 (1.1-4.5)</td>
</tr>
<tr>
<td>Acute renal failure</td>
<td>6.9 (3.3-14)</td>
<td>3.7 (1.6-8.6)</td>
</tr>
<tr>
<td>Acute respiratory failure</td>
<td>4.2 (2.2-8.1)</td>
<td>2.7 (1.3-5.7)</td>
</tr>
<tr>
<td>Acidosis</td>
<td>4.3 (2.2-8.4)</td>
<td>-</td>
</tr>
</tbody>
</table>

Note: Data were analysed by logistic regression analysis with backward stepwise elimination. Only variables which were significant on univariate analysis are shown. Only those remaining in the final model are shown in the multivariate analysis column. Odds ratios are given to 2 significant figures except for continuous dependent variables, where 3 significant figures are used.

a. Met SIRS criteria within a 24h period in the first 48h of hospital admission. Comparator= 2 SIRS criteria. b. Comparator=age ≤44 years. c. Lowest serum albumin concentration within first 24 hours of hospitalisation. Comparator=Albumin≥35 mg/dL. d. Defined as usual serum creatinine >150µMol/L or receiving chronic haemodialysis or peritoneal dialysis. e. Highest serum bilirubin concentration within first 24 hours of hospitalisation. Comparator=Bilirubin≤18. f. Compared with all other foci of infection.
5.6 Discussion

In the first description of the epidemiology of sepsis in tropical Australia, we have found that the incidence of sepsis is five-fold higher than that in temperate Australia, North America and Europe. Most of this difference is accounted for by the extremely high incidence rates in Indigenous Australians. Furthermore, sepsis accounted for a substantially higher proportion of hospital and ICU admissions than has been reported from elsewhere.

It is unclear why sepsis incidence is four-fold higher in Indigenous than non-Indigenous people. The design of this study was unable to determine community-based risk factors for sepsis, however Indigenous people in this study had an excess of several comorbidities which have been previously shown to increase the risk of sepsis or severe infections; these include diabetes [30], excessive alcohol use [31], chronic liver disease [32] and end-stage renal disease [33]. Other factors which are likely to contribute to the high burden of sepsis in Indigenous people include poor housing with a lack of health hardware [34] and overcrowding [35].

There are no previous published studies describing the population-based epidemiology of sepsis in predominantly indigenous populations. However, high rates of infectious morbidity have been reported in Indigenous populations in North America, Australia and New Zealand [15]. Emerging reports also suggest a disproportionate burden of severe infection with 2009 pandemic H1N1 influenza in indigenous populations [36]. The high rate of sepsis found in the present study may reflect the high incidence of infections in Indigenous people rather than a tendency to develop sepsis in response infection, but this hypothesis remains to be tested.
Table 5-6 Demographics, mortality, and causative organisms in severe sepsis requiring ICU admission, compared with other studies.

<table>
<thead>
<tr>
<th></th>
<th>Tropical NT&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Temperate Australia [10]&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Canada&lt;sup&gt;c&lt;/sup&gt; [5]</th>
<th>Europe&lt;sup&gt;c&lt;/sup&gt; [6]</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>150</td>
<td>691</td>
<td>458</td>
<td>898</td>
</tr>
<tr>
<td>Age (Mean, sd)</td>
<td>49.0 (15.0)</td>
<td>60.7 (17.2)</td>
<td>59.1 (16.6)</td>
<td>61.7 (16.7)</td>
</tr>
<tr>
<td>Male (%)</td>
<td>52.0</td>
<td>56.9</td>
<td>52.7</td>
<td>61.0</td>
</tr>
<tr>
<td>APACHE II score</td>
<td>19.5 (14-25)</td>
<td>21 (16-26)</td>
<td>26.0 (9.3)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>SOFA score Day 1</td>
<td>6.5 (3.4)</td>
<td>-</td>
<td>-</td>
<td>6.6 (4.2)</td>
</tr>
<tr>
<td>Hospital Mortality (%)</td>
<td>21.3</td>
<td>37.5</td>
<td>28.0</td>
<td>36.0</td>
</tr>
<tr>
<td>Identified organism (%)</td>
<td>62.0</td>
<td>57.8</td>
<td>46.1</td>
<td>56.0</td>
</tr>
<tr>
<td>Gram positive (%)</td>
<td>38.7</td>
<td>48.3</td>
<td>40.8</td>
<td>37.0</td>
</tr>
<tr>
<td>Gram negative (%)</td>
<td>54.0</td>
<td>38.5</td>
<td>20.9</td>
<td>34.0</td>
</tr>
<tr>
<td>Fungi (%)</td>
<td>0.1</td>
<td>5.9</td>
<td>3.8</td>
<td>16.0</td>
</tr>
<tr>
<td>Other (%)</td>
<td>7.2</td>
<td>7.3</td>
<td>14.1</td>
<td>13.0</td>
</tr>
<tr>
<td>Most common (%)</td>
<td><em>E. coli</em> (20.4%)</td>
<td><em>S. aureus</em> (28%)</td>
<td>-</td>
<td><em>S. aureus</em> (27%)</td>
</tr>
<tr>
<td>2&lt;sup&gt;nd&lt;/sup&gt; most common (%)</td>
<td><em>S. aureus</em> (11.8%)</td>
<td><em>E. coli</em> (9.3%)</td>
<td>-</td>
<td><em>E. coli</em> (12%)</td>
</tr>
<tr>
<td>3&lt;sup&gt;rd&lt;/sup&gt; most common (%)</td>
<td><em>Acinetobacter</em> spp. (10.8%)</td>
<td>Pseudomonas spp (8.6%)</td>
<td>-</td>
<td>Pseudomonas spp (12%)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Data only included for community-onset severe sepsis requiring ICU admission.  
<sup>b</sup> Data comprise all severe sepsis patients requiring ICU admission.  
<sup>c</sup> Data extracted to include only community-onset severe sepsis requiring ICU admission.

Published studies describing sepsis epidemiology have been conducted nearly exclusively in non-tropical areas. Recent publications from Brazil [9] and Thailand [8] considered only ICU admissions (where admission policies vary widely), included both nosocomial and community-onset sepsis, and did not determine population-based incidences, and are thus difficult to compare with the current study.

We cannot exclude the possibility that the higher incidence of sepsis in tropical Australia compared to estimates elsewhere reflects methodological differences. The comparator studies for sepsis incidence were all retrospective and based on discharge coding [3, 4, 18], a study design that is likely to substantially under-estimate the true incidence of sepsis elsewhere [19]. However, the comparator study for severe sepsis requiring ICU admission in temperate Australia was prospective [10], and used very similar inclusion criteria and
definitions, suggesting that the observed difference in incidence in the two studies is a true phenomenon.

The costs we estimated were similar to those reported for severe sepsis from the USA [1] (a mean cost of US$20,370 in the present study compared with $22,100 in the US study). The method we used for cost estimation was based on median costs for a patient in ICU or the hospital wards rather than individual-level data. We thus may have underestimated the true cost by failing to include specific expensive items such as high-cost drugs or investigations. However, a study in German ICUs found that the majority of all sepsis-related costs were fixed costs, and that the use of bed-day costs were thus appropriate in sepsis cost estimation [2]. We did not calculate indirect costs of sepsis, which include lost productivity due to early death or time away from work and impacts upon carers and family. A European study estimated that the direct costs of sepsis comprise only 28% of total costs [37]. If this were the case in Australia, the total cost of sepsis per year in the northern NT would be US$55,790,000 or $440 per capita per year.

The relatively low mortality rate in ICU patients with severe sepsis in this study (21.5%) is consistent with those previously reported in severe sepsis from RDH ICU (21-25% [38] [39]), and is lower than predicted mortality based on this cohort’s median APACHE II score (25.6%). This may be explained by the younger population in the tropical NT compared to temperate Australia and elsewhere; if so, similar APACHE II scores despite younger age imply either more severe physiological disturbance or more comorbidities in the present population compared with others.

There are several potential limitations of this study. We did not capture cases of sepsis not requiring hospital admission, making it likely that we have underestimated the true incidence of sepsis. Our population may not be representative of those in other tropical areas, and it is unclear if these results are generalisable to these areas. The strengths of our study include its prospective design, the capturing of an entire cycle of seasons over a year, and the inclusion of all hospitalised sepsis patients rather than only ICU patients.

In conclusion, the incidence of sepsis in tropical Northern Australia is substantially higher than that for temperate Australia and other countries, and this difference is largely explained by higher rates in Indigenous people. Efforts at decreasing this burden should focus on improving housing and access to health services, and addressing comorbidities,
alcohol and tobacco use. A considerable proportion of patients with severe sepsis are not admitted to ICU, and the short-term mortality in this group of patients is over 10%. Studies describing sepsis epidemiology should not be limited to patients who require ICU admission. Sepsis incurs a major cost and has a disproportionate impact on Indigenous people. Prospective studies are needed in indigenous populations globally to define the burden of sepsis and to inform appropriate resourcing of health services and community-based treatment and prevention strategies.
5.7 References


Chapter 6. Comparison of APACHE II scores for predicting mortality in patients with sepsis admitted to the intensive care unit or hospital wards.
6.1 Preamble
The data reported in Chapter 5 confirm that sepsis is a common problem in the Top End of the NT, and that the majority of patients with sepsis are never admitted to an ICU. Several risk stratification scoring systems have been described and are commonly used for outcome prediction in sepsis patients admitted to ICU. However, it is unclear how to predict the risk of mortality in patients admitted to hospital wards with sepsis. This chapter presents an analysis of data taken from the PRESTO cohort addressing this question. It has been written as a manuscript for submission to a peer-reviewed journal.
6.2 Abstract

Background
The APACHE II scoring system was developed and validated for predicting mortality in patients admitted to intensive care units (ICU), but it is unclear if it is useful in hospitalised patients who are not admitted to ICU.

Methods
We performed a prospective cohort study of all adult patients admitted to an Australian teaching hospital with sepsis over a 12-month period. Clinical, demographic and outcome data were collected from medical records and hospital databases. APACHE II scores were calculated based on the worst values in the first 24 hours of admission. Score performance was compared between ICU and non-ICU patients using the area under the receiver operator characteristic curve (AUROCC).

Results
There were 1,063 eligible patients admitted with sepsis, of whom 900 were not admitted to ICU and 163 were. Median (IQR) APACHE II scores were 18 (11-23) in the ICU group and 6 (3-10) in the non-ICU group. Hospital and one-year mortality were 21.5% and 29.2% respectively in the ICU group, and 1.6% and 8.2% respectively in those not admitted to ICU. The APACHE II score performed significantly better in the non-ICU group (AUROC [95% CI] = 0.88 [0.79-0.96]) than in the ICU group (AUROC = 0.71 [95% CI = 0.60-0.81], p=0.02).

Conclusions
In this population of adults hospitalised with sepsis, APACHE II scores performed well for predicting hospital mortality in those not admitted to ICU. The wider use of APACHE II scores outside the setting of ICU should be further investigated.
6.3 Introduction

The Acute Physiology and Chronic Health Evaluation (APACHE) scoring system, first described in 1981 [1], was designed to quantify severity of illness and predict risk of death in cohorts of patients admitted to intensive care units (ICU). Although the APACHE score has been serially refined and updated, as APACHE II in 1985 [2], APACHE III in 1991 [3] and APACHE IV in 2006 [4], the APACHE II score remains widely used both for clinical and research applications [5, 6]. The APACHE II score includes an acute physiology score (based on the worst values of twelve parameters in the first 24 hours in the ICU), as well as points for age and severe comorbidities.

The use of APACHE scores for indications beyond those for which they were originally intended is commonly practiced but controversial; these include predicting mortality in individual patients [7], as entry criteria for clinical trials [8], and for patients managed outside ICU. The APACHE II score has not been validated for use outside ICUs. The few studies which have investigated its use in such patients have been in very specific groups, such as those with cirrhosis [9, 10], chronic lung disease [11], pancreatitis [12] and acute tubular necrosis [13]. The performance of APACHE scores for patients with sepsis has not previously been compared in patients admitted to ICU and those managed on hospital wards.

The majority of prospective studies of sepsis epidemiology and outcomes include only patients admitted to ICU [14-18]. However, a significant proportion of patients with sepsis is admitted to general hospital wards, and these patients have a substantial mortality. For example in a multicentre prospective study from Spain, only 12% of patients with sepsis and 33% of those with severe sepsis were admitted to an ICU, and the mortality of severe sepsis managed outside ICU (25.7%) was similar to that managed in the ICU (33%) [19]. A recent Australian study of severe sepsis attending hospital emergency departments had similar findings, with 52.4% of severe sepsis patients admitted to ICU, and with the mortality of non-ICU patients (21.4%) being similar to that of ICU patients (24.7%) [20]. Hence sepsis managed on general hospital wards is an important but under-studied problem.

We investigated the performance of a modified APACHE II score in a prospective cohort of consecutive patients with sepsis admitted to an Australian teaching hospital over a one
year period. Our primary hypothesis was that APACHE II scores would predict in-hospital mortality at least as well in sepsis patients not admitted to ICU as in those who were admitted to ICU. We also hypothesised that APACHE II score would perform as well for predicting one-year mortality in non-ICU as in ICU patients.

6.4 Methods

Setting and patient recruitment
Royal Darwin Hospital is a 350-bed Australian teaching hospital, with an 18-bed mixed ICU. The patients in this cohort have been previously described in a study defining the epidemiology of sepsis in the region (Chapter 5). Details of study design, patient recruitment and data collection have been previously reported (Chapter 5 and appendix 1). Briefly, we screened every hospital admission over a one-year period by admission diagnosis and collected demographic, clinical and outcome data on those patients who met criteria for sepsis. These criteria were proven or suspected infection, and the presence of at least two criteria for the systemic inflammatory response syndrome (SIRS) within the first 48 hours of admission [21].

The cohort included in the current analysis is a subset of the originally described cohort (Chapter 5). Patients were included in the original epidemiology cohort but, in a predefined analytical plan, were excluded from the present analysis if they had orders limiting life-sustaining treatment or if they were not expected to survive for 30 days because of severe underlying comorbidities. For those who had more than one episode of sepsis during the study period, only the first admission was included.

Mortality was determined from the state-wide hospital database, to which is reported every death in the Northern Territory of any patient who has been previously hospitalised. Hence the denominator for mortality at one year excluded any patients who were not residents of the Northern Territory. Comorbidities were quantified using the Charlson comorbidity index [22]. “Severe sepsis” was used to mean at least one sepsis related acute organ dysfunction within the first 48 hours of admission, as defined in the PROWESS study [23].

Calculation of APACHE II scores
APACHE II scores were calculated by one of two trained study staff, using the worst physiological values in the first 24 hours of hospital admission, according to a modification
of the method originally described by Knaus and others [2]. The modification applied only to those patients in whom arterial blood gas analysis was not performed in the first 24 hours of admission. In these patients, pulse oximetry-derived arterial oxygen saturation whilst breathing room air were used for the oxygenation component of the score. This was estimated from the standard oxygen haemoglobin dissociation curve as follows: saturation on room air of 91-94% = 1 point (equivalent PaO₂ = 61-71mmHg), 89-91% = 3 points (equivalent PaO₂ = 55-60mmHg), and <89% = 4 points (equivalent PaO₂ < 55mmHg). If data were not available for any of the components of the APACHE II score, a score was not calculated and was coded as missing.

**Statistical analysis**

Performance of APACHE scores between groups was compared using the area under the receiver operator characteristic curve (AUROCC), which assesses the discrimination of a score, and the Hosmer-Lemeshow goodness of fit Chi-Squared statistic, which assesses calibration (how well the model [predicted mortality] fits the data [observed mortality]). The Hosmer-Lemeshow goodness of fit Chi-Squared statistic was calculated by first fitting a logistic regression model with mortality as the dependent and APACHE II score as the independent variable, followed by comparing predicted with observed mortality in deciles as originally described [24]. We used the classification suggested by Hosmer and Lemeshow to describe the performance of a scoring system based on the AUROCC: an AUROCC of 0.8-0.9 was defined as “excellent” performance, of 0.7-0.8 as “acceptable” and of <0.7 as “poor” [25]. Predicted mortality was calculated from the APACHE II score as described by Knaus and colleagues [2]. Statistical analysis was conducted using Stata version 10 (Statacorp, Texas, USA) and Graphpad Prism version 5 (GraphPad Software, California, USA). AUROCC for different groups was compared using the Stata command “roccomp”. P values of less than 0.05 were considered significant.
### Table 6-1. Baseline characteristics, severity scores and mortality of sepsis patients, according to Intensive Care Unit admission

<table>
<thead>
<tr>
<th></th>
<th>Sepsis overall (n=1,063)</th>
<th>Admitted to ICU (n=163)</th>
<th>Not admitted to ICU (n=900)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong>a</td>
<td>46.5 (17.2)</td>
<td>49.1 (15.7)</td>
<td>46.0 (17.5)</td>
<td>0.03</td>
</tr>
<tr>
<td><strong>Male</strong>b</td>
<td>558 (52.5)</td>
<td>94 (57.6)</td>
<td>464 (51.6)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Charlson index&gt;=1</strong>b</td>
<td>518 (48.7)</td>
<td>104 (63.8)</td>
<td>414 (46.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Charlson index&gt;=3</strong>b</td>
<td>194 (18.3)</td>
<td>41 (25.2)</td>
<td>153 (17.0)</td>
<td>0.01</td>
</tr>
<tr>
<td><strong>APACHE II scorec,d</strong></td>
<td>7 (4-12)</td>
<td>18 (11-23)</td>
<td>6 (3-10)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Severe sepsis</strong>b</td>
<td>229 (21.5)</td>
<td>132 (81.0)</td>
<td>97 (10.8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Hospital length of stay (days)c</strong></td>
<td>5 (3-11)</td>
<td>13 (7-26)</td>
<td>4 (3-8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Hospital mortality</strong>b</td>
<td>49 (4.6)</td>
<td>35 (21.5)</td>
<td>14 (1.6)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>1-year mortality</strong>b,e</td>
<td>115 (11.5)</td>
<td>45 (29.2)</td>
<td>70 (8.2)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Notes: ICU=Intensive Care Unit. P value=comparison between ICU group and non-ICU group. Charlson index=Charlson comorbidity index. Severe sepsis was defined as at least one sepsis-related acute organ dysfunction.

a. Mean (standard deviation)  b. n(%)  c. Median (interquartile range)  d. APACHE scores were available in 1,052 of the 1,063 patients  e. Denominator for 1-year mortality=1,004
6.5 Results

Over the 12-month study period, there were 1,090 patients admitted to the hospital with sepsis. Twenty-seven patients were excluded because of one or both of active orders limiting life-sustaining treatment (n=26), and not being expected to survive more than 30 days due to severe underlying comorbidities (n=6), leaving 1,063 patients in the cohort for analysis. Of these, 900 were not admitted to ICU during the hospitalisation (non-ICU group) and 163 were (ICU group). The ICU group were older, had more comorbidities and a longer length of stay than the non-ICU group (table 6-1). Nearly half (42%) of the patients with severe sepsis were not admitted to ICU.

Figure 6-1. Receiver operator characteristic curve assessing the discrimination of APACHE II scores for predicting hospital mortality in hospitalised sepsis patients admitted to ICU compared with general hospital wards
Table 6-2. Mortality in ICU and non-ICU groups compared with APACHE II scores

<table>
<thead>
<tr>
<th>APACHE II Score</th>
<th>28-day mortality (Admitted to ICU)</th>
<th>28-day mortality (Not admitted to ICU)</th>
<th>Predicted mortality*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-5</td>
<td>0/4 (0%)</td>
<td>0/398 (0%)</td>
<td>4.1%</td>
</tr>
<tr>
<td>6-10</td>
<td>2/31 (6.5%)</td>
<td>3/297 (1.0%)</td>
<td>8.2%</td>
</tr>
<tr>
<td>11-15</td>
<td>3/25 (12%)</td>
<td>3/124 (2.4%)</td>
<td>15.6%</td>
</tr>
<tr>
<td>16-20</td>
<td>9/27 (33%)</td>
<td>5/63 (7.9%)</td>
<td>27.6%</td>
</tr>
<tr>
<td>21-25</td>
<td>4/31 (12.9%)</td>
<td>0/6 (0%)</td>
<td>44.2%</td>
</tr>
<tr>
<td>26-30</td>
<td>8/20 (40%)</td>
<td>3/9 (33.3%)</td>
<td>62.2%</td>
</tr>
<tr>
<td>31-35</td>
<td>2/3 (67%)</td>
<td>N/A</td>
<td>77.3%</td>
</tr>
<tr>
<td>&gt;35</td>
<td>3/5 (60%)</td>
<td>N/A</td>
<td>87.6%</td>
</tr>
</tbody>
</table>

The mortality for the group as a whole was 4.6% at hospital discharge and 11.5% one year following admission (table 6-1). Although the non-ICU group had a low in-hospital mortality of 1.6%, their mortality at one year was substantial, at 8.2% overall and 19% in the subgroup with severe sepsis.

An APACHE II score was not available for 11 of the 1,063 patients. Of the remaining 1,052 patients, results of an arterial blood gas analysis were available in 165, with the other 887 having a modified APACHE II score calculated. Table 6-2 shows the expected and observed number of deaths according to APACHE II score. When using the APACHE II score to predict in-hospital mortality, the area under the ROC curve was significantly higher in the non-ICU group (excellent, at 0.88 [95% CI = 0.79-0.96]) than in the ICU group (acceptable, at 0.71 [95% CI = 0.60-0.81]), p=0.02 (Table 6-3, figure 6-1). The Hosmer-Lemeshow goodness of fit Chi squared statistic was similar for the ICU and non-ICU groups. The APACHE II score did not perform as well for predicting one-year mortality as for hospital mortality, but there was a non-significant trend towards better performance in the non-ICU group (AUROC 0.77) than in the ICU group (AUROC 0.69) (Table 6-3).
Table 6-3. Performance characteristics of the APACHE II score patients hospitalised with sepsis

<table>
<thead>
<tr>
<th></th>
<th>Sepsis Overall</th>
<th>Patients admitted to ICU</th>
<th>Patients not admitted to ICU</th>
<th>P value (^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Area under the Receiver Operator Curve (95% CI)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In-hospital mortality</td>
<td>0.89 (0.85-0.93)</td>
<td>0.71 (0.60-0.81)</td>
<td>0.88 (0.79-0.96)</td>
<td>0.02</td>
</tr>
<tr>
<td>One-year mortality</td>
<td>0.79 (0.75-0.84)</td>
<td>0.69 (0.59-0.79)</td>
<td>0.77 (0.71-0.83)</td>
<td>0.25</td>
</tr>
</tbody>
</table>

**Hosmer-Lemeshow goodness of fit Chi-squared statistic (p-value)\(^b\)**

|                         |                |                          |                             |              |
|-------------------------|----------------|--------------------------|                             |              |
| In-hospital mortality   | 7.51 (0.48)    | 7.54 (0.48)              | 6.16 (0.63)                 | -            |
| One-year mortality      | 11.51 (0.18)   | 10.89 (0.21)             | 8.08 (0.43)                 | -            |

\(^a\) P value comparing AUROC of ICU group with that of non-ICU group
\(^b\) Lower Chi-squared values and higher p-values represent a better fit between predicted and observed mortality within the group being assessed. A p-value of <0.05 suggests a poor fit between predicted and observed mortality.

6.6 Discussion

In the first study to have addressed this question, we have shown that the APACHE II score performs significantly better in patients with sepsis managed outside the ICU than in those admitted to ICU. The discrimination of the APACHE II score in non-ICU patients was excellent for predicting hospital mortality, and acceptable for predicting one year mortality.

Furthermore, we found that a considerable proportion of patients with severe sepsis were not admitted to ICU, similar to previous studies in Australia [20, 26] and Europe [19], and that this group has a substantial mortality. These findings are strengthened by the fact that, unlike other relevant studies [19, 20, 26], we excluded patients who were unlikely to be considered for ICU admission, meaning that the non-ICU sepsis cohort was more comparable to the ICU sepsis cohort than it would be if we had included such patients.

It is difficult to compare our findings with those of previous studies because of differences in study design. Nguyen and colleagues examined the performance of APACHE II scores in 246 patients attending a US emergency department with severe sepsis, and found only...
moderate discrimination for hospital mortality, with an AUROCC of 0.71 [27]. However, this study is not comparable with the present one, because although it was not ICU-based, all of the patients were eventually admitted to ICU. Goel and colleagues found that APACHE II scores were useful for predicting long-term survival in patients admitted to general hospital wards with exacerbations of chronic lung disease, but they did not report AUROCC, and did not examine hospital mortality [11]. An international study pooling data from 14,745 critically ill patients admitted to 137 ICUs found that the AUROCC for the APACHE II score for predicting hospital mortality was 0.85 [28]. This is similar to the findings from the present study in all sepsis patients combined (AUROCC=0.89), but higher than that within the ICU group (AUROCC=0.71).

It is unclear why the score did not perform as well in ICU patients as in those admitted to hospital wards. One possible reason is the relatively low mortality in this ICU (for example, the overall mortality in those with severe sepsis was 21%), consistent with that noted in several previous studies in this ICU [29, 30]. This may be due to the relatively young age of this cohort, or to excellent management of sepsis in this ICU compared with others where the incidence of sepsis is not as high.

This study has several limitations. Most patients did not have an arterial blood gas analysis, and thus modified APACHE scores were calculated. However, in this study, this did not appear to affect either the discrimination or the calibration of the score, and these data may allow APACHE II scores to be used pragmatically in settings where arterial blood gas analysis is rarely performed or is not available. The population described in this study may not be representative of that of other regions or countries, given their younger age, and a high proportion of Indigenous patients (Chapter 5) [31]. Hence this question should be addressed in larger numbers of patients and in different populations.

In conclusion, a substantial proportion of severe sepsis admissions never enter an intensive care unit, and methods for risk stratification and mortality prediction are needed in such patients. Although it has not previously been validated outside the setting of intensive care units, the APACHE II scoring system performed well in a cohort of adults admitted to general hospital wards with sepsis. Confirmation in other settings may allow broader application of the APACHE II scoring system outside intensive care units.
6.7 References


Chapter 7. Pneumonia risk stratification in tropical Australia
7.1 Preamble

Pneumonia was the most common focus of infection in those with severe sepsis in both the PRESTO (Chapter 5) and FRESH (Chapter 9-10) cohorts. Risk stratification tools are commonly used to inform management decisions regarding individual patients with pneumonia, including selection of antibiotic therapy, and whether to send a patient from the emergency department home, to the general ward or to the ICU [1-5]. During the planning phase of the PRESTO study, a new pneumonia severity scoring system (called SMARTCOP) was described by authors from temperate Australia, although it was not published until the following year [6]. Thus an a priori plan was made to collect the data necessary for the calculation of the SMARTCOP score for analysis at the end of the study. Considering the population described in Chapter 5 differed substantially from sepsis populations in temperate Australia, the aim of this study was to determine if the SMARTCOP score performed adequately in the tropical Northern Territory. This manuscript was published in the Medical Journal of Australia.
7.2 Pneumonia risk stratification in tropical Australia: does the SMARTCOP score apply? - Manuscript as published in the Medical Journal of Australia
Pneumonia risk stratification in tropical Australia: does the SMART-COP score apply?

Joshua S Davis, Gail B Cross, Patrick G P Charles, Bart J Currie, Nicholas M Anstey and Allen C Cheng

ABSTRACT

Objective: To examine the performance in tropical northern Australia of SMART-COP, a simple scoring system developed in temperate Australia to predict the need for intensive respiratory or vasopressor support (IRVS) in pneumonia patients.

Design, setting and patients: A prospective observational study of patients admitted to Royal Darwin Hospital in the Northern Territory with sepsis between August 2007 and May 2008. Chest x-rays were reviewed to confirm pneumonia, and each patient’s SMART-COP score was assessed against the need for IRVS.

Results: Of 206 patients presenting with radiologically confirmed pneumonia, 184 were eligible for inclusion. The mean age of patients was 50.1 years, 65% were Indigenous and 56% were men. Overall, 38 patients (21%) required IRVS, and 18 patients (10%) died by Day 30. A SMART-COP score of ≥3 had a sensitivity of only 71% for predicting the need for IRVS and 67% for 30-day mortality. As the variables most strongly associated with IRVS were serum albumin level <35 g/L (odds ratio, 6.8) and Indigenous status (odds ratio, 2.3), we tested a modified scoring system (SMARTACOP) that used a higher weighting for albumin and included Indigenous status. A SMARTACOP score of ≥3 had a sensitivity of 97% for IRVS and 100% for 30-day mortality.

Conclusions: The SMART-COP score underestimates the severity of pneumonia in tropical northern Australia, but can be improved by using locally relevant additions.

METHODS

Royal Darwin Hospital (RDH) is the only tertiary referral hospital for the tropical Northern Territory and serves a population of about 150 000 spread across an area of over 500 000 km². We conducted a prospective observational study of adult patients with sepsis (infection plus at least two criteria for systemic inflammatory response syndrome [SIRS]) admitted to RDH between August 2007 and May 2008. The study was approved by the Human Research Ethics Committee of the NT Department of Health and Families and Menzies School of Health Research.

Inclusion criteria were at least two symptoms suggestive of pneumonia (new cough, fever, rigors, chest discomfort, new-onset dyspnoea) and a chest radiograph or computed tomography scan taken within 24 hours of admission demonstrating acute pneumonia. All chest radiographs were reviewed by a radiologist and, where the report was inconclusive or ambiguous, radiographs were also viewed by an infectious diseases physician. Exclusion criteria were: immunosuppression, active orders limiting life-sustaining treatment, and direct admission to the intensive care unit (ICU).

We calculated SMART-COP scores as defined in the Australian Community-Acquired Pneumonia Study (ACAPS) (Box 1). We used univariate logistic regression analysis to examine the individual components of the SMART-COP score against the need for intensive respiratory or vasopressor support (IRVS). We then assessed the performance of the SMART-COP score and evaluated several variations of it, primarily on the basis of its negative predictive value (NPV), because of the need to identify patients who did not require IRVS and could thus be safely managed in a general ward. Statistical analysis was performed using Intercooled Stata, version 10 (StataCorp, College Station, Tex, USA). A significance level of 0.05 was used.

RESULTS

During the study period, 246 patients were admitted with sepsis and a clinical diagnosis of pneumonia. Of these, 40 did not have radiological evidence of pneumonia and 22 met exclusion criteria (immunosuppression, active orders limiting life-sustaining treatment, direct admission to the ICU), leaving 184 eligible patients (Box 2). Of the total group, 111 patients (60%) were in the low-risk SMART-COP group (score ≤2), and 11 of these (10%) required IRVS (Box 3, Box 4). As a predictor of need for IRVS, a SMART-COP score of ≥3 had a considerably lower sensitivity, NPV and area under the receiver operator characteristic curve (AUROC) in our RDH group than in the ACAPS cohort (P = 0.05, Box 5).
The SMART-COP score also had poor sensitivity for predicting 30-day mortality:

- **Low risk (0–2)**: 111 cases, 11 deaths (5%)
- **Moderate risk (3–4)**: 48 cases, 15 deaths (31%)
- **High risk (5–6)**: 22 cases, 10 deaths (46%)
- **Very high risk (≥ 7)**: 3 cases, 2 deaths (67%)

**SMARTACOP**

- **Low risk (0–2)**: 68 cases, 1 death (2%)
- **Moderate risk (3–4)**: 59 cases, 14 deaths (24%)
- **High risk (5–6)**: 39 cases, 13 deaths (33%)
- **Very high risk (≥ 7)**: 18 cases, 10 deaths (56%)

Of the SMART-COP score components, new-onset confusion (odds ratio [OR], 22.0; 95% CI, 2.5–194.4) and serum albumin level < 35 g/L (OR, 6.8; 95% CI, 2.9–15.9) were the strongest predictors of the need for IRVS (Box 6). However, confusion was present in only six of the 184 patients (3%), making it less clinically useful in predicting those at risk of needing IRVS. Indigenous status was also associated with the need for IRVS (OR, 2.3; 95% CI, 1.0–5.5). Contrary to expectations, none of the comorbidities that were assessed were significantly associated with the need for IRVS (Box 6).

Given that the SMART-COP score performed poorly at the low-risk end in the RDH cohort, we designed and tested a modified score, SMARTACOP, which increased the weighting for albumin to 2 points and included Indigenous status as a variable (Box 1). These changes improved the performance of the scoring system, largely due to better discrimination of patients in the low-risk group. Of the 111 patients in this group according to the SMART-COP score, 43 were recategorised as moderate risk, of whom 10 (23%) required IRVS. The specificity of both the SMART-COP and SMARTACOP scores was poor (Box 5), and thus they should not be used for their positive predictive value.

The SMART-COP score also had poor sensitivity for predicting 30-day mortality.
six patients (5%) with a low-risk SMART-COP score died (sensitivity, 67%), compared with none with a low-risk SMARTACOP score (sensitivity, 100%). The AUROC for 30-day mortality was 0.74 for SMART-COP and 0.77 for SMARTACOP.

**DISCUSSION**

In this study, we have demonstrated that the SMART-COP scoring system underestimates the need for intensive supportive treatment and the risk of death in a tropical Australian population hospitalised for pneumonia.

Our study population differs substantially from those involved in previous studies of pneumonia risk. Compared with patients in the ACAPS cohort used to derive the SMART-COP score, patients in this study were younger and more likely to be Indigenous, drink hazardous amounts of alcohol, smoke, and have chronic liver disease. In addition, the causative organism in those with an identified pathogen was a gram-negative bacillus in more than half the patients in this study, compared with 18% in the ACAPS study. More than 20% of our patients required IRVS, compared with 18% in the ACAPS study. This may account for a proportion of the decrease in NPV for the SMART-COP score in the RDH cohort compared with the ACAPS cohort, but is unlikely to be solely responsible for the observed difference in NPV.

Low serum albumin level appears to be more strongly associated with the requirement for IRVS in this study than in the original derivation study. Albumin decreases in response to acute inflammation and thus may be a surrogate marker for late presentation, as well as severity of inflammation. Chronic liver disease and poor nutrition associated with hazardous alcohol intake may also be contributors to low serum albumin levels.

We found Indigenous status to be associated with the requirement for IRVS and with mortality. It is likely that Indigenous status is a surrogate marker for poor health and social disadvantage, and these factors are likely to contribute more to poor outcomes than any possible genetic susceptibility. Hazardous levels of alcohol use were present in the majority of Indigenous patients in this study; however, this was not a risk factor for IRVS or mortality. For pneumonia generally, it is likely that an interaction between severe infection and decreased physiological reserve due to multiple underlying comorbidities is what puts an individual patient at risk.

An alternative strategy to improve the performance of the SMART-COP scoring system would be to change the cut-off value used to define low risk. However, changing this cut-off score from 3 to 2 is not as effective as modifying the score; the sensitivity for IRVS changes from 71% to 89% with a cut-off of 2 (compared with 97% at a cut-off of 3 for the modified score), and four of 38 patients who required IRVS would be misclassified using this strategy.

There are several limitations to our study. We only considered patients admitted to hospital; however, it is unlikely that patients not admitted would require IRVS. We also included only patients meeting SIRS criteria, and cannot exclude the possibility that patients without SIRS initially may develop worsening pneumonia later. This study was limited to a single centre, albeit one with the only ICU servicing northern Australia between the Kimberley region in Western Australia and the Queensland border. The number of patients in this study meant that we had limited statistical power to make comparisons or perform multiple logistic regression analysis. The high proportions of Indigenous Australians and of patients with hazardous levels of alcohol use mean that the results of this study may not be generalisable to tropical regions in other countries. We did not collect information on antibiotic use and thus could not control for this in our analysis; however, considering our hospital uses established antibiotic protocols with a high level of staff compliance, we would not expect this to vary between groups.

We have demonstrated that the current SMART-COP scoring system does not adequately identify patients requiring IRVS in this tropical setting. We propose some minor modifications that improve its performance, particularly its NPV. We intend to validate this modified scoring system prospectively in patients presenting to the emergency department at RDH.

**5 Performance characteristics of the SMART-COP score in the ACAPS and RDH cohorts and the SMARTACOP score in the RDH cohort**

<table>
<thead>
<tr>
<th></th>
<th>ACAPS</th>
<th>RDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMART-COP score ≥ 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AUROC</td>
<td>0.87 (0.83–0.91)</td>
<td>0.75 (0.66–0.83)</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>92% (85%–97%)</td>
<td>71% (54%–85%)*</td>
</tr>
<tr>
<td>Specificity</td>
<td>62% (59%–66%)</td>
<td>69% (60%–76%)</td>
</tr>
<tr>
<td>PPV</td>
<td>22% (18%–27%)</td>
<td>37% (26%–50%)</td>
</tr>
<tr>
<td>NPV</td>
<td>99% (97%–99%)</td>
<td>91% (83%–94%)*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SMARTACOP score ≥ 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AUROC</td>
<td>0.77 (0.70–0.85)</td>
<td>0.97 (0.86–1.00)</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>97% (86%–100%)</td>
<td>94% (83%–95%)</td>
</tr>
<tr>
<td>Specificity</td>
<td>46% (38%–54%)</td>
<td>27% (24%–41%)</td>
</tr>
<tr>
<td>PPV</td>
<td>32% (24%–41%)</td>
<td>99% (92%–100%)</td>
</tr>
<tr>
<td>NPV</td>
<td>99% (92%–100%)</td>
<td>99% (92%–100%)</td>
</tr>
</tbody>
</table>

Data are shown with 95% confidence intervals. ACAPS = Australian Community-Acquired Pneumonia Study; RDH = Royal Darwin Hospital. AUROC = area under the receiver operator characteristic curve. PPV = positive predictive value. NPV = negative predictive value. *P < 0.05 compared with the ACAPS.
## 6 Univariate logistic regression with need for IRVS as dependent variable

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>No. (%)</th>
<th>OR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic BP &lt; 90 mmHg</td>
<td>9 (5%)</td>
<td>2.3 (1.2–4.6)</td>
<td>0.02</td>
</tr>
<tr>
<td>&gt; 1 lobe involved on CXR</td>
<td>93 (51%)</td>
<td>2.6 (1.2–5.5)</td>
<td>0.02</td>
</tr>
<tr>
<td>Serum albumin level &lt; 35 g/L</td>
<td>82 (45%)</td>
<td>6.8 (2.9–15.9)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Tachypnoea</td>
<td>70 (38%)</td>
<td>1.4 (0.7–2.9)</td>
<td>0.34</td>
</tr>
<tr>
<td>Tachycardia</td>
<td>41 (22%)</td>
<td>1.9 (0.8–4.1)</td>
<td>0.13</td>
</tr>
<tr>
<td>New-onset confusion</td>
<td>6 (3%)</td>
<td>22.0 (2.5–194.4)</td>
<td>0.005</td>
</tr>
<tr>
<td>Hypoxaemia</td>
<td>49 (27%)</td>
<td>1.4 (0.9–2.0)</td>
<td>0.11</td>
</tr>
<tr>
<td>Arterial pH &lt; 7.35</td>
<td>8 (4%)</td>
<td>2.0 (1.0–4.2)</td>
<td>0.05</td>
</tr>
<tr>
<td>Male</td>
<td>103 (56%)</td>
<td>1.5 (0.8–3.2)</td>
<td>0.23</td>
</tr>
<tr>
<td>Indigenous</td>
<td>120 (65%)</td>
<td>2.3 (1.0–5.5)</td>
<td>0.05</td>
</tr>
<tr>
<td>Remote-dwelling</td>
<td>51 (28%)</td>
<td>2.0 (0.9–4.2)</td>
<td>0.07</td>
</tr>
<tr>
<td>Homeless</td>
<td>17 (9%)</td>
<td>1.1 (0.9–1.1)</td>
<td>0.1</td>
</tr>
<tr>
<td>Hazardous alcohol use</td>
<td>76/134 (57%)</td>
<td>1.0 (0.9–1.2)</td>
<td>0.96</td>
</tr>
<tr>
<td>Chronic renal disease</td>
<td>33 (18%)</td>
<td>1.6 (0.7–3.8)</td>
<td>0.3</td>
</tr>
<tr>
<td>Chronic liver disease</td>
<td>26 (14%)</td>
<td>1.9 (0.8–5.8)</td>
<td>0.17</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>42 (23%)</td>
<td>0.9 (0.4–2.1)</td>
<td>0.8</td>
</tr>
<tr>
<td>Smoking</td>
<td>93/151 (62%)</td>
<td>0.8 (0.7–1.1)</td>
<td>0.08</td>
</tr>
<tr>
<td>Admitted during wet season</td>
<td>126 (68%)</td>
<td>1.0 (0.5–2.1)</td>
<td>0.74</td>
</tr>
<tr>
<td>COPD</td>
<td>39 (21%)</td>
<td>1.7 (0.8–3.9)</td>
<td>0.34</td>
</tr>
<tr>
<td>Malignancy</td>
<td>7 (4%)</td>
<td>0.6 (0.1–5.4)</td>
<td>0.68</td>
</tr>
</tbody>
</table>

IRVS = intensive respiratory or vasopressor support. OR = odds ratio. BP = blood pressure. CXR = chest x-ray. COPD = chronic obstructive pulmonary disease.

## ACKNOWLEDGEMENTS

We thank Mark McMillan and Alex Humphrey for data collection and entry; Luke Diolosa and Dianne Stephens for assistance with intensive care databases; and staff in the radiology department for their assistance. This study was funded by the National Health and Medical Research Council (Program Grant 496600, Fellowships to Allen Cheng and Nicholas Anstey, and a scholarship to Joshua Davis).

## COMPETING INTERESTS

None identified.

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(Received 29 Apr 2009, accepted 23 Aug 2009)
7.3 Implications of and questions arising from this paper

7.3.1 Prospective validation study
As a result of the findings reported in this paper, a further study was designed to prospectively validate the SMARTACOP score. This is a 12-month study based in the emergency department of Royal Darwin Hospital, and is currently nearing completion of recruitment. It will add to the findings of the MJA paper, and will address some of its limitations. Firstly, all patients with pneumonia, not just those meeting sepsis criteria will be enrolled. Secondly, this study will enrol all pneumonia patients attending the emergency department, not only those who were admitted to hospital. Finally, data will also be collected to calculate the more cumbersome PSI (pneumonia severity index), as well as clinicians’ impressions of severity for comparison with the SMARTCOP and SMARTACOP scores.

7.3.2 Updating of hospital and national protocols
The writing group for the respiratory infections chapter of the Therapeutic Guidelines: Antibiotic has considered the findings reported here and have discussed referring to them in the next version of these guidelines (noting that the SMARTCOP score may not apply in tropical Australia). However, we (the authors of the MJA paper) have suggested that data be awaited from the subsequent validation study prior to making such a change.

The Royal Darwin Hospital pneumonia treatment protocol currently uses the categories “mild”, “moderate” and “severe” pneumonia to guide antibiotic choice, but these categories are not defined. A new protocol is currently being developed using the SMARTACOP scoring system to define severity and thus to determine antibiotic choice and early ICU referral of appropriate patients.
7.4 References


Section C. Endothelial and Microvascular Function in Sepsis

“Slowly the poison the whole blood stream fills.
It is not the effort nor the failure tires.
The waste remains, the waste remains and kills.”

William Empson (1906-1984)
Chapter 8. STOPWATCH – Separation Time of Plasma: Whether Amino acids are Temperature and Time Critical.
8.1 Preamble

Determination of plasma concentrations of arginine and other amino acids was a key part of the research plan for the studies of endothelial function in sepsis which are described in Chapters 9-13 and 17. The original laboratory protocols for processing of blood in these studies allowed for the separation of peripheral blood mononuclear cells (PBMCs) from whole blood at the time of plasma separation and prior to any cooling or freezing of the specimens. The PBMCs are being used for in-vitro investigations of the role of amino acids in T-cell function in sepsis, but these immunology studies are beyond the scope of this thesis and will not be reported here. Because PBMCs may be damaged by refrigeration, blood was left at room temperature until plasma separation (by centrifugation) and freezing at -80°C. Because of the logistics of the research laboratory and patient recruitment, specimens sometimes sat at room temperature for several hours or overnight prior to plasma separation (table 9-2).

Thus we became concerned about the effect of these delays on the accuracy of our amino acid determinations. A review of the literature did not provide sufficient detail to quantify the effect of delayed processing on plasma amino acid concentrations. The following manuscript describes the study which was carried out to try to answer these questions and refine our laboratory procedures for current and future studies.
8.2 Ex-vivo changes in amino acid concentrations from blood stored at room temperature or on ice: implications for arginine and taurine measurements. Manuscript as published in BMC Clinical Pathology.
Ex-vivo changes in amino acid concentrations from blood stored at room temperature or on ice: implications for arginine and taurine measurements

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Abstract

Background: Determination of the plasma concentrations of arginine and other amino acids is important for understanding pathophysiology, immunopathology and nutritional supplementation in human disease. Delays in processing of blood samples cause a change in amino acid concentrations, but this has not been precisely quantified. We aimed to describe the concentration time profile of twenty-two amino acids in blood from healthy volunteers, stored at room temperature or on ice.

Methods: Venous blood was taken from six healthy volunteers and stored at room temperature or in an ice slurry. Plasma was separated at six time points over 24 hours and amino acid levels were determined by high-performance liquid chromatography.

Results: Median plasma arginine concentrations decreased rapidly at room temperature, with a 6% decrease at 30 minutes, 25% decrease at 2 hours and 43% decrease at 24 hours. Plasma ornithine increased exponentially over the same period. Plasma arginine was stable in blood stored on ice, with a < 10% change over 24 hours. Plasma taurine increased by 100% over 24 hours, and this change was not prevented by ice. Most other amino acids increased over time at room temperature but not on ice.

Conclusion: Plasma arginine concentrations in stored blood fall rapidly at room temperature, but remain stable on ice for at least 24 hours. Blood samples taken for the determination of plasma amino acid concentrations either should be placed immediately on ice or processed within 30 minutes of collection.

Background

Quantification of plasma amino acids is not routinely offered by clinical laboratories and thus plasma often needs to be transported to research or reference laboratories for testing. In order to accurately assess the concentration of plasma amino acids, it is important to know their

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stability in human blood which has been stored or transported prior to testing. Previous studies addressing this question have been small and the rate of degradation has not been precisely quantified.

Arginine, the precursor of nitric oxide (NO) [1], is important for endothelial [2] and immunological [3] function and is acutely decreased in sepsis [4,5], malaria [6] and trauma [7], and was thus the focus of this study. The major routes for arginine metabolism in humans are metabolism by arginase to urea and ornithine; use for creatine synthesis; and metabolism by nitric oxide synthase to NO and citrulline [8]. Both red blood cells (RBCs) [9] and macrophages [10] are rich in arginase. In stored packed RBCs, arginase is released and the resulting degradation of plasma arginine is thought to be a mechanism of transfusion-associated immunosuppression [9,11]. Other amino acids which are commonly added to supplementary nutrition for critically ill patients may also play an important role in immune function including tryptophan [12] glutamine [13] and taurine [14,15].

Hainque and colleagues studied eight healthy volunteers and found a "significant degradation" of plasma arginine following 4 hours at room temperature but this was not quantified and no other time points were reported [16]. Schaefer et al. studied one volunteer and found a 50% decrease in plasma arginine after 6 hours at room temperature compared with a 10% decrease after 6 hours at 4 degrees centigrade, with earlier time points not reported [17]. Nutall and colleagues reported time profile data from one volunteer, which showed an approximate 33% decrease in plasma arginine by 2 hours at room temperature [18].

To determine the impact of delayed processing we undertook a study to estimate the rate of arginine degradation in human plasma at room temperature and on ice. We hypothesised that this degradation would be primarily due to plasma arginase activity and that there would be less than 10% degradation at 2 hours in samples placed immediately on ice. We also sought to determine the effect of delayed separation and freezing of plasma on the concentration of other amino acids.

**Methods**

The study was considered by the Chair of the Human Research Ethics Committee of the Menzies School of Health Research and Northern Territory Department of Health and Families, and was approved as a laboratory quality assurance activity which did not require full ethical review. Following written informed consent, six healthy normotensive fasting volunteers had venous blood collected into 12 × 2 mL lithium heparin tubes (Vacutainer, Becton Dickinson, Franklin Lakes, New Jersey) using a 21 gauge needle and vacutainer system. For each subject, the first six tubes were immediately placed into an ice slurry and the second six were left at room temperature (25° Celsius (C)) in an air conditioned laboratory. After intervals of 0 minutes, 30 minutes, 2 hours, 4 hours, 8 hours and 24 hours from the time of venepuncture, the tubes were centrifuged at 3000 rpm for 10 minutes (either at 4°C or at room temperature as appropriate) and the plasma immediately separated and stored at -80°C.

Subsequently, following thawing, plasma amino acids were extracted with ethanol, then derivatized with AccQ-Fluor (Waters, Milford, MA). Amino acid concentrations were then determined by reverse-phase high performance liquid chromatography (HPLC; Shimadzu corporation, Kyoto, Japan) with UV (250 nm) and fluorescence (excitation 250 nm, emission 395 nm) detection, using a method modified from van Wandelen and Cohen [19].

The data were analysed using Stata 10 (Statacorp, College Station, Texas) and GraphPad Prism 5 (Graphpad software, San Diego, California). Due to the small number of subjects, data were summarized using median and interquartile range. Median amino acid concentrations over time were compared using a paired Wilcoxon test, with a p-value of < 0.05 considered significant. The arginine degradation curve was fitted using a one-phase exponential decay model. The sample size was determined using data from an earlier experiment (unpublished data), which found that there was 31.8% (std dev = 14%) degradation of arginine at room temperature by 2 hours. Using a power of 80% and a significance level of 5%, five subjects in each group would be needed to detect a difference of 22% degradation at 2 hours, meaning less than 10% degradation in the ice group. To allow for sample wastage and errors, we recruited six subjects.

**Results**

Of the six study subjects, half were male, and the median age was 37.5 years, with a range of 19-47 years (table 1). All were healthy, of normal weight and normotensive, and none had cardiovascular disease or diabetes mellitus. The median baseline plasma arginine concentration was 74.9 μmol/L, similar to previously reported mean plasma arginine concentrations from healthy volunteers, the majority of which are between 60 and 80 μmol/L [20].

**Arginine and ornithine time profiles at room temperature**

Plasma arginine concentration decreased rapidly at room temperature (Figures 1a, 2, Table 2) with 6% degradation within 30 minutes, 25% degradation within 2 hours and 43% degradation within 24 hours. A non-linear model of the plasma arginine profile over time was defined by the equation $Y = \left((Y_0-P) e^{-kt}\right) + P$, where $t$ = time in hours, $P =$
the plateau value, $Y_0 = \text{initial value}$. The parameters of the model were $Y_0 = 81.3$, $P = 37.8$, and $k = 0.6273$. This model fitted the data well, with an $R^2$ of 0.73. Plasma ornithine concentration increased exponentially at room temperature (Table 1, Figure 2), with a 4% increase at 30 minutes, a 62% increase at 2 hours, and a 183% increase at 24 hours.

### Arginine time profile on ice compared with room temperature

Plasma arginine was very stable on ice, with a less than 10% change over a 24 hour period. At 2 hours, the median plasma arginine concentration had decreased by 6% in the ice specimens compared with 25% in the room temperature specimens ($p < 0.001$) (Figure 1). At 24 hours, the change in arginine was negligible for the ice specimens compared with a 43% decrease at room temperature ($p < 0.001$). Ornithine was also more stable on ice, with a 24% increase over the 24 hour period, compared with a 183% increase at room temperature.

### Time profile of other amino acids

For the majority of other amino acids, concentrations increased by > 10% over 24 hours at room temperature (Table 3). The majority of these changes were largely or completely prevented in the blood that was placed on ice. The most notable room temperature concentration increases at 24 hours were seen with taurine (which doubled) and glutamate (which increased more than five-fold). The change in taurine was unusual in that it was more marked in the blood placed on ice (a 126% increase) than the room temperature specimens (a 100% increase), suggesting that the increase in taurine may be due to release from lysed cells rather than to an enzymatic

### Table 1: Characteristics of study subjects

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age (years)</th>
<th>Gender</th>
<th>Ethnicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>36</td>
<td>F</td>
<td>Caucasian</td>
</tr>
<tr>
<td>2</td>
<td>39</td>
<td>M</td>
<td>Caucasian</td>
</tr>
<tr>
<td>3</td>
<td>47</td>
<td>F</td>
<td>Caucasian</td>
</tr>
<tr>
<td>4</td>
<td>27</td>
<td>F</td>
<td>Caucasian</td>
</tr>
<tr>
<td>5</td>
<td>19</td>
<td>M</td>
<td>Caucasian</td>
</tr>
<tr>
<td>6</td>
<td>44</td>
<td>M</td>
<td>Caucasian</td>
</tr>
</tbody>
</table>

Figure 1

**Plasma arginine time profile at room temperature and on ice.** Each curve represents an individual subject. Figure 1a depicts results from whole blood stored at room temperature ($25^\circ$C). Figure 1b depicts results from aliquots of the same blood samples which were stored in an ice slurry.

Figure 2

**Time profile of median plasma arginine and ornithine concentrations in blood stored at room temperature.** Each point represents the median value for that time, and the error bars represent the interquartile range. Median plasma arginine is indicated by triangles, and ornithine by solid circles.
process (Figure 3). Tryptophan was very stable both at room temperature and on ice.

**Discussion**

Plasma arginine concentration decreases rapidly in whole blood held at room temperature, and this decrease is greatly attenuated by placing the blood on ice. Ornithine, the metabolic product of arginine metabolism by arginase, rises exponentially at room temperature, and this rise does not occur on ice, suggesting that it is due to an enzymatic process. Thus, it is likely that arginase is the primary mechanism of arginine degradation in ex-vivo blood samples. This arginase could come from either lysed RBCs or lysed leucocytes, but we did not evaluate the source of arginase, and thus cannot determine which of these was more important. In-vitro hemolysis is difficult to measure, as the released cell-free haemoglobin is immediately bound by haptoglobin. While we have not proven this hypothesis, our observations strongly suggest it.

Most other amino acids increase at room temperature but not on ice, which also implies an enzymatic reaction. Tryptophan is very stable both at room temperature and on ice. Taurine and glutamine are unusual, in that they increase markedly both at room temperature and on ice; this may be due to cellular release rather than enzymatic catabolism.

The rate of decrease of plasma arginine which we found in blood held at room temperature is similar to that found by Nuttall and colleagues in the only published paper to have reported plasma arginine concentrations at room temperature at more than two time points [18]. The lack of early time points in other papers makes it difficult to estimate the rate of decline and whether it is linear or exponential. Nuttall et al. reported data in graphical form, from a single subject up to 2.5 hours post venepuncture. They found a fall from 89 \( \mu \text{mol/L} \) to approximately 60 \( \mu \text{mol/L} \) at 2 hours (a 33% drop), similar to our reported decrease of 25% at 2 hours.

The large increases seen in taurine and glutamate in our study have not previously been reported. Sahai et al. measured amino acid levels in whole blood from twenty-two volunteers, stored on ice for 1 hour or 2 hours, and found a less than 10% decrease in plasma taurine and glutamate at 1 and 2 hours [21]. Shaeffer et al. reported a

**Table 2: Median (IQR) arginine and ornithine plasma concentrations over time from blood stored at room temperature compared with stored on ice**

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>30 minutes</th>
<th>2 hours</th>
<th>4 hours</th>
<th>8 hours</th>
<th>24 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Arginine RT</strong>a</td>
<td>74.9</td>
<td>70.3</td>
<td>49.6</td>
<td>40.4</td>
<td>37.3</td>
<td>42.6</td>
</tr>
<tr>
<td></td>
<td>73.2-87.8</td>
<td>63.4-75.5</td>
<td>46.0-53.6</td>
<td>35.8-45.8</td>
<td>32.1-42.6</td>
<td>25.5-42.8</td>
</tr>
<tr>
<td><strong>Arginine Ice</strong></td>
<td>79.6</td>
<td>77.1</td>
<td>74.8</td>
<td>78.6</td>
<td>80.4</td>
<td>81.0</td>
</tr>
<tr>
<td></td>
<td>76.8-93.0</td>
<td>74.6-90.8</td>
<td>73.4-86.9</td>
<td>74.6-86.1</td>
<td>79.4-86.7</td>
<td>79.9-83.0</td>
</tr>
<tr>
<td><strong>Ornithine RT</strong></td>
<td>44.7</td>
<td>45.6</td>
<td>72.6</td>
<td>87.4</td>
<td>101.6</td>
<td>114.1</td>
</tr>
<tr>
<td></td>
<td>32.9-60.8</td>
<td>39.5-69.8</td>
<td>58.7-94.2</td>
<td>69.1-112.3</td>
<td>79.4-125.9</td>
<td>100.9-153.6</td>
</tr>
<tr>
<td><strong>Ornithine Ice</strong></td>
<td>38.6</td>
<td>31.6</td>
<td>39.2</td>
<td>40.5</td>
<td>36.3</td>
<td>43.1</td>
</tr>
<tr>
<td></td>
<td>29.4-57.3</td>
<td>38.3-59.2</td>
<td>30.2-60.0</td>
<td>30.5-61.9</td>
<td>32.8-61.5</td>
<td>36.2-68.1</td>
</tr>
</tbody>
</table>

**Figure 3**

Time profile of median plasma taurine concentrations in blood stored at room temperature and on ice. Each point represents the median value for that time, and the error bars represent the interquartile range. Median plasma taurine at room temperature is represented by solid circles, and median plasma taurine on ice is represented by triangles.
Table 3: Change in amino acid concentrations in whole blood after 24 hours at room temperature and on ice.

<table>
<thead>
<tr>
<th></th>
<th>% Change at 24 h at RT&lt;sup&gt;a, b&lt;/sup&gt;</th>
<th>% Change at 24 h on ice&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group 1 - ≤ 10% change at RT&lt;sup&gt;a&lt;/sup&gt; over 24 h</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citrulline</td>
<td>-4 (-6, 4)</td>
<td>-7 (-9, -4)</td>
</tr>
<tr>
<td>Glutamine</td>
<td>-10 (-13, -10)</td>
<td>-5 (-5, -4)</td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>8 (7,9)</td>
<td>-3 (-4, -3)</td>
</tr>
<tr>
<td>Methionine</td>
<td>0 (-2, 1)</td>
<td>1 (1, 6)</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>7 (5, 8)</td>
<td>4 (4, 6)</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>8 (5, 12)</td>
<td>-2 (-3, -1)</td>
</tr>
<tr>
<td>Valine</td>
<td>8 (4, 11)</td>
<td>1 (0, 1)</td>
</tr>
<tr>
<td><strong>Group 2 - &gt; 10% increase at RT&lt;sup&gt;a&lt;/sup&gt; over 24 h</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>18 (16,20)</td>
<td>0 (-1, 0)</td>
</tr>
<tr>
<td>Asparagine</td>
<td>17 (12, 21)</td>
<td>0 (-1, +3)</td>
</tr>
<tr>
<td>Glutamate</td>
<td>593 (563, 612)</td>
<td>38 (92, 186)</td>
</tr>
<tr>
<td>Glycine</td>
<td>26 (24, 34)</td>
<td>3 (2, 4)</td>
</tr>
<tr>
<td>Histidine</td>
<td>23 (17, 27)</td>
<td>1 (0, 1)</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>16 (10, 21)</td>
<td>0 (-1, 2)</td>
</tr>
<tr>
<td>Leucine</td>
<td>23 (17, 34)</td>
<td>2 (1, 5)</td>
</tr>
<tr>
<td>Lysine</td>
<td>19 (18, 19)</td>
<td>2 (1, 5)</td>
</tr>
<tr>
<td>Ornithine</td>
<td>183 (180, 224)</td>
<td>24 (23,25)</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>15 (14,22)</td>
<td>1 (1, 3)</td>
</tr>
<tr>
<td>Proline</td>
<td>11 (6,13)</td>
<td>1 (-1,2)</td>
</tr>
<tr>
<td>Serine</td>
<td>18 (17,28)</td>
<td>6 (2,6)</td>
</tr>
<tr>
<td>Taurine</td>
<td>100 (94, 102)</td>
<td>126 (120, 147)</td>
</tr>
<tr>
<td>Threonine</td>
<td>11 (10, 14)</td>
<td>-2 (-5, 0)</td>
</tr>
<tr>
<td><strong>Group 3 - &gt; 10% decrease at RT&lt;sup&gt;a&lt;/sup&gt; over 24 h</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arginine</td>
<td>-43 (-65, -43)</td>
<td>-1 (-5, 4)</td>
</tr>
</tbody>
</table>

a. RT - Room temperature  
b. Expressed as median % change (Interquartile range)  
Note - % change values are an increase (positive change) unless otherwise specified.
< 10% decrease in plasma taurine and glutamate at 6 hours in blood held at room temperature from one healthy volunteer [17]. The reason for this discrepancy is unclear. Both papers used different methods for amino acid quantification than we did. Sahai et al did not measure time points beyond 2 hours, and most of the increase in both taurine and glutamine in our study occurred beyond 2 hours. However, until this finding is reproduced by other investigators, it should be regarded with caution.

The primary limitations of this study are the relatively small number of subjects and the lack of subjects suffering from sepsis, trauma or other conditions of interest. A larger number of subjects would allow a more accurate estimate of the time profile of arginine degradation over time. Considering arginase activity is increased in severe sepsis [22] and trauma [23], it is unclear if blood from patients with these conditions would yield the same results as we observed. We did not directly measure arginase activity in blood or plasma, and thus our inference that plasma arginase is primarily responsible for the observed ex-vivo arginine degradation is based on indirect evidence. However, the only other significant mechanism for arginine degradation likely to occur ex-vivo is the breakdown of arginine to NO and citrulline by nitric oxide synthase, which accounts for less than 5% of arginine metabolism in healthy humans [24].

One potential implication of these data is that whole blood stored for the purpose of transfusion is likely to contain non-physiological concentrations of amino acids, which may have unintended immunosuppressive effects. These data also reinforce the importance of accurate methodological descriptions in papers reporting plasma amino acid levels. In a hospital setting, it is not always possible to process samples within 30 minutes of collection. It is therefore essential to note the time between collection and freezing when reporting concentrations of plasma amino acids. This is particularly important if the sample cannot be kept on ice - for example, if the blood is to be used for both peripheral blood mononuclear cell (PBMC) collection and amino acid analysis. As PBMCs are aged by freezing, these samples must be kept at room temperature and processed as soon as possible to allow accurate analysis of both PBMC function and amino acid concentrations. Furthermore, where plasma amino acids are being measured for clinical applications, our data emphasise the importance of timely separation and freezing of plasma to avoid potential diagnostic errors.

**Conclusion**

In conclusion, arginine undergoes rapid ex-vivo degradation at room temperature but this does not occur on ice; plasma tryptophan is stable for at least 24 hours both at room temperature and on ice; plasma taurine concentrations show large increases both at room temperature and on ice. Blood collected for the purposes of plasma amino acid determination should be placed immediately on ice; if this is not possible, plasma should be frozen with 30 minutes of collection.

**Abbreviations**

HPLC: High Performance Liquid Chromatography; NO: Nitric Oxide; PBMC: Peripheral Blood Mononuclear Cell; IQR: Interquartile range.

**Competing interests**

The authors declare that they have no competing interests.

**Authors’ contributions**

All authors took part in study design and contributed to the final draft of the paper. In addition, JSD participated in interpretation of HPLC results, performed the data analysis and wrote the first draft of the paper. CJD, KP, and TW performed sample preparation. YM performed and analysed the HPLC. NA secured the funding. All authors read and approved the final manuscript.

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**References**


Pre-publication history
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http://www.biomedcentral.com/1472-6890/9/10/prepub
8.3 Implications of the STOPWATCH study

Following completion of this study, the following changes to laboratory procedures were made:

1) Analysis of L-arginine concentrations excluded any samples processed more than 30 minutes after blood collection.

2) For ongoing and future studies at Royal Darwin Hospital, an extra 2ml tube of blood will be collected and placed immediately on ice, and every effort will be made to process blood specimens within 30 minutes of collection. This still allows extraction of intact PBMCs from the blood held at room temperature, but provides an extra aliquot for accurate amino acid determination.

3) Future studies assessing most other amino acids can include blood specimens which have undergone delayed separation if necessary.

4) An estimated decay curve of arginine in healthy human plasma was developed which could be used to estimate plasma L-arginine from blood separated at any time post collection. We decided not to do this for the FRESH studies (chapters 9-13), and took the more conservative approach of excluding specimens with delayed processing.
Chapter 9. Finger Reactive Hyperaemia to measure Endothelial function in Sepsis and in Health: Background to the FRESH study
9.1 Introduction

The following four manuscripts (Chapters 10-13) are all derived from data from this cohort. Hence some detail about study background and procedures will be provided here, whilst trying not to repeat information given in the manuscripts.

9.1.1 The study site

The study was conducted in the intensive care unit (ICU) and hospital wards of Royal Darwin Hospital, which have been previously described in chapter 5.

9.1.2 Summary of state of knowledge about endothelial function in human sepsis at the time of commencement of the FRESH study

This has been covered in more detail in Chapter 3. In brief, few papers reporting functional measurements of endothelial function in septic humans had been published prior to 2006, and those that had been published contained small numbers of subjects and used complex or invasive techniques. Seven papers had reported reactive hyperaemia in septic adults [1-7]. Each of these studies enrolled between six and twenty-three patients with sepsis, and used venous plethysmography (three studies), laser Doppler flowmetry of skin (two studies) and invasive laser Doppler flowmetry of muscle (two studies). Five of these studies reported impaired reactive hyperaemia responses in septic patients and two found no impairment. None of them compared functional measurements with plasma concentrations of arginine, inflammatory cytokines or markers of endothelial activation, and all were cross-sectional, with no longitudinal component. Two papers examining reactive hyperaemia in the skin of septic neonates (n=12 for both studies) had both found it to be increased rather than impaired compared with controls [8, 9].

Thus, although the role of the endothelium was being increasingly recognised in sepsis pathophysiology, a practical means of measuring it at the bedside had not been described. In addition, it was unclear what the prevalence, implications and correlates of impaired microvascular reactivity were in patients with sepsis, or how it changed over time.

Peripheral arterial tonometry had been used widely for assessment of endothelial function in ambulatory patients and was FDA approved in the USA as a tool for cardiovascular risk assessment [10-17]. Our research group at the Menzies School of Health Research had previously used peripheral arterial tonometry in patients with malaria in Papua, Indonesia.
Apart from this, peripheral arterial tonometry had not previously been used in patients with acute infections or sepsis.

### 9.2 Aims and hypotheses of the FRESH study.

The FRESH study was designed to supplement and expand the body of knowledge about endothelial and microvascular function in sepsis, particularly with regard to relationships with plasma arginine concentrations and markers of endothelial activation; longitudinal changes; and use of a simpler and more practical method for estimation of endothelial function at the bedside.

**9.2.1 Aims of the FRESH study:**

1) To assess the feasibility of peripheral arterial tonometry as a bedside tool for estimating endothelial function in patients with sepsis

2) To compare RH-PAT index, a measure of NO-dependent microvascular reactivity and thus an estimate of endothelial function, in patients with and without sepsis, both at baseline, and longitudinally over the first two to four days of illness.

3) To examine the relationship between RH-PAT index and the following factors:
   - i) The severity and outcome of sepsis
   - ii) The degree of endothelial activation
   - iii) Plasma concentrations of L-arginine and dimethylarginines.

4) To assess plasma concentrations of the amino acids arginine and tryptophan against disease severity, and immunological and vascular pathology in sepsis.

**9.2.2 Hypotheses of the FRESH study:**

1) Baseline endothelial function (as measured by RH-PAT index) is impaired in patients with sepsis compared with non-septic controls.

2) The change in RH-PAT index over the first 48-72 hours of illness correlates with disease severity and outcome.

3) Plasma concentrations of ICAM-1, E-selectin, angiopoietin-2 and VEGF will be elevated in patients with sepsis compared with controls, with the degree of elevation correlating with the degree of endothelial dysfunction.
4) The degree of endothelial dysfunction (as estimated by RH-PAT index) will correlate with the severity of illness and the risk of death.

9.3 Methods of the FRESH study

9.3.1 Study recruitment

The FRESH study was a prospective observational cohort study, with enrolment conducted between March 2006 and November 2007. Sepsis patients were recruited both from the wards and ICU, by daily screening rounds of these areas. Screening was primarily conducted by two senior nurses: the ICU research co-ordinator (Ms Jane Thomas) in ICU, and the study's research assistant, Mr Mark McMillan on the wards. Dr Josh Davis also screened and enrolled patients in both of these areas. Control patients were recruited primarily from the Hospital in the Home unit, after patients had completed treatment and were about to be discharged. Thus the control patients were drawn from the same population as the study patients (people living in the drainage area of Royal Darwin Hospital who had needed to attend the hospital), and more importantly had a similar frequency of risk factors for endothelial dysfunction (such as diabetes and smoking). Around half of eligible patients were not enrolled in the study, primarily due to difficulty obtaining consent due to confusion or obtundation and lack of access to appropriate next of kin within the time-frame (Figure 9-1).

Figure 9-1. Patient recruitment for the FRESH study.
9.3.2 Ethics approval and study procedures

The study was prospectively approved by the human research ethics committee of the Menzies School of Health Research and the Northern Territory Department of Health and Families (approval number 06/09 “Endothelial function and arginine in sepsis”). Following written informed consent, microvascular reactivity was measured by peripheral arterial tonometry and blood was drawn. This was mainly done by Dr Davis, Ms Thomas, and Mr McMillan, but some measurements were taken by one of four others (two ICU registrars, an Infectious Diseases registrar and a Hospital in the Home nurse). All of these study staff were trained in the use of the Endopat apparatus as below.

Inclusion and exclusion criteria are listed in the methods sections of chapters 10-13. One of the exclusion criteria, coagulopathy, requires further explanation. Coagulopathy was defined as one or more of INR≥2.0, APTT≥70 or platelet count<20 x 10^9/L. Because the RH-PAT measurement requires a blood pressure cuff on the forearm to be inflated to suprasystolic pressures for 5 minutes, we were concerned about the possibility of causing bruising to the forearm of study subjects. This is primarily a theoretical possibility; in the patients who were included, we observed no bruising or subcutaneous bleeding, even in those with platelet counts of between 20 and 50 x 10^9/L. Figure 9-1 contains the details of the number of patients excluded. Of 191 patients who met inclusion criteria, 106 were excluded, of whom 77 were due to inability to gain consent, and 18 were due to coagulopathy.

9.3.3 Peripheral arterial tonometry

Microvascular reactivity was measured by peripheral arterial tonometry (RH-PAT), using a commercial device, the “Endopat 2000” (Itamar Medical, Caeserea, Israel). All measurements were taken according to the manufacturer’s instructions. Details of the technique and device are given in Chapter 3 and in the manuscripts which follow. The device and its use are shown in figures 9-2 and 9-3.

9.3.4 Training of study staff in peripheral arterial tonometry and inter-observer variation.

Dr Davis was trained in the use of the Endopat 2000 apparatus by a previously experienced user, Dr Tsin Yeo. All other users of the apparatus were trained by Dr Davis. The correct use
of the apparatus was explained and demonstrated to all study staff, who then took three measurements under supervision prior to independent use of the apparatus. Since the manufacturer’s software calculates the RH-PAT index according to an automated algorithm, one would not expect any user-dependence or inter-individual variation of the measurements. This expectation was borne out on eventual analysis of the data, which showed that there was no consistent difference between measurements taken by different observers. Table 9-1 shows the number of observations taken by each of 7 study staff; figures 9-2a-c show the median RH-PAT index generated by each observer, according to patient group. Kruskal-Wallis tests comparing RH-PAT index across all observers within each group was not significant (p=0.80 in controls, p=0.42 in sepsis without organ dysfunction, and p=0.67 in severe sepsis patients).
Table 9-1. Number of RH-PAT measurements taken by each trained observer, according to group.

<table>
<thead>
<tr>
<th>Observer</th>
<th>Severe sepsis</th>
<th>Sepsis without organ dysfunction</th>
<th>Controls</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>MM</td>
<td>26</td>
<td>49</td>
<td>22</td>
<td>97</td>
</tr>
<tr>
<td>JT</td>
<td>66</td>
<td>1</td>
<td>0</td>
<td>67</td>
</tr>
<tr>
<td>JSD</td>
<td>4</td>
<td>3</td>
<td>15</td>
<td>22</td>
</tr>
<tr>
<td>KB</td>
<td>19</td>
<td>0</td>
<td>0</td>
<td>19</td>
</tr>
<tr>
<td>AVA</td>
<td>5</td>
<td>4</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>ST</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>PK</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>
Figure 9-2. Box-plots of RH-PAT indices generated by different observers in healthy controls (11-2a), sepsis without organ dysfunction (11-2b) and severe sepsis (11-2c).
Figure 9-3. The Endopat 2000 and lap-top computer, set-up as used in the FRESH study

Figure 9-4. Measuring microvascular reactivity in a study subject by reactive hyperaemia peripheral arterial tonometry

The probes on each index finger transduce pulsatile pressure changes (tonometry) into volume changes (plethysmography). The resulting pulse wave amplitude signals are sent to an attached laptop computer. The blue foam anchors on the middle fingers prevent any external pressure on the finger probes. The left hand also has a pulse oximetry probe on the ring finger, which is not part of the peripheral arterial tonometry measurement. Note the blood pressure cuff on the right forearm (study arm), ready to be inflated following a 5-10 minute baseline recording.
9.3.5 Laboratory procedures and reason for differing subject numbers in the following manuscripts.

Just prior to the recording of peripheral arterial tonometry, 10 ml of blood was collected into lithium heparin vacutainer tubes from the patient’s arterial line if one was present. Otherwise, blood was collected from a central venous line, or by venepuncture. The blood was transported to the research laboratory (in a separate building on the same campus) as soon as possible, but was left at room temperature until plasma separation and freezing. This is because peripheral blood mononuclear cells (PBMCs) were to be harvested from this whole blood and freezing can damage these cells.

If blood was collected in the evening, it was left at room temperature until the next morning prior to processing. As a result of the experiment reported in Chapter 8, all specimens which were processed more than 30 minutes after collection were excluded from analysis with regard to arginine concentration [20]. Following a review of the literature, specimen processing delays were accepted of up to 30 minutes for cytokines [21], up to 24 hours for angiopoietin-2 [22], and up to 2 hours for asymmetric dimethyl arginine (unpublished data). Table 9-1 shows the proportion of blood specimens separated and frozen within these cut-offs. These data explain why the numbers of subjects differ in the four manuscripts which follow.

Table 9-2. Number of baseline blood specimens separated and frozen according to time elapsed following blood collection

<table>
<thead>
<tr>
<th></th>
<th>≤30 min</th>
<th>≤60 min</th>
<th>≤120 min</th>
<th>&gt;120 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Severe sepsis (n=54)</td>
<td>30</td>
<td>32</td>
<td>38</td>
<td>16</td>
</tr>
<tr>
<td>Sepsis without organ failure (n=31)</td>
<td>26</td>
<td>30</td>
<td>30</td>
<td>1</td>
</tr>
<tr>
<td>Control (n=45)</td>
<td>28</td>
<td>30</td>
<td>32</td>
<td>13</td>
</tr>
</tbody>
</table>
9.3.6 Data management

Clinical data were collected on standardised clinical research forms (CRFs) (Appendix 2). All data forms were hand-checked by Dr Davis and missing or erroneous data were corrected. All peripheral arterial tonometry traces were examined for errors or technical problems and RH-PAT index generated using the manufacturer’s software. Data were entered into an ACCESS 2000 (Microsoft, California, USA) database by Dr Davis and Mr McMillan. A random 10% of entries were then checked against the CRFs, with a resulting error rate of <1% of fields. Data were then transferred to Stata version 10 (Statacorp, Texas, USA) for analysis, merged with laboratory datasets, and checked for outliers.

9.3.7 Erratum in the FRESH paper

On page 6, in Results, under Longitudinal changes in RH-PAT and L-arginine.

“Mean plasma L-arginine concentrations increased from baseline to day 2 to 4 (95% CI: 38.2 to 49.9 µmol/L)” – should say: ... (Mean [95% CI]: 38.2 µmol/L [33.7-42.6] to 49.9 [39.2-60.6], p=0.01)

This has been reported to the editor of Critical Care.
9.4 References


Chapter 10. Impaired endothelium-dependent microvascular reactivity in sepsis
10.1 Sepsis-associated microvascular dysfunction measured by peripheral arterial tonometry: an observational study. Manuscript as published in Critical Care.
Research

Sepsis-associated microvascular dysfunction measured by peripheral arterial tonometry: an observational study

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Abstract

Introduction Sepsis has a high mortality despite advances in management. Microcirculatory and endothelial dysfunction contribute to organ failure, and better tools are needed to assess microcirculatory responses to adjunctive therapies. We hypothesised that peripheral arterial tonometry (PAT), a novel user-independent measure of endothelium-dependent microvascular reactivity, would be impaired in proportion to sepsis severity and related to endothelial activation and plasma arginine concentrations.

Methods Observational cohort study in a 350-bed teaching hospital in tropical Australia. Bedside microvascular reactivity was measured in 85 adults with sepsis and 45 controls at baseline and 2-4 days later by peripheral arterial tonometry. Microvascular reactivity was related to measures of disease severity, plasma concentrations of L-arginine (the substrate for nitric oxide synthase), and biomarkers of endothelial activation.

Results Baseline reactive hyperaemia index (RH-PAT index), measuring endothelium-dependent microvascular reactivity; (mean [95% CI]) was lowest in severe sepsis (1.57 [1.43-1.70]), intermediate in sepsis without organ failure (1.85 [1.67-2.03]) and highest in controls (2.05 [1.91-2.19]; P<0.00001. Independent predictors of baseline RH-PAT index in sepsis were APACHE II score and mean arterial pressure, but not plasma L-arginine or markers of endothelial activation. Low baseline RH-PAT index was significantly correlated with an increase in SOFA score over the first 2-4 days (r = -0.37, P = 0.02).

Conclusions Endothelium-dependent microvascular reactivity is impaired in proportion to sepsis severity and suggests decreased endothelial nitric oxide bioavailability in sepsis. Peripheral arterial tonometry may have a role as a user-independent method of monitoring responses to novel adjunctive therapies targeting endothelial dysfunction in sepsis.

Introduction

Mortality from severe sepsis remains high, despite advances in its management [1]. Organ failure commonly occurs despite the achievement of normal haemodynamics in response to fluid resuscitation, vasopressors and the treatment of infection. This may be due to impaired vasomotor regulation of the microcirculation [2]. In sepsis, the endothelium has key roles in regulating vascular tone and permeability and its activation is pivotal in initiating both the inflammatory and coagulation cascades [3].

Endothelial function is assessed clinically by the ability of blood vessels to vasodilate in response to pharmacological stimuli or to shear stress, and is primarily dependent on endothelial nitric oxide (NO) production [4]. As a result, many clinical studies investigating the endothelium in sepsis have...
measured circulating endothelial activation markers, as a surrogate for endothelial function. Current techniques for measurement of endothelial function, such as laser Doppler, plethysmography and flow-mediated dilatation of the brachial artery, require skilled operators and are technically difficult to perform at the bedside. Some studies have assessed endothelial function by measuring reactive hyperaemia in human sepsis using these operator-dependant techniques [5-10]. These studies have generally shown normal baseline blood flow and impaired reactive hyperaemic responses in sepsis, but have been small (n = 8 to 45) and have not correlated reactive hyperaemia with L-arginine or circulating markers of endothelial activation. More recently, investigators using dynamic near-infrared spectroscopy (NIRS) have found impaired microvascular responses in sepsis; however, the nature of the relation between NIRS and endothelial NO activity is unclear [11].

Reactive hyperaemia peripheral arterial tonometry (RH-PAT) is a novel, simple and user-independent bedside technique used to measure microvascular endothelial function [12] (Figure 1). It is increasingly being used to measure endothelial function as a cardiovascular risk assessment tool in ambulatory patients [12-16], including in the third-generation Framingham Heart Study cohort [17]. RH-PAT has been shown to be at least 50% dependent on endothelial NO activity [18]. RH-PAT uses finger probes to measure digital pulse wave amplitude detected by a pressure transducer, and has been validated against the operator-dependent flow-mediated dilatation method [19,20] and with endothelial function in other vascular beds, including the coronary arteries [13]. Using RH-PAT, we have demonstrated endothelial dysfunction in subjects with severe malaria [21] but it has not previously been evaluated in subjects with sepsis.

Vasodilatory shock in sepsis has been hypothesized to reflect a state of NO excess. However, several recent isotope studies have shown no net increase in NO synthesis in humans with sepsis [22-24]. To explain this, it has been proposed that sepsis may be a state of imbalance between the NOS isoforms inducible NOS and endothelial NOS in the microvasculature [25]. This could lead to a relative deficiency of endothelial NO, which is required to maintain the microvascular endothelium in a healthy, quiescent state.

Another possible reason for endothelial NO deficiency is decreased availability of L-arginine, the substrate for NOS and the precursor for NO [26]. Sepsis has been hypothesised to be an arginine-deficient state [27], although plasma L-arginine levels in humans with sepsis have been variably reported to be high [28], normal [29,30] or low [22,31,32]. Decreased

Figure 1

Representative normal and abnormal peripheral arterial tonometry traces. The tracings represent the pulse wave amplitude from a fingertip over a 15-minute period. The y axis is pulse wave amplitude in arbitrary units (derived from millivolts). The top trace was taken from a control subject whose reactive hyperaemia peripheral arterial tonometry; (RH-PAT) index was 1.98, and the bottom from a severe sepsis subject whose RH-PAT index was 1.16. The horizontal axis is time. The first shaded section is averaged as a baseline signal. The middle section is arterial occlusion, with consequent loss of the pulse wave signal. The final section is the pulse wave amplitude following release of the cuff. The random vertical spikes are movement artefacts. In the top trace there is reactive hyperaemia, with an increase in average pulse wave amplitude. The shaded post-occlusion section is compared with the shaded baseline section to give a ratio -- the RH-PAT index.
plasma L-arginine has been linked to decreased NO production in animal and in vitro models [33].

We hypothesised that RH-PAT would be a feasible technique to measure microvascular reactivity in sepsis and that microvascular reactivity would be impaired in subjects with sepsis in proportion to disease severity. Our secondary hypotheses were that microvascular reactivity would correlate with plasma L-arginine and measures of endothelial activation, and that plasma L-arginine concentrations would be decreased in sepsis.

**Materials and methods**

**Study design and setting**

We performed a prospective observational cohort study in a 350-bed teaching hospital in tropical northern Australia, with an 18-bed mixed intensive care unit (ICU). Approval was obtained from Human Research Ethics Committee of the Menzies School of Health Research and the Department of Health and Community Services, Darwin. Written informed consent was obtained from all participants or next of kin.

**Participants**

Between March 2006 and November 2007, all adult subjects (≥ 18 years) admitted to the hospital were screened regarding eligibility for the study. Inclusion criteria for sepsis subjects were: suspected or proven infection; presence of two or more criteria for the systemic inflammatory response syndrome within the preceding four hours [34]; and admission to ICU within the preceding 24 hours or to the wards within the preceding 36 hours. Exclusion criteria were coagulopathy (platelets ≤ 20 × 10^9/L, activated partial thromboplastin time ≥ 70 seconds, international normalized ratio ≥ 2.0); smoking of tobacco within the preceding four hours; and current administration of intravenous nitrates. Control subjects were recruited from hospital patients with no clinical or laboratory evidence of inflammation or infection, and who had not met systemic inflammatory response syndrome criteria within the preceding 30 days. Severe sepsis was defined as sepsis with organ dysfunction or shock at the time of enrolment according to American College of Chest Physicians/Society of Critical Care Medicine consensus criteria [34,35].

**Measurement of microvascular reactivity**

Sepsis subjects underwent standardised demographic and clinical data collection, bedside RH-PAT measurement (Endopat 2000, Itamar Medical, Caesarea, Israel), and blood collection at days 0 and 2 to 4. All studies were performed after resuscitation and at least one hour of haemodynamic stability (defined as no change in vasopressor dose or need for fluid boluses) in a quiet room at 25°C, with the patient recumbent. Control subjects had the same assessment at a single time point.

In this study, probes were placed on the index fingers of both hands of all patients, or on other fingers if the index fingers were not suitable. Digital pulse wave amplitude was recorded from both hands for a resting baseline period of five minutes and then a blood pressure cuff was rapidly inflated on the study arm up to 200 mmHg, or 50 mmHg above systolic blood pressure, whichever was greater. After five minutes ± 10 seconds, the cuff was deflated. Pulse wave amplitude was then recorded for a further five minutes. An automated computerised algorithm provided by the manufacturer (Endo-PAT 2000 software version 3.1.2, Itamar Medical, Caesarea, Israel) was used to calculate a post occlusion-pre occlusion ratio (RH-PAT index), thus making the measurements user independent. The software also normalises the RH-PAT index to the control arm to correct for changes in systemic vascular tone (Figure 1).

There was no systematic difference between RH-PAT indices generated by different observers. We have previously examined the reproducibility of RH-PAT measurements by repeating them after 0.5 to 0.75 hours in 37 healthy adults [21]. Reproducibility was acceptable according to the method of Bland and Altman [36], and was comparable with previous reproducibility results for RH-PAT [37] and with those obtained with the flow-mediated dilatation method [38].

**Laboratory assays**

Blood was collected in lithium heparin tubes at each time point and the plasma was frozen. Plasma arginine concentrations were determined using high-performance liquid chromatography, with a method modified from van Wandelen and Cohen [39]. To assess circulating measures of endothelial activation, intra-cellular adhesion molecule-1 (ICAM1) and E-selectin were measured by ELISA (R&D Systems, Minneapolis, Minnesota, USA). Plasma IL-6 was measured by flow cytometry using a cytokine bead array (BD Biosciences, San Jose, California, USA). *Ex vivo* plasma arginine activity causes significant degradation of L-arginine at room temperature [40], thus only L-arginine levels derived from blood frozen within 30 minutes of collection were included in the analysis.

**Statistical methods**

Predefined groups for analysis were sepsis without organ failure, severe sepsis and controls. Continuous variables were compared using Student’s t-test and analysis of variance or Mann Whitney U test for parametric and non-parametric variables, respectively. Categorical variables were compared using Fisher’s exact test. Correlates with baseline RH-PAT index were determined using Pearson’s (parametric) or Spearman’s (non-parametric) coefficient for univariate analysis. For multivariate analysis, linear regression with backward selection was used. To examine longitudinal correlations, linear mixed-effects models were used. A two-sided *P* value of <0.05 was considered significant. All analyses were performed using Stata version 10 (Stata Corp, College Station, Texas, USA).
Results

Participants

Over the 19-month study period, 85 subjects with sepsis and 45 control subjects were enrolled. Of the sepsis subjects, 54 had organ failure due to sepsis at baseline (severe sepsis group) and 31 did not (sepsis without organ failure). The three groups were well matched in terms of risk factors for endothelial dysfunction and other baseline characteristics (Table 1). Of the 85 sepsis subjects, 92% had community-acquired sepsis, with no preceding trauma or surgery, and pneumonia was the most common focus of infection.

Baseline microvascular reactivity

Baseline microvascular reactivity was impaired in sepsis subjects compared with controls ($P < 0.0001$; Table 2). Mean RH-PAT index was lowest in the severe sepsis group (1.57, 95% confidence interval (CI): 1.43 to 1.70), intermediate in the sepsis without organ failure group (1.85, 95% CI: 1.67 to 2.03), and highest in the control group (2.05, 95% CI: 1.91 to 2.19; $P < 0.00001$; Figure 2). Subjects with severe sepsis were more likely to have endothelial dysfunction than control subjects (odds ratio (OR) 9.4, 95% CI: 3.5 to 25.0). This relation persisted after controlling for known associations with and risk factors for endothelial dysfunction (diabetes, smoking, ischaemic heart disease, chronic renal disease, hypercholesterolaemia, hypertension, statin use and age; adjusted OR 17.0, 95% CI: 5.0 to 58.0). Within the severe sepsis group, mean RH-PAT index was not significantly different in the 27 subjects requiring vasopressors (1.48, 95% CI: 1.30 to 1.66) than in those not requiring vasopressors (1.64, 95% CI: 1.39 to 1.89; $P = $ not significant (NS)). In those receiving noradrenaline ($n = 25$), there was no correlation between RH-PAT index and noradrenaline dose ($r = 0.19$, $P = NS$). There was also no relation between body temperature and RH-PAT index. Males

Table 1

<table>
<thead>
<tr>
<th>Baseline characteristics of participants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Severe sepsis</td>
</tr>
<tr>
<td>N</td>
</tr>
<tr>
<td>Age$^b$</td>
</tr>
<tr>
<td>Male n (%)</td>
</tr>
<tr>
<td>Diabetic n (%)</td>
</tr>
<tr>
<td>Smoker n (%)</td>
</tr>
<tr>
<td>IHD n (%)</td>
</tr>
<tr>
<td>On statin n (%)</td>
</tr>
<tr>
<td>APACHE II$^c$</td>
</tr>
<tr>
<td>SOFA score$^c$</td>
</tr>
</tbody>
</table>

Focus of infection -- n (%)

| Pleuropulmonary n (%) | 26 (48) | 16 (52) |
| Skin/soft tissue n (%) | 9 (17) | 9 (29) |
| Intra-abdominal n (%) | 6 (11) | 1 (3) |
| Urinary n (%) | 4 (7) | 3 (10) |
| Other n (%) | 9 (17) | 2 (6) |

Causative organism

| None cultured n (%) | 25 (46) | 20 (65) |
| Gram positive bacterium n (%) | 15 (28) | 5 (16) |
| Gram negative bacterium n (%) | 14 (26) | 6 (19) |

Origin of sepsis

| Community-acquired n (%) | 47 (87) | 30 (97) |
| Nosocomial n (%) | 7 (13) | 1 (3) |

---

a. For difference between all three groups by one way analysis of variance
b. Mean (95% confidence interval)
c. Median (interquartile range)
APACHE II = Acute Physiology and Chronic Health Evaluation II; IHD = ischaemic heart disease; NS = not significant; SOFA = Sequential Organ Failure Assessment score
(1.76, 95% CI: 1.62 to 1.89) had higher baseline microvascular reactivity than females (1.50, 95% CI: 1.32 to 1.68; \(P = 0.02\)).

RH-PAT was well tolerated by all subjects. In 18 of 227 measurements (8%), a result was not obtainable. This occurred in 15 of 182 measurements (8%) in sepsis subjects and 3 of 45 (7%) in controls and was due either to inability to obtain a baseline pulse wave reading, or failure to completely occlude forearm blood flow due to oedema.

Plasma markers of endothelial activation (ICAM-1 and E-selectin) were both significantly raised in sepsis subjects compared with controls (Table 2); however, they did not correlate with RH-PAT index. Blood lactate levels were routinely measured only in subjects with severe sepsis, in whom the baseline median lactate was 1.6 mmol/L (range 0.5 to 12.7; interquartile range (IQR) 1.0 to 2.3). Among severe sepsis subjects, lactate correlated inversely with RH-PAT index, but this was not statistically significant (\(r = -0.28, P = 0.06\)).

Among all sepsis subjects, baseline RH-PAT index correlated with mean arterial pressure (MAP; \(r = 0.55, P < 0.0001\) ) and serum albumin (\(r = 0.27, P = 0.03\)), and was inversely related

### Table 2

<table>
<thead>
<tr>
<th>RH-PAT index and related variables at time of initial measurement</th>
<th>Severe sepsis</th>
<th>Sepsis without organ failure</th>
<th>Control</th>
<th>(P) value pooled sepsis v control</th>
<th>(P) value severe sepsis vs SWOF</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>54</td>
<td>31</td>
<td>45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RH-PAT index (a)</td>
<td>1.57 (1.43-1.70)</td>
<td>1.85 (1.67-2.03)</td>
<td>2.05 (1.91-2.19)</td>
<td>&lt; 0.00001</td>
<td>0.01</td>
</tr>
<tr>
<td>Plasma L-arginine ((\mu\text{mol/L}))</td>
<td>35.8 (30.2-41.4)</td>
<td>40.9 (33.5-48.3)</td>
<td>80.4 (72.3-88.6)</td>
<td>&lt; 0.00001</td>
<td>NS</td>
</tr>
<tr>
<td>MAP (mmHg) (a)</td>
<td>77 (74-81)</td>
<td>89 (83-95)</td>
<td>83 (79-87)</td>
<td>NS</td>
<td>0.0006</td>
</tr>
<tr>
<td>Receiving vasopressors n (%)</td>
<td>27 (50)</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Noradrenaline dose ((\mu\text{g/kg/min})b, c)</td>
<td>0.08 (0.03-0.42)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Receiving assisted ventilation n (%)</td>
<td>20 (37)</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CVP (cmH2O) (a)</td>
<td>12.2 (10.3-14.1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma ICAM-1 (ng/ml)b</td>
<td>811 (500-1502)</td>
<td>507 (368-673)</td>
<td>323 (252-397)</td>
<td>&lt; 0.00001</td>
<td>0.003</td>
</tr>
<tr>
<td>Plasma E-selectin (ng/ml)b</td>
<td>329 (138-502)</td>
<td>90 (51-164)</td>
<td>38 (26-63)</td>
<td>&lt; 0.00001</td>
<td>0.0003</td>
</tr>
<tr>
<td>Plasma IL 6 (pg/ml)b</td>
<td>385 (124-996)</td>
<td>148 (46-315)</td>
<td>5 (2-8)</td>
<td>&lt; 0.00001</td>
<td>0.009</td>
</tr>
<tr>
<td>White blood cell count (a)</td>
<td>16.7 (14.2-19.2)</td>
<td>15.5 (13.3-17.7)</td>
<td>8.4 (6.9-9.8)</td>
<td>&lt; 0.00001</td>
<td>NS</td>
</tr>
<tr>
<td>C-reactive protein (b)</td>
<td>190 (131-255)</td>
<td>102 (84-234)</td>
<td>7 (3-24)</td>
<td>&lt; 0.00001</td>
<td>NS</td>
</tr>
</tbody>
</table>

\(a\). mean (95% confidence interval)

\(b\). Median (interquartile range)

\(c\). Of 27 patients receiving vasopressors, 25 were receiving noradrenaline

\(d\). Severe sepsis n = 30, sepsis without organ failure n = 26, control n = 2.

CVP = central venous pressure; ICAM = intra-cellular adhesion molecule 1; NS = not significant; MAP = mean arterial pressure; RH-PAT = reactive hyperaemia peripheral arterial tonometry; SWOF = sepsis without organ failure.
to Acute Physiology and Chronic Health Evaluation (APACHE II) score ($r = -0.36, P = 0.002$), C-reactive protein ($r = 0.30, P = 0.02$) and the cardiovascular component of the Sequential Organ Failure Assessment (SOFA) score ($r = -0.29, P = 0.01$), but not with total SOFA score. Independent predictors of baseline RH-PAT index on multivariate analysis were APACHE II score ($\beta = -0.014, P = 0.03$) and MAP ($\beta = 0.012, P < 0.0001$).

**Baseline plasma L-arginine**

In the subjects whose blood samples were processed within 30 minutes of collection, baseline mean plasma L-arginine concentration was significantly lower in sepsis subjects (38.6 μmol/L, 95% CI: 34.2 to 43.1; $n = 56$) than in controls (80.3 μmol/L, 95% CI: 72.5 to 88.1; $n = 27$; $P < 0.0001$). There was no significant difference in L-arginine levels between severe sepsis and sepsis without organ failure (Figure 3). When all subjects including controls were considered, baseline plasma L-arginine correlated with baseline RH-PAT index ($r = 0.32, P = 0.007$); however, this association was no longer significant when stratified by disease severity.

**Longitudinal changes in RH-PAT and L-arginine**

Longitudinal RH-PAT readings were only available in 70% of subjects. There was no difference in disease severity, as measured by APACHE II score, in those with (median 14, IQR 8 to 23) and without (median 15.5, IQR 8.5 to 20.5; $P = NS$) longitudinal data. In sepsis subjects, there was no statistically significant change in mean RH-PAT index from baseline to day 2 to 4 (95% CI: 1.67 to 1.85, $P = NS$; Figure 3). The same was true in the severe sepsis subgroup (95% CI: 1.57 to 1.76, $P = NS$). In contrast, mean plasma L-arginine concentrations significantly increased from baseline to day 2 to 4 (95% CI: 38.2 to 49.9 μmol/L, $P = 0.01$). In a mixed-effects linear regression model, change in microvascular reactivity over the first 2 to 4 days of treatment correlated significantly with increasing MAP and decreasing C-reactive protein, but not with change in plasma L-arginine.

**Subject outcomes**

Low baseline RH-PAT index was significantly correlated with an increase in SOFA score over the first 2 to 4 days ($r = -0.37, P = 0.02$). In subjects whose SOFA score worsened over the first 2 to 4 days, the median RH-PAT index was 1.54, compared with 1.74 in those whose SOFA score improved or did not change ($P = 0.01$). At both hospital discharge and 28-day follow-up, 8 of 85 (9%) subjects with sepsis had died. Among those with septic shock at baseline, 6 of 29 (21%) had died at 28-day follow-up. The mean baseline RH-PAT index was 1.67 among survivors and 1.60 among non-survivors ($P = NS$). The strongest baseline predictors of death on univariate analysis were APACHE II score ($P = 0.008$), SOFA score ($P = 0.002$) and IL-6 level ($P = 0.004$).

**Discussion**

To the authors’ knowledge, this is the largest published study to date assessing reactive hyperaemia in human sepsis and the first to use peripheral arterial tonometry. We have found that endothelium-dependent microvascular reactivity is impaired in sepsis, in proportion to disease severity, even after controlling for known associations with endothelial dysfunction, suggesting that sepsis itself is the explanation for the observed impairment in microvascular reactivity, rather than traditional cardiovascular risk factors. Furthermore, the degree of impairment of baseline microvascular reactivity predicted subsequent deterioration in organ function.

RH-PAT proved to be a practical and feasible method of measuring microvascular reactivity at the bedside in critically ill septic subjects, with a low proportion of technical failures, which were no more common in sepsis subjects than in controls, and which showed no relation with noradrenaline dose. The findings of this study are generally consistent with those of the previous small studies of reactive hyperaemia in adult subjects with sepsis using other methods, which were generally user-dependent and of limited availability.
Plasmodymographic measures of forearm blood flow in sepsis have found a post occlusion-pre occlusion ratio of 1.6 [9] and forearm skin laser Doppler studies have found a ratio of 1.4 [5]. These results are very similar to our observed ratio of 1.57, suggesting that the finding of impaired reactive hyperaemia in adults with sepsis is a true phenomenon, which is independent of the method used to measure it.

Compared with laser Doppler flowmetry, venous plethysmography and flow-mediated dilatation of the brachial artery, PAT requires less staff training and simpler equipment, has less potential for inter-observer variability, and is easier to perform on uncooperative patients. PAT has also been validated with regards to accuracy [13,19,20] and reproducibility [37,41]. Disadvantages of PAT include the expense of disposable finger probes.

Because RH-PAT is at least 50% NO-dependent [18], impaired RH-PAT responses in sepsis suggest reduced endothelial NO bioavailability. Our results are in accord with increasing data from radiolabelled arginine flux studies suggesting that NO synthesis is decreased in sepsis [22-24]. Impaired RH-PAT has been demonstrated to be reversible with L-arginine infusion in malaria caused by Plasmodium falciparum, providing direct evidence for NO dependence in acute inflammatory states [21]. However, we cannot exclude contributions by other mechanisms, including impaired production of prostacyclin and endothelium-derived hyperpolarizing factor [42,43].

There was a significant correlation between plasma L-arginine and microvascular reactivity when all subjects were considered together, but this was not significant within groups. Furthermore, the improvement of plasma L-arginine over the first 2 to 4 days was not significantly correlated with change in microvascular reactivity. These findings suggest that NO production and endothelial function in sepsis are influenced by other factors in addition to circulating L-arginine. Such factors may include an increase in competitive inhibitors of NOS, such as asymmetric dimethylarginine [44]; deficiency of NOS cofactors such as tetrahydrobiopterin; NO quenching by microvascular reactive oxygen intermediates [45]; and the enhanced local expression and activity of endothelial cell arginase [46]. The observation of higher microvascular reactivity in males compared with females is an unexpected finding; previous studies have found better microvascular function in females than males, both in non-inflammatory states [47] and in response to infusion of lipopolysaccharide [48]. However, gender-specific microvascular function has not previously been reported in sepsis.

The marked hypoargininaemia, which we found in subjects with sepsis, supports the hypothesis that L-arginine is decreased in sepsis, independent of trauma [27]. This finding is strengthened by the fact that we only included subjects within 24 to 36 hours of admission, with standardised sepsis criteria and with more than 90% having community-acquired sepsis.

Targeting tissue oxygen delivery [49] or the splanchnic microcirculation [50] as resuscitation goals in sepsis have not been shown to improve outcomes. What, then, is the significance of monitoring the microvascular endothelium in sepsis? Endothelial cells have multiple roles in sepsis pathophysiology, including the regulation of microcirculatory vasomotor tone and the regulation of coagulation, immune and inflammatory responses and microvascular barrier function. Preliminary studies aimed at increasing endothelial NO bioavailability in sepsis have shown promising results [51] and the interventions which have been demonstrated to improve outcomes in sepsis (activated protein C [52], early goal directed therapy [53] and intensive insulin therapy [54]) could all potentially be mediated, at least in part, via attenuation of endothelial cell dysfunction [55]. Thus, monitoring of microvascular and endothelial function are likely to be important components of future trials of adjunctive treatments in sepsis.

Our study has several potential limitations. Baseline blood flow measurements were not available, and it is possible that the apparent decrease in reactive hyperaemia in sepsis is an artefact of marked baseline vasodilatation. This could potentially limit the subjects’ ability to respond to ischaemia by increased blood flow, because they already have near-maximal vasodilatation. This is unlikely to be the case because baseline forearm blood flow in septic subjects has been found to be normal or decreased by multiple investigators [6,7,10,56]. Furthermore, skeletal muscle has the capacity to increase blood flow by up to 10-fold [57], which greatly exceeds the increase seen in both healthy and septic subjects in this and other studies.

Although we controlled for the major factors influencing endothelial function, we cannot exclude minor influences of altered thyroid or adrenal function. Due to variations in sample processing time, we were unable to determine accurate plasma arginine values for all subjects. Thus the reported arginine values may not be fully representative of the groups as a whole. Of the subjects who had an initial measurement of RH-PAT index, 70% had a repeat measurement 2 to 4 days later. Although those who were followed up had a similar baseline APACHE II score to those who were followed up, this may not have been a representative population, because subjects who rapidly improved and were discharged home did not have repeat measurements. Thus the observed degree of recovery in microvascular reactivity is likely to be an underestimate.

The mortality rate in this cohort was low (hospital and 28-day mortality 9% overall and 21% among those with septic shock). Although this is consistent with the relatively low mortality rate in severe sepsis previously documented in our ICU [35], it
does mean that the study may have been underpowered to
detect associations of measured variables with mortality.

Conclusions
In summary, we have found that peripheral arterial tonometry is a
feasible tool for measuring microvascular reactivity in sepsis,
and that it is impaired in sepsis in proportion to disease severity,
suggesting reduced endothelial function and decreased
endothelial NO bioavailability. Baseline RH-PAT was useful in
predicting subsequent deterioration in organ dysfunction,
although this should be reproduced by other investigators
before its clinical utility can be confirmed. Given the growing
interest in HMG CoA reductase inhibitors [58] and other
potential adjunctive therapies targeting the endothelium in
sepsis [55], better tools for monitoring the response of the
endothelium in clinical trials are needed. RH-PAT is an attrac-
tive option for such studies, as other current methods are user-
dependent and have limited availability.

Key messages
• Current tools for assessing endothelial function in
  patients with sepsis are generally user dependant and
  are not widely available.
• Peripheral arterial tonometry, a simple, user-independ-
  ent technique for measuring endothelium-dependent
  microvascular reactivity is feasible in patients with
  sepsis.
• Endothelium-dependent microvascular reactivity is
  impaired in sepsis, in proportion to disease severity, and
  may predict subsequent deterioration in organ function.

Competing interests
DC has received research support (as equipment) from Itamar
Medical, the manufacturer of the RH-PAT device, and has
received speaker’s fees (less than US$1000 per year) for
speaking at Itamar-sponsored educational events. The other
authors have no competing interests.

Authors’ contributions
Study design was performed by JSD, NMA, TWY, DPS and
DSC. Patient recruitment was carried out by JHT, MM, JSD
and DPS. The data was processed by JSD and MM, and was
analysed by JSD with help from ACC, TWY and NMA. Labo-
roatory sample processing and HPLC assays were performed
by CJD and YRM. The manuscript was drafted by JSD and
NMA. All authors had access to all data and contributed to the
final draft of the paper. All authors read and approved the final
manuscript.

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Joseph McDonnell for statistical advice; and the medical and nursing
staff of the Royal Darwin Hospital Intensive Care and Hospital in the
Home units.

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The funding source played no role in the design or conduct of the study,
nor in the drafting of the manuscript or the decision to submit it for
publication.

References
1. Angus DC, Pereira CA, Silva E: Epidemiology of severe sepsis


Available online http://ccforum.com/content/13/5/R155
10.2 Previously unpublished Supplementary figures for the Crit Care paper

Figure 10-1. Baseline plasma L-arginine concentration is decreased in sepsis.

Circles represent individual patients. Horizontal lines represent mean values for the group. P values indicate pair-wise comparisons between groups.
Figure 10-2. Baseline plasma soluble E-selectin and Intercellular adhesion molecule-1 (ICAM-1) across disease categories.

Circles represent individual patients. Horizontal lines represent median values for the group. P values indicate pair-wise comparisons between groups.
10.3 Implications of and questions arising from this paper.

1) Peripheral arterial tonometry is a feasible, practical and user-independent method of monitoring microvascular reactivity in patients with both non-severe and severe sepsis, including those receiving vasopressor infusions.
   - RH-PAT is an alternative to the more complex and user-dependent methods of flow-mediated dilatation of the brachial artery, venous plethysmography and laser Doppler flowmetry in studies of endothelial pathophysiology in sepsis.
   - Preliminary data from this study were used to inform the study design and sample-size analysis of a randomised trial of atorvastatin to improve endothelial dysfunction in severe sepsis, described in chapter 17.

2) These data significantly expand the existing literature on reactive hyperaemia in adult sepsis at the time of study commencement, adding data from a further eighty-five patients with sepsis to a total previously reported eighty-four, and confirming that post-ischaemic microvascular reactivity is impaired in patients with sepsis in proportion to disease severity. The fact that peripheral arterial tonometry is at least 50% NO-dependent strengthens the evidence that endothelial NO bioavailability is decreased in sepsis.
   - This challenges the accepted wisdom that sepsis is a state of global NO excess, and identifies an important area to target with novel adjunctive sepsis therapies

3) The study confirms that plasma L-arginine is acutely decreased in early community-acquired sepsis, independent of trauma (Figure 10-1), and makes a substantial contribution to the systematic review of plasma L-arginine concentrations in sepsis reported in Chapter 4.

4) The finding that impaired microvascular reactivity may predict subsequent deterioration in organ function suggests that peripheral arterial tonometry may be a useful prognostic test in sepsis, but this needs to be confirmed in larger numbers and other cohorts.
5) Neither ICAM-1 nor E-selectin correlated with RH-PAT index in this study, nor have they been shown to do so with other clinical measures of endothelial function. This has two major implications:

- Studies of endothelial function in sepsis should employ functional measures rather than just blood tests
- There may be better plasma markers of endothelial activation than ICAM-1 and E-selectin. Hence in Chapter 12, the relationship between angiopoietin-2, a novel plasma marker of endothelial activation, disease severity and microvascular reactivity is explored.

6) Contrary to expectation, plasma L-arginine (the precursor of nitric oxide) did not correlate with RH-PAT index, a measure of endothelial NO bioavailability.

- One of the main possible explanations for this is competitive inhibition by asymmetric dimethyl arginine, a naturally occurring inhibitor of nitric oxide synthase. This hypothesis is explored in Chapter 11.
Chapter 11. Asymmetric dimethyl arginine, endothelial function and mortality in sepsis.
11.1 Preamble

Although the data reported in Chapter 10 support the hypothesis that microvascular reactivity (as measured by RH-PAT) would be impaired in sepsis, they did not show the expected relationship between microvascular reactivity and plasma L-arginine concentration. Since plasma L-arginine is the substrate for nitric oxide synthase (NOS) and the precursor of NO, and RH-PAT is an estimate of endothelial nitric oxide bioavailability, one would expect a relationship between them.

There are several possible explanations for the observed lack of correlation: Plasma arginine might not reflect the concentration of L-arginine inside endothelial cells; Since RH-PAT index is only 50% NO-dependent, the FRESH study might be underpowered to detect such a correlation; Factors other than L-arginine concentration, such as NO-quenching by oxidative stress might be more important in determining endothelial NO bioavailability in sepsis; Finally, competitive inhibition of NOS by asymmetric dimethyl arginine (ADMA), an endogenous NOS inhibitor, might account for the lack of observed correlation between plasma L-arginine and RH-PAT index.

ADMA has been extensively studied in chronic vascular disease as a contributor to endothelial dysfunction and as a cardiovascular risk marker [1]. However, its role in critical illness and acute infections is only beginning to emerge. Some [2], but not other [3, 4] studies have shown that ADMA is associated with mortality in critical illness and in severe sepsis. Hence we carried out the following study, using data and plasma from the FRESH cohort, to address the hypotheses that plasma L-arginine : ADMA ratio would be a stronger correlate of RH-PAT index than plasma L-arginine alone, and that raised plasma ADMA concentrations would be predictive of mortality.

The manuscript presented in this chapter has been submitted to Intensive Care Medicine.
11.2 Abstract

Introduction
Plasma concentrations of asymmetric dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide synthase, are raised in patients with chronic vascular disease, causing increased cardiovascular risk and endothelial dysfunction, but the role of ADMA in acute inflammatory states is less well defined.

Methods
Prospective longitudinal study in 67 patients with sepsis and 31 controls in the intensive care unit and wards of an Australian teaching hospital. Digital microvascular reactivity was measured by peripheral arterial tonometry and blood was collected at baseline and 2-4 days later. Plasma ADMA concentrations were determined by high performance liquid chromatography (HPLC).

Results
Baseline plasma ADMA was not significantly elevated in sepsis patients (Median [IQR] 0.53 [0.39-0.66] µmol/L) compared with controls (0.57 [0.50-0.62] µmol/L), but it had increased significantly by day 2-4 (0.64 [0.51-0.78], p=0.002). Baseline plasma ADMA was independently associated with 28-day mortality (Odds ratio [95% CI] for death in those in the highest quartile ≥0.66µmol/L=20.8 [2.2-195.0], p=0.008). Plasma L-arginine: ADMA ratio was inversely associated with sepsis severity, and correlated with microvascular reactivity more strongly than did plasma L-arginine concentration. Baseline plasma ADMA was independently correlated with severity of organ failure (SOFA score) and increase in ADMA over time correlated with increase in SOFA score and decrease in microvascular reactivity.

Conclusions
Plasma ADMA is a predictor of mortality in sepsis and correlates with the degree of organ failure. Impaired endothelial and microvascular function due to decreased endothelial NO bioavailability is a potential mechanism linking increased plasma ADMA with organ failure and death in sepsis.
11.3 Introduction

Asymmetric dimethylarginine (ADMA), an endogenous non-specific nitric oxide synthase (NOS) inhibitor, is associated with chronic endothelial dysfunction [5] and increased cardiovascular risk [1], but its role in the setting of acute infections has been less well characterised.

Severe sepsis is the leading cause of death in intensive care units in the USA [6], and is increasing in incidence globally [7]. Microvascular and endothelial dysfunction are key contributors to organ failure and death in sepsis but the mechanisms linking sepsis with vascular dysfunction remain incompletely understood [8]. A relative deficiency of constitutively expressed endothelial nitric oxide (NO), essential to maintain a quiescent and functional endothelium, may underlie sepsis-associated endothelial and microvascular dysfunction [9, 10]. NO is produced by NOS from its primary substrate, L-arginine. ADMA competitively inhibits the production of NO by NOS and additionally, along with symmetrical dimethylarginine (SDMA) and L-lysine, competes with L-arginine for transport across the cell membrane [11]. Hence the L-arginine : ADMA ratio is considered a better indicator of the availability of L-arginine to NOS than is plasma L-arginine concentration alone [12].

Infusion of ADMA in both rats [13] and humans [14] acutely decreases NO production, resulting in endothelial dysfunction. Plasma ADMA concentrations are increased in patients with chronic renal disease [15], hypertension [16], diabetes mellitus [17] and peripheral vascular disease [18]. Furthermore, ADMA has been shown to be an independent predictor of cardiovascular events in patients with existing coronary artery disease [19] and end-stage renal disease [20].

In contrast, few studies have examined the role of ADMA in sepsis, and none have reported L-arginine : ADMA ratios or examined microvascular reactivity in this context. The few clinical studies that have reported plasma ADMA concentrations during acute infection have had conflicting results [3, 4, 21]. Using peripheral arterial tonometry, we have previously shown that digital microvascular reactivity, a measure of endothelial NO bioavailability [22], is decreased in patients with sepsis [10]. However, we did not find a correlation between concentrations of plasma L-arginine and microvascular reactivity. We also found that despite an increase in plasma L-arginine concentrations over time, there
was no corresponding improvement in microvascular reactivity. A potential explanation for these findings in sepsis is competitive inhibition of NOS by ADMA.

We hypothesised that plasma ADMA concentrations would correlate with disease severity, would predict mortality and would be raised in patients with sepsis. We also hypothesised that the L-arginine : ADMA ratio would correlate more strongly with reactive hyperaemia peripheral arterial tonometry (RH-PAT) index, an in vivo measure of endothelial NO bioavailability, than would L-arginine alone.

11.4 Methods

Study design and setting

We performed a prospective observational study at a 350-bed Australian teaching hospital, with an 18-bed mixed intensive care unit (ICU). Approval was obtained from the Human Research Ethics Committee of the Menzies School of Health Research and the Department of Health and Community Services. Written informed consent was obtained from all participants or next of kin where necessary.

Participants

Study subjects were adults (≥18 years) hospitalised with sepsis, who were enrolled in a previously-reported study of microvascular reactivity [10]; more detail of subject recruitment and study procedures are provided in this paper [10].

Sepsis was defined as a proven or suspected infection plus at least two criteria for the systemic inflammatory response syndrome (SIRS) present within the last 4 hours [23]. Septic patients were eligible for enrolment within 24 hours of their admission to the ICU, or within 36 hours of admission to the ward. Control subjects were adults recruited from hospitalised patients with no clinical or laboratory evidence of inflammation or infection, and who had not met SIRS criteria within the last 30 days. Septic patients were classified as septic shock, or sepsis without shock. Septic shock was defined at the time of enrolment as systolic blood pressure <90mmHg or a reduction of ≥ 40mmHg from baseline despite adequate fluid resuscitation, or the need for vasopressors to maintain these targets [23].
Laboratory assays

Blood from arterial lines if present, or venepuncture if not, was collected in lithium heparin tubes at baseline and 2-4 days later, and plasma was separated and stored at -70°C within 2 hours of blood collection. Control patients had blood collected at baseline only. ADMA and SDMA were measured by reverse phase HPLC with simultaneous fluorescence and UV-visible detection, as previously described [24]. IL-6 and TNFα were measured by flow cytometry using a cytokine bead array (BD Biosciences, CA, USA).

Measurement of microvascular reactivity

Microvascular reactivity was measured at the bedside by RH-PAT (Itamar Medical, Caesarea, Israel), a non-invasive method of assessing endothelial function [25] [26] which is at least 50% dependent on endothelial NO production [22]. Peripheral arterial tonometry (PAT) was measured in a fingertip before and after a 5-minute ischemic stress at the forearm, generating an RH-PAT index, normalized to the control arm, as previously reported [10].

Statistical methods

Continuous variables were compared using Mann Whitney U test, and categorical variables using Fisher’s exact test. Correlates with baseline ADMA were determined using Spearman’s coefficient for univariate analysis. Day 2 values were compared with baseline values using paired Wilcoxon signed-rank test. The relationship between baseline ADMA and mortality among sepsis patients was examined using logistic regression with ADMA divided into quartiles, as previously described [2]. To examine longitudinal correlations, linear mixed-effects models were used. A 2-sided p-value of <0.05 was considered significant. All analyses were performed using Intercooled Stata 10 (Statacorp, Texas).

11.5 Results

There were 20 subjects with septic shock, 47 with sepsis without shock and 31 controls. The three groups were well-matched in terms of age, sex and known associations with chronically raised ADMA (Table 11-1).

Baseline plasma ADMA concentrations

Median [IQR] baseline plasma ADMA concentrations were not significantly different in pooled sepsis patients (0.53 [0.39-0.66] µmol/L) or in septic shock patients (0.64 [0.54-0.85] µmol/L) than in controls (0.58 [0.50-0.62] µmol/L) (Table 11-2, figure 11-1). However,
patients with sepsis without shock had significantly lower baseline ADMA (0.47 [0.38-0.57] µmol/L) than both septic shock patients (p=0.008) and controls (p=0.002). Plasma L-arginine: ADMA ratio was significantly lower in sepsis patients than controls, with septic shock patients having approximately three-fold lower L-arginine: ADMA ratios than control patients (Table 11-2, figure 11-2).

**ADMA, SDMA and outcomes**

Six of 67 sepsis patients (9%) had died by day 28 of follow-up, five of whom were in the septic shock subgroup. Median [IQR] baseline ADMA was approximately twice as high in those who died (1.07 [0.75-1.31]) as in survivors (0.51 [0.39-0.61]), p=0.001. Sepsis patients with a baseline plasma ADMA concentration in the highest quartile (≥0.66 µmol/L) had an odds ratio for death of 20.8 (95% CI 2.2-195.0, p=0.008). In a multivariate model incorporating SOFA score, age, gender and IL-6 concentration, baseline ADMA was the only significant predictor of death (p=0.04).

**Table 11-1. Baseline characteristics of study participants**

<table>
<thead>
<tr>
<th></th>
<th>Septic Shock</th>
<th>Sepsis without shock</th>
<th>Controls</th>
<th>p value&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>20</td>
<td>47</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>Age&lt;sup&gt;b&lt;/sup&gt;</td>
<td>51.5 (12.0)</td>
<td>52.5 (14.4)</td>
<td>45.4 (12.7)</td>
<td>NS</td>
</tr>
<tr>
<td>Male&lt;sup&gt;c&lt;/sup&gt;</td>
<td>11 (55)</td>
<td>30 (63)</td>
<td>24 (75)</td>
<td>NS</td>
</tr>
<tr>
<td>Diabetic&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6 (30)</td>
<td>13 (27)</td>
<td>10 (31)</td>
<td>NS</td>
</tr>
<tr>
<td>Smoker&lt;sup&gt;c&lt;/sup&gt;</td>
<td>8 (40)</td>
<td>22 (46)</td>
<td>14 (44)</td>
<td>NS</td>
</tr>
<tr>
<td>IHD&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4 (20)</td>
<td>8 (17)</td>
<td>4 (13)</td>
<td>NS</td>
</tr>
<tr>
<td>Hypertension&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5 (25)</td>
<td>17 (35)</td>
<td>9 (28)</td>
<td>NS</td>
</tr>
<tr>
<td>Hyperlipidemia&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4 (20)</td>
<td>11 (22)</td>
<td>11 (34)</td>
<td>NS</td>
</tr>
<tr>
<td>Chronic disease renal disease&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4 (20)</td>
<td>4 (8)</td>
<td>3 (10)</td>
<td>NS</td>
</tr>
<tr>
<td>APACHE II score&lt;sup&gt;d&lt;/sup&gt;</td>
<td>20.0 (16-23)</td>
<td>10.0 (6-16)</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>SOFA score&lt;sup&gt;d&lt;/sup&gt;</td>
<td>6 (3-9)</td>
<td>2.0 (0.5-4.0)</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> – by Chi<sup>2</sup> test for difference between all 3 groups  
<sup>b</sup> – Mean (sd)  
<sup>c</sup> – n (%)  
<sup>d</sup> – Median (Interquartile range)
SDMA was highest in septic shock, intermediate in sepsis without shock and lowest in controls (Table 11-2). SDMA, which is predominantly renally excreted [27], correlated strongly with serum creatinine (r=0.70, p<0.001), whereas ADMA did not (r=0.16, p=NS). On univariate analysis, sepsis patients with a plasma SDMA concentration in the highest quartile (≥1.30 µmol/L) had an odds ratio for death of 8.12 (95% CI 1.33-50.0), however this became insignificant on controlling for disease severity (using either IL-6 or SOFA score).
Table 11-2. Plasma asymmetric dimethylarginine and related variables at time of initial measurement

<table>
<thead>
<tr>
<th></th>
<th>All sepsis</th>
<th>Septic shock</th>
<th>Sepsis without shock</th>
<th>Control</th>
<th>p value pooled sepsis vs control</th>
<th>p value septic shock vs control</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>67</td>
<td>20</td>
<td>47</td>
<td>31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma ADMA(µmol/L)</td>
<td>0.52 (0.39-0.65)</td>
<td>0.64 (0.54-0.85)</td>
<td>0.47 (0.38-0.57)</td>
<td>0.57 (0.50-0.62)</td>
<td>0.10</td>
<td>0.09</td>
</tr>
<tr>
<td>Plasma L-arginine (µmol/L)</td>
<td>35.5 (27.3-51.2)</td>
<td>31.0 (23.7-40.4)</td>
<td>38.1 (29.4-51.7)</td>
<td>81.8 (68.9-91.3)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Plasma L-arginine/ADMA ratio</td>
<td>63.2 (45.3-103.4)</td>
<td>43.4 (33.6-73.3)</td>
<td>91.4 (55.5-108.3)</td>
<td>142.9 (123.0-165.7)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Plasma SDMA(µmol/L)</td>
<td>0.66 (0.50-1.29)</td>
<td>1.05 (0.77-1.45)</td>
<td>0.56 (0.45-0.80)</td>
<td>0.47 (0.43-0.65)</td>
<td>0.002</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Plasma lysine(µmol/L)</td>
<td>128 (100-171)</td>
<td>129 (90-190)</td>
<td>128 (104-162)</td>
<td>184 (157-216)</td>
<td>&lt;0.001</td>
<td>0.006</td>
</tr>
<tr>
<td>Receiving mechanical ventilation</td>
<td>14 (21)</td>
<td>9 (47)</td>
<td>5 (26)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>RH-PAT index</td>
<td>1.70 (0.47)</td>
<td>1.47 (0.40)</td>
<td>1.78 (0.47)</td>
<td>2.05 (0.46)</td>
<td>0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Plasma Interleukin 6 (pg/ml)</td>
<td>223 (76.6-563)</td>
<td>885 (298-2412)</td>
<td>148 (46.0-322)</td>
<td>4.7 (2.2-9.5)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>White blood cell count</td>
<td>15.2 (10.1-20.2)</td>
<td>17.5 (11.0-27.8)</td>
<td>15.2 (9.1-17.8)</td>
<td>7.7 (5.7-9.0)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>C-reactive protein</td>
<td>180 (87.3-259)</td>
<td>202 (126-297)</td>
<td>143(84-259)</td>
<td>7 (4-22)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

a. median (Interquartile range); b. n=septic shock 19, sepsis without shock 37, controls 27; c. n (%) d. mean (sd)
Figure 11-1. Baseline plasma concentration of asymmetric dimethylarginine according to disease category

P values represent comparisons between groups. Solid circles represent individual sepsis subjects and solid triangles represent individual control subjects. Open circles represent subjects with a fatal outcome at 28 day follow-up. Horizontal lines represent median group values, and error bars represent interquartile range.
Baseline correlations of ADMA with microvascular reactivity and disease severity.

There was no correlation between plasma L-arginine and RH-PAT index at baseline \((r=0.14, p=0.35)\). However, L-arginine: ADMA ratio, a better measure of substrate availability to NOS, correlated more strongly with RH-PAT index \(r=0.34\) \((p=0.02)\). Models incorporating SDMA and lysine the major competitors of L-arginine for the CAT transporter, did not improve the strength of this correlation. ADMA also correlated with total SOFA score \((r=0.45, p<0.001)\), and with the liver component of SOFA score \((r=0.45, p<0.001)\) but not with IL-6, lactate or CRP.

Longitudinal changes in ADMA and microvascular reactivity.

Over the first 2-4 days of follow up, ADMA increased in the sepsis patients \((0.53 \text{ to } 0.64, p=0.002)\) (Table 11-3), and also in the septic shock subgroup \((0.64 \text{ to } 0.85, p=0.03)\). Plasma L-arginine concentrations also increased, but due to the increase in ADMA, there was no significant change in the L-arginine: ADMA ratio, which may explain the lack of significant increase in the RH-PAT index over this time. In a mixed effects linear regression model examining change from baseline to day 2-4, increase in ADMA over time significantly correlated with increase in SOFA score \((p<0.001)\) and decrease in RH-PAT index \((p=0.03)\), but not with change in IL-6 or CRP. It also correlated with increase in the liver \((p<0.001)\) but not the renal \((p=0.09)\) components of the SOFA score.
Figure 11.2. a - Ratio of L-arginine to asymmetric dimethylarginine in baseline plasma samples, according to disease category; b - Baseline microvascular reactivity according to disease category.

**Figure**: The top diagram shows the ratio of L-arginine to asymmetric dimethylarginine in baseline plasma samples across different disease categories. The bottom diagram illustrates baseline microvascular reactivity. Both figures display statistical significance denoted by p-values.

**Legend**:
- Solid circles represent individual sepsis subjects and solid triangles represent individual control subjects. Horizontal lines represent median group values, and error bars represent interquartile range.
- Solid circles represent mean group values for sepsis subjects, and the solid triangle for control subjects. Error bars represent standard error of the mean.

**Note**:
- P values represent comparisons between groups.
- Solid circles represent individual sepsis subjects and solid triangles represent individual control subjects. Horizontal lines represent median group values, and error bars represent interquartile range.
- Solid circles represent mean group values for sepsis subjects, and the solid triangle for control subjects. Error bars represent standard error of the mean.
Table 11-3. Longitudinal results in subjects with sepsis

<table>
<thead>
<tr>
<th></th>
<th>Day 0</th>
<th>Day 2</th>
<th>P Day 0 to 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>67</td>
<td>47</td>
<td></td>
</tr>
<tr>
<td>ADMA</td>
<td>0.53 (0.39-0.66)</td>
<td>0.64 (0.51-0.78)</td>
<td>0.002</td>
</tr>
<tr>
<td>L-arginine</td>
<td>35.5 (27.3-51.2)</td>
<td>47.2 (30.8-58.1)</td>
<td>0.03</td>
</tr>
<tr>
<td>L-arginine : ADMA ratio</td>
<td>63.2 (45.3-103.4)</td>
<td>63.0 (41.7-108.0)</td>
<td>NS</td>
</tr>
<tr>
<td>RH-PAT index</td>
<td>1.70 (1.57-1.82)</td>
<td>1.81 (1.65-1.96)</td>
<td>NS</td>
</tr>
<tr>
<td>SDMA</td>
<td>0.66 (0.50-1.30)</td>
<td>0.71 (0.47-1.36)</td>
<td>NS</td>
</tr>
<tr>
<td>IL-6</td>
<td>223 (78.2-530)</td>
<td>54.5 (16.1-201)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>SOFA score</td>
<td>3 (1-7)</td>
<td>2 (1-7)</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Note: ADMA=Asymmetric dimethylarginine. RH-PAT index=Reactive hyperaemia peripheral arterial tonometry index. SDMA=Symmetric dimethylarginine. IL-6=Interleukin 6. SOFA score=Sequential Organ Failure Assessment Score.

11.6 Discussion

Plasma ADMA correlates with the degree of organ failure and predicts mortality in this cohort of patients with sepsis. Increase in ADMA over time was associated with worsening microvascular reactivity and organ dysfunction. Our results suggest a possible mechanism underlying these associations: impairment of microvascular function due to inhibition of endothelial NO production by ADMA.

Impaired microvascular and endothelial function have been shown to be important contributors to organ dysfunction and death in animals and humans with sepsis [28]. ADMA causes both acute [14] and chronic [1] endothelial dysfunction by inhibiting NOS and decreasing endothelial NO bioavailability. In previous clinical studies, O’Dwyer and colleagues enrolled 47 patients with severe sepsis and found that baseline ADMA was increased compared with controls, but that it did not correlate with mortality [3]. Zoccoli studied 17 patients with bacterial infections and raised C-reactive protein (CRP), but no organ failure, and found that ADMA was not raised compared with controls, but that it significantly increased after resolution of fever [4]. Finally, Nakamura studied 10 patients with septic shock and found that ADMA concentrations were increased and correlated with mortality [21].

Raised plasma ADMA has also been found to predict short-term mortality in critically ill surgical patients with multiple organ failure [2]. We have found that L-arginine: ADMA ratio correlates with RH-PAT index, a measure of NO-dependent microvascular reactivity in septic patients. This suggests that ADMA may contribute to decreased endothelial NO
bioavailability in sepsis, leading to endothelial and microvascular dysfunction and subsequently organ failure. This may provide a mechanistic explanation for the observed association of plasma ADMA concentrations with organ failure and death in this and other studies [2, 21].

ADMA, derived from protein catabolism, is metabolised by dimethylarginine dimethylaminohydrolase (DDAH) to citrulline [29]. Healthy humans generate approximately 300 µmol of ADMA per day, of which 250 µmol is metabolised by DDAH and 50 µmol is renally excreted [30]. Although plasma ADMA was significantly raised in those with a fatal outcome, it was not raised in sepsis patients overall compared with controls; moreover, plasma ADMA concentrations were lower in patients with less severe sepsis (sepsis without shock) than in controls. This finding was unexpected and has not been previously reported. This results in a roughly U-shaped relationship between sepsis severity and plasma ADMA levels, with highest concentrations in patients with a fatal outcome, followed by septic shock patients, lowest concentrations in sepsis without shock, and moderate concentrations in control subjects.

A possible explanation for this is that early sepsis is a hyperdynamic state, with increased cardiac output and liver and kidney blood flow [31, 32]. This may lead to increased degradation of ADMA in the liver by DDAH and, to a lesser extent, increased renal excretion. This hypothesis is supported by a study which found that the liver fractional extraction rate for ADMA is significantly higher and circulating ADMA is significantly lower in endotoxaemic rats compared to controls [33]. Patients with septic shock generally have developed multiple organ failure and down-regulation of cellular functions [34] and thus hepatic metabolism and renal excretion of ADMA may drop back to baseline levels. This hypothesis is supported by our finding that ADMA concentrations inversely correlate with liver function, both at baseline and longitudinally.

This is the first study to report the L-arginine : ADMA ratio in patients with sepsis, and it mirrors what was found in a study of experimental human endotoxaemia [35] – an acutely decreased L-arginine : ADMA ratio due to decreased plasma L-arginine concentrations. The increase in plasma ADMA concentrations over time may in part explain the lack of significant improvement in microvascular reactivity as patients recover, despite an increase in plasma L-arginine; this may be because the L-arginine : ADMA ratio (and thus the availability of L-arginine to NOS inside endothelial cells) does not change over time. The
mechanism behind the change in ADMA over time cannot be determined from these data, however there are several possibilities. Protein catabolism in patients with sepsis could lead to progressive release of methylated L-arginine residues into the plasma. However, this is unlikely to be the case because endogenous leucine flux (a measure of protein catabolism) does not correlate with plasma ADMA concentrations in septic humans [36]. NO causes direct inhibition of DDAH activity by S-nitrosylation of an active cysteine residue [37]. Thus it is possible that as patients recover from sepsis and endothelial NO bioavailability increases, DDAH activity is inhibited, resulting in an increase in plasma ADMA concentrations. Finally, the longitudinal inverse association between liver function and plasma ADMA suggests that worsening liver function due to sepsis progression, and thus decreased metabolism of ADMA, may also explain these findings.

*In-vitro* studies have shown that pro-inflammatory cytokines [38], oxidative stress [39] and lipopolysaccharides (LPS) [40] inhibit the breakdown of ADMA by DDAH. Nevertheless, and in contrast to the two previous studies in severe sepsis [3, 21], ADMA was not significantly raised in septic shock patients at baseline. Potential explanations for this difference include a higher mortality and greater illness severity in these previous studies. Moreover, one of these studies [3] used an ELISA rather than HPLC to determine ADMA levels, and used serum rather than plasma, both of which can artefactually elevate measured ADMA concentrations [12]. The only other published study to have enrolled non-severe sepsis patients also found no difference in plasma ADMA concentrations between sepsis and control patients [4].

This study has several limitations. Although it is at least 50% dependent on endothelial NO [22], peripheral arterial tonometry is not a direct measure of NO activity. Other factors may contribute to endothelial NO bioavailability besides the L-arginine : ADMA ratio, including CAT transport inhibitors (such as lysine and SDMA) and oxidative stress resulting in NO-quenching. The 67 sepsis patients were not all followed up on day 2-4, largely because of hospital discharge; thus the longitudinal results may underestimate the degree of improvement in microvascular and organ function.

Raised plasma ADMA concentrations are a strong predictor of death in patients with sepsis and thus may be useful as a prognostic marker. Impaired endothelial and microvascular function due to decreased endothelial NO production may be a mechanism linking ADMA with organ dysfunction and mortality. The DDAH-ADMA axis is a potential therapeutic
target and may be important in individual tailoring of therapy. Agents which compete with ADMA for NOS (such as L-arginine) or which potentiate DDAH activity should be further investigated in sepsis.
11.7 References


Chapter 12. Angiopoietin-2 and endothelial nitric oxide bioavailability in sepsis
12.1 Preamble

The data presented in Chapter 10 support the hypothesis that microvascular reactivity is impaired in patients with sepsis due to decreased endothelial nitric oxide bioavailability. However, the expected relationship between ICAM-1, E-selectin and RH-PAT index was not apparent. Angiopoietin-2 is a relatively recently described circulating marker of endothelial activation, which is elevated in patients with sepsis, correlates with mortality, and worsens endothelial cell damage. In this chapter, the relationship of angiopoietin-2 with estimated endothelial nitric oxide bioavailability and sepsis severity is explored.
12.2 Abstract

Introduction
Angiopoietin-2 (ang-2), an angiogenic peptide released by endothelial cell Weibel-Palade bodies (WPBs), increases endothelial activation and vascular permeability. Ang-2 is raised in severe sepsis but the mechanisms underlying this are not known. Nitric oxide (NO) inhibits WPB exocytosis, and bioavailability of endothelial NO is decreased in sepsis. We hypothesized that endothelial NO bioavailability would be inversely correlated with ang-2 concentrations in sepsis.

Methods
Plasma ang-2, vascular endothelial growth factor (VEGF) and endothelial-active cytokines were assessed in 83 patients with early sepsis and 41 hospital controls, and related to reactive hyperaemia-peripheral arterial tonometry, RH-PAT, a measure of endothelial NO bioavailability.

Results
Plasma Ang-2 was elevated in sepsis (median [IQR], ng/ml: severe sepsis 12.4 [8.5-33.4], sepsis without organ failure 6.1 [5.0-10.4], controls 2.7 [2.2-3.6], p<0.0001). It correlated inversely with RH-PAT (r=-0.38, p<0.0001) and positively with IL-6 (r=0.57, 0<0.0001) and degree of organ failure (sequential organ function assessment score) (r=0.58, p<0.0001). The correlation of ang-2 with RH-PAT persisted after controlling for sepsis severity. In a longitudinal mixed-effects model, recovery of RH-PAT over time was associated with decline in ang-2.

Conclusions
Ang-2 is elevated in proportion to sepsis severity, and inversely correlated with NO-dependent microvascular reactivity. Impaired endothelial NO bioavailability may contribute to increased endothelial cell release of ang-2, endothelial activation and capillary leak. Agents that increase endothelial NO bioavailability or inhibit WPB exocytosis and/or Ang-2 activity may have therapeutic potential in sepsis.
12.3 Introduction

Microvascular and endothelial dysfunction are central to the pathophysiology of sepsis, contributing to organ dysfunction even in the setting of normal post-resuscitation haemodynamics [1]. Angiopoietin-2 (ang-2), an angiogenic peptide, activates endothelial cells and increases vascular inflammation. It functions as an autocrine mediator of the endothelium and is stored predominantly in endothelial cells [2]. Ang-2 is a ligand of the tyrosine kinase receptor, Tie-2, and antagonises the angiopoietin 1- induced Tie-2 receptor autophosphorylation responsible for maintenance of endothelial cell quiescence [3]. This results in endothelial cells being sensitized to the effects of pro-inflammatory cytokines and vascular endothelial growth factor (VEGF), resulting in a loss of endothelial cell quiescence and an increase in vascular activation and inflammation.

Circulating ang-2 has been shown to be raised in human sepsis [4-6] and, more recently, to correlate with mortality [7-9] and pulmonary vascular leak [10, 11]. Despite a growing interest in ang-2 in sepsis, the mechanisms underlying elevated ang-2 levels in sepsis are unclear. Ang-2 is co-packaged with von Willebrand Factor (vWF) within endothelial cell Weibel-Palade bodies (WPBs) and is immediately released upon endothelial cell stimulation and WPB exocytosis [12]. *In-vitro* studies demonstrate that exocytosis of WPBs can be triggered by multiple secretagogues, including thrombin, histamine, epinephrine, VEGF and hypoxia [13]. However, there are only two known inhibitors of WPB release: nitric oxide (NO) and hydrogen peroxide (H$_2$O$_2$), of which NO is thought to be the most important [14].

We have recently demonstrated impaired microvascular reactivity in patients with sepsis by reactive hyperaemia peripheral arterial tonometry (RH-PAT) [15], which is at least 50% NO-dependent and which thus provides an estimate of endothelial NO bioavailability [16]. In contrast to earlier hypotheses suggesting major overproduction of NO in sepsis [17], there is now increasing evidence that systemic NO production is normal or decreased in humans with sepsis [18, 19]. Impaired endothelial NO bioavailability may underlie increased WPB exocytosis in sepsis, and thus the release of ang-2 from endothelial cells. However, the relationship between endothelial NO bioavailability and measures of WPB release in sepsis has not been determined. We hypothesized that plasma ang-2 levels in patients with sepsis would be raised in proportion to disease severity and would be inversely related to endothelial NO bioavailability, as estimated by RH-PAT.
12.4 Methods

Study design and setting

We performed a prospective observational study at a 350-bed teaching hospital in tropical Australia, with an 18-bed mixed intensive care unit. Prior approval was obtained from the Human Research Ethics Committee of the Menzies School of Health Research and the Department of Health and Community Services. Written informed consent was obtained from all participants or next of kin where necessary.

Participants

The study subjects were adults (≥18 years) hospitalised with sepsis, who were enrolled in a previously-reported study of microvascular reactivity; more detail of subject recruitment, patient characteristics and study procedures are provided in this paper [15]. Some of the data included in the current paper have been previously reported (RH-PAT index, ICAM1, E-selectin and IL6), but are included here for comparison with angiopoietin-2.

Sepsis was defined as a proven or suspected infection plus at least two criteria for the systemic inflammatory response syndrome present within the last 4 hours [20]. Septic patients were eligible for enrolment within 24 hours of their admission to the ICU, or within 36 hours of admission to the ward. Exclusion criteria were: coagulopathy (Platelets ≤ 20x10^9/L, APTT≥70 seconds, INR≥2.0); smoking of tobacco within the preceding 4 hours; and receipt of intravenous nitrates. Control subjects were adults recruited from hospital patients with no clinical or laboratory evidence of inflammation or infection, and who had not met SIRS criteria within the last 30 days. Septic patients were prospectively classified as severe sepsis, or sepsis without organ failure. Severe sepsis was defined as sepsis with organ dysfunction or shock at the time of enrolment according to American College of Chest Physicians/Society of Critical Care Medicine consensus criteria [15, 20].

Laboratory assays

Venous blood was collected in lithium heparin tubes at baseline and 2-4 days later, and plasma was frozen at -80°C. Control patients had blood collected at baseline only. Plasma concentrations of VEGF, ICAM-1 and E-selectin were measured by ELISA (R&D Systems, Minneapolis, MN, USA), according to the manufacturer’s instructions. The ELISA used to determine plasma angiopoietin-2 concentrations (R&D Systems, Minneapolis, MN, USA) reports a lower limit of detection of 8.3 pg/ml (0.0083 ng/ml), with coefficients of variation.
for intra-assay and inter-assay precision of 4.2% and 7.4% respectively IL-6 and TNFα were measured by flow cytometry using a cytokine bead array (CBA) (BD Biosciences, CA, USA).

**Measurement of microvascular reactivity/endothelial NO bioavailability.**

Microvascular reactivity was measured at the bedside by reactive hyperaemia - peripheral arterial tonometry (RH-PAT [Itamar Medical, Caesarea, Israel]), a non-invasive method of assessing endothelial function [21] which is at least 50% dependent on endothelial NO production [16]. We have previously reported internal validation and repeatability of RH-PAT in acute inflammatory states [22]. PAT was measured in a fingertip before and after a 5-min ischemic stress at the forearm, generating an RH-PAT index, normalized to the control arm, as previously described [15]. All studies were performed after resuscitation and at least an hour of hemodynamic stability in a quiet room at 25°C, with the patient recumbent.

**Statistical methods**

All analyses were hypothesis-based and were specified *a priori*. Continuous variables were compared using Student’s t-test/ANOVA or Mann Whitney U test/Kruskal-Wallis test for parametric and non-parametric variables respectively. Categorical variables were compared using Fisher’s exact test. For the ELISA and CBA assays, values below the lower limit of detection were assigned a value of halfway between zero and the lower limit of detection for the purposes of analysis. Correlates with baseline ang-2 were determined using Spearman’s coefficient. For multivariate analysis, linear regression with backward selection was used. All independent variables with a Wald p-value of <0.10 on univariate analysis, or which were considered biologically important were included in the initial model. Variables with a Wald p-value of ≥0.05 were sequentially dropped from the model. The natural logarithm of angiopoietin-2 was used as the dependent variable, since angiopoietin-2 was right-skewed and log transformation lead to a normal distribution.

To control for covariates in the relationship between angiopoietin-2 and RH-PAT index, the covariate in question was added to a linear regression model with log angiopoietin-2 as the independent variable and RH-PAT index as the dependent variable. To examine longitudinal correlations, linear mixed-effects models were used. A 2-sided p-value of <0.05 was considered significant. All analyses were performed using Stata version 10 (Stata Corp, Texas)
12.5 Results

Participants

85 patients with sepsis and 45 control patients were enrolled. Two sepsis patients and 4 controls were excluded from further analysis because they refused blood collection. Of the remaining 83 sepsis patients, 52 had organ dysfunction due to sepsis at baseline (severe sepsis group) and 31 did not (sepsis without organ failure). The three groups were well matched in terms of risk factors for endothelial dysfunction and other baseline characteristics (table12-1).

Baseline Ang-2 and VEGF

Plasma ang-2 concentrations were raised in sepsis in proportion to disease severity (Table 12-2, Figure 12-1a). Median ang-2 concentrations (ng/ml [IQR]) were 2-fold higher in patients with severe sepsis (12.4 [8.5-33.4]), than in those with sepsis without organ failure (6.1 [5.0-10.4]) p<0.0001, and 4.5-fold higher than in controls (2.7 [2.2-3.6]), p<0.0001. The difference in ang-2 between sepsis without organ failure and controls was also significant (p<0.0001). VEGF was also raised in sepsis patients compared with controls (p=0.0001, Table 12-2, Figure 12-1b), but the difference in VEGF between severe sepsis and sepsis without organ failure was not significant.
# Table 12-1. Baseline characteristics of participants

<table>
<thead>
<tr>
<th></th>
<th>Severe sepsis</th>
<th>Sepsis without organ failure</th>
<th>Control</th>
<th>p value across all groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>52</td>
<td>31</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>Age (years)³</td>
<td>52.8 (48.6-56.9)</td>
<td>50.8 (46.5-55.2)</td>
<td>47.3 (43.2-51.6)</td>
<td>NS³</td>
</tr>
<tr>
<td>Male n (%)</td>
<td>31 (60)</td>
<td>21 (68)</td>
<td>28 (68)</td>
<td>NS</td>
</tr>
<tr>
<td>Diabetic n (%)</td>
<td>17 (33)</td>
<td>7 (23)</td>
<td>13 (32)</td>
<td>NS</td>
</tr>
<tr>
<td>Smoker n (%)</td>
<td>26 (50)</td>
<td>12 (39)</td>
<td>16 (39)</td>
<td>NS</td>
</tr>
<tr>
<td>IHD³ n (%)</td>
<td>9 (17)</td>
<td>6 (19)</td>
<td>5 (12)</td>
<td>NS</td>
</tr>
<tr>
<td>On a statin n (%)</td>
<td>13 (25)</td>
<td>9 (29)</td>
<td>11 (27)</td>
<td>NS</td>
</tr>
<tr>
<td>APACHE II³</td>
<td>19 (15-25)</td>
<td>8 (5-11)</td>
<td>11 (27)</td>
<td>0.0001f</td>
</tr>
<tr>
<td>SOFA score³</td>
<td>6 (3-9)</td>
<td>1 (0-2)</td>
<td>11 (27)</td>
<td>0.0001f</td>
</tr>
</tbody>
</table>

- a. Mean (95% confidence interval)
- b. Ischemic Heart Disease
- c. APACHE II - Acute Physiology and Chronic Health Evaluation II score. SOFA – Sequential Organ Failure Assessment score. Median (Interquartile range)
- d. By oneway ANOVA
- e. By Fisher’s exact test across all three groups
- f. By Kruskal-Wallis test
**Ang-2 and disease severity**

Ang-2 correlated with sepsis severity (Table 12-3), as measured by APACHE II score (r=0.46, p<0.0001), SOFA score (r=0.58, p<0.0001), number of organ failures (r=0.48, p<0.0001) and arterial lactate (r=0.41, p=0.003), whereas VEGF did not correlate with any of these parameters. Since neutrophils release hydrogen peroxide, we also examined the relationship between neutrophil counts and plasma ang-2 concentrations, and found no significant correlation (r=0.16, p=0.15).

**Ang-2 and NO-dependent microvascular reactivity.**

On univariate analysis, ang-2 was inversely correlated with RH-PAT index, an estimate of endothelial NO bioavailability (r=-0.38, p<0.0001), and positively correlated with markers of endothelial activation (ICAM-1 r=0.58 p=<0.0001, E-selectin r=0.53 p<0.0001). VEGF did not correlate with endothelial NO bioavailability, endothelial activation or plasma ang-2. The relationship between log angiopoietin-2 and RH-PAT index remained significant after controlling for disease severity using SOFA score.

In a longitudinal analysis, plasma ang-2 concentrations decreased significantly between day 0 (median [IQR] 10.16 [5.32-19.39]) and day 2-4 (8.72 [5.38-15.73]), p=0.01, Table 4. RH-PAT index increased over the same time period, but the change was not statistically significant (Day 0 mean index 1.67 [95%CI: 1.55-1.78], Day 2-4=1.85 [1.70-2.00]). In a mixed-effects linear regression model, increase in RH-PAT index over the first 2-4 days correlated significantly with fall in ang-2 (r=0.45, p<0.0001); change in RH-PAT index over time did not correlate with VEGF, ICAM-1, E-selectin, SOFA score, or IL-6.
### Table 12-2. Baseline measurements according to disease category

<table>
<thead>
<tr>
<th></th>
<th>Severe sepsis</th>
<th>Severe sepsis</th>
<th>Sepsis without organ failure</th>
<th>Control</th>
<th>p value across all groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>52</td>
<td>31</td>
<td>41</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Angiopoietin 2 (ng/ml)</td>
<td>12.44 (8.47-33.44)</td>
<td>6.11 (4.59-10.37)</td>
<td>2.71 (2.15-3.61)</td>
<td>&lt;0.0001&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>VEGF (pg/ml)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>98.4 (56.4-142.6)</td>
<td>80.8 (57.5-147.3)</td>
<td>52.3 (31.8-73.5)</td>
<td>&lt;0.0007&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Plasma ICAM-1 (ng/ml)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>846 (523-1483)</td>
<td>501 (368-672)</td>
<td>323 (265-393)</td>
<td>&lt;0.0001&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Plasma E-selectin (ng/ml)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>200.5 (113-478)</td>
<td>87.0 (50.8-164.4)</td>
<td>38.4 (26.9-58.2)</td>
<td>&lt;0.0001&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>RH-PAT index&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.57 (1.44-1.71)</td>
<td>1.85 (1.67-2.03)</td>
<td>2.07 (1.93-2.22)</td>
<td>&lt;0.0001&lt;sup&gt;e&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Plasma Interleukin 6 (pg/ml)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>385.1 (124.2-996.0)</td>
<td>148.3 (45.9-315.0)</td>
<td>5.0 (2.2-8.1)</td>
<td>&lt;0.0001&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Plasma TNFα ≥2.8 pg/ml (n, %)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>8 (22)</td>
<td>4 (14)</td>
<td>5 (17)</td>
<td>NS&lt;sup&gt;f&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

- a. Median (Interquartile range)
- b. Mean (95% confidence interval)
- c. 2.8pg/ml is the lower limit of detection for the assay used for TNFα.
- d. By Kruskal-Wallis test
- e. By one-way ANOVA
- f. By Fisher’s exact test across all three groups
**Ang-2 and markers of inflammation**

Plasma TNFα was below the lower limit of detection in the majority of patients in both the sepsis and control groups (Table 2). In those in whom it was detectable (>2.8 pg/ml), there was no relationship between angiopoietin-2 and TNFα (r=0.24, p=0.44). Ang-2 correlated with IL-6 (r=0.57, p<0.0001), but not with C-reactive protein or white blood cell count.

**Multivariate analysis of correlates of angiopoietin-2**

The variables included in the initial multivariate linear regression model, with log angiopoietin-2 as the dependent variable, were: RH-PAT index, serum albumin, APACHE and SOFA scores, plasma concentrations of E-selectin, ICAM-1, IL-6 and IL-10, and peripheral blood platelet and white blood cell counts. The independent variables which remained significant in the final model, along with their β coefficients [95% CI] were: RH-PAT index (β = -0.35 [-0.64 to -0.06]), ICAM-1 (ng/ml, β=3.8 x 10^-4 [1.4 to 6.3 x 10^-4]), IL-6 (pg/ml, β=1.7 x 10^-4 [0.05-2.9 x 10^-4]), platelet count ( x 10^9/L, β= -2x10^-3 [-3.2 to -0.90 x 10^-3]), and white blood cell count (x 10^9/L, β= 3.2x 10^-2 [1.1 to 5.2 x 10^-2]).

**Outcomes**

The median [IQR] length of stay in the ICU among all sepsis patients was 5.4 [3.0-8.4] days, and this was significantly correlated with baseline ang-2 (r=0.30, p=0.03). Of the 83 patients with sepsis, only eight had died at 28 day follow-up (10%). Seven of these were from the severe sepsis group (28-day mortality 13%) and one was from the sepsis without organ failure group (mortality 3%). Baseline levels of ang-2 were not significantly different (p=0.32) in those with fatal (11.46 [7.09-45.12]) and non-fatal outcomes (10.04 [5.26-18.96]).
Table 12-3. Correlations of Baseline Plasma Angiopoietin-2 in sepsis patients

<table>
<thead>
<tr>
<th>Markers of Endothelial function and activation</th>
<th>Spearman’s rho</th>
<th>p</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>RH-PAT index</td>
<td>-0.38</td>
<td>&lt;0.0001</td>
<td>74</td>
</tr>
<tr>
<td>VEGF</td>
<td>-0.04</td>
<td>NS</td>
<td>80</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>0.58</td>
<td>&lt;0.0001</td>
<td>83</td>
</tr>
<tr>
<td>E-Selectin</td>
<td>0.53</td>
<td>&lt;0.0001</td>
<td>83</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Markers of inflammation</th>
<th>Spearman’s rho</th>
<th>p</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>0.57</td>
<td>&lt;0.0001</td>
<td>66</td>
</tr>
<tr>
<td>C-Reactive Protein</td>
<td>0.16</td>
<td>NS</td>
<td>82</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Markers of disease severity</th>
<th>Spearman’s rho</th>
<th>p</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOFA score</td>
<td>0.58</td>
<td>&lt;0.0001</td>
<td>82</td>
</tr>
<tr>
<td>APACHE II score</td>
<td>0.46</td>
<td>&lt;0.0001</td>
<td>83</td>
</tr>
<tr>
<td>Number of organ failures</td>
<td>0.48</td>
<td>&lt;0.0001</td>
<td>83</td>
</tr>
<tr>
<td>Arterial Lactate</td>
<td>0.41</td>
<td>0.003</td>
<td>51</td>
</tr>
</tbody>
</table>
12.6 Discussion

Plasma ang-2 is raised in sepsis, in proportion to disease severity and endothelial cell activation, and is inversely associated with estimated endothelial NO bioavailability both at baseline and during recovery. This finding supports the hypothesis that impaired endothelial NO bioavailability in sepsis leads to increased exocytosis of WPBs, release of ang-2, and thus to further endothelial cell sensitisation and activation. This hypothesis is also supported by recent findings in severe malaria, where an increase in endothelial NO bioavailability over time (also measured by RH-PAT) was significantly associated with falling plasma ang-2 [23].

While we demonstrate for the first time the relationship between estimated endothelial NO bioavailability and plasma ang-2 concentrations in sepsis, there is substantial recent evidence underpinning this hypothesis. In vitro, NO is the only substance demonstrated to reduce exocytosis of WPBs and release of Ang-2 apart from high concentrations of hydrogen peroxide [13, 14]. NO reduces WPB exocytosis by facilitating the S-nitrosylation of N-ethyl-maleimide sensitive factor (NSF), which results in the inability of the WPB membrane to fuse with the plasma membrane [2, 12, 13]. Furthermore, contrary to previously accepted theories, there is increasing evidence that systemic NO production is normal or decreased rather than increased in sepsis [18, 19], and that sepsis is a state of imbalance between the endothelial and inducible isoforms of NO synthase in the microvasculature, resulting in a relative deficiency of endothelial NO [24, 25]. The fact that non-specific NO inhibitors increase mortality in sepsis [26] supports this idea, and it is possible that increased angiopoietin-2 release is one of the mechanisms underlying this finding.

Clinical studies investigating the endothelium in sepsis commonly use circulating markers of endothelial cell activation as a surrogate measure of endothelial function. We have previously shown that ICAM-1 and E-selectin, two of the most commonly used markers of endothelial activation in sepsis, do not correlate with endothelial function as measured by RH-peripheral arterial tonometry [15]. In contrast, ang-2 correlates with endothelial function as measured by RH-PAT, both at baseline and longitudinally. Thus ang-2 is a more meaningful biomarker of endothelial cell function in sepsis than currently used surrogate measures.
Endothelial cell activation in sepsis increases vascular leak, triggers a pro-coagulant state, up-regulates adhesion molecule expression, and further drives the inflammatory response [27]. Together, these processes cause regional hypoperfusion and acute organ dysfunction. By mediating autocrine activation of local endothelial cells, ang-2 may exacerbate tissue hypoperfusion and inflammation, providing a plausible mechanism for its independent association with organ failure and mortality in sepsis [7].

Table 12-4. Change in Angiopoietin-2 and other variables over time

<table>
<thead>
<tr>
<th></th>
<th>Day 0</th>
<th>Day 2-4</th>
<th>p value a</th>
</tr>
</thead>
<tbody>
<tr>
<td>RH-PAT b</td>
<td>1.67 (1.55-1.78)</td>
<td>1.83 (1.67-1.99)</td>
<td>NS</td>
</tr>
<tr>
<td>SOFA score c</td>
<td>4 (2-8)</td>
<td>3 (1-7)</td>
<td>0.07</td>
</tr>
<tr>
<td>Ang-2 (ng/ml) c</td>
<td>10.16 (5.32-19.39)</td>
<td>8.72 (5.38-15.73)</td>
<td>0.01</td>
</tr>
<tr>
<td>VEGF (pg/ml) c</td>
<td>94.8 (57.0-143.8)</td>
<td>73.6 (48.6-167.3)</td>
<td>NS</td>
</tr>
<tr>
<td>ICAM-1 (ng/ml) c</td>
<td>655.6 (448.0-1084.1)</td>
<td>694.0 (450.3-1258.1)</td>
<td>NS</td>
</tr>
<tr>
<td>E-selectin (ng/ml) c</td>
<td>154.5 (69.1-396.3)</td>
<td>120.3 (63.9-199.2)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>IL-6 (pg/ml) c</td>
<td>224.4 (75.0-595.5)</td>
<td>58.1 (17.2-255.8)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

a. Mean (95% confidence interval)
b. Median (Interquartile range)
c. Wilcoxon’s paired signrank test

In-vitro studies of ang-2 demonstrate that in the absence of ang-2, a TNFα concentration of ≥40 pg/ml is required to independently activate endothelial cells, whereas in the presence of ang-2 at a concentration of 2000 pg/ml, a TNFα concentration of ≥5 pg/ml is able to cause endothelial activation [28]. Plasma TNFα levels in sepsis patients in this study were relatively low, with only 1 of 69 sepsis patients having TNFα levels of ≥40 pg/ml, similar to the low levels reported in other sepsis studies [29, 30]. The concentrations of ang-2 in this and other human sepsis studies [4, 5, 7] are higher than those used in in-vitro studies, and may sensitise endothelial cells to lower concentrations of TNFα. Furthermore, local microvascular TNFα concentrations may be higher than we and others have found in plasma. Nevertheless, taken together our results support the hypothesis that in sepsis, ang-2 sensitises endothelial cells to the effects of cytokines that may otherwise cause only minimal or no endothelial activation [28].
The factors triggering ang-2 release from WPBs in sepsis are not known. Thrombin [12], VEGF [31] and, in some [31, 32], but not other studies [4, 12], TNFα, cause WPB release \textit{in-vitro}. However, we found that neither TNFα nor VEGF correlated with ang-2. Although we found a strong independent association between IL-6 and ang-2, IL-6 has not been shown to cause exocytosis of WPBs or secretion of ang-2 \textit{in-vitro}. IL-6 is an important pro-inflammatory cytokine and correlates with disease severity in sepsis. Bacterial lipopolysaccharide increases ang-2 levels [33] and drives IL-6 expression [30], and such factors may account for this association.

Although ang-2 correlated with length of stay in this study, it did not correlate with mortality. Despite a median APACHE II score of 19, and a consequent predicted mortality of 34.8% [34], there were few deaths in our study (eight in total, 13% within the severe sepsis group). This is consistent with the previously reported low mortality from severe sepsis in our ICU [35], and suggests that our study was under-powered to examine the relationship between ang-2 and mortality. However, in studies with higher numbers of deaths, Siner et al. and Kumpers et al. both found a clear association between plasma ang-2 levels and risk of mortality [7, 8].

Although we did not directly measure endothelial cell NO concentrations (which is not possible in septic patients), RH-PAT index is an indirect measurement of NO bioavailability and is at least 50% NO-dependent in healthy volunteers [16]. Other methods of measuring NO in sepsis, such as plasma NO metabolites, are not specific to the endothelium and are confounded by nitrate retention in renal failure [36]. Thus it is not possible to directly confirm the relationship between endothelial nitric oxide bioavailability and plasma angiopoietin-2 using currently available methods in humans with sepsis.

The correlation between angiopoietin-2 and RH-PAT index was statistically significant but was not strong. We cannot exclude an alternative explanation for the inverse association between ang-2 and endothelial NO bioavailability: that increased ang-2 release in sepsis leads to decreased NO bioavailability as a consequence of upregulated endothelial cell inflammation and superoxide-mediated NO quenching. Nevertheless the clear \textit{in-vitro} evidence for NO being the major inhibitor of WPB exocytosis and Ang-2 release, and the findings in other disease settings such as malaria [23], make it more likely that impaired NO bioavailability is a significant contributor to Ang-2 release in sepsis.
Von Willebrand Factor (vWF) is co-packaged with ang-2 in WPBs but is also released by activated platelets, and is thus less specific for endothelial cells. While not measured in our study, plasma vWF activity is known to be increased in patients with sepsis, and to correlate with mortality [37]. Like other markers of endothelial cell activation, vWF has not been compared with measures of endothelial NO bioavailability in sepsis. However, in non-septic patients with risk factors for cardiovascular disease, vWF is raised, correlates with endothelial activation as measured by E-selectin [38], and is inversely proportional to endothelial NO bioavailability as estimated by flow-mediated dilatation of the brachial artery [39]. Furthermore, plasma vWF is raised in proportion to plasma ang-2 in patients with sepsis and acute lung injury [11]. Because our results suggest that impaired endothelial NO bioavailability exacerbates WPB release, they provide a plausible explanation for the increase in both ang-2 and vWF in sepsis.

In conclusion, ang-2 is raised in sepsis in proportion to disease severity and correlates with endothelial activation and inversely with NO-dependent microvascular reactivity, both at baseline and over the first 2-4 days of treatment. This suggests that decreased endothelial NO bioavailability may contribute to ang-2 release by reducing negative feedback on WPBs, thus augmenting endothelial cell activation and contributing to organ dysfunction. Adjunctive therapies which improve endothelial NO, decrease WPB release, or antagonise ang-2 may have roles in reducing organ dysfunction and improving mortality in sepsis.
12.7 References


14. Matsushita K, Morrell CN, Cambien B, Yang SX, Yamakuchi M, Bao C, Hara MR, Quick RA, Cao W, O’Rourke B et al: Nitric oxide regulates exocytosis by S-nitrosylation of N-ethylmaleimide-sensitive factor. *Cell* 2003, 115(2):139-150.


Chapter 13. Tryptophan metabolism and immune and microvascular function in sepsis
13.1 Preamble

Chapters 3 and 8-10 have demonstrated that endothelial and microvascular function are central aspects of sepsis pathophysiology. Immune function is a closely related and pivotal aspect of sepsis pathophysiology. The immunopathology of sepsis is, in general, beyond the scope of this thesis. However, during our analyses of plasma amino acid concentrations in patients with sepsis, we made an intriguing observation which is a potential link between endothelial and immunological function in sepsis: increased catabolism of tryptophan to its toxic metabolite kynurenine. This reaction is catalysed by indoleamine 2,3-dioxygenase (IDO), which inhibits nitric oxide synthase. This chapter presents an analysis of IDO activity from patients in the FRESH cohort. This manuscript has been submitted to *Infection and Immunity.*
13.2 Abstract

Introduction

Both endothelial and immune dysfunction contribute to mortality in sepsis, but the underlying mechanisms are unclear. Activation by interferon-γ of indoleamine 2,3-dioxygenase (IDO) results in catabolism of the essential amino acid tryptophan to the toxic metabolite kynurenine. IDO is predominantly expressed in immune and endothelial cells, the two key cell types involved in sepsis pathophysiology. Increased IDO activity causes T cell apoptosis and inhibits nitric oxide (NO) synthase, a key determinant of endothelial function. We hypothesized that IDO activity in sepsis would be related to plasma interferon-γ and disease severity and would be inversely related to T lymphocyte counts and NO-dependent microvascular reactivity.

Methods

In an observational cohort study of 80 sepsis patients and 40 hospital controls, we determined the relationship between IDO activity (plasma KT ratio) and selected plasma cytokines, sepsis severity, NO-dependent microvascular reactivity and lymphocyte subsets. Patients meeting sepsis criteria were enrolled within 24 hours of admission to ICU or within 36 hours of admission to the wards. Blood was collected at enrolment, day 2-4 and day 7 until discharge from the hospital or death. Plasma amino acids were measured by high performance liquid chromatography and microvascular reactivity by peripheral arterial tonometry.

Results

The plasma KT ratio was significantly increased in sepsis (median 141 µmol/mmol [IQR 64-235]) compared to controls (36 µmol/mmol [28-52]); p<0.0001), and correlated with sepsis severity as measured by APACHE II and SOFA scores. In sepsis, plasma KT ratio correlated with plasma interferon-γ and interleukin-10 and inversely with total lymphocyte count and both CD8+ and CD4+ T lymphocytes. There was an inverse relationship between plasma KT ratio and NO-dependent microvascular reactivity in sepsis, independent of sepsis severity.

Conclusions

IDO-mediated tryptophan catabolism is increased in proportion to sepsis severity, and may contribute to impaired microvascular reactivity and immune dysfunction. IDO-inhibitors may have therapeutic potential in sepsis.
13.3 Introduction

Sepsis is a systemic inflammatory response to infection [1]. Despite advances in its management, severe sepsis still has a mortality rate of 30-50% [2-4]. Both immune and endothelial dysfunction are thought to contribute to the high mortality rate in sepsis [5, 6], however the underlying mechanisms are not completely understood.

Tryptophan is an essential amino acid that is central to cellular respiration [7] and neurotransmission [8], and is a key immune mediator. During inflammation, tryptophan is metabolised by the interferon-γ induced enzyme, indoleamine 2,3-dioxygenase (IDO), to the toxic metabolite kynurenine. IDO activity is measured by the ratio of kynurenine to tryptophan (the KT ratio). IDO is predominately expressed in immune [9, 10] and endothelial [11] cells, the two key cell types involved in the pathophysiology of sepsis. IDO activity regulates a number of immune responses. Increased IDO activity inhibits T cell function [12] and proliferation [13-15] and contributes to T cell apoptosis [16]. Furthermore, elevated IDO activity inhibits both the expression and the activity of nitric oxide (NO) synthase [17-19]. Recent isotope studies have also shown that systemic NO synthesis is either reduced or unchanged in human sepsis [20-22].

NO is also essential for normal endothelial function. NO-dependent microvascular reactivity has been previously shown to be impaired in patients with sepsis, in proportion to disease severity [23, 24]. As IDO activity reduces NO availability, increased tryptophan catabolism may also be associated with impaired NO-dependent microvascular reactivity.

IDO activity correlates with disease severity in patients with chronic inflammatory diseases such as human immunodeficiency virus [25], systemic lupus erythematosus [26] and malignancy [27], but little is known about IDO activity in acute inflammatory states. A raised KT ratio has recently been reported in patients with bacteraemia [28]. We investigated whether the KT ratio correlated with disease severity in sepsis. We hypothesised that the KT ratio would be related to IFN-γ and IL10, and that the KT ratio would be inversely related to both T cell lymphopenia and NO-dependent microvascular reactivity.
13.4 Methods

Participants
We evaluated patients with sepsis and hospital controls who were part of a previously reported study of endothelial function in sepsis [23]. Sepsis patients had suspected or proven infection and the presence of two or more criteria for the systemic inflammatory response syndrome (SIRS) within the last 4 hours [1]. Severe sepsis patients had organ dysfunction or shock at the time of enrolment according to criteria from the American College of Chest Physicians/Society of Critical Care Medicine [1, 29].

Sepsis severity was estimated using the Acute Physiology and Chronic Health Evaluation (APACHE) II score from the first 24 hours of admission and daily modified Sequential Organ Failure Assessment (SOFA) score [30]. Patients were enrolled within 24 hours of ICU admission or within 36 hours of ward admission. Control subjects were recruited from hospital patients who had not met SIRS criteria within the last 30 days and who had no clinical or laboratory evidence of inflammation or infection. Written informed consent was obtained from all participants or next of kin. The study was approved by the Human Research Ethics Committee of Menzies School of Health Research and the Department of Health and Community Services.

Blood collection and lymphocyte counts
Venous blood was collected in lithium heparin tubes at enrolment, day 2-4, and day 7 until discharge from the hospital or death. Whole blood differential white cell counts were measured by Coulter Counter. Lymphopenia was defined as an absolute lymphocyte count less than 1.2 x10^9/μL [31]. Plasma was separated and stored at -80°C.

Lymphocytes were analysed in more detail in a subset of patients in whom samples could be processed within 30 minutes of collection, matched for age and gender. Peripheral blood mononuclear cells were separated using Ficoll-Paque™ Plus (GE Healthcare Biosciences, Uppsala, Sweden) and cryopreserved in fetal calf serum and dimethyl sulfoxide. Cells were thawed and stained with appropriate antibodies and read on a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, MA, USA). Antibodies were sourced from Biolegend, California, USA (CD3, CD16 and CD56) or BD Biosciences Pharmingen, California, USA (CD4 and CD8). Results were analysed using
Flow Jo software (Tree Star, Oregon, USA). T cells were defined as CD3+ lymphocytes and natural killer cells were defined as CD3-CD16+CD56+ lymphocytes.

**Tryptophan and kynurenine measurements**

Plasma tryptophan and kynurenine concentrations were measured by High Pressure Liquid Chromatography (HPLC; Shimadzu, Kyoto, Japan) with UV (250 nm) and fluorescence (excitation 250 nm, emission 395 nm) detection, using a method modified from van Wandelen and Cohen [32]. The kynurenine to tryptophan (KT) ratio was calculated by dividing the kynurenine concentration (μmol/L) by the tryptophan concentration (μmol/L) and multiplying the quotient by 1000 [25, 33, 34].

**Plasma cytokine measurements**

Concentrations of plasma IFN-γ, IL6 and IL10 were determined using a cytometric bead array (Human Th1/Th2 Cytokine Kit II, BD Biosciences Pharmingen, CA, USA) and a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, MA, USA). Results were analysed using FCAP array version 1.0.1 (Soft Flow Hungary for Becton Dickinson Biosciences). The lower limits of detection (LLD) of the assay were 2.5 pg/mL for IFN-γ and 10 pg/mL for IL6 and IL10. Values below the LLD were assigned a value halfway between zero and the LLD for statistical analysis. Cytokines were only measured if plasma had been frozen within 2 hours of collection.

**Measurement of endothelial function**

Sepsis patients underwent serial bedside reactive hyperaemia peripheral arterial tonometry (RH-PAT) measurements at enrolment, day 2-4, and day 7 [23]. Control patients had the same assessment at a single time point. RH-PAT (Itamar Medical, Caesarea, Israel) is a non-invasive method of assessing endothelial function that is at least 50% NO-dependent [35]. RH-PAT uses finger probes to measure digital pulse wave amplitude detected by a pressure transducer [36], and correlates with the more operator-dependent flow-mediated dilatation method [37] and with endothelial function in other vascular beds [38].

**Statistical methods**

Predefined groups for analysis were severe sepsis, non-severe sepsis (meaning sepsis without evidence of organ dysfunction or shock at enrolment), and hospital controls. Continuous parametric variables were compared using Student’s t-test or ANOVA while continuous non-parametric variables were compared using Mann-Whitney, Kruskal-Wallis
or Wilcoxon tests as appropriate. Correlations were examined using Pearson’s or Spearman’s tests for parametric and non-parametric data respectively. As SOFA score was highly right-skewed and no transformation gave a normal distribution, Kendall’s tau coefficient for partial correlation was used for multivariate analysis involving SOFA [39]. Linear mixed-effects models were used to examine longitudinal correlations. A 2-sided p-value of <0.05 was considered significant. Analyses were performed using Stata version 10.0 (Stata Corp TX, USA) and Prism version 5.01 (GraphPad Software, CA, USA).

13.5 Results

Patients

The study included fifty patients with severe sepsis, thirty with non-severe sepsis and forty hospital controls. The three groups did not differ significantly in age or gender (Table 13-1). Ninety percent of severe sepsis patients and all non-severe sepsis patients were either orally or enterally fed at the time of enrolment; none were receiving parenteral nutrition.

IDO activity and sepsis severity

Plasma tryptophan concentrations were significantly reduced in patients with sepsis (p<0.0001, Figure 13-1 and Table 13-2). In all sepsis patients, plasma tryptophan was inversely related to SOFA score (r=-0.45, p<0.0001). There was no difference in the baseline plasma tryptophan concentrations among severe sepsis patients who were orally fed (n=29), enterally fed (n=16) or who were nil by mouth (n=5).
Table 13-1. Baseline clinical characteristics of participants

<table>
<thead>
<tr>
<th></th>
<th>Severe sepsis</th>
<th>Non-severe sepsis</th>
<th>Controls</th>
<th>p value&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subjects (n)</td>
<td>50</td>
<td>30</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Age&lt;sup&gt;b&lt;/sup&gt;</td>
<td>52 (48-57)</td>
<td>50 (46-55)</td>
<td>48 (44-52)</td>
<td>NS</td>
</tr>
<tr>
<td>Male – n (%)</td>
<td>29 (58%)</td>
<td>20 (67%)</td>
<td>27 (68%)</td>
<td>NS</td>
</tr>
<tr>
<td>Diabetic – n (%)</td>
<td>16 (32%)</td>
<td>7 (23%)</td>
<td>13 (33%)</td>
<td>NS</td>
</tr>
<tr>
<td>MAP&lt;sup&gt;c&lt;/sup&gt;</td>
<td>74 (70-82)</td>
<td>88 (77-104)</td>
<td>80 (73-93)</td>
<td>0.001</td>
</tr>
<tr>
<td>Systolic BP&lt;sup&gt;c&lt;/sup&gt;</td>
<td>113 (105-132)</td>
<td>123 (110-140)</td>
<td>115 (110-128)</td>
<td>NS</td>
</tr>
<tr>
<td>Diastolic BP&lt;sup&gt;c&lt;/sup&gt;</td>
<td>60 (54-68)</td>
<td>70 (60-90)</td>
<td>60 (60-75)</td>
<td>0.002</td>
</tr>
<tr>
<td>APACHE II&lt;sup&gt;c&lt;/sup&gt;</td>
<td>19 (15-23)</td>
<td>7 (5-12)</td>
<td></td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>SOFA score</td>
<td>6 (3-9)</td>
<td>1 (0-2)</td>
<td></td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>RH-PAT index&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.59 (1.45-1.73)</td>
<td>1.86 (1.67-2.05)</td>
<td>2.04 (1.91-2.18)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

*MAP=Mean arterial pressure. BP=Blood pressure.

<sup>a</sup>For difference between all 3 groups by one way analysis of variance

<sup>b</sup>Mean (95% confidence interval)

<sup>c</sup>Median (interquartile range)
Conversely, plasma kynurenine concentrations were elevated in sepsis patients compared to hospital controls (p<0.0001, Figure 13-1 and Table 13-2), and correlated with SOFA score (r=0.34, p=0.005). As kynurenine is renally excreted and accumulates in renal failure,[40, 41] kynurenine concentrations were tested for relationships with renal impairment. Kynurenine concentrations were significantly higher in patients requiring continuous renal replacement therapy (CRRT) (median 4.5 µmol [IQR 4-5.3]) than in patients not receiving CRRT (2.8 µmol [2.1-4.4]; p=0.03). In all sepsis patients, kynurenine concentration correlated with plasma creatinine (r=0.41, p=0.0002). Nevertheless, the association between plasma kynurenine concentration and SOFA score remained significant even after controlling for creatinine (ktau=0.24, p<0.01).

IDO activity was significantly increased in sepsis patients (median KT ratio 141 µmol/mmol [IQR 64-235]) compared to controls (36 µmol/mmol [28-52]) (p<0.0001) and in severe sepsis compared to non-severe sepsis (p=0.0006, Table 13-2). The baseline KT ratio correlated with APACHE II (r_s=0.51, p<0.0001) and SOFA scores (r_s=0.54, p<0.0001) in sepsis patients. The baseline KT ratio was higher in non-survivors than survivors (median 270 µmol/mmol [IQR 102-431] versus 138 µmol/mmol [63-232]) but this difference was not statistically significant. In all sepsis patients, there was a weak association between the baseline KT ratio and mean arterial pressure (r_s=-0.29, p=0.009) and diastolic blood pressure (r_s=-0.29, p=0.01) but not with systolic blood pressure.

In longitudinal analysis in those severe sepsis, the KT ratio significantly decreased between day 0 (median 162 µmol/mmol [IQR 100-286]) and day 7 (89 µmol/mmol [65-139]), p=0.0006); Figure 13-1D. Among all sepsis patients, decrease in KT ratio correlated with decrease in SOFA score over time (p<0.0001).
Figure 13-1. Plasma markers of tryptophan catabolism in sepsis

Plasma tryptophan (Fig 1A), kynurenine (Fig 1B) and the KT ratio (Fig 1C) and D shows the KT ratio in severe sepsis patients on admission (n=50), day 2 (n=34) and day 7 (n=16). Horizontal lines represent median values for the group.
**IDO activity and plasma cytokines**

Plasma IFN-γ, IL6 and IL10 were all significantly increased in patients with sepsis (Table 13-2). Plasma concentrations of interleukin-1, interleukin-2, interleukin-4 and tumour necrosis factor-α were not significantly increased in this cohort and were not analysed further. Both IL6 and IL10 positively correlated with SOFA score ($r_s=0.55$, $p<0.0001$ and $r_s=0.55$, $p<0.0001$ respectively) but there was no association between IFN-γ and SOFA score.

In sepsis patients, the KT ratio correlated with plasma IFN-γ ($r_s=0.44$, $p=0.0002$), IL6 ($r_s=0.49$, $p<0.0001$) and IL10 ($r_s=0.62$, $p<0.0001$). The associations between KT ratio and IL6 and IL10 remained significant after controlling for SOFA score (ktau=0.30, $p<0.003$ and ktau=0.45, $p<0.0001$ respectively).

In a univariate mixed effects model, the decrease in KT ratio over time correlated with the decrease in IL6 ($p<0.0001$) and IL10 ($p<0.0001$) between day 0 and day 7. In a multivariate model, these relationships remained significant after controlling for change in SOFA score (IL6 $p=0.009$; IL10 $p=0.02$).

**IDO activity and lymphocyte count**

Sepsis patients had significantly higher total white blood cell ($p<0.0001$) and neutrophil ($p<0.05$) counts than hospital controls (Table 13-2). Conversely, sepsis patients had significantly lower total lymphocyte counts compared with hospital controls ($p<0.0001$, Table 13-2). In all sepsis patients the baseline KT ratio was weakly associated with absolute lymphocyte count ($r_p=0.26$, $p=0.02$). In a linear mixed effects model, absolute lymphocyte count increased as the KT ratio decreased over time ($p=0.001$). This relationship persisted after controlling for SOFA score ($p=0.008$). When all subjects were grouped according to lymphopenia, lymphopenic patients ($n=63$) had a median KT ratio of 128 µmol/mmol [IQR 63-236], compared with 59 µmol/mmol [33-86] in non-lymphopenic patients ($n=57$) ($p<0.0001$).

As IDO activity contributes to T cell apoptosis [16], we examined the relationship between KT ratio and lymphocyte subsets. Peripheral blood mononuclear cells were analysed from twenty-three of the eighty sepsis patients whose blood had been processed within 30 minutes of collection. This subset of patients was representative of the cohort in terms of age, gender distribution, total lymphocyte count and KT ratio. In this subset of patients, the
KT ratio negatively correlated with absolute numbers of lymphocytes ($r_p=-0.54$, $p=0.007$), T cells ($r_p=-0.53$, $p=0.01$), CD4+ T cells ($r_p=-0.50$, $p=0.01$), CD8+ T cells ($r_p=-0.49$, $p=0.02$) and natural killer cells ($r_p=-0.46$, $p=0.03$) (Table 13-2).
Table 13-2. Immunological characteristics of participants

<table>
<thead>
<tr>
<th></th>
<th>Severe sepsis N=50</th>
<th>Non-severe sepsis N=30</th>
<th>Controls N=40</th>
<th>p (Sepsis vs Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptophan μmol/L</td>
<td>21 (13-29)</td>
<td>31 (23-37)</td>
<td>49 (40-55)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Kynurenine μmol/L</td>
<td>3.5 (2.4-5.2)</td>
<td>2.3 (1.9-3.9)</td>
<td>1.9 (1.5-2.3)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>KT ratio</td>
<td>162 (100-286)</td>
<td>82 (55-159)</td>
<td>36 (28-52)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Plasma IFN-γ pg/mL</td>
<td>8 (1.3-20.1)</td>
<td>27 (3-84)</td>
<td>1.3 (1.3-7)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Plasma IL6 pg/mL</td>
<td>380 (121-979)</td>
<td>136 (44-320)</td>
<td>5 (5-5)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Plasma IL10 pg/mL</td>
<td>23 (13-64)</td>
<td>5 (4-25)</td>
<td>5 (5-5)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Neutrophils x10^9/μL</td>
<td>13.5 (8.7-20.4)</td>
<td>14.1 (9.2-16.3)</td>
<td>5.1 (3.2-6.5)</td>
<td>0.049</td>
</tr>
<tr>
<td>Lymphocytes x10^9/μL</td>
<td>0.9 (0.5-1.2)</td>
<td>1.0 (0.7-1.3)</td>
<td>2.1 (1.2-2.2)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Lymphocyte subsets b</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T cells x10^9/μL</td>
<td>0.65 (0.34-1.8)</td>
<td>0.67 (0.34-1.0)</td>
<td>1.49 (1.0-1.7)</td>
<td>NS</td>
</tr>
<tr>
<td>CD4+ T cells x10^9/μL</td>
<td>0.35 (0.17-0.85)</td>
<td>0.35 (0.17-0.59)</td>
<td>0.89 (0.52-1.2)</td>
<td>NS</td>
</tr>
<tr>
<td>CD8+ T cells x10^9/μL</td>
<td>0.18 (0.07-0.72)</td>
<td>0.16 (0.10-0.33)</td>
<td>0.46 (0.31-0.61)</td>
<td>NS</td>
</tr>
<tr>
<td>NK cells x10^9/μL</td>
<td>0.07 (0.03-0.12)</td>
<td>0.06 (0.03-0.17)</td>
<td>0.08 (0.04-0.20)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Tryptophan and Kynurenine concentrations are from subjects' plasma.
a. All sepsis vs controls, Mann Whitney test
b. Severe sepsis n=11, non-severe sepsis n=12, control n=4
**IDO activity and endothelial function**

In sepsis, the KT ratio at baseline correlated inversely with NO-dependent microvascular reactivity \((r=-0.45, \ p=0.001)\) even after controlling for disease severity (SOFA score; \(p=0.001\)). In a multivariate mixed effects model controlling for SOFA score, improvement in KT ratio between day 0 and day 7 correlated with improvement in microvascular reactivity \((p=0.001)\).

**13.6 Discussion**

IDO activity is increased in sepsis and correlates with disease severity both at baseline and longitudinally. IFN-\(\gamma\) and IL10 were associated with, and may contribute to, increased IDO activity in sepsis. The independent inverse association between the KT ratio and NO-dependent microvascular reactivity suggests that IDO activity may also contribute to impaired endothelial function in sepsis. Similarly, the inverse association with total and T cell lymphocyte counts suggests a potential role in sepsis-associated lymphopenia.

A high KT ratio has been previously described in trauma [34, 42] and recently in bacteraemia [28]. One of the strengths of our study was enrolment of patients early in their hospitalization, within 24 hours of ICU admission or within 36 hours of ward admission with sepsis. We have shown that the KT ratio in sepsis decreases rapidly between day 0 and day 2. The median baseline KT ratio in sepsis of 141 \(\mu\)mol/mmol is higher than that reported previously in bacteraemia (89.9 \(\mu\)mol/mmol) [28]. This difference may be because the bacteraemia study used the highest KT ratio measured within 4 days following hospital admission, with few measurements taken during the first 2 days.

A limitation of this study is that we did not directly measure IDO expression. However, the KT ratio is an established measure of IDO activity [25, 43] with previous studies measuring both IDO expression and KT ratio reporting good correlation [44, 45]. It is unlikely that nutritional deficiency and renal impairment accounted for the differences we found, because controlling for these factors made no difference to our results.

Increased expression of IFN-\(\gamma\) [46], IL6 [47, 48] and IL10 [14] have been separately associated with increased tryptophan catabolism by IDO in other disease states. In sepsis patients in our study, IFN-\(\gamma\) concentration correlated with the KT ratio only at baseline, whereas IL6 and IL10 correlated with the KT ratio both at baseline and longitudinally. These findings agree with the in vitro literature, where IFN-\(\gamma\) induces IDO [46, 49] and IL10
stabilises IDO expression [14]. Our data suggest that the high IFN-γ associated with early sepsis [50] may lead to increased IDO activity while high IL10 may sustain that IDO activity throughout the course of the disease. IL6 is not known to affect IDO expression, however a low tryptophan environment stabilises IL6 mRNA [51]. As the KT ratio correlates with IL6 at baseline and longitudinally, high IDO activity may sustain IL6 expression in sepsis. Past studies have shown higher infection-related mortality when IL6 and IL10 are elevated concurrently than when either is elevated alone [52].

The high KT ratio in sepsis is associated with a decreased lymphocyte count independent of disease severity, a finding similar to that found in patients with trauma [34], human immunodeficiency virus [25] and cancer [53]. Previous studies in sepsis have associated lymphopenia with disease severity [54], duration of ICU stay [54] and mortality [55] in sepsis and prevention of lymphocyte apoptosis improves survival in animal models of sepsis [56-59]. High kynurenine concentrations induce lymphocyte apoptosis [16] suggesting a potential mechanism through which increased IDO activity may contribute to lymphopenia and its deleterious consequences in sepsis.

There is significant cross-talk between IDO and NOS, with IDO activity inhibiting both expression and activity of NOS [17-19]. NO is an important regulator of endothelial function, however the vascular effects of IDO are not well characterised in vivo. Increased IDO activity has been linked to chronic vascular disease [60] though its vascular effects in acute inflammatory diseases are not known. We found the KT ratio is inversely associated with microvascular reactivity as measured by RH-PAT, which is at least 50% dependent on endothelial NO production [61]. IDO activity may contribute to endothelial dysfunction in sepsis through the inhibition of NO. Conversely, NO also suppresses IDO activity, and decreased endothelial NO bioavailability in sepsis [23] may further exacerbate the high KT ratio in sepsis. Increased plasma kynurenine concentrations may have other deleterious effects in sepsis, with kynurenine also mediating adhesion of monocytes and neutrophils to the vascular endothelium [62].

The generation of a low tryptophan environment may be a maladaptive host response to infection. While growth of some bacterial species is inhibited by low tryptophan [63], most can synthesize tryptophan [64] and others have specialized tryptophan transport systems [65]. Blockade of IDO increases survival in a murine model of sepsis [66] and the KT ratio is significantly higher in bacteraemic patients with fatal outcome [28]. We demonstrate that
the KT ratio is associated with disease severity in sepsis. Together this evidence supports the hypothesis that increased IDO activity is a deleterious host response in human sepsis. A broad range of IDO inhibitors is being considered as potential adjunctive cancer treatments [67] and these treatments may also have therapeutic potential in sepsis.

In conclusion, IDO activity is elevated in sepsis and associated with disease severity, T cell lymphopenia and microvascular dysfunction. Because excessive IDO activity is associated with both immune and endothelial dysfunction, the increased tryptophan catabolism we have described may link these two key aspects of sepsis pathophysiology. Modulation of IDO activity warrants investigation as a therapeutic strategy in sepsis.
13.7 References


tryptophan availability to the brain in the elderly and increased serum interleukin-6 in DAT. Aging (Milano) 1998, 10(4):316-323.


Section C - Part 2

The use of near infrared spectroscopy as a tool to assess endothelial and microvascular function in sepsis
Chapter 14. Single versus repeated use of near-infrared spectroscopy sensors
14.1 Preamble

In addition to peripheral arterial tonometry, near-infrared spectroscopy has been included as an outcome measure in the study protocol for the randomised controlled trial described in chapter 19. In this trial, we are using the Hutchinson InSpectra 650 NIRS monitor to measure dynamic tissue oxygenation in the thenar eminence at several time points (days 0, 1, 2 and 7) in each patient. This monitor requires the use of a sensor which is applied using an adhesive shield to the skin of the thenar eminence, and transmits a signal along a cable to the monitor (figure 14-1).

Figure 14-1. Hutchinson Near Infrared Spectroscopy Sensor
(taken directly from [1])

The manufacturer states that the sensors are for single use only, and should not be reused once removed [1]. Because the sensors are expensive (A$220.50 each at the time of writing), we planned to use a single sensor per patient, to be removed following the baseline measurement, and reapplied on days 1, 2 and 7.

We communicated with several researchers overseas who had used these sensors for critically ill patients, and found that reusing sensors was a common practice (I am unable to quote sources here as they do not wish to be named), but that there were no available data to support this practice. Although the manufacturer states in the product information that the probes are single use only, they do not give any justification for this recommendation [1]. Discussion with a company representative revealed that the manufacturers were concerned about the accuracy of reused sensors, due to the possibility of poor
adhesiveness and thus stray light contaminating the signal (Dr Dean Myers, personal communication, 2008).

This chapter describes a small study performed to examine the agreement between dynamic StO₂ measurements taken using sensors in the standard manner as compared with removing and reapplying them for each measurement. This has been written for submission as a short report to *Intensive Care Medicine*; it has been reformatted for this thesis, but the text is unchanged.
14.2 Abstract

Introduction
Dynamic near infrared spectroscopy, an emerging technique for the measurement of microcirculatory responses in critical illness, uses adhesive sensors applied to the thenar eminence. The manufacturer states that these sensors are for single use only, and should not be reapplied once removed. Longitudinal studies of microvascular function may require daily measurements in a given subject. Since the cost of each sensor is considerable, we wished to investigate the effect of sensor reuse on the accuracy of measurements.

Methods
In each of two healthy male volunteers, we determined microvascular reactivity using dynamic near infrared spectroscopy on eight occasions. On each occasion, we performed a 5 minute vascular occlusion test on one hand using a standard sensor, followed by a measurement on the contralateral hand using a reused sensor. The primary outcome measure was the agreement in the $\text{StO}_2$ recovery rate between standard and reused sensors.

Results
The mean [sd] $\text{StO}_2$ recovery rate (RR) (%/sec) was no different with reused sensors (5.13 [1.21]) than standard sensors (4.93 [1.41]), $p=\text{NS}$, and there was good correlation between these two groups ($r$ for $\text{StO}_2$ RR=0.81, $p=0.002$). A Bland-Altman plot suggested acceptable agreement, with a mean within-pair difference of close to zero (0.29%/sec) and all values falling within the limits of agreement (-1.47 to +2.03 %/sec).

Conclusions
Removal and reuse of thenar sensors for near infrared spectroscopy has acceptable agreement compared with standard sensor use and represents a considerable cost saving.
14.3 Introduction

Microcirculatory dysfunction and tissue hypoperfusion are important factors contributing to organ dysfunction and mortality in sepsis [2], trauma [3] and other critical illnesses [4]. Near infrared spectroscopy (NIRS) is an emerging non-invasive technique for assessing tissue oxygenation in the clinical setting [5]. NIRS uses the comparative light-absorption properties of oxygenated and deoxygenated haemoglobin to estimate tissue oxygen saturation [6].

The most commonly used NIRS apparatus in published studies of sepsis and trauma uses a flat adhesive sensor to estimate muscle oxygen saturation in the thenar eminence [5]. This sensor contains light emitting and sensing elements, an adhesive shield which excludes all ambient light, and a cable which connects it to the monitor. The manufacturer states that these sensors are intended for single use only, and should not be reapplied once removed [1].

However, studies investigating the microcirculation in critical illness often collect repeated measurements over time [7], and because of the complexity of their care and the use of other monitoring devices it is not always practical in critically ill patients to leave a thenar eminence NIRS sensor in-situ for days on end. Because the sensors are expensive, it is important to determine if they can be reused in a given individual without compromising the accuracy of StO$_2$ measurements. There are no previously published studies addressing this question.

We aimed to determine if there is acceptable agreement between post-ischaemia StO$_2$ recovery rate (a commonly used measure of microvascular reactivity in critical illness) measured using reused sensors compared with sensors which were left continuously applied between readings (standard sensor use).
14.4 Methods

Subjects
We enrolled two healthy male volunteers, who were non-smokers, were free of vascular disease, and had no history of hypertension, diabetes or hyperlipidaemia. They were taking no medications at the time of the study. The subjects gave informed consent prior to the study, which was approved by the chair of the local human research ethics committee as a quality assurance activity which did not require full ethical review.

Dynamic near-infrared spectroscopy (dNIRS) measurements.
Dynamic NIRS measurements were performed using a tissue spectrometer and 15mm reflectance sensor (Inspectra 650, Hutchinson Technology, Minnesota, USA). Each vascular occlusion test was performed according to the following protocol: following 15 minutes of rest in a quiet, air-conditioned room, thenar muscle StO$_2$ was continuously recorded from a recumbent subject for a baseline period of 5 minutes. Data were streamed continuously to a laptop computer. A blood pressure cuff was rapidly inflated on the study arm up to 200 mm Hg; after a further 5 minutes, the cuff was deflated and StO2 recorded for a final five minutes. The resulting StO$_2$ trace was analysed using the manufacturer’s software (Inspectra researcher’s analysis software, version 4.01, Hutchinson Technology, Minnesota, USA).

Baseline StO$_2$ was the mean value over the 5 minutes prior to cuff inflation. Deoxygenation rate (%/minute) was the slope of the curve for the first 2 minutes following cuff inflation. StO$_2$ recovery rate (%/second) was the slope of the curve form the time of cuff deflation until StO$_2$ had recovered to 85% of the baseline value. Delta StO$_2$ was the difference between the baseline StO$_2$ and the peak reperfusion StO$_2$. Slopes were determined by least-squares regression (see [5] for a visual representation of these parameters).

Each subject underwent eight pairs of vascular occlusion tests (VOT) over three days, at least one hour apart. To control for diurnal variation in endothelial function [8], and the effect of diet [9, 10], all measurements were taken in the morning, following a caffeine-free, low-fat breakfast. On each morning, the subject had a new sensor applied to one hand (standard sensor) and this was left in place until all tests were complete. The other hand had a sensor applied and removed (reuse sensor) for each measurement. An individual sensor was reused up to six times and then discarded. For each pair, a vascular occlusion
test was performed using either a reused or standard sensor on one hand, followed immediately by a repeat VOT on the opposite hand, using the other type of sensor. The order of sensor use was equally balanced across all readings. This was done to ensure that any possible effect of ischaemic pre-conditioning on endothelial responses [11] would be balanced across the two groups.

**Statistical analysis**

The primary outcome of interest was the agreement in StO$_2$ recovery rate between reused and standard probes. StO$_2$ recovery rate is the most widely reported primary outcome measure in studies using dNIRS in critically ill patients [7, 12-15], and was thus selected as the primary indicator for comparison in this study. Secondary outcomes were agreement in baseline StO$_2$, delta StO$_2$ and StO$_2$ deoxygenation rate between reused and standard probes. For StO$_2$ recovery rate, agreement between the two groups was assessed by i) a Mann-Whitney U test, ii) A scatter plot with Spearman correlation coefficient and iii) A Bland-Altman plot. The Bland-Altman limits of agreement were calculated as the mean difference +/- 1.96 x the standard deviation of the differences [16].

For secondary outcome measures, agreement was assessed using only a Mann-Whitney U test between the two groups. Possible degradation of sensor performance was assessed by graphing StO$_2$ recovery rate against the usage number of the sensor. All statistical analysis was conducted using Stata version 10 (Statacorp, Texas, USA) and Graphpad Prism version 5 (GraphPad Software, California, USA). P values of less than 0.05 were considered significant.

### 14.5 Results

One of the eight pairs of measurements in Subject 2 did not provide usable data due to an error in data streaming to the computer, and all data from this pair were excluded from analysis. Results of the remaining measurements are presented in **table 14-1**.

The mean [sd] StO$_2$ recovery rate (%/sec) was no different with reused sensors (5.13 [1.21]) than standard sensors (4.93 [1.41]), p=0.68. There was also no significant difference in baseline StO$_2$, delta StO$_2$ or StO$_2$ deoxygenation rate between the reuse and standard groups, either within each individual, or with all observations combined. There was strong correlation between StO$_2$ recovery rate in the reuse and standard groups (**figure 14-2**, r=0.81, p=0.002).
Table 14-1. Results of vascular occlusion tests in two healthy volunteers, using standard sensors compared with reused sensors.

<table>
<thead>
<tr>
<th>Subject 1</th>
<th>Baseline StO$_2$ (%)</th>
<th>StO$_2$ RR (%/Sec)</th>
<th>ΔStO$_2$ (%)</th>
<th>StO$_2$ Deox (%/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td>Pair 1</td>
<td>77.0</td>
<td>84.1</td>
<td>3.64</td>
<td>3.07</td>
</tr>
<tr>
<td>Pair 2</td>
<td>77.0</td>
<td>77.6</td>
<td>3.57</td>
<td>3.85</td>
</tr>
<tr>
<td>Pair 3</td>
<td>82.3</td>
<td>80.7</td>
<td>4.95</td>
<td>5.32</td>
</tr>
<tr>
<td>Pair 4</td>
<td>78.9</td>
<td>81.7</td>
<td>4.40</td>
<td>3.52</td>
</tr>
<tr>
<td>Pair 5</td>
<td>76.0</td>
<td>79.0</td>
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<td>2.81</td>
</tr>
<tr>
<td>Pair 6</td>
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<td>77.6</td>
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<td>Pair 7</td>
<td>76.4</td>
<td>74.8</td>
<td>4.89</td>
<td>4.50</td>
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<tr>
<td>Pair 8</td>
<td>74.8</td>
<td>80</td>
<td>4.63</td>
<td>3.98</td>
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<tr>
<td>Mean</td>
<td>78.2</td>
<td>79.4</td>
<td>4.20</td>
<td>3.97</td>
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<table>
<thead>
<tr>
<th>Subject 2</th>
<th>Baseline StO$_2$ (%)</th>
<th>StO$_2$ RR (%/Sec)</th>
<th>ΔStO$_2$ (%)</th>
<th>StO$_2$ Deox (%/min)</th>
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<tbody>
<tr>
<td>R</td>
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<td>R</td>
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<tr>
<td>Pair 1</td>
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<td>5.02</td>
<td>4.32</td>
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<td>89</td>
<td>6.66</td>
<td>7.20</td>
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<td>6.16</td>
<td>6.80</td>
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<td>Pair 7</td>
<td>79.5</td>
<td>79.8</td>
<td>6.66</td>
<td>7.01</td>
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<tr>
<td>Mean</td>
<td>85.7</td>
<td>83.8</td>
<td>6.19</td>
<td>6.03</td>
</tr>
<tr>
<td>Overall</td>
<td>81.7</td>
<td>81.5</td>
<td>5.13</td>
<td>4.93</td>
</tr>
</tbody>
</table>

| Mean$^a$ | p-value$^b$ | 0.76 | 0.68 | 0.54 | 0.37 |

Notes: StO$_2$=Tissue oxygen saturation. StO$_2$ RR=Tissue oxygen recovery rate following release of the blood pressure cuff. ΔStO$_2$ =Change in tissue oxygen saturation from baseline to peak reperfusion StO$_2$. StO$_2$ Deox=Tissue deoxygenation rate following inflation of the blood pressure cuff.

a. Mean of all observations from both subjects combined. b. P-value comparing all observations in the reuse group with those in the standard group, from both subjects combined.
Figure 14-2. Scatter plot of StO2 recovery rate measured with standard sensor use compared with reused sensors

StO2 recovery rate is expressed in %/second. 
$r=$ the Spearman correlation coefficient. $P=$ the $p=$ value associated with $r$. Each black circle represents a pair of observations. The solid line is the least-squares linear regression line of best fit.

Figure 14-3 is a Bland-Altman plot of the mean difference between pairs of StO2 recovery rate measurements against the average of that pair. This shows that the mean of the differences is close to zero, at 0.29%/sec; that there is no relationship between the differences and the mean difference; and that all values fall within the limits of agreement, which are -1.47 to +2.03%/sec. Hence the two methods (standard use versus reuse of sensors) show an acceptable degree of agreement. Furthermore, within the reuse group, there was no consistent change in mean (sd) StO2 recovery rate between the first use and the final (sixth) use of the sensor (figure 14-4).
Figure 14-3. Bland Altman plot comparing pairs of measurements of StO2 made with reused sensors compared with standard sensor use.

The y axis is the difference within each pair of measurements. The x axis is the average of the two readings from each pair of measurements. The horizontal dashed lines are the limits of agreement. Each circle represents a pair of measurements.

Figure 14-4. Mean StO2 recovery rate against number of uses of the NIRS sensor

The black circles represent mean values. The vertical error bars represent standard deviation.
14.6 Discussion

This small study suggests that, in healthy volunteers, there is no systematic difference between dNIRS parameters measured using sensors which have been removed and reapplied on up to six occasions, compared with sensors which are left continuously applied, or which are only used on one occasion. This has important implications for cost and convenience in studies investigating longitudinal changes in dNIRS responses in critically ill patients.

There are two reasons why investigators might choose not to use NIRS sensors in the standard manner, which means leaving them continuously applied for the duration of the study, with a new sensor applied every 72 hours. The major reason is convenience. The forearm and hand of critically ill patients are often occupied with radial arterial lines (with accompanying splints and dressings), digital oxygen saturation probes, peripheral venous cannulae or wound dressings. Less severely ill patients may be conscious and thus wish to use their hands to eat. It thus may be inconvenient or impossible to have a NIRS sensor constantly in-situ, particularly if it is being used only for research purposes. The second reason is expense. If a study is following up NIRS responses every 1 to 2 days for more than 72 hours, several sensors may have to be used per subject.

The alternative strategy is to remove the NIRS sensor following each vascular occlusion test and reapply either the same or a new sensor each time the test is repeated. For a hypothetical study measuring dNIRS responses each day for 7 days in patients with critical illness, using a new sensor on each occasion would cost more than US$1000 per subject, compared with approximately US$150 for the reuse strategy. It is important to point out that, like all disposable equipment in intensive care units, sensors should not be reused on any patient apart from the patient on whom they were originally used, to avoid the possibility of the transmission of multiresistant skin bacteria.

This study has several important limitations. Only two subjects were studied. Given that there was substantial inter-subject variation in dNIRS responses, our findings should be reproduced in a larger number of subjects. We only studied healthy subjects, and these data may not be generalisable to critically ill patients. Finally, although there was no significant difference in mean dNIRS parameters between standard and reuse observations, the limits of agreement were larger than we expected. This may be due to the substantial
within subject variability in dNIRS responses, irrespective of which type of sensor was used. Most of this variability is likely to be due to actual variability in endothelial and microvascular function, which has been shown to vary by as much as 25% per hour [8]. In conclusion, these data suggest that removal and reuse of NIRS sensors is an acceptable strategy in longitudinal studies of dNIRS responses, but these findings need to be confirmed in a larger number of subjects, and in critically ill patients.
14.7 References

Chapter 15. Dynamic near-infrared spectroscopy compared with peripheral arterial tonometry to measure microvascular reactivity in severe sepsis.
15.1 Preamble

We have found that reactive hyperaemia peripheral arterial tonometry (RH-PAT) responses are impaired in severe sepsis in proportion to disease severity, but that there are several potential problems with RH-PAT in critically ill patients: a technical failure rate of approximately 10%, significant variability between and within individuals, and a failure to predict mortality [1]. Thus we added dynamic near infrared spectroscopy (dNIRS) (see Chapter 3 for more detail about dNIRS) to the study protocol for a planned randomized controlled trial of atorvastatin to improve endothelial and microvascular function in severe sepsis (Chapter 17).

Although dNIRS has been more extensively studied in severe sepsis than RH-PAT, less is known about what it is actually measuring. RH-PAT has been widely studied in cardiovascular disease [2-4], has been shown to correlate with endothelial function measured by other means [4], and to be at least 50% dependent on nitric oxide-derived responses [5]. Dynamic NIRS has primarily been studied in sepsis [6-8] and trauma [9, 10], and it is unknown whether it correlates with endothelial function measured by other means, and what proportion of observed dNIRS responses are NO-dependent. Dynamic NIRS appears to be a promising tool for quantifying microvascular responses in severe sepsis, as it provides more information than RH-PAT: specifically tissue oxygen utilization can be derived from the thenar muscle deoxygenation rate, in addition to reactive hyperaemia from the thenar muscle \( \text{StO}_2 \) recovery rate [11].

The following paper analyses preliminary data from a randomized controlled trial of atorvastatin to improve endothelial function in severe sepsis (Chapter 17) to explore this question. At the time of writing, the atorvastatin trial continues to recruit. Since it is a double-blind trial, we have only analysed baseline data taken before the administration of study drug. Hence this paper concerns severe sepsis but not the effect of atorvastatin. Moreover, because these data derive from an RCT with only two arms (atorvastatin and placebo), there is no healthy control group and longitudinal data could not be included as they would have been influenced by the study drug. However, because no comparison of RH-PAT and dNIRS has been previously published (either in sepsis or any other condition), we examined our baseline data to learn more about the relationships between dNIRS and RH-PAT in severe sepsis.
15.2 Abstract

Introduction
Dynamic near-infrared spectroscopy (dNIRS) and reactive hyperaemia peripheral arterial tonometry (RH-PAT) are two recently described methods for measuring microvascular reactivity in severe sepsis, but they have not previously been compared. We hypothesised that RH-PAT index would correlate with dNIRS-derived tissue oxygen (StO\textsubscript{2}) recovery rate in patients with severe sepsis.

Methods
We simultaneously measured microvascular reactivity in the finger tips (using RH-PAT) and thenar muscle (using dNIRS) of 22 patients with severe sepsis, within 24 hours of onset of organ failure. An ischaemic stress was created by placing a blood pressure cuff on the forearm and inflating to suprasystolic pressures for 5 minutes. RH-PAT index was derived as a ratio of hyperaemic-phase and baseline digital pulse wave amplitude. StO\textsubscript{2} recovery rate was the slope of the StO\textsubscript{2} curve from cuff release until recovery to 85% of baseline.

Results
Five patients did not have usable RH-PAT data due to technical failures. Among the remaining seventeen, median [IQR] RH-PAT index (1.40 [1.28-1.57]) and mean [SD] StO\textsubscript{2} recovery rate (3.7 [1.8] %/sec) were both impaired, but there was no correlation between them (r=0.30, p=0.25).

Conclusions
Simultaneous RH-PAT and dNIRS-derived estimates of microvascular reactivity do not correlate in severe sepsis, suggesting they may be measuring different aspects of the same phenomenon. Although these findings need to be reproduced in larger numbers of patients, these data suggest that RH-PAT and dNIRS may provide complementary information as outcome measures in clinical trials targeting the endothelium in severe sepsis.
15.3 Introduction

Microvascular and endothelial dysfunction are pivotal components of the pathophysiology of sepsis [12] and contribute to organ dysfunction and mortality [13] but are difficult to measure in the clinical setting. Dynamic near infrared spectroscopy (dNIRS) and reactive hyperaemia peripheral arterial tonometry (RH-PAT) are emerging techniques for bedside measurement of microvascular function using post-ischaemic changes in the thenar eminence and fingers respectively [1, 6, 11, 14].

NIRS uses reflectance spectroscopy to measure haemoglobin oxygen saturation in small blood vessels whereas RH-PAT uses plethysmography to determine changes in finger-tip pulse wave amplitude [15, 16]. RH-PAT has been extensively investigated in cardiovascular disease [2-4], is at least 50% nitric oxide (NO)-dependent [5], and correlates with flow-mediated dilatation of the brachial artery (FMD) [4] and coronary artery endothelial function [2]. RH-PAT has recently been used to demonstrate impaired microvascular reactivity in severe sepsis [1] and severe malaria [17]. Dynamic NIRS-derived post-ischaemic StO$_2$ recovery rate is also impaired in sepsis in proportion to disease severity [6-8]. However, it is unknown whether dNIRS responses are dependent on endothelial NO release and dNIRS has not been directly compared with other methods of assessing microvascular or endothelial function.

At the time of enrolment into a phase two randomised controlled trial (RCT) targeting the endothelium in severe sepsis, we measured simultaneous microvascular responses using RH-PAT and dNIRS. We hypothesised that digital RH-PAT index would correlate with thenar muscle StO$_2$ recovery rate.
15.4 Methods

Study setting and subjects

The study was carried out in the intensive care unit of an Australian teaching hospital. Subjects were enrolled in a RCT of atorvastatin in adults with severe sepsis (Australian clinical trials registry number ACTRN012607000393459). Inclusion criteria were suspected or proven infection; three or more criteria for the systemic inflammatory response syndrome (SIRS) within the last 48 hours [18]; and sepsis-related organ failure commencing within the last 24 hours [19]. Exclusion criteria were pregnancy, rhabdomyolysis, severe acute hepatitis, decompensated cirrhosis, and coagulopathy.

Study procedures

The study was approved by the institutional human research ethics committee. Following written informed consent, patients underwent bedside measurement of microvascular reactivity following randomisation but prior to receipt of the first dose of study drug.

NIRS and PAT measurements

Digital microvascular reactivity was measured using RH-PAT (Endopat 2000, Itamar medical, Caeserea, Israel). Probes were placed on the index fingers of both hands, and digital pulse wave amplitude was recorded for a baseline period of 5 minutes (baseline phase); a blood pressure cuff was rapidly inflated on the study arm up to 200 mm Hg (ischaemic phase); after 5 minutes, the cuff was deflated and pulse wave amplitude recorded for a further 5 minutes (hyperaemic phase). A computerised algorithm provided by the manufacturer (Endo-PAT 2000 software version 3.1.2) was used to calculate a post-pre occlusion ratio (RH-PAT index). StO$_2$ was measured in the thenar eminence with a tissue spectrometer and 15mm reflectance probe (Inspectra 650, Hutchinson Technology, Minnesota, USA).

Continuous StO$_2$ measurements were recorded from the same hand as, and simultaneously with the digital PAT readings. The resulting StO$_2$ trace was analysed using the manufacturer’s software (Inspectra researcher’s analysis software, version 4.01, Hutchinson Technology, Minnesota, USA). Baseline StO$_2$ was the mean value over the 5 minutes prior to cuff inflation. Deoxygenation rate (%/minute) was the slope of the curve for the first 2 minutes following cuff inflation. StO$_2$ recovery rate (%/second) was the slope of the curve between cuff deflation until StO$_2$ had recovered to 85% of the baseline value. Slopes were determined by least-squares regression.
Due to the small number of subjects, all data were analysed using non-parametric approaches. Continuous variables were compared using Spearman’s correlation and categorical using Fisher’s exact test. A p value of <0.05 was considered significant. All analyses were carried out using Stata v10 (Statacorp, Texas, USA).

15.5 Results

Participants

Twenty-two patients were included. Five did not have analysable RH-PAT traces for technical reasons (two poor baseline digital pulse waveform, three excessively noisy signal), but all had analysable dNIRS traces, leaving seventeen analysable pairs. Haemodynamic parameters indicated a well-resuscitated group of patients, with median MAP of 75mmHg and CVP of 12 cm H_2O (Table 15.1). Of the twenty-two patients, four had died (18%) by 28-days of follow up, but only one of these were among the seventeen having complete data.

Table 15.1. Baseline characteristics and variables at the time of measurement (n=17)

<p>| | |</p>
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<tbody>
<tr>
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</tr>
<tr>
<td><strong>Age b</strong></td>
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</tr>
<tr>
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<td><strong>Hypertension a</strong></td>
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<td><strong>Central venous pressure (cm H_20) b</strong></td>
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</tr>
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<td><strong>APACHE II score b</strong></td>
<td>18 (16-22)</td>
</tr>
<tr>
<td><strong>SOFA score b</strong></td>
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</tr>
<tr>
<td><strong>Receiving invasive ventilation a</strong></td>
<td>8 (50)</td>
</tr>
<tr>
<td><strong>Receiving vasopressors a</strong></td>
<td>12 (75)</td>
</tr>
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<td><strong>Noradrenaline dose b,c</strong></td>
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<tr>
<td><strong>Blood lactate (units) b</strong></td>
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<tr>
<td><strong>Serum C-reactive protein (units) b</strong></td>
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</tr>
<tr>
<td><strong>Haemoglobin (g/dL) b</strong></td>
<td>118 (105-126)</td>
</tr>
<tr>
<td><strong>White blood cell count (x 10^9/L) b</strong></td>
<td>17.4 (15.8-20.9)</td>
</tr>
</tbody>
</table>

a. n (%)
b. Median (Interquartile range)
c. All 12 patients on vasopressors were receiving noradrenaline alone
**Microvascular and endothelial function**

Median RH-PAT index was impaired at 1.40, compared with previously reported values of 1.57 in severe sepsis and 2.05 in healthy controls (Table 15-2) [1]. Mean StO₂ Recovery rate was 3.7% per second, consistent with previously reported values in severe sepsis [6-8, 20]. There was no correlation between RH-PAT index and StO₂ recovery rate, either when analysed as continuous variables \( r=0.30, p=0.25; \) Figure 15-1, or when broken into quartiles \( p=0.25 \) by 4x4 table and Fisher’s exact test). There was also no correlation between RH-PAT index and deoxygenation rate \( r=-0.11, p=0.67 \).

**Correlations with disease severity and vasopressor dose**

RH-PAT index correlated inversely with APACHE II score \( r=-0.62, p=0.01 \), but not SOFA score \( r=0.16, p=0.54 \). None of the dynamic NIRs-derived variables correlated with either APACHE II or SOFA scores. There was also no correlation of either RH-PAT index \( r=0.03, p=0.92 \) or StO₂ recovery rate \( r=0.34, p=0.28 \) with noradrenaline dose.
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<tbody>
<tr>
<td>N</td>
<td>17</td>
<td>54</td>
<td>45</td>
<td>72</td>
<td>18</td>
<td>10</td>
<td>6</td>
<td>15</td>
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<td>RH-PAT index</td>
<td>1.40</td>
<td>(1.28-1.57)</td>
<td>1.57</td>
<td>(1.43-1.70)</td>
<td>2.05</td>
<td>(1.91-2.19)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline StO₂ (%)</td>
<td>81</td>
<td>(9)</td>
<td>72</td>
<td>(11)</td>
<td>78</td>
<td>(7)</td>
<td>75</td>
<td>(15)</td>
</tr>
<tr>
<td>Deoxygenation rate</td>
<td>9.2</td>
<td>(4.3)</td>
<td>11.2</td>
<td>(2.4)</td>
<td>13.2</td>
<td>(3.6)</td>
<td>10.4</td>
<td>(7.8-13.3)</td>
</tr>
<tr>
<td>StO₂ Recovery rate</td>
<td>3.7</td>
<td>(1.8)</td>
<td>2.6</td>
<td>(1.5)</td>
<td>4.8</td>
<td>(1.6)</td>
<td>2.3</td>
<td>(1.0)</td>
</tr>
</tbody>
</table>

All values are expressed as mean (standard deviation) or median (25th centile – 75th centile).

a. Forearm blood flow was occluded for 3 minutes rather than 5 minutes
b. Forearm blood flow was occluded until StO₂ fell to 40% rather than for a fixed period of time.
15.6 Discussion

In the first study to have compared dynamic NIRS with peripheral arterial tonometry in patients with sepsis, or indeed any disease state, we found no correlation between these two measures of microvascular reactivity, although both techniques showed impaired responses. RH-PAT and dNIRS both measure microvascular responses to an ischaemic insult in the hand, but despite this fact, simultaneous measurement found no correlation. This suggests that these techniques are measuring different aspects of the same phenomenon. RH-PAT is derived from changes in digital pulse amplitude, and thus from responses in a mixture of digital arteries, arterioles, venules and capillaries. dNIRS reads changes in skeletal muscle oxygen saturation, rather than in blood flow or pulse amplitude. Using a 15mm probe spacing, the infrared beam is reflected from a mean depth of 7.5mm, and the signal is primarily derived from skeletal muscle blood vessels of <1mm in diameter, similar to those in the soft tissues of the finger tip [15]. Thus the size and type of blood vessels measured by the two techniques have significant overlap, but peripheral arterial tonometry includes input from small arteries in addition to the microvasculature. Another reason for the lack of correlation may be the way the data are analysed and the indices derived. The RH-PAT index is derived from the average pulse-wave amplitude from 60 to 120 seconds after cuff release, compared with a baseline value [21]. In contrast dNIRS-derived StO₂
recovery rate is derived from the first 10-20 seconds following cuff release, and is independent of baseline measurements [6].

 Whilst RH-PAT is known to be at least 50% NO-dependent [5], and to correlate with endothelial function in other vascular beds across multiple disease states [2-4], much less is known about the vascular physiological changes underlying NIRS responses. While this study was underpowered to detect correlations with disease severity, RH-PAT index correlated with APACHE II score whereas none of the NIRS-derived parameters did so. This suggests RH-PAT may parallel disease severity more closely than dNIRS, but this needs to be confirmed in a larger number of subjects.

 There was an unexpectedly high proportion of technical failures with RH-PAT in severe sepsis (23%). In the only previously published paper evaluating RH-PAT in sepsis, the technical failure rate was 18 of 227 measurements (8%) overall and 12 of 124 (10%) in the severe sepsis subgroup [1]. In a large community-based study of endothelial function using RH-PAT in adult outpatients, the technical failure rate was 207 out of 2,217 (9.3%) [3]. Thus NIRS appears to be more robust to disturbed peripheral perfusion and peripheral vasoconstriction in sepsis than RH-PAT, but this also needs to be confirmed in larger numbers.

 This study has several limitations. The sample size is relatively small; however there is no previously published comparison of dNIRS and RH-PAT. We could not include healthy controls because this study was part of a randomised trial – thus it is unclear whether the same lack of correlation would be found in non-septic patients who do not have impaired microvascular reactivity. Finally the exclusion of patients with technical failures means that the population may not be representative of severe sepsis patients as a whole, since those with technical failures may have been more severely ill. The strengths of this study include the inclusion of only patients with early (<24 hours) severe sepsis, and the simultaneous measurement of RH-PAT and dNIRS on the same hand, which has not been reported previously.

 In conclusion, in this small study of patients with early severe sepsis, there was no correlation between digital reactive hyperaemia measured by peripheral arterial tonometry and thenar muscle oxygen recovery rate as measured by dynamic near infrared spectroscopy, although both methods found impaired responses. Until more is known, in
trials of adjunctive therapies targeting the endothelium and microcirculation in sepsis, investigators should consider using peripheral arterial tonometry in addition to dynamic NIRS as complementary measures of microvascular reactivity.
15.7 References


Section D
Assessing statins as a potential adjunctive treatment in sepsis

“Aye, in the very temple of Delight
Veiled melancholy has her sov’reign shrine,
Though seen of none save him whose strenuous tongue
Can burst Joy’s grape against his palate fine”

John Keats (1795-1821)
16.1 Preamble

As discussed in Chapter 3, multiple cohort studies have suggested that statin use is protective against the development of and death from sepsis. However, not all of these studies have been positive, and none have previously been published from Australia. Given that the population of the Top End is different in several important respects from those included in previously published studies, the following cohort study was undertaken. These data were taken from the PRESTO cohort, as described in Chapter 5. The following manuscript has been written as a short report for submission to a peer-reviewed journal.
16.2 Abstract

Introduction

HMG Co-A reductase inhibitors (statins) usually used for their lipid-lowering effect, have additional “pleiotropic” effects which may be of benefit in patients with sepsis, and their use has been associated with decreased sepsis-related mortality in multiple cohort studies. No studies investigating their effects on sepsis outcomes have been published from Northern Australia, a tropical region with a high proportion of Indigenous Australians. We hypothesised that statin use, after adjusting for comorbidities and disease severity, would be associated with decreased 28-day mortality in patients hospitalised with sepsis.

Methods

Prospective cohort study enrolling all patients admitted to Royal Darwin Hospital with sepsis over a 12-month period. Data on clinical and demographic features, statin use and outcomes were collected from medical records and hospital databases. Prognostic factors which differed between the statin and no statin groups were included in a multivariate logistic regression model relating mortality to statin use.

Results

1,090 patients were admitted with sepsis, of whom 167 (15%) were taking a statin at hospital admission, despite 323 (29%) patients having indications for statin therapy. The statin group was older, had higher APACHE II scores and more comorbidities than the no statin group. At 28-days and 1-year of follow up, the mortality rates were 5.4% and 11.6% respectively in the statin group, and 6.0% and 15% in the no statin group (p=NS at both time points). After adjusting for age, disease severity and comorbidities, there was a significant negative association between statin use and risk of death, at both 28 days (Odds Ratio [95%CI] = 0.36 [0.15 – 0.85]) and 1 year (OR [95%CI] = 0.48 [0.27-0.84]).

Conclusions

After adjusting for important differences between the statin and no statin groups, pre-existing statin use is associated with decreased 28-day and 1-year mortality from sepsis in tropical northern Australia.
16.3 Introduction

HMG CoA reductase inhibitors, or “statins” are the most commonly prescribed medications in Australia [1] and are used primarily for their lipid-lowering effect. However, statins have other “pleiotropic” effects which may be beneficial to patients with sepsis [2]. These include anti-inflammatory [3] and immunomodulatory [4] effects as well as improvement of endothelial and vascular function [5, 6]. Statins have been shown to be of benefit both in animal models of sepsis [7, 8] and experimental endotoxaemia in human volunteers [9, 10]. Multiple prospective and retrospective cohort studies have evaluated the effect of statin use on infection-related mortality [11]. These studies have shown protective effects of statin use against the development of sepsis [12-14], and against mortality [15-20], or disease progression [21, 22] in those hospitalised with bacteraemia or sepsis. On the contrary, Yang and colleagues failed to find a protective effect of statins on mortality from sepsis in a Taiwanese cohort, and hypothesised that this might be due to racial and demographic differences in their patient population compared with those from previously published studies [23].

The Northern Territory is a tropical area of northern Australia with a high proportion of Indigenous and remote-dwelling residents. Indigenous Australians have a high prevalence of premature vascular disease and cardiovascular risk factors, including diabetes, chronic renal disease, smoking and microalbuminuria [24, 25]. Thus one would expect statin use to be common in this population. Furthermore, the incidence of sepsis in this region is approximately five times higher than that in temperate Australia, North America or Europe (Chapter 6). The effect of statins on sepsis-related mortality has not previously been investigated in Northern Australia. We performed a prospective cohort study of patients admitted to hospital with sepsis. Our primary hypothesis was that, after adjusting for comorbidities and sepsis severity, patients taking a statin at the time of hospital admission would have lower 28-day mortality than those not on a statin. Secondary hypotheses were that statin use would be common in this population and would have a protective effect on mortality one year after hospital admission.
16.4 Methods

Between May 2007 and May 2008 inclusive, we performed a prospective cohort study including every adult (≥15 years) admitted to Royal Darwin Hospital (RDH) with sepsis. Details of the study setting and procedures have been previously described (Chapter 6). The study was approved by the human research ethics committee of the Menzies School of Health Research and the Northern Territory Department of Health and Families.

In brief, all acute admissions were screened by admission diagnosis and those with possible or probable infections had data collected from their medical record, hospital databases and the treating clinician if necessary. All patients who met pre-defined criteria for infection (Chapter 6) and who met two or more criteria for the systemic inflammatory response syndrome (SIRS) [26] within the first 48 hours of the hospital admission were considered to have sepsis and were enrolled in the study. Patients could be enrolled more than once within the study period if they had repeat admissions, but only the first admission for a given patient was included in this analysis. A subject was considered to be receiving a statin at the time of hospitalisation if a statin had been prescribed for that patient within the last 30 days. This information was derived from the patient’s medical admission notes, referral letters from general practitioners, medication records from community clinic databases, and the hospital pharmacy database.

Parametric and non-parametric continuous variables were compared using Student’s t test and Mann-Whitney U test respectively. Categorical variables were compared using Pearson’s chi-squared test. To control for covariates in the relationship between statin use and mortality, logistic regression modelling was used with mortality as the dependent variable. Predetermined criteria were used for choosing covariates to include in the models; namely, covariates were included if they differed between the statin and no statin group and were independently related to mortality (as reported in Chapter 6). The following independent variables met these criteria and were included in the model for 28-day mortality: age, SOFA score, APACHE score, Charlson comorbidity index [27] and number or SIRS criteria met within the first 48 hours of hospitalisation [26]. For the model with 1 year mortality as the dependent variable, the following covariates met these criteria and were included: age, Indigenous status, Charlson comorbidity index, APACHE II score and chronic liver disease. Two-sided p values of <0.05 were considered significant. All data analysis was performed using Stata version 10 (Statacorp, Texas, USA)
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<th>On a statin (n=167)</th>
<th>P value</th>
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<td>Male n(%)</td>
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<td>84 (50)</td>
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<td>Chronic renal disease</td>
<td>64 (7)</td>
<td>60 (35)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>End-stage renal failure</td>
<td>24 (3)</td>
<td>25 (15)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hyperlipidaemia</td>
<td>18 (2)</td>
<td>91 (55)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hazardous alcohol use</td>
<td>273/577 (47%)</td>
<td>19/91 (21%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Smoking</td>
<td>333/624 (53%)</td>
<td>40/108 (37%)</td>
<td>0.002</td>
</tr>
<tr>
<td>On other lipid therapy</td>
<td>62 (7)</td>
<td>5 (3)</td>
<td>NS</td>
</tr>
<tr>
<td>Characteristics of Infection</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Focus=pneumonia</td>
<td>271 (30)</td>
<td>60 (36)</td>
<td>NS</td>
</tr>
<tr>
<td>Focus=Urosepsis</td>
<td>106 (12)</td>
<td>21 (13)</td>
<td>NS</td>
</tr>
<tr>
<td>Focus=Skin and soft tissue</td>
<td>332 (33)</td>
<td>57 (32)</td>
<td>NS</td>
</tr>
<tr>
<td>Focus=Abdominal</td>
<td>112 (11)</td>
<td>13 (7.2)</td>
<td>0.12</td>
</tr>
<tr>
<td>No organism isolated</td>
<td>506 (55)</td>
<td>82 (49.1)</td>
<td>0.16</td>
</tr>
<tr>
<td>Gram positive infection</td>
<td>223/414 (54%)</td>
<td>48/85 (56%)</td>
<td>NS</td>
</tr>
<tr>
<td>Gram negative infection</td>
<td>174/414 (42%)</td>
<td>35/85 (41%)</td>
<td>NS</td>
</tr>
</tbody>
</table>
16.5 Results

Study subjects
There were 1,191 admissions for sepsis in 1,090 patients over the 12-month study period. Of the 1,090 patients, 167 had been prescribed a statin within the last 30 days (statin group), 920 had not (no statin group), and data were missing for three patients. The mean age was 46.9 years, 47% of patients were Indigenous, and 53% were male. The most common focus of infection was skin and soft tissue (40%), followed by pneumonia (31%) and urosepsis (12%).

Details of statin use
Statin users were older, more likely to be Indigenous, had more comorbidities, worse acute organ dysfunction and a higher APACHE II score than those not on a statin (table 16-1). However, statin users were less likely to report hazardous alcohol consumption or smoking, and were less likely to have liver disease. There was no difference in the characteristics of infection between the statin group and the no statin group (table 16-1). Of the 167 patients on a statin, 125 (75%) were on atorvastatin, 39 (23%) simvastatin, and 3 (2%) were on pravastatin. According to national Australian guidelines [28], 323 (29%) of the cohort had indications for statin therapy (one or more of hyperlipidemia, vascular disease, and diabetes plus either age>60 years or indigenous status), but only 167 (15%) had actually been prescribed a statin (p<0.001). Chronic renal disease (OR [95% CI] = 1.9 [1.2-3.2]) was positively associated with concordance with guidelines on statin prescribing, and chronic liver disease (OR [95% CI] = 0.23 [0.06-0.81]) was negatively associated. There was no difference between Indigenous status, remote dwelling, age or sex among patients who were guideline concordant and those who were not.

<table>
<thead>
<tr>
<th>Table 16-2. Univariate analysis of the association between statin use at hospital admission and mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not on a statin (n=920)</td>
</tr>
<tr>
<td>28-day mortality</td>
</tr>
<tr>
<td>1 year mortality</td>
</tr>
</tbody>
</table>

28-day and 1-year mortality
Sixty (5.5%) and 132 (12.1%) of the patients had died by 28 days and 1 year post admission respectively. On univariate analysis, there was no significant difference in the mortality rate according to statin use at either 28 days or 1 year following hospital admission (Table 2).
However, when adjusting for all variables associated with mortality which differed between the statin group and the no statin group using multivariate models, statin use was associated with a decreased risk of mortality at both 28-days (Odds ratio for death [95% CI]= 0.36 [0.15 – 0.85]) and 1 year (OR for death [95% CI] 0.48 = [0.27-0.84] Table 3).

Table 16-3. Multivariate analysis of the association between statin use at hospital admission and mortality

<table>
<thead>
<tr>
<th></th>
<th>Odds Ratio</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>28-day mortality</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unadjusted</td>
<td>1.11</td>
<td>0.55-2.23</td>
</tr>
<tr>
<td>Adjusted(^a)</td>
<td>0.36</td>
<td>0.15-0.85</td>
</tr>
<tr>
<td><strong>1 year mortality</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unadjusted</td>
<td>1.34</td>
<td>0.83-2.14</td>
</tr>
<tr>
<td>Adjusted(^b)</td>
<td>0.48</td>
<td>0.27-0.84</td>
</tr>
</tbody>
</table>

Derived from logistic regression models with mortality as the outcome variable and statin use as the first dependent variable.
\(^a\) Adjusted for age, SOFA score, APACHE score, Charlson comorbidity index and number of SIRS criteria met within the first 48 hours of hospitalisation.
\(^b\) Adjusted for age, Indigenous status, Charlson comorbidity index, APACHE II score and chronic liver disease

16.6 Discussion

In the first study to examine the relationship between statin use and sepsis outcomes in Northern Australia, we have found a significant association between statin use and decreased mortality from sepsis, both at 28 days and 1 year of follow up. The lack of effect of statin use on mortality on univariate analysis may be explained by the substantial difference in several important factors between the statin and no statin groups. These include age, comorbidities and the severity of acute illness, all of which are important determinants of mortality from sepsis [29]. When these factors were adjusted for, the degree of association between statin use and decreased mortality (odds ratio 0.36) was similar to that observed in most observational studies of statins in patients hospitalised with sepsis or infection (odds ratios for short-term mortality of 0.13 [15]; 0.27 [18]; 0.39 [16]; 0.42 [17]; 0.43 [21]; and 0.48 [30]) or with pneumonia (odds ratios of 0.33 [31]; 0.36 [32]; and 0.46 [33]). It is also consistent with a summary odds ratio for mortality from a recent meta-analysis of nine such studies, which was 0.55 (95% CI 0.36-0.86) [11].

Of the eight published cohort studies which have specifically assessed the effect of statins on mortality in patients hospitalised with sepsis or bacteraemia, six found decreased
mortality in the statin group [15-18, 21, 30] and two did not [19, 23]. One of these two found decreased mortality at 180 days of follow up, but not at 30 days [19]. The prevalence of statin use in the populations of these studies ranged from 3.2% [19] to 32% [17], with a mean of 17.2%, similar to the 15% in the present study. Moreover, all of these studies except for one [17] found significant differences in baseline characteristics between the statin and no statin groups, primarily with respect to comorbidities.

The study by Yang and colleagues found no difference in 30 day mortality among statin users compared with non-users among 454 Taiwanese people hospitalized with sepsis. The authors suggest this discrepancy with other published studies might be due to the fact that theirs is the only study to have included “Oriental people” [23]. All other comparable published studies to date have been conducted in North America or Western Europe. It is certainly possible that racial differences in genetic polymorphisms influencing sepsis susceptibility [34, 35] or drug metabolism [36] could account for such differences, supporting the need to study the association between statin use and sepsis outcomes in other racial groups such as Indigenous Australians.

The prevalence of statin use in this population was lower than expected, with less than half of those with indications for statin therapy having been prescribed it. There are several possible explanations for this finding. Firstly, we have studied a cohort of patients hospitalized with sepsis, who may not be representative of the population as a whole, particularly with regard to their high prevalence of hazardous alcohol use (46.3%) and chronic liver disease (9.3%). Secondly, since we relied on documentation of a statin having been prescribed in the last 30 days, it is possible that we have underestimated statin use in this population. However, since all patients from remote communities (25% of the cohort) have details of prescribed drugs included on databases which are accessible to hospital staff on admission, this appears unlikely, at least for these patients. Finally, there is a disconnect between clinical guidelines on the indications for statin therapy, and the approved indications for government subsidy of statin prescriptions. Even allowing for the above issues, it is concerning that only half of those for whom a statin is indicated were actually receiving one. It is encouraging to note that Indigenous status or remote-dwelling were not associated with guideline-discordance for statin use. More work needs to be done across the local population to determine factors influencing statin prescribing by doctors, and to improve the uptake of this simple but effective intervention for the prevention of vascular disease-associated mortality.
Observational studies assessing the association between statin use and mortality, including the present study, all share a fundamental flaw: an inability to prove a causative association. This has led some to hypothesise that statins do not have any protective effect on infection-related mortality, and that all of the observational studies have been biased by the “healthy-user effect” [37]. Moreover, a meta-analysis of observational studies assessing statins for the prevention and treatment of infections found evidence of possible publication bias (Egger test p=0.07), as well as substantial heterogeneity [11]. These concerns underline the need for randomized controlled trials of statins in patients with sepsis, several of which are currently underway [11, 38]. The only RCT to have been published to date found a significant decrease in pro-inflammatory cytokines in patients with bacterial infections randomised to simvastatin, but was underpowered to assess effects on mortality [39].

In conclusion, in this prospective cohort study in Northern Australia, statin use was associated with a substantial decrease in all-cause mortality among patients hospitalised with sepsis, consistent with the findings of the majority of similar studies from Europe and North America. Statins appear to be under-prescribed in this population with a high incidence of vascular disease and sepsis, and the reasons behind this need to be further investigated. The current study adds to the growing weight of published observational data suggesting a protective effect of statin use on sepsis mortality, but the results of randomized trials should be awaited before definitive conclusions can be drawn.
16.7 References


Chapter 17. Targeting the endothelium in sepsis: genesis of and protocol for a randomised controlled trial of atorvastatin in patients with severe sepsis
17.1 Genesis of the STREAMS study and its relationship with the STATInS study

17.1.1 Original plans for a randomised trial of atorvastatin for severe sepsis at Royal Darwin Hospital.

Part of the original research plan for my PhD was to develop and commence a randomised controlled trial (RCT) of atorvastatin for patients with sepsis at Royal Darwin Hospital (RDH). I had developed a preliminary protocol for this study during 2006, prior to the commencement of my PhD in February 2007. The primary outcome measure was going to be duration of organ dysfunction, and the secondary outcome measures included endothelial function as measured by RH-PAT. The plan was for this to be a phase II, “proof of concept” study, with an eventual subsequent funding application to move on to a multicentre phase III study with mortality as an endpoint.

However, prior to this local RCT commencing, the Australian New Zealand Clinical Trials Group (ANZICS CTG) endorsed a multicentre study (the Study of Atorvastatin Therapy in Sepsis, or the STATInS study) which substantially overlapped with the local RCT. Furthermore, the RDH ICU decided to take part in the multicentre STATInS study, meaning that the local RCT would now be impossible to conduct. Hence following discussions with my supervisor, and negotiations with my collaborators in the RDH ICU, and with the management committee of the STATInS study (primarily Dr Peter Kruger), we salvaged the plan by deciding to nest a local substudy (STREAMS) within the STATInS study at RDH.

Local HREC approval was gained for both the national study and the substudy; participants are able to consent to just the STATInS study, or to both the STATInS and STREAMS studies.
17.1.2 Summary of the STATInS protocol

17.1.2.1 My role in the STATInS study
I am not a member of the management committee of the STATInS study and was not an author of its protocol. However, I attended the start-up meeting for the study (In Noosa, March 2007), and contributed to discussions regarding study design both at this meeting and subsequently, and thus made several minor contributions. Along with Dr Dianne Stephens, the director of RDH ICU, I am the principal site investigator for the STATInS study at RDH.

17.1.2.2 Australia New Zealand Clinical trial registry numbers

- STATInS - ACTRN12607000028404
- STREAMS - ACTRN12607000393459

17.1.2.3 Overview and hypotheses
The STATInS study is being funded by an NH&MRC project grant, and the ANZIC Research Centre is providing infrastructure and administrative support. It is a randomised, double-blind, placebo controlled, phase II trial. There are approximately fifteen ICUs in Australia and New Zealand enrolling patients in the study.

The hypotheses of the STATInS study are as follows:

In a heterogeneous population of ICU patients with severe sepsis:

- Continuation of pre-existing statin therapy will result in lower levels of inflammatory markers and improved clinical outcome compared with those in whom statin therapy is discontinued
- Commencement of de-novo atorvastatin therapy in patients not previously on statin therapy will result in lower levels of inflammatory markers and improved clinical outcome compared with placebo.
- The degree of reduction in inflammatory markers will be related to the serum levels of atorvastatin (efficacy-response relationship).
- There will be variation in pharmacokinetics of atorvastatin depending on patient gastric emptying, the presence of renal and hepatic function, and the use of agents known to interact with the Cytochrome P450 3A system.
17.1.2.4 Outcome measures of the STATInS study

Primary outcome measure:
- Change in serum IL-6 concentrations

Secondary outcome measures:
- Biological
  - Levels of C-Reactive Protein (CRP [marker of inflammation])
  - Plasma 8-isoprostane level (marker of oxidative stress)
  - Plasma nitrate/nitrite ratio (marker of NO production)
  - Levels of AT III
  - Lipid profile – Including total cholesterol, HDL, Triglycerides and calculated LDL
  - Lipoprotein profile – Apo A1, Apo B, lipoprotein a
  - Immunoglobulin levels
- Clinical
  - Days alive and free of organ dysfunction
  - Percentage of patients on vasopressor agents at 72 hours.
  - ICU and hospital length of stay and mortality
- Pharmacological
  - Incidence of adverse events
  - Serum levels of atorvastatin and population pharmacokinetics

17.1.2.5 STATInS study recruitment

The STATInS study aims to enrol 250 patients in total.

Inclusion criteria are all four of the following:
- Strongly suspected or confirmed focus of sepsis
- Three or more SIRS criteria within the last 48 hours
- A newly-developed sepsis-related organ dysfunction (defined in a similar manner to the PROWESS study)
- Randomisation achieved within 24 hours of the onset of severe sepsis

Exclusion criteria are any of the following:
- Age < 18 years and > 90 years
- Death is imminent (<24 hours)
- Pregnancy or breast feeding
- Known history of intolerance to a statin agent
- Acute liver failure (INR > 2.5, that in the clinician’s opinion is due to liver dysfunction)
- Severe liver disease - Child - Pugh’s classification C (see appendix 4)
- The most recent serum ALT > 5 x ULN
- Any other liver disease which in the opinion of the treating clinician would potentially predispose the patient to adverse effects from the study medication
- Rhabdomyolysis (CK > 10 times the upper limit of normal.
- Enteral administration of the study drug not possible
- Any factor which would prevent informed consent being obtained from the patient (where possible) or the person responsible / next of kin/ legal guardian.
- Patient’s family, physician or both not in favour of aggressive treatment of the patient or the presence of an advance directive to withhold life-sustaining treatment
- Patient not expected to survive 28 days because of an irreversible medical condition such as poorly controlled neoplasm or other end-stage disease.
- Human Immunodeficiency Virus (HIV) infection with a CD4 count <50/mm3
- Patients on statin therapy prior to hospital admission that either:
  - was commenced less than two weeks prior to randomisation; OR
  - has been stopped for more than 72 hours prior to randomisation.
- Patient is unable to tolerate caffeine
- Concomitant fibrate medication which will not cease at randomisation

17.1.2.6 STATInS study procedures
The study medication is 20mg atorvastatin, or 20mg of identical placebo. Participants will be given a dose of study medication each morning (PO, NG or via PEG), and have blood tests as described in table 17-1. The study will continue for 14 days or until the patient is discharged from ICU. Study drug will be permanently discontinued if the ALT exceeds 5 times the upper limit of normal (ULN), or if CK exceeds 10 times ULN.

17.1.3 Progress of the STATInS and STREAMS studies at the time of writing
The STATInS (national) study commenced enrolment in July 2007. In March 2010, a total of 211 patients have been enrolled, with a target of 250. An interim safety analysis has been performed by the DSMB, who found no safety concerns and recommended continuing the study.
The STREAMS (local RDH) substudy commenced enrolment in September 2007. By March 2010, 36 patients had been enrolled, with a target of 78. The recruitment rate, both locally and nationally has been significantly slower than anticipated. The primary reasons for this are the exclusion criteria. A high proportion of patients as RDH ICU with severe sepsis have liver disease or rhabdomyolysis. Recruitment is currently continuing in both the national STATInS study and the local STREAMS study.

Note that the attached STREAMS study protocol was written in early 2007, prior to the completion of the FRESH study (Chapters 9-13), and prior to the completion of the updated literature reviews detailed in Chapters 2 to 4. Minor amendments were made in 2009. The protocol has been reformatted for inclusion in the thesis, but has not been altered from the original version, so that it can be assessed in context. The introductory literature review, and the data upon which are based the power analysis are now out of date, but are included in their original forms.
17.2 A protocol for a randomised controlled trial of atorvastatin in patients with severe sepsis.

STREAMS (STatinS to Reduce Endothelial dysfunction
Adjuvant Management in Sepsis)

A STATInS SUBSTUDY

A PILOT RANDOMISED CONTROLLED TRIAL OF ATORVASTATIN THERAPY AND ITS IMPACT ON ENDOTHELIAL FUNCTION IN PATIENTS WITH SEPSIS.

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This study has been endorsed by the management committee of the STATInS study.
17.2.1 Preamble

Royal Darwin Hospital (RDH) ICU will be one of fifteen ICUs in Australasia taking part in the ANZICS CTG STATInS study, a randomised controlled trial of atorvastatin versus placebo in ICU patients with severe sepsis. In an extension of our previous work on endothelial function in malaria and sepsis, we plan to conduct a substudy (STREAMS) on all consenting locally enrolled patients. Study design and procedures will be identical to those in STATInS, apart from an additional non-invasive measurement of endothelial and microvascular function as well as 6mls of additional blood collection on days 0, 1, 2 and 7. These measurements will be taken in all consenting patients, including those who have been discharged from ICU/HDU but remain in hospital. The STREAMS study has a target enrolment of 78 patients and will have two stages – the first will be a substudy of STATInS. The second will be a post-STATInS stage, a single-centre continuation until the target recruitment is met.

17.2.2 Introduction and rationale

17.2.2.1 Overview

Severe sepsis (defined as sepsis associated with organ dysfunction) is a common reason for admission to the intensive care unit (ICU). A recent national inception cohort study found an incidence of severe sepsis of 0.77/1000 population, corresponding to more than 15,000 new cases each year in Australian ICUs [1]. This common condition carries a hospital mortality of 37.5% and consumes vast health care resources. The cost per episode of severe sepsis has been estimated at 22,800 Euros ($39,300 AUD) [2].

The treatment of severe sepsis includes fluid resuscitation and/or vasopressor therapy to restore circulatory stability, diagnostic studies to identify the causative organism, administration of appropriate antibiotics and source control measures [3]. This approach to treatment has remained essentially unaltered for the last three decades and mortality remains high; thus there is an urgent need for new adjunctive (non-antibiotic) therapies for sepsis. In Australia, the Therapeutic Goods Administration has granted approval for the use of only one agent as adjunctive treatment of severe sepsis or septic shock: activated protein C (drotrecogin alpha) [4]. However, this drug is expensive (approx. AUD $10-12,000/patient) and its efficacy is the subject of intense controversy.
Severe sepsis is characterised by major dysfunction of the inflammatory response to infection and efficacious new adjunctive therapies are likely to possess immune modulating properties [5]. Increasing evidence from animal studies and observational human studies suggests that statins, as a class of drugs, possess anti-inflammatory, antioxidant, endothelial function modulating and immune modulating effects [6, 7] However, balancing such potential benefits are concerns about the increased risk of toxicity in the critically ill [8]. As a result, current prescribing guidelines recommend that statin therapy should be ceased in acute illness. However, there are minimal pharmacokinetic data and no controlled studies to assess the risk-benefit profile of continuing or commencing statin therapy in ICU patients with severe sepsis.

Although there is a growing body of evidence to suggest that statin therapy is likely to be beneficial in sepsis, the mechanisms of this benefit remain to be elucidated. Multiple potential mechanisms have been proposed [7], but their relative importance is unclear. A salutary effect on endothelial function is one of the proposed mechanisms of benefit, and this is one of the few which is measurable at the bedside.

Microcirculatory and endothelial dysfunction are invoked to explain the paradox of organ failure in sepsis despite adequate blood pressure, cardiac output and fluid status [9]. However, many questions remain about microcirculatory dysfunction, primarily around its therapeutic implications. Endothelial dysfunction is well characterised in septic animals, but has only been studied in small numbers of septic humans [10-15]. A better understanding of the pathophysiological and prognostic significance of endothelial dysfunction in sepsis will help to define a new potential target for adjuvant therapies in sepsis – the endothelium [16].

17.2.2.2 Endothelial Function in Sepsis.

The endothelium is an active organ which plays roles in vasoregulation, thromboresistance, barrier function and co-ordination of the inflammatory response [17]. One way that sepsis causes organ damage and death is through alterations in small blood vessel blood flow to vital organs [18]. Factors contributing to this include reversible narrowing of very small blood vessels (vasoconstriction), injury and dysfunction of the cells lining blood vessels (endothelial cell dysfunction) and formation of small clots in blood vessels [18].
The endothelium becomes “activated” early in sepsis, in response to pro-inflammatory cytokines (e.g. IL-1, TNF-a). This activated state changes the endothelial cell from an anticoagulant to a procoagulant state and also causes it to express adhesion molecules which allow passing white blood cells to adhere and migrate into tissues. Endothelial cell activation is probably an adaptive response to infection, aiming to prevent spread of a local infection. However, in severe sepsis, this cell activation can progress to cell damage, dysfunction and apoptosis [19]. Animal models of sepsis have demonstrated endothelial dysfunction [20, 21]. Endothelial cell activation can be difficult to distinguish from dysfunction, and there is no generally accepted way of doing so in septic humans. Both states result in increased serum levels of soluble adhesion molecules (e.g. ICAM-1, VCAM-1, E-selectin). Endothelial dysfunction implies not just a change in the endothelial cell phenotype, but a decrease in the cells’ ability to prevent fluid leakage into tissues and to regulate blood flow at a microvascular level. Functional and physiological measurements of the endothelium, rather than just biochemical, are necessary to differentiate dysfunction from activation, and to truly understand the endothelium in sepsis.

Endothelial function is defined as the ability of blood vessels to dilate in response to either Acetyl Choline (ACh) or shear stress. Traditionally this was measured in the coronary circulation, by angiography before and after intra-arterial ACh. In 1992, Celermajer described flow-mediated dilatation of the brachial artery, as measured by ultrasound [22]. This method has since been adopted as the gold standard non-invasive technique for measuring endothelial function (EF). However, it is highly user-dependent, subjective, and difficult to perform in patients who cannot lie completely still.

The “Endopat” (Itamar Medical, Caeserea, Israel) is a portable device that uses finger peripheral arterial tonometry (PAT) to measure pulse wave amplitude (PWA) before and after a 5 minute ischaemic stress. It then calculates the post:pre PWA ratio after controlling for systemic changes using the contralateral arm (PAT index). This has been shown to correlate well with established techniques for measuring endothelial function [23, 24]. The quantification of reactive hyperaemia by this method reflects endothelial function and has been shown to be at least 50% nitric-oxide dependent [25]. Our research group has successfully used PAT to show that patients with severe falciparum malaria have significant impairment of endothelial function, which is associated with measures of endothelial activation (plasma ICAM-1) and blood lactate [26].
Endothelial function in septic humans has not been well characterized in the past. Several small studies (n=8-16) have previously shown it to be impaired [10-15]. These studies used complex and impractical methodologies (e.g. venous plethysmography and invasive tissue laser Doppler) and did not have significant longitudinal or correlative components. To our knowledge PAT has not been used in septic humans before, but our group has found it to be a safe and effective for measuring EF in both severe malaria and severe sepsis.

17.2.2.3 Preliminary results from our observational study of endothelial function in sepsis.

We are conducting an ongoing observational study of sepsis based in the Royal Darwin Hospital, using the Endopat to measure EF in patients with sepsis over time [27]. We have recently undertaken analysis of data from over 80 patients. The mean (95% CI) PAT index at day 0 in 28 healthy controls was 2.03 (1.87, 2.19). This compares with 1.56 (1.41, 1.71) in 38 ICU patients with sepsis and 1.72 (1.52, 1.92) in 19 non-ICU patients with sepsis. In summary, endothelial function is severely impaired in the ICU sepsis patients at baseline, compared with control patients (p=0.0001). It is moderately impaired in the less severely ill ward sepsis patients compared with healthy controls (p=0.02)

We also found that endothelial function showed only a minor, non-significant improvement in ICU sepsis patients over the first 48 hours of hospitalisation, but improved completely to normal in the less severely ill ward sepsis patients.

17.2.2.4 Arginine and its metabolites in sepsis.

Arginine is the precursor for the generation of nitric oxide by endothelial cells. Endothelial production of nitric oxide is part of normal healthy blood vessel function and assists in maintaining blood flow to organs. We have shown that the impaired endothelial cell function in patients with malaria is partially reversible with intravenous arginine and this is associated with increased nitric oxide production [26]. Plasma concentrations of plasma arginine are known to be similarly low in severe sepsis. This may contribute to impaired endothelial function in sepsis. Asymmetric dimethyl arginine (ADMA) is an arginine metabolite which competes with arginine as a substrate for nitric oxide synthase (NOS), thus preventing the production of nitric oxide. Even with normal arginine concentrations, a raised plasma ADMA level may be associated with endothelial dysfunction. Raised ADMA
levels have been shown to be independently associated with increased mortality in critical illness [28].

17.2.2.5 Near Infrared Spectroscopy (NIRS)

Near-infrared spectroscopy (NIRS) is a technology for measuring tissue oxygen content [29]. It has been used to measure muscle and brain oxygenation in the settings of neuro- and cardiac surgery, trauma resuscitation and, more recently, sepsis. NIRS can be used to provide a measurable target for resuscitation after trauma and other acute illness.

In addition, combining NIRS with an ischaemic stress can measure the rate of tissue oxygen consumption and tissue reperfusion. This provides vital information to characterize microcirculatory function. Patients with severe sepsis can experience organ failure, lactic acidosis and death despite adequate blood pressure and oxygenation. Important questions remain about the relative contribution to this problem of shunting at the level of the microcirculation, or of “mitochondrial dysoxia”, an inability of the cell to efficiently use oxygen. NIRS can help answer these questions.

The “InSpectra StO₂”, (Hutchinson technology, Minnesota USA) is a portable monitoring device which uses an adherent flat probe placed on the thenar eminence to give a continuous reading of tissue oxygen content (measured as oxygen saturation of haemoglobin). This technology has been previously used in patients with severe sepsis, and has found a significantly slower reoxygenation rate of the thenar eminence after release of an ischaemic stress in septic patients versus healthy controls [30]. In other words, the tissue oxygen content increases more slowly over the first 14 seconds post cuff release in septic patients than in healthy patients. This is primarily a measure of reactive hyperaemia. A slower reoxygenation rate in septic patients has also been shown to correlate with increased mortality and more organ dysfunction [30, 31].

17.2.2.6 Statins

In addition to their effects on cholesterol metabolism, statins are thought to have a number of pleiotropic effects that may be beneficial in modulating the inflammatory response to sepsis [6]. Statins reduce the production of reactive oxygen species and inhibit the respiratory burst of phagocytes [32]. They also reduce C-reactive protein (CRP), modulate nitric oxide production and reduce monocyte adhesion to vascular endothelium [7].
addition, atorvastatin has recently been shown to decrease circulating ADMA concentrations [33].

The putative beneficial effects of statins in sepsis have been tested in several animal models. In a murine model of caecal ligation and perforation, Merx and colleagues demonstrated that mice pre-treated with statins had a 4-fold increase in mean survival compared to controls [34]. More importantly, the same investigators subsequently found that statin therapy commenced after induction of sepsis also significantly improved survival time (23+/−1.2 hours for placebo versus 40+/−4.2 hours for atorvastatin) [35]. In addition, pre-treatment with intravenous cerivastatin in mice with lipopolysaccharide-induced sepsis attenuated production of TNFα and IL1β [36] and simvastatin pre-treatment has been shown to reduce pulmonary inflammatory cell infiltrates in *Chlamydia pneumoniae* infection in mice [37].

Further evidence for a beneficial effect of statin therapy in septic states comes from observational human studies. In a retrospective study of 388 bacteraemic patients, Liappis and co-workers reported that overall mortality was reduced from 28% to 6% (p=0.002) [38]. Kruger and co-workers similarly found that all-cause hospital mortality was reduced from 23.1% to 10.6% in 438 patients with bacteraemia [39]. Thus far, eleven cohort studies assessing the effect of statins on mortality from sepsis have been published, including a total of 92,059 patients. Of the eleven studies, nine found a significant protective effect of statin use on development of sepsis or death from sepsis. The findings from all of these investigations have all the inherent limitations of observational studies. Importantly, they cannot fully account for the effect of greater illness severity leading to cessation of statin therapy, thus biasing study outcomes. It is also possible that the beneficial effects suggested for prior statin therapy in sepsis are related to the “healthy user effect” [40].

Statins have been shown to improve chronic endothelial dysfunction in patients with cardiovascular risk factors, with this effect evident within 4 hours of administration [41]. There is also evidence from *in-vitro* and animal models that statins improve nitric oxide availability and down-regulate excessive nitric oxide production [42]. Finally, studies in human volunteers have shown that statins blunt the acute endothelial dysfunction caused by intravenous endotoxin (which causes inflammation and mimics infection) [43, 44]. There are no published studies examining the effect of statins on endothelial function in patients with sepsis.
In summary, there is strong theoretical and \textit{in-vitro} evidence that statins have an anti-inflammatory effect and can improve endothelial function. Furthermore, there is a strong signal from multiple observational studies in humans that statins can prevent and attenuate sepsis in chronic users. Whether statins will be safe and effective if given to humans after the onset of sepsis is an important question which remains to be answered, as is the mechanism of such a benefit.

\textbf{17.2.3 Methods}

STREAMS will be a prospective, double blind, randomised controlled trial. It will be a single-centre substudy of a national multicentre RCT Inclusion criteria, randomisation, drug preparation and administration, data safety monitoring and study procedures will all be identical to those in STATInS (refer to the STATInS protocol for details). Any differences from or additions to the STATInS protocol are detailed below.

The trial will be conducted in two stages. The first stage will be a substudy of STATInS. The second stage will be a stand-alone local continuation of the study.

\textbf{17.2.3.1 Aims of STREAMS}

In addition to the aims of the STATInS trial:

To prospectively assess, in adult patients with severe sepsis in ICU:

- The effect of atorvastatin therapy on endothelial and microvascular function and biomarkers related to endothelial function
- The association between the degree of endothelial dysfunction and plasma arginine: ADMA ratio, and the level of selected plasma and cellular markers of inflammation and endothelial cell activation.

\textbf{17.2.3.2 Primary endpoint}

- Change in RH-PAT (reactive hyperaemia peripheral arterial tonometry) index at 24 hours and 48 hours post enrolment.
### 17.2.3.3 Secondary outcome measures

As for STATInS, with the following additions

- Change in microvascular function using tissue oxygen recovery rate at 24 and 48 hours (as measured by NIRS)
- Change in basal thenar muscle tissue oxygen saturation over the first 7 days
- Proportion of patients with endothelial dysfunction (RH-PAT index<1.67) at 24h, 48h and 7 days post enrolment.
- Severity of organ dysfunction (as measured by SOFA scores) over first 7 days
- Change in plasma arginine:ADMA ratio over the first 7 days
- Change in serum markers of endothelial activation over the first 7 days

### 17.2.3.4 Study population

#### 17.2.3.4.1 Power analysis

In total, STREAMS will aim to recruit a total of 78 patients with severe sepsis over an 24 month period. Preliminary work at RDH has shown that in patients with sepsis, mean (95% CI) PAT index at day 0 (n=26) is 1.66 (1.47-1.85) and at day 2 (n=26) is 1.76 (1.54-1.98) p=0.33 by paired t-test. In contrast, ward patients (as opposed to those in ICU) with sepsis had a baseline index of 1.84 (1.55-2.14) and this improved to 2.30 (1.99-2.61) by day 2 (p=0.0004 by paired t test). 28 healthy control patients had an index of 2.03 (1.87-2.19). In other words, the PAT index (a marker of endothelial function) improves by only a small, non-significant margin (0.10) in ICU patients over the first 48 hours, but improves completely to normal (increment 0.46) in ward patients with sepsis, who are less severely ill.

In light of these figures, we would consider a further increment of PAT index at day 2 of 0.35 (a 20% improvement) in the ICU statin group to be clinically significant. Assuming an alpha of 0.05 and a power of 80%, 28 patients would be required in each of the two groups to be able to detect such a difference. Considering only 70% of patients thus far have had follow-up measurements, we need to multiply this by 1.4. This gives a total sample size of 78 patients. It is estimated that RDH will contribute 35-50 patients to STATInS (and thus to STREAMS). This is based on a recruitment rate of 3-4 patients per month, taken from recent severe sepsis studies in our ICU, and a recruitment period of 12-18 months. Thus at the conclusion of the STATInS multicentre trial, there will remain 30-40 patients to be recruited into the STREAMS study.
17.2.3.4.2 Inclusion and Exclusion Criteria

Inclusion criteria will be identical to those for STATInS (Adult ICU patients with severe sepsis of less than 24 hours duration)

Additional exclusion criteria will be

- Known latex allergy
- Platelet count <20x10^9/L
- INR >2.0
- APTT >70 seconds
- Receiving intravenous GTN or sodium nitroprusside

The rationale for these extra exclusion criteria are:

- To minimize the risk of bruising or bleeding into the skin as a result of the blood pressure cuff being inflated on the forearm (though these have not been found in our studies of RH-PAT in severe malaria patients with thrombocytopenia)
- The finger cuffs on the Endopat contain latex
- GTN and sodium nitroprusside are direct NO donors and are likely to affect endothelial function.

17.2.3.5 Study procedures

Identical to those in STATInS with the following additions (see table 17-1):

- A measurement of endothelial function, using the Endopat apparatus, taken on days 0, 1, 2 and 7
- A measurement of microvascular function using NIRS on days 0, 1 and 2
- 8mls of additional blood will be taken on the above days, at the time of the Endopat measurement. (changed Oct 08 from 1x 6ml LiHep tube to 1x6ml LiHep tube at room temperature plus 1x2ml LiHep tube put straight on ice)
- Endopat and NIRS measurements will be taken at the above time points in all patients, including those who have ceased study drug, either due to discharge from ICU/HDU, or for other reasons. If a patient withdraws consent for ongoing involvement in the study, further readings will not be taken, but permission will be sought to use the existing data for that patient.
Table 17-1. STREAMS study procedures according to day
(adapted from STATInS study protocol).

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17.2.3.6 Laboratory Procedures

17.2.3.6.1 Specimen collection
Non-invasive Endopat and NIRS measurements and blood for STREAMS will be collected prior to the daily dose of atorvastatin whenever possible (around 11am). This is because caffeine will be co-administered with atorvastatin on days 1, 3, 5, 7, 10 and 14. Caffeine may affect endothelial function.

On days 0, 1 and 7, 5x3ml tubes of blood will be collected for the pre-dose STATInS central laboratory specimens. On these days, 2 additional lithium heparin tubes will be collected for STREAMS at the same time – 1 x 6ml tube (left at room temperature with the rest) and 1 x 2ml tube, which will be immediately put on ice.

On day 2, pre-dose bloods are not being collected for STATInS. On this day, 2 additional lithium heparin tubes will be collected for STREAMS – 1 x 6ml tube (left at room temperature) and 1 x 2ml tube, which will be immediately put on ice.

17.2.3.6.2 Central laboratory blood processing
All of these specimens (as well as the blood tubes taken on days when extra STREAMS bloods are not collected) will be transferred to the laboratory at the Menzies School of Health Research, as soon as possible (within 30 minutes of collection). They will be centrifuged and the plasma and cells separated.

The plasma from the 5 central laboratory specimen tubes will be transferred to pre-labelled aliquot tubes as detailed in the STATInS protocol. These tubes will be stored at -70 degrees centigrade and will be shipped to the central laboratory in Queensland at the end of the study, or earlier if there is insufficient freezer space. Any residual white cells cells/plasma following centrifugation of blood collected for STATInS will be stored and assayed as outlined in STREAMS below (5.4.3).

17.2.3.6.3 STREAMS specimen processing
17.2.3.6.3.1 Plasma
On days 0, 1, 2 and 7, the 6ml lithium heparin tube which has been collected for STREAMS will be centrifuged. The plasma from this tube will be stored in one or more aliquot tubes in a -70 degrees centigrade freezer, in a separate location from the central laboratory
samples. At an interim analysis and at the end of the study, these samples will be thawed and processed at Menzies. They will undergo testing for measures of sepsis severity, including cytokines measured by bead array, tetrahydrobiopterin, and markers of endothelial activation measured by ELISA (ICAM-1, e-selectin and angiopoietin 2)

The 2ml LiHep tube which was put straight on ice will be used for HPLC to measure plasma amino acids. It will initially be centrifuged and then the plasma stored at -70 in the same way as the other blood specimens, but they will be clearly labelled as having been put straight on ice.

17.2.3.6.3.2 Cells

Only room temperature samples can be used for PBMC extraction. On days 0 and 7, there will be three such STATInS lithium heparin tubes. The infranatant from these tubes, along with that from the 6ml STREAMS tube will be combined. On days 0 and 7, these cells will be processed to separate the lymphocytes. The lymphocytes will then be stored in liquid nitrogen for later analysis.

After thawing of PBMCs, T-Cell function will be assessed as a hypothesis-generating investigation. Flow cytometry will be used to determine the proportion of T-cells which fail to express the zeta subunit of the T-cell receptor. This proportion will be compared in the atorvastatin versus placebo group on days 0, 2, and 7. It will also be correlated with the Arginine:ADMA ratio.

On day 2, there will only be a single 6ml room temperature tube. Although there will be a lower number of cells available, lymphocytes will be separated and stored from this day 2 blood.

So plasma will be stored for STREAMS on days 0, 1, 2 and 7; PBMCs will be stored for STREAMS on days 0, 2 and 7

Once a patient is randomised and data collection is about to commence, Menzies lab staff will be notified. Samples will then be retrieved from RDH by Menzies lab staff. On Monday to Saturday, all blood samples will be processed ASAP. On Sunday, STREAMS bloods will be processed as usual but samples which are exclusively non-STREMS bloods (i.e. day 3, 5, 10 or 14) will be stored in the ICU blood fridge (at 4 degrees) until first thing Monday morning.
17.2.3.7 Data management and statistical analysis

17.2.7.1 Data management and publication.
There will be two parallel databases for STATInS and STREAMS. All data required for STATInS will be sent to the central data collection site. These will be deidentified data. All additional data required for STREAMS will be kept in a local database at the Menzies School of Health Research. No identified data will leave Royal Darwin Hospital. Data for STREAMS will be double-entered into a password-protected ACCESS database located on the Menzies server.
The STATInS database will be managed by the ANZIC research centre at Monash University. The STREAMS database will be managed by Dr Josh Davis, at Menzies.

The exact nature of the contribution of the ANZICS CTG, the ANZIC RC and the STATInS investigators will be acknowledged and made clear when STREAMS is published. Data from STREAMS will not be published prior to that from STATInS.

17.2.7.2 Statistical Considerations
17.2.7.2.1 Planned Interim Analysis
STREAMS will continue as a stand-alone study at RDH after the completion of STATInS until the target recruitment is met. At the conclusion of STATInS, an interim analysis of STREAMS will be conducted by the independent DSMB. The investigators will not have access to the data used for this analysis and will not take part in the analysis.

The purposes of this interim analysis will be:

- Safety – the STREAMS DSMB will consider the report of the STATInS DSMB regarding safety and will decide whether it is safe and ethical to continue the trial locally in light of this report.
- Efficacy – The DSMB will consider stopping the trial early if the primary end point has been reached at a highly significant level (defined as a p-value of <0.001, according to a conservative alpha-spending function).
- Examining trial conduct, validating assumptions and refining power analysis – The DSMB will examine data integrity and trial conduct. They will refine the power analysis based on the existing data and target recruitment may be altered, in discussion with the investigators.
17.2.7.2.2 Final Data Analysis

Any patient who has been randomised, has received at least one dose of study drug and has had PAT readings taken at 24 and 48h will be included in the final analysis for the primary end point, according to a modified intention to treat principle.

The RH-PAT index at 24 and 48 hours will be compared in the atorvastatin and placebo groups. Specifically, the increment of RH-PAT index over the first 24 and 48 hours (a continuous variable) will be compared in the two groups by unpaired student’s t-test. Data will be first checked for normality, and if the data is skewed or otherwise non-normal and not transformable, non-parametric methods of analysis will be used (Mann-Whitney U test). If major differences in baseline characteristics between the groups are found, statistical adjustment will be made using multivariate analysis. A p value of 0.05 will be considered significant. A priori subgroup analysis will be conducted for pre-existing statin use.

17.2.3.8 Ethical considerations

The RDH ethics application for the STATInS study included STREAMS and the patient information and consent forms for STATInS include an additional section for the substudy. Patients or their surrogate will be able to consent for the STATInS study and decline the substudy. Ethical concerns regarding consent and the risk/benefit ratio of the intervention will be identical to those for STATInS.

The additional ethical concerns for STREAMS are:

- Measuring endothelial function using Endopat and NIRS involves inflation of a blood-pressure cuff on the forearm for 5 minutes. This has been well tolerated by all 107 patients recruited into the FRESH study thus far, as well as around 200 patients we have studied in Timika with severe malaria. We will exclude patients at high risk of bleeding so as to minimize the chance of bruising being caused by the blood pressure cuff.
- An additional 8ml of blood will be collected on four occasions over the first 7 days. This 32ml of blood over two weeks is a modest amount and does not present risk to the subject.
17.2.4 The local continuation stage of the STREAMS study

It is anticipated that 30-50 patients will be enrolled at RDH as part of the STATInS multicentre RCT. When the STATInS study ceases enrolment, the STREAMS study will continue at RDH as a single-centre continuation, until target recruitment is met. We will no longer have the benefit of the infrastructure and resources of the STATInS study in this stage. The solution to these problems is detailed below.

It is important to note that if the STATInS study finds atorvastatin to be harmful, then the continuation phase of STREAMS will be cancelled, and the study will be stopped early.

17.2.4.1 Randomisation
Randomisation will be performed locally using a computer-generated list in random permuted blocks with variable block sizes of 4 and 6. Randomisation will also be stratified by pre-existing statin use, and will allocate patients to either atorvastatin or placebo in a 1:1 ratio. The randomisation list will be held by the hospital pharmacy. Once patients have met eligibility criteria and informed consent has been obtained, the ICU pharmacist will be contacted, and he/she will use the list to allocate patients to either atorvastatin or placebo.

17.2.4.2 Study medication
This will continue to be supplied by Wickham House pharmacy, Queensland (purchased by the STREAMS study). It will be identical to that used in the STATInS trial, i.e. amber glass bottles each containing 16 gelatine capsules of finely crushed atorvastatin or of methylcellulose.

17.2.4.3 Study population
Inclusion and exclusion criteria and patient recruitment methods will not change for the post-STATInS stage of the trial.

17.2.4.4 Study procedures
17.2.6.4.1 Data collection
Specific simplified clinical research forms (CRFs) will be used for the post-STATInS stage of the study.

17.2.6.4.2 Pharmacokinetics
There will not be a pharmacokinetic component to the post-STATInS stage of the study. Thus we will not be administering caffeine tablets and we will not be performing post-dose blood collection.
17.2.6.4.3 Blood collection and testing

There will be limited central laboratory blood test collection in the post-STATInS stage of the study; we will not be testing for 8-iso prostane, nitrite/nitrate concentrations, antithrombin III or immunoglobulins.

Atorvastatin levels will continue to be sent to the central laboratory, in order to build a pharmacokinetic/pharmacodynamic (PK/PD) model of the effect of atorvastatin on endothelial function.

Local laboratory testing of CRP, ALT and CK will continue. Lipid profiles will not be tested in the post-STATInS stage of the study. Blood collection amounts and frequencies are as follows:

**STATInS stage**

Central pre-dose bloods - 15mls of blood on days 0, 1, 3, 5, 7, 10 and 14
Menzies pre-dose bloods – 8mls on days 0, 1, 2 and 7
Central post-dose bloods – 2mls on days 1, 3, 5, 7, 10 and 14.
Local bloods (CRP, Lipids) – 2-3mls on days 0, 1, 3, 5, 10, and 14.
Local safety bloods (CK and ALT) – 2-3mls on days 0, 1, 3, 5, 7, 10, 14 and 1-3 days after cessation of study drug.

**Post-STATInS stage**

Central pre-dose bloods – None
Menzies pre-dose bloods – 12 mls (10mls room temp plus 2ml on ice) on days 0, 1, 2 and 7.
Central post-dose bloods – None
Local bloods including safety bloods – Will be taken as part of routine ICU blood collection on days 0, 1, 3, 5, 7, 10, 14 and 1-3 days after cessation of study drug, with the exception of lipids which will not be tested in this stage of the study.

17.2.4.4 Data Safety and Monitoring Board and Safety Issues

For the duration of the STATInS study, there will not be a separate DSMB for STREAMS. After STATInS concludes, a local DSMB will continue to monitor the trial. This board will consist of three experts who are not involved with the STREAMS or STATInS studies – one biostatistician, one clinical trials expert and one intensivist.
This DSMB will continue to use the same definitions, guidelines and protocols as the STATInS DSMB. The local DSMB will also conduct the interim analysis, as discussed below. Adverse events and serious adverse events will continue to be monitored and will be reported to Dr Davis and Dr Stephens. If they are thought to be possibly, probably or likely related to the study drug, they will be reported to the DSMB, the TGA and the local HREC.

17.2.4.5 Ethical considerations
There will be no additional ethical implications for the post-STATInS stage of the trial. However, new patient information and consent forms will be created and these will be submitted to the local HREC for approval prior to the commencement of that stage of the trial.
17.2.5 References


40. Thomsen RW: **The lesser known effects of statins: benefits on infectious outcomes may be explained by "healthy user" effect.** *Bmj* 2006, 333(7576):980-981.


Section E – Conclusions and Future Directions

“Science has explained nothing; the more we know the more fantastic the world becomes and the profounder the surrounding darkness.”

Aldous Huxley (1894-1963)

Sepsis is an enigmatic, chaotic and maladaptive host response to infection. The maelstrom that is sepsis impacts upon most aspects of the host’s physiology, and dysfunction of the endothelium is a unifying theme of this disturbance. Every question that has been answered by previous research about sepsis pathophysiology has led to yet more questions of increasing complexity. The research presented in this thesis has confirmed many of the originally posed hypotheses, and has generated several new hypotheses. The key originally proposed questions, and the answers provided by this thesis will be discussed below.

18.1 Is the incidence of sepsis higher in tropical northern Australia than elsewhere?
Although the epidemiology of sepsis in the Top End of the Northern Territory has not previously been described, clinical experience suggests that sepsis is more common in the Top End than elsewhere, and that patients are both younger and more severely ill than elsewhere.

The data presented here have confirmed that the incidence of both sepsis and severe sepsis requiring ICU admission is substantially higher in the Top End than elsewhere, but that the majority of this difference is accounted for by extremely high rates in Indigenous Australians. Patients with sepsis in the Top End are younger than elsewhere, but they are not more severely ill, and furthermore their mortality is less than that reported elsewhere in Australia. However, because of the high incidence rates, the population-based mortality rates are higher than elsewhere.

These data confirm that sepsis is an important public health problem in the Top End of the Northern Territory, but they raise several important questions. Why are incidence rates so much higher in Indigenous than non-Indigenous people? More importantly, how can these incidence rates be decreased? The study reported in Chapters 5-7 was not able to determine risk factors for developing sepsis and further study is required to clarify this. It is hoped that the data presented here will help to address the problem of sepsis, by contributing to future study design, research funding applications and health policy.
18.2 What are the key predictors of death in patients hospitalised with sepsis?
Patients with chronic renal disease, older age and hypoalbuminaemia are at highest risk of dying of sepsis. Hypoalbuminaemia is likely to be a surrogate marker of poor nutrition, liver disease and late presentation. The key risk factors for being re-admitted with a second or subsequent episode of sepsis are chronic renal disease, chronic liver disease, and Indigenous status. Thus efforts at prevention or early aggressive treatment of sepsis should target these risk groups.

The nationally and internationally validated SMARTCOP score for predicting severe progression of pneumonia does not function well in this population, but a local variation of this score improves its performance, and this variation is currently undergoing prospective validation.

Contrary to expectation, the APACHE II score performed better in sepsis patients admitted to the hospital wards than those admitted to ICU. The use of APACHE II scores for severity assessment and risk stratification should be studied further in patients admitted to hospital wards with sepsis.

18.3 Is peripheral arterial tonometry a feasible tool in sepsis, and is endothelial function impaired in patients with sepsis?
The technique of peripheral arterial tonometry has not previously been applied to patients with sepsis, and the data presented here confirm that it is a feasible tool in this setting, and that endothelial function is impaired in patients with sepsis in proportion to disease severity. This builds upon previous small studies which had similar findings using different techniques. More importantly, it provides a tool for the monitoring of endothelial responses to adjunctive therapies in sepsis.

18.4 Do circulating markers of endothelial activation correlate with endothelial function and disease severity in sepsis?
ICAM-1 and E-selectin, two of the most commonly used circulating markers of endothelial activation, do not correlate with endothelial function as measured by peripheral arterial tonometry, nor have they been shown elsewhere to correlate with other clinical measures of endothelial function. Angiopoietin-2, however, does correlate with endothelial function, and correlates more strongly with markers of disease severity than ICAM-1 or E-selectin.
This leads to the hypothesis that decreased endothelial nitric oxide bioavailability may be the mechanism for raised plasma angiotensin-2 in sepsis (which causes further endothelial cell damage and possibly organ failure); it also provides a rationale for the therapeutic potential of attempting to increase endothelial nitric oxide bioavailability in sepsis.

Asymmetric dimethyl arginine, an endogenous NOS inhibitor, not only correlates with endothelial dysfunction in sepsis, but is a predictor of mortality. Thus ADMA may be a link between endothelial dysfunction, organ failure and death in sepsis, and this hypothesis merits further exploration.

18.5 Do statins improve endothelial function and decrease mortality in patients with sepsis?
The cohort study reported in Chapter 16 adds to the existing literature suggesting that statin use is protective against sepsis-related mortality. However, a randomised trial is required to definitively address this question. Several randomised trials of statins in patients with sepsis are currently underway around the world. The STREAMS study (Chapter 17), designed as a part of this thesis, will hopefully shed light on the mechanisms of any benefit of statins in patients with sepsis.

18.6 Future Directions
A significant proportion of Indigenous Australians in the Northern Territory live in remote communities. Efforts to further understand and improve sepsis incidence and outcomes in the Northern Territory should include community-based studies of sepsis epidemiology and pre-hospital care. Consideration should be given to designing and conducting randomised trials of sepsis prevention strategies in people with chronic renal disease and chronic liver disease. These could include statins, immunisations, prophylactic antibiotic therapy, or protocolised early aggressive treatment of infection in the community. Further attention should be paid to the prevention of infection through improvement of health hardware and basic infrastructure in Aboriginal communities.

The management of patients hospitalised with sepsis could also be improved. The knowledge gained from this work, including the risk factors for death, microbial epidemiology and value of various severity scoring systems will be used to inform local management guidelines for community-acquired pneumonia and sepsis.
The previously accepted dogma that sepsis is a state of global NO excess is being increasingly challenged. Recently published data, along with the data reported in this thesis support the hypothesis that sepsis is a state of NO imbalance, with decreased endothelial nitric oxide bioavailability. Thus future studies of adjunctive therapies in sepsis should aim not to block excessive NO production, but to boost endothelial nitric oxide bioavailability, for example through the use of NO precursors (L-arginine), direct NO donors (inhaled NO, nitrates) or endothelial cell stabilisers (angiopoietin 1). Peripheral arterial tonometry and dynamic near-infrared spectroscopy should be considered as tools for monitoring endothelial responses to such therapies.

### 18.7 Conclusions in summary

Sepsis is an important cause of morbidity and mortality in tropical Northern Australia, particularly in Indigenous people, who have among the highest reported incidence in the world. Efforts to improve Indigenous health should incorporate strategies for prevention and early effective treatment of sepsis. Endothelial function is impaired in sepsis, in proportion to disease severity, and peripheral arterial tonometry is a practical and accessible means of measuring it. Dynamic near-infrared spectroscopy may be more reliable than peripheral arterial tonometry in those with poor peripheral perfusion, and may provide complementary information to peripheral arterial tonometry. Manipulation of the L-arginine/ADMA/nitric oxide system, the angiopoietin-1/angiopoietin-2/Tie-2 pathway and the tryptophan/IDO/kynurenine pathway are all potential therapeutic strategies to improve outcomes in sepsis via improved endothelial and immunological function. Statins may be an effective adjunctive treatment in sepsis; the work presented in this thesis has developed tools and a protocol to help determine this. To quote Winston Churchill, although the pathophysiology of sepsis remains a “riddle wrapped in a mystery inside an enigma”, its mystery is slowly beginning to be unravelled.
Section F. Appendices
Appendix 1. Clinical research forms used for the PRESTO study.
1.1 INCLUSION CRITERIA

Admission diagnosis on Caresys _________________________________ None given __

1.1.1 Age criteria

- Age ≥ 15 years __

1.1.2 Infection Criteria

Any one of the following, which is thought to be the cause of the SIRS (mark all that apply):

- PMNLs in a normally sterile body fluid __
- Positive culture of pathogen from a sterile site __
- Chest X-Ray changes consistent with pneumonia __ (with consistent clinical Hx and/or examination)
- Syndrome associated with a high risk of infection __ __
- Visually identified focus of infection __ __
- Other (Discuss with Dr Davis) __ __

AND

1.1.3 SIRS Criteria

Two or more of the following within a 24h period in the first 48 hours post admission (mark all that apply)

Y N 1.1.3.1 Fever

Core temperature <36 degrees centigrade OR Core temperature >38 degrees centigrade - add 0.5 degrees if axillary)

Y N 1.1.3.2 Tachycardia

Heart Rate >90 bpm

Y N 1.1.3.3 Tachypnoea

Respiratory rate >20 breaths/minute AND/OR PaCO₂ <32 mmHg AND/OR Need for mechanical ventilation

Y N 1.1.3.4 Abnormal white cell count

>12.0 x 10⁹ cells/litre OR <4.0 x 10⁹ cells/litre AND/OR >10% band forms

Are all inclusion criteria met? Y N

If no, complete this form, have a cup of coffee (then find the next patient!). If yes, allocate study number, and then commence form 2.

Study Number __ __ __ __ __
1.2  How was patient first identified?

1.2.1 Jadecare/Careys admission list  
1.2.2 Laboratory sterile site isolate data  
1.2.3 Clinical team  
1.2.4 Active case finding  
1.2.5 Other __________________________

Date of assessment  __/__/____ (DD/MM/YYYY)  Initials  __________________
Time of assessment  __:__
2.1 DEMOGRAPHICS

2.1.1 Date of Birth  [ ] [ ] [ ] [ ]/ [ ] [ ] [ ] [ ] (DD/MM/YYYY)

2.1.2 Gender  [M] [F]

2.1.3 Aboriginal  [Y] [N] [U]

2.1.4 Community/Suburb/City  ____________________________  Unknown [ ] Overseas [ ]

2.1.5 Longrasser currently  [Y] [N] [U]

2.2 DETAILS OF ADMISSION

2.2.1 Treating Team  (at time of assessment)

Medicine [ ]  Surgery [ ]  O&G [ ]  Other [ ]

2.2.2 Source  [ ]

Self presentation  [ ]  T/F from Clinic/Community  [ ]  Referred by GP  [ ]

Interhospital transfer  [ ]  Residential Care Facility  [ ]

2.2.3 Health-Care Associated Infection Indicators?  [Y]  [N] (skip to 2.3)

HITH patient in last 30 days  [ ]

≥48h in hospital in last 90 days  [ ]

Haemodialysis patient  [ ]

Outpatient chemotherapy in last 30 days  [ ]

Lives in residential care facility  [ ]

2.2.4 Date and time of hospital admission  Date  [ ] [ ] [ ]/ [ ] [ ] [ ]  Time  [ ] [ ] [ ] [ ] [ ]

2.5 KNOWN COMORBIDITIES

No Available information about past health  [ ]

Yes  [ ]  No  [ ]

CHARLSON 1

2.5.1 Myocardial Infarction  [ ]  [ ]

2.5.2 Congestive cardiac failure  [ ]  [ ]

2.5.3 Peripheral vascular disease  [ ]  [ ]

2.5.4 Cerebrovascular disease  [ ]  [ ]

2.5.5 Dementia  [ ]  [ ]

2.5.6 Chronic lung disease  [ ]  [ ]

2.5.7 Rheumatologic disease  [ ]  [ ]

2.5.8 Peptic ulcer disease  [ ]  [ ]
<table>
<thead>
<tr>
<th>Condition</th>
<th>Yes</th>
<th>No</th>
<th>Not recorded</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5.9 Non-severe chronic liver disease</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5.10 Diabetes mellitus (uncomplicated)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CHARLSON 2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5.11 Diabetes with chronic complications</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5.12 Hemiplegia or paraplegia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5.13 Chronic renal disease</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5.14 Any malignancy (not metastatic)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CHARLSON 3</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5.15 Severe liver disease</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CHARLSON 6</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5.16 Metastatic solid tumour</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5.17 AIDS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>STATIN Ix</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5.17 Hyperlipidaemia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5.18 Ischaemic heart disease</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>SUBSTANCE USE</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5.19 Hazardous alcohol use</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5.19.1 Alcohol binge last 48h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5.20 Regular kava use</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5.21 Current smoking</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5.21.1 Ex-smoker</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>ICU ADMISSION MODIFIERS</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5.23 Not expected to survive 28 days</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5.24 Active orders limiting life-sustaining treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>OTHER</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5.25 HIV</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5.26 Iatrogenic Immunosuppression</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5.27 Long-term central venous line</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5.28 Other indwelling tube</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5.29 End stage renal failure</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.6 STATIN USE

2.6.1 Prescribed a statin in the last 1 month  Y □  N □ If no, skip to 2.6.4  U □

2.6.2 Name of statin (circle one)  1 □ Atorvastatin  2 □ Simvastatin  3 □ Pravastatin  4 □ Fluvastatin  5 □ Rosuvastatin  99 □ Unknown statin

2.6.3 Dose of statin (mg/day) □ □

2.6.3.1 Statin continued this admission  Y □  N □

2.6.4 Prescribed another lipid-lowering agent in the last 3 months  Y □  N □ If no, skip to 2.7  U □

2.6.5 Name of other agent(s)  1 □ Gemfibrozil  2 □ Clofibrate  3 □ Fenofibrate  4 □ Ezetimibe  5 □ Nicotinic acid  6 □ Fish oil  7 □ Colestipol  8 □ Cholestyramine  9 □ Psyllium  99 □ Unknown other agent

2.7 ADMISSION DIAGNOSIS

2.7.1 Initial clinical diagnosis
If available, use diagnosis documented on consultant’s post-take ward round

None given □

2.4 SOFA SCORE (Fill in physiological value or tick box in left column if not available/not measured)

NM

☐ 2.4.1 Respiration  PaO2 □ □  Fio2(%) □ □ □ If no ABG available, Sats on Room Air □ □ □

☐ 2.4.2 Coagulation  Platelet Count (x10^9/L) □ □ □

☐ 2.4.3 Liver

Bilirubin  (μmol / L) □ □

Albumin  g/L □ □

☐ 2.4.4 Cardiovascular  MAP (mmHg) □ □ □ OR Systolic BP □ □ □ AND Diastolic BP □ □ □

Inotropes  None □  Dobutamine □  Ad/NAd <0.1 □  Ad/NAd >0.1 □

☐ 2.5.5 Renal  Creatinine (μmol / L) □ □ □ If no Cr, U/O last 24 hours □ □ □

☐ 2.4.6 New Confusion (recorded in notes)  Yes □  No □

☐ 2.4.7 Band forms > 5%  Yes □  No □
2.9 SMART COPS DATA

2.9.1 Initial systolic BP (mmHg) □□□

2.9.2 Initial respiratory rate (bpm) □□

2.9.3 Initial heart rate (bpm) □□□

2.9.4.1 If ABG done in ED  
Initial FiO2 □□□  
Initial Pao2 □□□  
Initial pH □□□

2.9.4.2 If no ABG done in ED  
Initial O₂ Sats on room air □□□  OR  Not available □

2.3 ORGAN DYSFUNCTION  
Y□  N□ (skip to 2.4)  
(Due to the infection and within the first 48h post-admission – mark all that apply)

2.3.1 Cardiovascular  □  (SBP<90 or MAP<70 for ≥1 hour despite fluid resuscitation OR vasopressors)

2.3.2 Renal  □  (Urine output<0.5ml/kg/hr for ≥1 hour)

2.3.4 Respiratory  □  (P:F ratio ≤250, or ≤200 if lung is the only failing organ. If no ABG, Sats<87% on R/A or <77% on R/A if lung is the only failing organ)

2.3.5 Haematological  □  (Plt<80 or a drop of>50% in last 3 days)

2.3.6 Acidosis  □  (pH≤7.3 OR (Lactate>2.9 and BE≥-5). if no ABG: Bicarb<16 OR Lactate>4.0)

P.T.O
# 2.8 APACHE II SCORE

<table>
<thead>
<tr>
<th>PHYSIOLOGIC VARIABLE</th>
<th>High Abnormal Range</th>
<th>Low Abnormal Range</th>
<th>APS Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature – rectal (°C)</td>
<td>+ 4 39 – 40.9</td>
<td>+ 3 38.5 – 38.9</td>
<td>+ 2 36 – 38.4</td>
</tr>
<tr>
<td>Mean arterial pressure – mmHg</td>
<td>≥ 160 130 - 159</td>
<td>+ 1 110 – 129</td>
<td>+ 2 70 - 109</td>
</tr>
<tr>
<td>Heart rate (ventricular response)</td>
<td>≥ 180 140 – 179</td>
<td>+ 1 110 – 139</td>
<td>+ 2 70 - 109</td>
</tr>
<tr>
<td>Respiratory rate (non-ventilated or ventilated)</td>
<td>≥ 50 35 – 49</td>
<td>+ 1 25 - 34</td>
<td>+ 2 12 - 24</td>
</tr>
<tr>
<td>Oxygenation: A - aDO, or PaO₂ (mmHg)</td>
<td>&gt; 500 350 – 499</td>
<td>+ 1 200 – 349</td>
<td>+ 2 &lt; 200 Sats ≤ 95%</td>
</tr>
<tr>
<td></td>
<td>a. if FiO ≥ 0.5 record A - aDO₂</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>b. if FiO &lt; 0.5 record only PaO₂</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arterial pH</td>
<td>≥ 7.7 7.6 – 7.69</td>
<td>+ 1 7.5 – 7.59</td>
<td>+ 2 7.33 – 7.49</td>
</tr>
<tr>
<td>Serum sodium (mMol/L)</td>
<td>≥ 180 160 – 179</td>
<td>+ 1 155 – 159</td>
<td>+ 2 150 - 149</td>
</tr>
<tr>
<td>Serum potassium (mMol/L)</td>
<td>≥ 7 6 – 6.9</td>
<td>+ 1 5.5 – 5.9</td>
<td>+ 2 3.5 – 5.4</td>
</tr>
<tr>
<td>Serum creatinine (mMol/L) (double point score for acute renal failure)</td>
<td>≥ 0.300 0.171-0.299</td>
<td>+ 1 0.121-0.17</td>
<td>+ 1 0.05-0.12</td>
</tr>
<tr>
<td>Haematocrit (%)</td>
<td>≥ 60 50 – 59.9</td>
<td>+ 1 46 – 49.9</td>
<td>+ 2 30 – 45.9</td>
</tr>
<tr>
<td>White blood count (total/mm³) (in 1,000s)</td>
<td>≥ 40 20 – 39.9</td>
<td>+ 1 15 – 19.9</td>
<td>+ 2 3 – 14.9</td>
</tr>
<tr>
<td>Glasgow Coma Score (GCS) (Score = 15 minus actual GCS)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Serum HCO₃ (venous – mMol/L) (Only use this if no ABGs available)</td>
<td>≥52 41 – 51.9</td>
<td>+ 1 32 – 40.9</td>
</tr>
</tbody>
</table>

## APACHE II SCORE - a sum of

A. APS points = ____  
B. Age points = ____  
C. Chronic Health points = ____  
Sum of A + B + C = (0 to 71)

### 2.8.1 APACHE II Score

- [ ] Available
- [x] Not available

APACHE modified because no ABG

---

**Date of assessment**: [ ] [ ] [ ] [ ] [ ] [ ] (DD/MM/YYYY)  
**Initials**: [ ] [ ] [ ]

**Time of assessment**: [ ] [ ] [ ]
## INSPIRED OXYGEN CONVERSION CHART

### NASAL CANNULAE
- 1 LPM = 24%
- 2 LPM = 28%
- 3 LPM = 32%
- 4 LPM = 36%

### HUDSON OXYGEN MASK
- 5 - 6 LPM = 40%
- 6 - 7 LPM = 50%
- 7 - 8 LPM = 60%

### VENTURI MASK
- 28%, 35%, 50%, 60% - Check colour/type of mask

### NON-REBREATHING BAG
- 8 - 15 LPM = 90-99%

## APACHE AGE POINTS

<table>
<thead>
<tr>
<th>AGE</th>
<th>POINTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤44</td>
<td>0</td>
</tr>
<tr>
<td>45-54</td>
<td>2</td>
</tr>
<tr>
<td>55-64</td>
<td>3</td>
</tr>
<tr>
<td>65-74</td>
<td>5</td>
</tr>
<tr>
<td>≥75</td>
<td>6</td>
</tr>
</tbody>
</table>

Josh 28836 OR 0425239237 OR Pager#778
Mark 27844 OR 0412884028
Nick 28932
Study Number □□□□ Patient Initials □□

3.1 MICROBIOLOGY  (Samples collected within 24h prior to onset of sepsis and 48h after onset)

3.1.1 Blood significant isolate Y □ N □

3.1.2 Name of blood isolate 1
Date collected □□/□□/□□□□

3.1.3 Name of blood isolate 2
Date collected □□/□□/□□□□

3.1.4 Urine significant isolate Y □ N □

3.1.5 Name of urine isolate 1
Date collected □□/□□/□□□□

3.1.6 Name of urine isolate 2
Date collected □□/□□/□□□□

3.1.7 Other significant isolate Y □ N □

3.1.8 Name of other isolate 1
Date collected □□/□□/□□□□

3.1.9 Site of other isolate 1
Sputum □ CSF □
Synovial fluid □ Deep tissue □
Other □ Pus/Swab □

3.1.10 Name of other isolate 2
Date collected □□/□□/□□□□

3.1.11 Site of other isolate 2
Sputum □ CSF □
Synovial fluid □ Deep tissue □
Other □ Pus/Swab □

3.1.12 Significant non-culture-based/other test results
Type of test □□□□□□□□□□
Result □□□□□□□□□□
Date collected □□/□□/□□□□
3.2 FOCUS OF INFECTION AT FOLLOW-UP ASSESSMENT

3.2.1 Primary bloodstream infection  
3.2.2 Pleuropulmonary  
3.2.3 Urinary sepsis  
3.2.4 Skin/soft tissue infection  
3.2.5 Osteoarticular infection  
3.2.6 CNS Infection  
3.2.7 Intra-abdominal infection  
3.2.8 Gynaecological infection  
3.2.9 Cardiac  
3.2.10 IV Line-related  
3.2.11 ENT  
3.2.12 Other (Identified focus)  
3.2.13 No identified focus  
3.2.14 Unknown  

Source of follow-up diagnosis

Clinician caring for patient  
Discharge summary  
Medical Record  
Other  

3.2.14 Final diagnosis  

3.3 NEED FOR ICU/HDU

3.3.1 Admitted to ICU or HDU during admission  

3.3.2 Date of admission (DD/MM/YYYY)  

3.3.3 Date of ICU/HDU discharge  

3.3.4 Alive at ICU/HDU discharge  

3.6 TRANSFER FROM ED

3.6.1 Time of transfer from ED (DD/MM/YYYY)  

3.4 HOSPITAL DISCHARGE

3.4.1 Date of discharge   / / /
3.4.2 Alive at hospital discharge   Y   N   U
3.4.3 Destination   Home   Another hospital   Died   Unknown
3.4.4 If deceased, date of death   / / /
3.4.5 Cause of death (if known)  

3.5 DAY 28 FOLLOW-UP AND BEYOND

3.5.1 Late follow up 1   / / /
3.5.2 Alive at late follow-up 1   Y   N   U
3.5.3 Cause of death (if known)  
3.5.4 Source of information   NT BDM Register   National BDM Register
Hospital/THS contact   Other   

3.5.5 Late follow up 2   / / /
3.5.6 Alive at late follow-up 2   Y   N   U
3.5.7 Cause of death (if known)  
3.5.8 Source of information   NT BDM Register   National BDM Register
Hospital/THS contact   Other }
Appendix 2. Clinical research forms used for the FRESH study.
**Patient details**

<table>
<thead>
<tr>
<th>Patient initials</th>
<th>Hospital ID</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Date of birth</th>
<th>Age in Years</th>
</tr>
</thead>
</table>

**To be eligible, patients must be 18 years or over and admitted to ICU/HDU and have sepsis, severe sepsis or septic shock**

AGE 18 YEARS OR OVER  

A) SEPSIS  - - - - Y / N

Suspected or proven infection  

AND 2 or more of the following criteria for sepsis

1) **Fever:** temperature >38°C or <36°C  
   Y / N

2) **Tachycardia:** heart rate >90 beats/min  
   Y / N

3) **Tachypnoea:**  
   Respiratory rate >20 breaths/minute – and/or  
   \( P_{a}CO_2 <32 \text{ mmHg} \) – and/or  
   Mechanical ventilation  
   Y / N

4) **Abnormal white cell count:**  
   >12,000 cells/mL or  
   <4,000 cells/mL or  
   >10% band forms  
   Y / N

B) SEVERE SEPSIS  - - - - Y / N

One or more of the following:

**Metabolic acidosis** (pH<7.3 or base deficit ≥ 5)  

**Respiratory dysfunction** \( PaO_2/FiO_2 ≤299 \)  

**Renal dysfunction:**

Oliguria<30mL/hr for 3 hours - or  

Oliguria<700mL/24 hours – and/or  

Renal replacement therapy  

**Altered mental status** GCS<15 prior to intubation  

**Liver dysfunction:**

Billirubin > 20mg/dL - and/or  

AST>80 or ALT> 88  

**Coagulopathy:** platelets <150*10⁹/L  

Y / N

C) SEPTIC SHOCK  - - - - Y / N

Both of the following:

i) Systolic blood pressure <90mmHg  OR  

Fall of SBP>40mmHg from baseline  

AND

ii) BP not corrected with adequate fluid resuscitation OR  

Requirement for vasopressors/inotropes  

Y / N
Exclusion criteria (Patient NOT eligible if any ‘yes’ response ticked)

- KNOWN LATEX ALLERGY  Y / N
- RECEIVING IV GTN  Y / N
- RECEIVING IV SODIUM NITROPRUSSIDE  Y / N
- RECEIVING IV ACTIVATED PROTEIN C  Y / N
- PLATELETS ≤ 20x10^9/L  Y / N
- INR ≥ 2.0  Y / N
- APTT ≥ 70secs  Y / N
- Known Raynaud's disease  Y / N
- SMOKING IN LAST 4 HOURS  Y / N
- Expected survival of less than 24 hours with active orders limiting treatment  Y / N
- In ICU/HDU for >24 hours  Y / N

Consent

Has the patient understood and signed the consent form?  Y / N
If not, has the next-of-kin understood and signed the consent form?  Y / N

Is the patient eligible for inclusion

Does the patient meet the inclusion criteria?  Y / N
Have the exclusion criteria been answered?  Y / N
Has consent been obtained?  Y / N

Date of assessment  [ ]/ [ ]/ [ ] (DD/MM/YYYY)  Initials  [ ] (XXX)

AFTER COMPLETING THIS FORM CONTACT KIM AT MENZIES ON 28074 TO LET HER KNOW PATIENT STUDY ABOUT TO START AND WHEN BLOOD SAMPLES WILL BE READY TO BE COLLECTED
# FRESH

## Form 2: Baseline and Follow Up Data

<table>
<thead>
<tr>
<th>Study ID</th>
<th>Patient initials</th>
<th>Hospital ID</th>
</tr>
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</table>

| Date of birth | (DD/MM/YYYY) | Age in Years | (YY) |
|---------------|--------------|--------------|

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<thead>
<tr>
<th>Hospital admission date</th>
<th>(DD/MM/YYYY)</th>
<th>(HH:MM)</th>
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</table>

<table>
<thead>
<tr>
<th>ICU admission date/time</th>
<th>(DD/MM/YYYY)</th>
<th>(HH:MM)</th>
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<thead>
<tr>
<th>Study enrolment date/time</th>
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<th>ICU discharge date</th>
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<th>(HH:MM)</th>
<th>Alive</th>
<th>Y / N</th>
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</table>

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<th>(HH:MM)</th>
<th>Alive</th>
<th>Y / N</th>
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<th>(DD/MM/YYYY)</th>
<th>(HH:MM)</th>
<th>Home</th>
<th>Y / N</th>
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<table>
<thead>
<tr>
<th>Gender</th>
<th>(M/F)</th>
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<thead>
<tr>
<th>Aboriginal or Torres Strait origin</th>
<th>(Y/N)</th>
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</thead>
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**HANDEDNESS**

- Left / Right / Unknown

## Vascular / Endothelial Disease, Risk Factors, Medications (circle choice)

- **Diabetes**
  - Y / N / U

- **End-stage renal disease (Dialysis dependent)**
  - Y / N / U

- **Renal impairment (Not dialysis dependent)**
  - Y / N / U

- **Smoker (>5 cigarettes per day or equivalent in the last one month)**
  - Y / N / U

- **Hypertension**
  - Y / N / U

- **Hypercholesterolaemia**
  - Y / N / U

- **Ischaemic heart disease**
  - Y / N / U

- **Congestive cardiac failure**
  - Y / N / U

- **Family history ischaemic heart disease**
  - Y / N / U

- **Peripheral vascular disease**
  - Y / N / U

- **Chronic liver disease**
  - Y / N / U

- **Menopause**
  - Y / N / U / NA

- **STATINS**
  - Y / N / U

  If Y, which statin and dose________________________

- **ACE INHIBITORS**
  - Y / N / U

- **ANGIOTENSIN RECEPTOR BLOCKERS**
  - Y / N / U

- **BETA BLOCKERS**
  - Y / N / U

- **OTHER VASODILATORS**
  - Y / N / U

- **NICOTINE REPLACEMENT**
  - Y / N / U

- **ANTIPLATELET DRUGS**
  - Y / N / U
ADMISSION APACHE DIAGNOSIS: ______________________________

ADMISSION APACHE II SCORE: ______________________________

- SEPSIS  Y / N
- SEVERE SEPSIS  Y / N
- SEPTIC SHOCK  Y / N

POSITIVE CULTURE FOR CAUSATIVE ORGANISM  Y / N

PRIMARY ORGANISM: ______________________________

SITE OF POSITIVE CULTURE: ______________________________

FOCUS OF INFECTION/SYNDROME: ______________________________

Date of baseline  □□/□□/□□□□ (DD/MM/YYYY)  Initials  □□□□ (XXX)

Date of follow up  □□/□□/□□□□ (DD/MM/YYYY)  Initials  □□□□ (XXX)
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<th>2-4</th>
<th>7</th>
<th>30 (+/-2)</th>
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<tbody>
<tr>
<td>Date</td>
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<tr>
<td>Location</td>
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</tr>
<tr>
<td>Operator</td>
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<tr>
<td>Study arm (L/R – default to non-dominant) Avoid side with arterial line</td>
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<tr>
<td>Arterial line</td>
<td>(Y / N).</td>
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<tr>
<td>If present state site and side</td>
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<tr>
<td>Finger used (2-4)</td>
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<tr>
<td>Time probes activated and recording commenced (24 h+ secs)</td>
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<tr>
<td>Time BP cuff inflated (Aim 5-10 min after probes inflated)</td>
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<tr>
<td>Pressure of BP cuff (mmHg) (Aim 60mmHg above systolic)</td>
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<tr>
<td>Time BP cuff deflated (SHOULD BE 5 MIN +/- 10 sec)</td>
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<tr>
<td>Time probes deflated</td>
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<tr>
<td>Occlusion Time (from computer)</td>
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<tr>
<td>RH-PAT index</td>
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<tr>
<td>Diameter of forearm (cm)</td>
<td></td>
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<tr>
<td>Comments or Errors</td>
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**SOFA SCORE**

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<tr>
<th></th>
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<tbody>
<tr>
<td>Respiration</td>
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<tr>
<td>Coagulation</td>
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<tr>
<td>Liver</td>
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<tr>
<td>Cardiovascular</td>
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</tr>
<tr>
<td>Renal</td>
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<tr>
<td>TOTAL</td>
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</table>
# Inotrope requirements (at time of measurement)

<table>
<thead>
<tr>
<th>STUDY DAY</th>
<th>0</th>
<th>2-4</th>
<th>7</th>
<th>30 (+/-2)</th>
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</thead>
<tbody>
<tr>
<td><strong>ON ANY INOROPES/VASOPRESSORS</strong></td>
<td></td>
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<tr>
<td>Noradrenaline (µg/kg/min)</td>
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<tr>
<td>Adrenaline (µg/kg/min)</td>
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<tr>
<td>Dobutamine (µg/kg/min)</td>
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<tr>
<td>Vasopressin (units/hr)</td>
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<tr>
<td>Levosimendan (µg/kg/hr)</td>
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# BASELINE CLINICAL/LABORATORY INVESTIGATIONS (AT OR CLOSEST TO TIME OF MEASUREMENT)
IF PARAMETER NOT AVAILABLE, WRITE N/A.

<table>
<thead>
<tr>
<th>STUDY DAY</th>
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<th>2-4</th>
<th>7</th>
<th>30 (+/-2)</th>
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<tbody>
<tr>
<td>Blood pressure Systolic/ Diastolic (Mean)</td>
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<tr>
<td>Central venous pressure (cm H₂O)</td>
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<tr>
<td>Temperature (axillary)</td>
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<tr>
<td>SaO2 reliable trace (Y/N)</td>
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<tr>
<td>SaO2 (%)</td>
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<tr>
<td>Haemoglobin (g/L)</td>
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<tr>
<td>Platelets (x10^9/L)</td>
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<tr>
<td>White cell count (x10^9/L)</td>
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<tr>
<td>Creatinine (µmol/L)</td>
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<tr>
<td>Serum albumin (g/L)</td>
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<tr>
<td>CRP (mg/L)</td>
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<tr>
<td>Lactate (mmol/L)</td>
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<tr>
<td>PaCO2 (mmHg)</td>
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<tr>
<td>Swan-Ganz OR PiCCO in-situ</td>
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<tr>
<td>If neither, go to next section</td>
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<tr>
<td>Cardiac Index</td>
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<tr>
<td>Systemic vascular resistance index</td>
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<tr>
<td>Pulmonary artery wedge pressure</td>
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<tr>
<td>Extravascular lung water index</td>
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</table>
### OTHER ICU THERAPIES (AT TIME OF MEASUREMENT)

<table>
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<th>30 (+/-2)</th>
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<tbody>
<tr>
<td>Renal Replacement Therapy (Y/N)</td>
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</tr>
<tr>
<td>Nutrition</td>
<td></td>
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</tr>
<tr>
<td>1 – NBM</td>
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</tr>
<tr>
<td>2 – Oral</td>
<td></td>
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<tr>
<td>3 – Enteral</td>
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<tr>
<td>4 – Parenteral</td>
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<tr>
<td>Ventilation Therapy</td>
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</tr>
<tr>
<td>1 – Spontaneous</td>
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<tr>
<td>2 – Non-invasive</td>
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<tr>
<td>3 – Invasive</td>
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<tr>
<td>Inhaled nitric oxide Y/N. If Y, dose (ppm)</td>
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</tbody>
</table>
Appendix 3. Angiopoietin-2 is increased in sepsis and inversely associated with nitric oxide-dependent microvascular reactivity. Manuscript as published in Critical Care.
Angiopoietin-2 is increased in sepsis and inversely associated with nitric oxide-dependent microvascular reactivity

Joshua S Davis, Tsin W Yeo, Kim A Piera, Tonia Woodberry, David S Celermajer, Dianne P Stephens, Nicholas M Anstey

Abstract

Introduction: Angiopoietin-2 (ang-2), an angiogenic peptide released by endothelial cell Weibel-Palade bodies (WPBs), increases endothelial activation and vascular permeability. Ang-2 is raised in severe sepsis but the mechanisms underlying this are not known. Nitric oxide (NO) inhibits WPB exocytosis, and bioavailability of endothelial NO is decreased in sepsis. We hypothesized that endothelial NO bioavailability would be inversely correlated with ang-2 concentrations in sepsis.

Methods: Plasma ang-2, vascular endothelial growth factor (VEGF) and endothelial-active cytokines were assessed in 83 patients with early sepsis and 41 hospital controls, and related to reactive hyperaemia-peripheral arterial tonometry, RH-PAT, a measure of endothelial NO bioavailability.

Results: Plasma Ang-2 was elevated in sepsis (median [interquartile range (IQR)], ng/ml: severe sepsis 12.4 [8.5-33.4], sepsis without organ failure 6.1 [5.0-10.4], controls 2.7 [2.2-3.6], P < 0.0001). It correlated inversely with RH-PAT (r = -0.38, P < 0.0001) and positively with IL-6 (r = 0.57, P < 0.0001) and degree of organ failure (sequential organ function assessment score) (r = 0.58, P < 0.0001). The correlation of ang-2 with RH-PAT persisted after controlling for sepsis severity. In a longitudinal mixed-effects model, recovery of RH-PAT over time was associated with decline in ang-2.

Conclusions: Ang-2 is elevated in proportion to sepsis severity, and inversely correlated with NO-dependent microvascular reactivity. Impaired endothelial NO bioavailability may contribute to increased endothelial cell release of ang-2, endothelial activation and capillary leak. Agents that increase endothelial NO bioavailability or inhibit WPB exocytosis and/or Ang-2 activity may have therapeutic potential in sepsis.

Introduction

Microvascular and endothelial dysfunction are central to the pathophysiology of sepsis, contributing to organ dysfunction even in the setting of normal post-resuscitation haemodynamics [1]. Angiopoietin-2 (ang-2), an angiogenic peptide, activates endothelial cells and increases vascular inflammation. It functions as an autocrine mediator of the endothelium and is stored predominantly in endothelial cells [2]. Ang-2 is a ligand of the tyrosine kinase receptor, Tie-2, and antagonises the angiopoietin 1-induced Tie-2 receptor autophosphorylation responsible for the maintenance of endothelial cell quiescence [3]. This results in endothelial cells being sensitized to the effects of pro-inflammatory cytokines and vascular endothelial growth factor (VEGF), resulting in a loss of endothelial cell quiescence and an increase in vascular activation and inflammation.

Levels of circulating ang-2 have been shown to be raised in human sepsis [4-6] and, more recently, to correlate with mortality [7-9] and pulmonary vascular leak [10,11]. Despite a growing interest in ang-2 in sepsis, the mechanisms underlying elevated ang-2 levels in patients with sepsis are unclear. Ang-2 is co-packaged with von Willebrand Factor (vWF) within endothelial
cell Weibel-Palade bodies (WPBs) and is immediately released upon endothelial cell stimulation and WPB exocytosis [12]. In-vitro studies demonstrate that exocytosis of WPBs can be triggered by multiple secretagogues, including thrombin, histamine, epinephrine, VEGF, and hypoxia [13]. However, there are only two known inhibitors of WPB release: nitric oxide (NO) and hydrogen peroxide (H$_2$O$_2$), of which NO is thought to be the most important [14].

We have recently demonstrated impaired microvascular reactivity in patients with sepsis by reactive hyperaemia peripheral arterial tonometry (RH-PAT) [15], which is at least 50% NO-dependent and thus provides an estimate of endothelial NO bioavailability [16]. In contrast to earlier hypotheses suggesting major overproduction of NO in patients with sepsis [17], there is now increasing evidence that systemic NO production is normal or decreased in humans with sepsis [18,19]. Impaired endothelial NO bioavailability may underlie increased WPB exocytosis in sepsis, and thus the release of ang-2 from endothelial cells. However, the relation between endothelial NO bioavailability and measures of WPB release in sepsis has not been determined. We hypothesized that plasma ang-2 levels in patients with sepsis would be raised in proportion to disease severity and would be inversely related to endothelial NO bioavailability, as estimated by RH-PAT.

**Materials and methods**

**Study design and setting**

We performed a prospective observational study at a 350-bed teaching hospital in tropical Australia, with an 18-bed mixed ICU. Prior approval was obtained from the Human Research Ethics Committee of the Menzies School of Health Research and the Department of Health and Community Services. Written informed consent was obtained from all participants or their next of kin where necessary.

**Participants**

The study subjects were adults (≥18 years) hospitalized with sepsis, who were enrolled in a previously reported study of microvascular reactivity; more detail of subject recruitment, patient characteristics and study procedures are provided in this paper [15]. Some of the data included in the current paper have been previously reported (RH-PAT index, intra-cellular adhesion molecule-1 [ICAM1], E-selectin and IL-6), but are included here for comparison with Ang-2. Sepsis was defined as severe sepsis, or sepsis without organ failure. Severe sepsis was defined as sepsis with organ dysfunction or shock at the time of enrolment according to American College of Chest Physicians/Society of Critical Care Medicine consensus criteria [15,20].

**Laboratory assays**

Venous blood was collected in lithium heparin tubes at baseline and two to four days later, and plasma was frozen at -80°C. Control patients had blood collected at baseline only. Plasma concentrations of VEGF, ICAM-1 and E-selectin were measured by ELISA (R&D Systems, Minneapolis, MN, USA), according to the manufacturer’s instructions. The ELISA used to determine plasma Ang-2 concentrations (R&D Systems, Minneapolis, MN, USA) reports a lower limit of detection of 8.3 pg/ml (0.0083 ng/ml), with coefficients of variation for intra-assay and inter-assay precision of 4.2% and 7.4%, respectively.

IL-6 and TNFα were measured by flow cytometry using a cytokine bead array (CBA; BD Biosciences, Franklin Lakes, NJ, USA).

**Measurement of microvascular reactivity/endothelial NO bioavailability**

Microvascular reactivity was measured at the bedside by RH-PAT (Itamar Medical, Caesarea, Israel), a non-invasive method of assessing endothelial function [21], which is at least 50% dependent on endothelial NO production [16]. We have previously reported internal validation and repeatability of RH-PAT in acute inflammatory states [22]. PAT was measured in a fingertip before and after a five-minute ischemic stress at the forearm, generating an RH-PAT index, normalized to the control arm, as previously described [15]. All studies were performed after resuscitation and at least an hour of hemodynamic stability in a quiet room at 25°C, with the patient in a recumbent position.

**Statistical methods**

All analyses were hypothesis based and were specified a priori. Continuous variables were compared using Student’s t-test/analysis of variance or Mann Whitney U test/Kruskal-Wallis test for parametric and non-parametric variables, respectively. Categorical variables...
were compared using Fisher’s exact test. For the ELISA and CBA assays, values below the lower limit of detection were assigned a value of halfway between zero and the lower limit of detection for the purposes of analysis. Correlates with baseline Ang-2 were determined using Spearman’s coefficient. For multivariate analysis, linear regression with backward selection was used. All independent variables with a Wald $P$-value of less than 0.10 on univariate analysis, or which were considered biologically important were included in the initial model. Variables with a Wald $P$-value of 0.05 or more were sequentially dropped from the model. The natural logarithm of Ang-2 was used as the dependent variable, because Ang-2 was right-skewed and log transformation lead to a normal distribution. To control for covariates in the relationship between Ang-2 and RH-PAT index, the covariate in question was added to a linear regression model with log Ang-2 as the independent variable and RH-PAT index as the dependent variable. To examine longitudinal correlations, linear mixed-effects models were used. A two-sided $P$ value of less than 0.05 was considered significant. All analyses were performed using Stata version 10 (Stata Corp, College Station, TX, USA).

**Results**

**Participants**

Eighty five patients with sepsis and 45 control patients were enrolled in the study. Two sepsis patients and four controls were excluded from further analysis because they refused blood collection. Of the remaining 83 sepsis patients, 52 had organ dysfunction due to sepsis at baseline (severe sepsis group) and 31 did not (sepsis without organ failure). The three groups were well matched in terms of risk factors for endothelial dysfunction and other baseline characteristics (Table 1).

**Baseline Ang-2 and VEGF**

Plasma Ang-2 concentrations were raised in sepsis in proportion to disease severity (Table 2 and Figure 1a). Median Ang-2 concentrations (ng/ml (interquartile range (IQR)) were two-fold higher in patients with severe sepsis (12.4 (8.5 to 33.4)), than in those with sepsis without organ failure (6.1 (5.0 to 10.4); $P < 0.0001$), and 4.5-fold higher than in controls (2.7 (2.2 to 3.6); $P < 0.0001$). The difference in Ang-2 between sepsis without organ failure and controls was also significant ($P < 0.0001$). VEGF was also raised in sepsis patients compared with controls ($P = 0.0001$, Table 2 and Figure 1b), but the difference in VEGF between severe sepsis and sepsis without organ failure was not significant.

**Ang-2 and disease severity**

Ang-2 correlated with sepsis severity (Table 3), as measured by Acute Physiology and Chronic Health Evaluation II score ($r = 0.46$, $P < 0.0001$), Sequential Organ Failure Assessment (SOFA) score ($r = 0.58$, $P < 0.0001$), number of organ failures ($r = 0.48$, $P < 0.0001$) and arterial lactate ($r = 0.41$, $P = 0.003$), whereas VEGF did not correlate with any of these parameters. As neutrophils release H2O2, we also examined the relation between neutrophil counts and plasma Ang-2 concentrations, and found no significant correlation ($r = 0.16$, $P = 0.15$).

**Ang-2 and NO-dependent microvascular reactivity**

On univariate analysis, Ang-2 was inversely correlated with RH-PAT index, an estimate of endothelial NO bioavailability ($r = -0.38$, $P < 0.0001$), and positively correlated with markers of endothelial activation (ICAM-1 $r = 0.58$, $P ≤ 0.0001$, E-selectin $r = 0.53$, $P < 0.0001$). VEGF did not correlate with endothelial NO

---

**Table 1 Baseline characteristics of participants**

<table>
<thead>
<tr>
<th></th>
<th>Severe sepsis</th>
<th>Sepsis without organ failure</th>
<th>Control</th>
<th>$P$ value across all groups</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>N</strong></td>
<td>52</td>
<td>31</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>**Age (years)**a</td>
<td>52.8 (48.6-56.9)</td>
<td>50.8 (46.5-55.2)</td>
<td>47.3 (43.2-51.6)</td>
<td>NSc</td>
</tr>
<tr>
<td>Male n (%)</td>
<td>31 (60)</td>
<td>21 (68)</td>
<td>28 (68)</td>
<td>NSd</td>
</tr>
<tr>
<td>Diabetic n (%)</td>
<td>17 (33)</td>
<td>7 (23)</td>
<td>13 (32)</td>
<td>NSd</td>
</tr>
<tr>
<td>Smoker n (%)</td>
<td>26 (50)</td>
<td>12 (39)</td>
<td>16 (39)</td>
<td>NSd</td>
</tr>
<tr>
<td>IHD n (%)</td>
<td>9 (17)</td>
<td>6 (19)</td>
<td>5 (12)</td>
<td>NSd</td>
</tr>
<tr>
<td>On a statin n (%)</td>
<td>13 (25)</td>
<td>9 (29)</td>
<td>11 (27)</td>
<td>NSd</td>
</tr>
<tr>
<td>APACHE IIb</td>
<td>19 (15-25)</td>
<td>8 (5-11)</td>
<td>1 (0-2)</td>
<td>0.0001*</td>
</tr>
<tr>
<td>SOFA scoreb</td>
<td>6 (3-9)</td>
<td>1 (0-2)</td>
<td>0.0001*</td>
<td></td>
</tr>
</tbody>
</table>

a. Mean (95% confidence interval).

b. Median (Interquartile range).

c. By one-way analysis of variance.

d. By Fisher’s exact test across all three groups.

e. By Kruskal-Wallis test.

APACHE II, Acute Physiology and Chronic Health Evaluation II score; IHD, ischemic heart disease; NS, not significant; SOFA, Sequential Organ Failure Assessment score.
bioavailability, endothelial activation or plasma Ang-2. The relationship between log Ang-2 and RH-PAT index remained significant after controlling for disease severity using SOFA score.

In a longitudinal analysis, plasma Ang-2 concentrations decreased significantly between day 0 (median (IQR) 10.16 (5.32 to 19.39)) and day two to four (8.72 (5.38 to 15.73), \(P = 0.01\); Table 4). RH-PAT index increased over the same time period, but the change was not statistically significant (Day 0 mean index 1.67 (95% confidence interval (CI): 1.55 to 1.78), day two to four = 1.85 (1.70 to 2.00)). In a mixed-effects linear regression model, increase in RH-PAT index over the first two to four days correlated significantly with fall in Ang-2 (\(r = 0.45, P < 0.0001\)); change in RH-PAT index over time did not correlate with VEGF, ICAM-1, E-selectin, SOFA score, or IL-6.

Ang-2 and markers of inflammation
Plasma TNF\(\alpha\) was below the lower limit of detection in the majority of patients in both the sepsis and control groups (Table 2). In those in whom it was detectable (\(\geq 2.8\) pg/ml), there was no relation between Ang-2 and TNF\(\alpha\) (\(r = 0.24, P = 0.44\)). Ang-2 correlated with IL-6 (\(r = 0.57, P < 0.0001\)), but not with C-reactive protein or white blood cell count.

Multivariate analysis of correlates of angiopoietin-2
The variables included in the initial multivariate linear regression model, with log Ang-2 as the dependent variable, were: RH-PAT index, serum albumin, APACHE and SOFA scores, plasma concentrations of E-selectin, ICAM-1, IL-6 and IL-10, and peripheral blood platelet and white blood cell counts. The independent variables that remained significant in the final model, along with their \(\beta\) coefficients (95% CI) were: RH-PAT index (\(\beta = -0.35 (-0.64 \text{ to } -0.06)\)), ICAM-1 (ng/ml, \(\beta = 3.8 \times 10^{-4}\) (1.4 to 6.3 \times 10^{-4})), IL-6 (pg/ml, \(\beta = 1.7 \times 10^{-4}\) (0.05 to 2.9 \times 10^{-4})), platelet count (\(\times 10^{9}/L, \beta = -2 \times 10^{-3}\) (-3.2 to -0.90 \times 10^{-3})), and white blood cell count (\(\times 10^{9}/L, \beta = 3.2 \times 10^{-2}\) (1.1 to 5.2 \times 10^{-2})).

Outcomes
The median (IQR) length of stay in the ICU among all sepsis patients was 5.4 (3.0 to 8.4) days, and this was significantly correlated with baseline Ang-2 (\(r = 0.30, P = 0.03\)). Of the 83 patients with sepsis, only 8 had died at 28-day follow up (10%). Seven of these were from the severe sepsis group (28-day mortality 13%) and one was from the sepsis without organ failure group (mortality 3%). Baseline levels of Ang-2 were not significantly different (\(P = 0.32\)) in those with fatal (11.46 (7.09 to 45.12)) and non-fatal outcomes (10.04 (5.26 to 18.96)).

Discussion
Plasma Ang-2 concentrations are raised in patients with sepsis, in proportion to disease severity and endothelial cell activation, and are inversely associated with estimated endothelial NO bioavailability both at baseline and during recovery. This finding supports the hypothesis that impaired endothelial NO bioavailability in sepsis leads to increased exocytosis of WPBs, release of Ang-2, and thus to further endothelial cell sensitization and activation. This hypothesis is also supported by recent findings in patients with severe malaria, where an increase in endothelial NO bioavailability over time (also measured by RH-PAT) was significantly associated with falling plasma Ang-2 levels [23].

Table 2 Baseline measurements according to disease category

<table>
<thead>
<tr>
<th></th>
<th>Severe sepsis</th>
<th>Sepsis without organ failure</th>
<th>Control</th>
<th>(P) value across all groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>52</td>
<td>31</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>Angiopoietin 2 (ng/ml)*</td>
<td>12.44 (8.47-33.44)</td>
<td>6.11 (4.59-10.37)</td>
<td>2.71 (2.15-3.61)</td>
<td>0.0001*</td>
</tr>
<tr>
<td>VEGF (pg/ml)*</td>
<td>98.4 (56.4-142.6)</td>
<td>80.8 (57.5-147.3)</td>
<td>52.3 (31.8-73.5)</td>
<td>0.0007*</td>
</tr>
<tr>
<td>Plasma ICAM-1 (ng/ml)*</td>
<td>846 (523-1483)</td>
<td>501 (368-672)</td>
<td>323 (265-393)</td>
<td>0.0001*</td>
</tr>
<tr>
<td>Plasma E-selectin (ng/ml)*</td>
<td>200.5 (113-478)</td>
<td>87.0 (50.8-164.4)</td>
<td>38.4 (26.9-58.2)</td>
<td>0.0001*</td>
</tr>
<tr>
<td>RH-PAT index#</td>
<td>1.57 (1.44-1.71)</td>
<td>1.85 (1.67-2.03)</td>
<td>2.07 (1.93-2.22)</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Plasma interleukin 6 (pg/ml)*</td>
<td>385.1 (124-2996.0)</td>
<td>148.3 (45.9-315.0)</td>
<td>5.0 (2.2-8.1)</td>
<td>0.0001*</td>
</tr>
<tr>
<td>Plasma TNF(\alpha) (\geq 2.8) pg/ml (n,%)*</td>
<td>8 (22)</td>
<td>4 (14)</td>
<td>5 (17)</td>
<td>NS*</td>
</tr>
</tbody>
</table>

a. Median (Interquartile range).
b. Mean (95% confidence interval).
c. \(2.8\) pg/ml is the lower limit of detection for the assay used for TNF\(\alpha\).
d. By Kruskal-Wallis test.
e. By oneway analysis of variance.
f. By Fisher’s exact test across all three groups.

ICAM-1, intra-cellular adhesion molecule-1; NS, not significant; RH-PAT, reactive hyperemia peripheral arterial tonometry; TNF\(\alpha\), tumor necrosis factor \(\alpha\); VEGF, vascular endothelial growth factor.
Although we demonstrate for the first time the relation between estimated endothelial NO bioavailability and plasma Ang-2 concentrations in sepsis, there is substantial recent evidence underpinning this hypothesis. In vitro, NO is the only substance demonstrated to reduce exocytosis of WPBs and release of Ang-2 apart from high concentrations of H₂O₂ [13,14]. NO reduces WPB exocytosis by facilitating the S-nitrosylation of N-ethyl-malemide sensitive factor (NSF), which results in the inability of the WPB membrane to fuse with the plasma membrane [2,12,13]. Furthermore, contrary to previously accepted theories, there is increasing evidence that systemic NO production is normal or decreased rather than increased in sepsis [18,19], and that sepsis is a state of imbalance between the endothelial and inducible isoforms of NO synthase in the microvasculature, resulting in a relative deficiency of endothelial NO [24,25]. The fact that non-specific NO inhibitors increase mortality in patients with sepsis [26] supports this idea, and it is possible that increased Ang-2 release is one of the mechanisms underlying this finding.

Clinical studies investigating the endothelium in sepsis commonly use circulating markers of endothelial cell activation as a surrogate measure of endothelial function. In contrast, Ang-2 correlates with endothelial function as measured by RH-PAT [15].
measured by RH-PAT, both at baseline and longitudinally. Thus Ang-2 is a more meaningful biomarker of endothelial cell function in sepsis than currently used surrogate measures.

Endothelial cell activation in sepsis increases vascular leak, triggers a pro-coagulant state, up-regulates adhesion molecule expression, and further drives the inflammatory response [27]. Together, these processes cause regional hypoperfusion and acute organ dysfunction. By mediating autocrine activation of local endothelial cells, Ang-2 may exacerbate tissue hypoperfusion and inflammation, providing a plausible mechanism for its independent association with organ failure and mortality in sepsis [7].

In vitro studies of Ang-2 demonstrate that in the absence of Ang-2, a TNFα concentration of 40 pg/ml or more is required to independently activate endothelial cells, whereas in the presence of Ang-2 at a concentration of 2000 pg/ml, a TNFα concentration of 5 pg/ml or more is able to cause endothelial activation [28]. Plasma TNFα levels in sepsis patients in this study were relatively low, with only 1 of 69 sepsis patients having TNFα levels of 40 pg/ml or more, similar to the low levels reported in other sepsis studies [29,30]. The concentrations of Ang-2 in this and other human sepsis studies [4,5,7] are higher than those used in in vitro studies, and may sensitize endothelial cells to lower concentrations of TNFα. Furthermore, local microvascular TNFα concentrations may be higher than we and others have found in plasma. Nevertheless, taken together our results support the hypothesis that in sepsis, Ang-2 sensitizes endothelial cells to the effects of cytokines that may otherwise cause only minimal or no endothelial activation [28].

The factors triggering Ang-2 release from WPBs in sepsis are not known. Thrombin [12], VEGF [31] and, in some [31,32], but not other studies [4,12], TNFα, cause WPB release in vitro. However, we found that neither TNFα nor VEGF correlated with Ang-2. Although we found a strong independent association between IL-6 and Ang-2, IL-6 has not been shown to cause exocytosis of WPBs or secretion of Ang-2 in vitro. IL-6 is an important pro-inflammatory cytokine and correlates with disease severity in sepsis. Bacterial lipopolysaccharide increases Ang-2 levels [33] and drives IL-6 expression [30], and such factors may account for this association.

Although Ang-2 correlated with length of stay in this study, it did not correlate with mortality. Despite a median APACHE II score of 19, and a consequent predicted mortality of 34.8% [34], there were few deaths in our study (8 in total, 13% within the severe sepsis group). This is consistent with the previously reported low mortality from severe sepsis in our ICU [35], and suggests that our study was under-powered to examine the relation between Ang-2 and mortality. However, in studies with higher numbers of deaths, Siner and colleagues and Kumpers and colleagues both found a clear association between plasma Ang-2 levels and risk of mortality [7,8].

Although we did not directly measure endothelial cell NO concentrations (which is not possible in septic patients), RH-PAT index is an indirect measurement of NO bioavailability and is at least 50% NO-dependent in healthy volunteers [16]. Other methods of measuring NO in patients with sepsis, such as plasma NO metabolites, are not specific to the endothelium and are confounded by nitrate retention in renal failure [36]. Thus it is not possible to directly confirm the relation between endothelial NO bioavailability and plasma Ang-2 using currently available methods in humans with sepsis.

The correlation between Ang-2 and RH-PAT index was statistically significant but was not strong. We cannot exclude an alternative explanation for the inverse association between Ang-2 and endothelial NO bioavailability: that increased Ang-2 release in sepsis leads to decreased NO bioavailability as a consequence of upregulated endothelial cell inflammation and superoxide-mediated NO quenching. Nevertheless the clear in vitro evidence for NO as the major inhibitor of WPB exocytosis and Ang-2 release, and the findings in other disease settings such as malaria [23], make it more likely that impaired NO bioavailability is a significant contributor to Ang-2 release in sepsis.

vWF is co-packaged with Ang-2 in WPBs but is also released by activated platelets, and is thus less specific for endothelial cells. Although not measured in our study, plasma vWF activity is known to be increased in patients with sepsis, and to correlate with mortality [37]. Like other markers of endothelial cell activation, vWF has not been compared with measures of endothelial NO bioavailability in sepsis. However, in non-septic patients with risk factors for cardiovascular disease, vWF is raised, correlates with endothelial activation as measured by E-selectin [38], and is inversely proportional to endothelial NO bioavailability as estimated by flow-mediated dilatation of the brachial artery [39]. Furthermore, plasma vWF is raised in proportion to plasma Ang-2 in patients with sepsis and acute lung injury [11]. Because our results suggest that impaired endothelial NO bioavailability exacerbates WPB release, they provide a plausible explanation for the increase in both Ang-2 and vWF in patients with sepsis.

Conclusions

In conclusion, Ang-2 is raised in sepsis in proportion to disease severity and correlates with endothelial...
activation and inversely with NO-dependent microvascular reactivity, both at baseline and over the first two to four days of treatment. This suggests that decreased endothelial NO bioavailability may contribute to Ang-2 release by reducing negative feedback on WPBs, thus augmenting endothelial cell activation and contributing to organ dysfunction. Adjunctive therapies which improve endothelial NO, decrease WPB release, or antagonise Ang-2 may have roles in reducing organ dysfunction and improving mortality in sepsis.

Key messages
- Plasma concentrations of Ang-2, an angiogenic peptide, have been shown to be raised in patients with sepsis and to correlate with organ failure and mortality, but the underlying mechanisms are unclear.
- In-vitro, NO inhibits Ang-2 release from endothelial cells. In this study, plasma concentrations of Ang-2 in septic patients were raised in proportion to disease severity, and were inversely proportional to estimated endothelial NO bioavailability.
- Decreased endothelial NO bioavailability in sepsis may be the mechanism for raised Ang-2 concentrations, thus contributing to capillary leak and organ dysfunction.
- Adjunctive therapies that improve endothelial NO or antagonise Ang-2 may have roles in reducing organ dysfunction and improving mortality in sepsis.

Abbreviations
- Ang-2: angiopoietin-2
- APACHE: Acute Physiology and Chronic Health Evaluation
- CBA: cytokine bead array
- CI: confidence interval
- ELISA: enzyme-linked immunosorbent assay
- H2O2: hydrogen peroxide
- ICAM-1: intracellular adhesion molecule-1
- IL: interleukin
- IQP: interquartile range
- NSF: N-ethylmaleimide sensitive factor
- NO: nitric oxide
- RH-PAT: reactive hyperemia peripheral arterial tonometry
- SIRS: systemic inflammatory response syndrome
- SOFA: Sequential Organ Failure Assessment
- TNFα: tumour necrosis factor α
- VEGF: vascular endothelial growth factor
- VWF: von Willebrand factor
- WPB: Weibel Palade bodies

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4 Intensive Care Unit, Royal Darwin Hospital, Rocklands Drive, Tiwi, Darwin, NT, 0810, Australia.

Authors’ contributions
Study Design was performed by JSD, NMA, TWY, DPS and DSC. JSD and DPS contributed to patient recruitment. The data was processed by JSD, KP and TW, and was analysed by JSD. Laboratory sample processing was performed by KP and TW. The manuscript was drafted by JSD and NMA. All authors had access to all data and contributed to the final draft of the paper. All authors read and approved the final manuscript.

Competing interests
DC has received research support (as equipment) from Itamar Medical, the manufacturer of the RH-PAT device, and has received speaker’s fees (less than US$1000 per year) for speaking at Itamar-sponsored educational events. The other authors have no competing interests.

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14. Matsushita K, Morrell CN, Cambien B, Yang SX, Yamakuchi M, Bao C, Hara MR, Quick RA, Cao W, O’Rourke B, Lowenstein JM, Pevsner J, Wagner DD, Lowenstein CJ. Nitric oxide regulates exocytosis by S-nitrosylation of N-ethylmaleimide-sensitive factor. Cell 2003, 115:139-150.

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Is plasma arginine concentration decreased in patients with sepsis? A systematic review and meta-analysis

Joshua S. Davis, Nicholas M. Anstey

Introduction: L-arginine is a conditionally essential amino acid that plays an important role in immune and vascular function in sepsis. Plasma concentrations of L-arginine are decreased after trauma or surgery but have been variably reported to be normal or decreased in patients with sepsis.

Methods: We searched MEDLINE and Embase from database inception until January 2010 for the MESH terms “arginine,” “amino acids,” and “sepsis” and reviewed all studies that reported plasma arginine concentrations in humans with sepsis. Studies were grouped according to the presence or absence of trauma and surgery. We performed a pooled quantitative analysis on the subset of studies that reported appropriate data.

Results: We identified 285 citations, of which 16 met inclusion criteria and 10 were included in the quantitative analysis. Plasma arginine concentration was lower in sepsis patients compared with concurrent or historical controls in three of four studies of surgical sepsis, one of four of sepsis after trauma, and all eight studies of predominantly medical sepsis. In the quantitative analysis, mean plasma L-arginine concentration was 33.9 μmol/L (95% confidence interval, 41.2–26.6) lower in sepsis patients than in concurrent nonseptic controls (p < .001), which is a relative decrease of 41%.

Conclusion: Plasma concentrations of plasma L-arginine are substantially decreased in patients with sepsis in the absence of trauma or surgery. There are not enough studies of sufficient quality to determine whether this is also the case for trauma-associated or surgery-associated sepsis. (Crit Care Med 2011; 39: 000–000)

Key Words: L-arginine; sepsis; trauma; nitric oxide; systematic review

L-arginine, a nonessential amino acid in baseline physiologic states, becomes essential at times of physiologic stress such as sepsis, trauma, and surgery, and is thus a “conditionally essential” amino acid (1). L-arginine is the substrate for nitric oxide production by nitric oxide synthase (2). Baseline endothelial nitric oxide production is required to maintain the microcirculation, with inducible synthesis having antimicrobial effects (3, 4). Nitric oxide-independent effects of L-arginine include important roles in cell-mediated immune function (5, 6), protein synthesis (7), and wound healing (8, 9). Enteral nutrition with L-arginine-containing supplements is controversial in sepsis (10); various studies have shown either beneficial or harmful effects, but none has studied arginine administration alone (11).

Plasma L-arginine levels are generally (10, 12, 13), but not always (14, 15), described in review articles as being low in humans with sepsis. However, these articles selectively cite either other commentaries (12, 16, 17) or the same few clinical studies (18–20), and none of them critically assesses the available published evidence. Furthermore, several studies and commentaries suggest that plasma L-arginine concentrations are not decreased in sepsis (21, 22) or that they are more markedly decreased in trauma than in sepsis (10, 14, 15, 20). By far, the two most highly cited studies of plasma L-arginine concentrations in sepsis both reported no significant difference between plasma L-arginine concentrations in sepsis patients compared with healthy controls (21, 22).

Plasma L-arginine also has been shown to be low in humans with severe trauma (20, 22) and other severe states of physiologic stress (23). Macrophage arginine consumption and myeloid cell arginase production are increased after trauma (24) and surgery (25, 26). Because many patients with sepsis have also experienced recent trauma or surgery, it is unclear if sepsis alone is an arginine-deficient state.

Because of its important implications for understanding sepsis pathophysiology, and to clarify the potential role of arginine supplementation in sepsis, we conducted a systematic review of the published literature to address the question of whether plasma L-arginine concentration is decreased in patients with sepsis compared with healthy controls.

METHODS

We searched MEDLINE and Embase using the MESH headings “arginine,” “amino acids,” and “sepsis,” and the limits “human,” from database inception until January 2, 2010. We also manually searched the references of retrieved articles and review articles. Studies were included if they met all of the following predefined criteria: (1) included humans with a diagnosis of “sepsis”; (2) provided a definition of “sepsis”; (3) reported plasma L-arginine concentrations; (4) patients were receiving no specific amino acid supplements at the time of measurements (apart from standard enteral or parenteral nutrition); and (6) reported in English. Data were extracted and recorded on a predefined checklist.

To be included in the quantitative analysis, studies also had to: (1) report mean L-arginine concentrations (rather than expression in graph-
Table 1. Characteristics of published studies reporting plasma L-arginine concentrations in humans with sepsis

<table>
<thead>
<tr>
<th>Author</th>
<th>Subjects</th>
<th>Sepsis Duration*</th>
<th>Sepsis Definition</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freund (31)</td>
<td>15 adults with sepsis (9 surgical)⁶</td>
<td>Not stated</td>
<td>Infection plus fever and leucocytosis</td>
<td>None</td>
</tr>
<tr>
<td>Freund (18)</td>
<td>25 adults with sepsis (Mostly surgical, numbers not stated)</td>
<td>Not stated</td>
<td>Infection plus fever and leucocytosis</td>
<td>None</td>
</tr>
<tr>
<td>Askanazi (21)</td>
<td>10 adults with trauma (5 of whom also had sepsis)</td>
<td>Not stated</td>
<td>Fever plus infection</td>
<td>15 “normal”</td>
</tr>
<tr>
<td>Vente (23)</td>
<td>27 adults with sepsis 38 adults with “stress” (Trauma, pancreatitis, or ruptured aneurysm)</td>
<td>Not stated</td>
<td>Bacteraemia plus all of fever, tachycardia, and leucocytosis</td>
<td>None</td>
</tr>
<tr>
<td>Ochoa (22)</td>
<td>17 surgical sepsis 14 trauma 8 trauma and sepsis (All adults)</td>
<td>Not stated</td>
<td>Approximately corresponds to 1992 SCCM criteria for severe sepsis (58)</td>
<td>14 healthy volunteers</td>
</tr>
<tr>
<td>Sprung (37)</td>
<td>15 adults septic shock (12 with pneumonia)</td>
<td>Not stated</td>
<td>Approximately corresponds to 1992 SCCM criteria for septic shock plus acute confusion (58)</td>
<td>17 with infection (but with no confusion or shock)</td>
</tr>
<tr>
<td>Chiarla (32)</td>
<td>16 adults with trauma and sepsis⁶</td>
<td>Not stated</td>
<td>Infection plus fever and leucocytosis</td>
<td>None</td>
</tr>
<tr>
<td>Druml (36)</td>
<td>9 adults with severe sepsis (4 surgical)</td>
<td>Not stated</td>
<td>1992 SCCM criteria plus bacteraemia</td>
<td>8 healthy controls</td>
</tr>
<tr>
<td>Luiking (34)</td>
<td>8 adults severe sepsis (Focus not stated)</td>
<td>Within 48 hrs of sepsis diagnosis</td>
<td>Not stated</td>
<td>5 critically ill without sepsis</td>
</tr>
<tr>
<td>Villapando (35)</td>
<td>6 adults septic shock (All medical)</td>
<td>Within 12 hrs of septic shock criteria</td>
<td>1992 SCCM criteria</td>
<td>10 healthy volunteers</td>
</tr>
<tr>
<td>Chiarla (20)</td>
<td>9 adults with trauma and sepsis</td>
<td>Not stated</td>
<td>Infection plus fever and leucocytosis</td>
<td>None</td>
</tr>
<tr>
<td>Engel (33)</td>
<td>32 adults with trauma and sepsis</td>
<td>Not stated</td>
<td>1992 SCCM criteria</td>
<td>29 healthy volunteers</td>
</tr>
<tr>
<td>Van Waardenburg (38)</td>
<td>19 children with sepsis</td>
<td>Within 24 hrs of admission</td>
<td>1992 SCCM criteria</td>
<td>21 children with viral respiratory infections</td>
</tr>
<tr>
<td>Kao (40)</td>
<td>13 adults with sepsis (all medical)</td>
<td>Within 48 hrs of sepsis diagnosis</td>
<td>2003 Consensus criteria (59)</td>
<td>7 healthy volunteers</td>
</tr>
<tr>
<td>Luiking (39)</td>
<td>10 adults septic shock (7 medical, 3 surgical)</td>
<td>Within 48 hrs of septic shock diagnosis</td>
<td>2003 Consensus criteria (59)</td>
<td>16 healthy volunteers</td>
</tr>
<tr>
<td>Davis (28)</td>
<td>56 sepsis patients (50 community-acquired sepsis)</td>
<td>Within 36 hrs of admission</td>
<td>1992 SCCM criteria</td>
<td>27 healthy volunteers</td>
</tr>
</tbody>
</table>

SCCM, Society of Critical Care Medicine.

*Time elapsed between onset/diagnosis of sepsis and enrollment in the study; ⁶Surgical” sepsis refers to patients managed exclusively in surgical intensive care units or who have undergone an operation; ⁷Eight excluded from quantitative analysis because they were receiving nonstandard total parenteral nutrition with branched chain amino acids.
RESULTS

Search Results

Two hundred eighty-five citations were identified, of which 16 were included for analysis, and nine were included in the quantitative analysis (Fig. 1).

Characteristics of Included Studies

The 16 studies (Table 1) were divided into three groups (Table 2) based on the presence or absence of trauma or surgery in addition to sepsis. Group 1 comprised those studies with patients with predominantly surgical (postoperative) sepsis. Group 2 included those enrolling patients in whom trauma and sepsis coexisted. Group 3 comprised those studies enrolling patients with no recent trauma and with predominantly nonsurgical sepsis (such as pneumonia or urosepsis). The studies used varying sepsis definitions, and most did not report the time elapsed between the diagnosis of sepsis and collection of blood for analysis (Table 1). The quality scores were lower for the studies in groups 1 and 2 (mean quality score, 1.8) than for those in group 3 (mean quality score, 4.9; p = .003) (Table 2).

Group 1 (surgical sepsis) included four studies, three of which reported decreased L-arginine concentrations in sepsis patients compared with historical controls (18, 23, 31), and one of which reported no statistically significant difference in plasma L-arginine in sepsis patients compared with healthy controls (22). Three of four studies in group 2 (sepsis with trauma) reported no difference in plasma L-arginine in sepsis patients compared with concurrent controls (27, 34–40).

Excluded Studies

Twenty of the 36 retrieved studies were excluded, 12 did not report plasma L-arginine concentrations, three were review articles, two comprised patients with severe malaria without sepsis criteria (41, 42), one included patients receiving variable rates of intravenous amino acid infusions, and two small studies were not written in English (43, 44).

Quantitative Data Synthesis

Of the 16 included studies, ten reported sufficient information to be included in a pooled quantitative analysis. These ten studies included 192 patients with sepsis and 149 controls. With the exception of one study, all reported lower mean plasma L-arginine concentrations in sepsis patients than in controls. The pooled mean difference in L-arginine concentrations between sepsis and control groups was $-33.9\text{ mol/L}$ (95% confidence interval, $-41.2$ to $-26.6$), i.e., plasma L-arginine was, on average, 33.9 mol/L lower in sepsis patients than in controls ($p < .0001$), a 41% relative decrease from the pooled mean control plasma arginine concentration of 82.2 mol/L.

Sensitivity Analysis

Only four of the included studies had quality scores of five or more out of a...
This review demonstrates that plasma L-arginine concentrations are acutely decreased in patients with sepsis, independent of trauma or surgery. Furthermore, the degree of hypoargininaemia in sepsis is considerable, with a mean difference of at least 33 μmol/L compared with controls. Given that normal plasma L-arginine concentrations are approximately 70–80 μmol/L (45–47), this difference may be large enough to contribute to significant impairment of endothelial and immune function in patients with sepsis.

The signal from studies of sepsis associated with trauma or surgery was more mixed, with half of eight studies showing decreased L-arginine concentrations in sepsis and half showing no difference between controls and normal ranges. However, because this group of studies was of significantly lower quality than those concerning nonsurgical sepsis, they should be interpreted with caution. Because plasma arginine has been shown to decrease toward normal over the first 2–4 days in patients with sepsis (48) but to remain low for at least 7 days in patients with trauma (24), an important explanation for a lack of difference in these studies may be a prolonged time between sepsis onset and blood collection for plasma amino acid analysis. Of the four studies that did not find hypoargininaemia in sepsis patients, three provided no information about the time of specimen collection in relation to sepsis onset (20, 22, 32), and in the remaining study at least 3–4 days had elapsed before blood collection (21).

The findings of this systematic review provide an evidence base that supports the majority of review articles and editorials concerning hypoargininemia in sepsis (1, 10, 12, 13, 49). Furthermore, our findings are consistent with observations from both animal (50) and human (51) experimental models of sepsis, which both show an acute decrease in plasma L-arginine concentration after intravenous administration of bacterial endotoxin. Several mechanisms are likely to underlie hypoargininemia in sepsis, including decreased gastrointestinal absorption of arginine (52), increased arginase activity (53), and increased utilization of arginine for protein synthesis (12).

This study has several limitations. The overall quantitative analysis has significant heterogeneity ($I^2 = 70\%$), and most of the studies including patients after surgery or trauma were of poor quality for the purposes of comparing plasma L-arginine concentrations in sepsis with controls. Because some studies report plasma L-arginine concentrations as secondary outcomes, the search strategy may have missed some relevant studies. However, a funnel plot (not shown) of the mean difference vs. its standard error for each study was relatively symmetrical, which suggests that there are unlikely to be missing or unpublished studies that would alter the conclusions of this meta-analysis. Two non-English language articles were excluded. Although this may have affected the findings, their inclusion would have added only 39 patients to the 285 included, making this unlikely.

In conclusion, sepsis not associated with trauma or surgery is a profoundly hypoargininaemic state, but there are not enough high-quality studies to determine with certainty if this is also the case for sepsis associated with trauma or surgery. Although studies of arginine supplementation in human (11) and animal (54, 55) sepsis have had conflicting results, few studies have assessed the role of arginine alone as a therapeutic agent in human sepsis (56, 57). Given that plasma L-arginine concentrations are clearly decreased in humans with sepsis, the issue of exogenous arginine administration in sepsis should be revisited.

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Figure 3. A. Pooled quantitative analysis of studies reporting plasma arginine concentration in sepsis patients compared with healthy controls. B. Pooled quantitative analysis of studies reporting plasma arginine concentration in sepsis patients compared with unwell but nonseptic controls. The controls had either infection without sepsis (Sprung 1991 and van Waardenburg 2007) or critical illness without sepsis (Luiking 2003). Units for plasma arginine are μmol/L. SD, standard deviation; IV, inverse variance method; Random, random effects model; CI, confidence interval; df, degrees of freedom.
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