New Insights into the Mechanisms of Vascular Dysfunction in a Young and Aged Septic Murine Model

by

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A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Experimental Surgery

Department of Surgery

University of Alberta

Abstract

Background:

Sepsis is a dysregulated response to infection that results in life-threatening organ dysfunction. The elderly are disproportionately affected by sepsis and suffer an alarmingly high mortality rate. We hypothesized that increased mortality in the aged is due to a more highly dysfunctional vasculature secondary to loss of soluble guanylate cyclase signalling. As sepsis mortality is high, we aimed to provide insight into septic vascular dysfunction and discover a novel sepsis treatment that would be effective in the young and aged.

Methods:

In all studies included herein, hemodynamics and vascular function were assessed *in-vivo* and *ex-vivo*. In Study 1, young (3 to 4 months) and aged (22 to 24 months) mice were anesthetized and instrumented for hemodynamic assessments. Mice received an intraperitoneal injection of fecal slurry or an equivalent volume of vehicle. Systolic blood pressure, diastolic blood pressure, and heart rate were continuously recorded for a maximum of 600 minutes. A subset of mice received norepinephrine treatment. All mice received intravenous methacholine every 30 minutes to measure vascular reactivity. Following euthanization, tissues and arteries were collected for analysis of cGMP production and wire myography. Study 2 examined the young septic murine response to the soluble guanylate cyclase activator cinaciguat using the same protocol as above while Study 3 examined aged mice. The cytokine Oncostatin M is released in sepsis and plays a role in hemodynamic stability. As such, its receptor deficiency in both young males and

females was investigated using a model of Oncostatin M receptor deficiency combined with the above septic protocol (Study 4).

Results:

In study 1, aged mice had impaired non-septic and septic vasorelaxation responses *in-vivo* compared to the young. Non-septic aged mice were more responsive to methacholine, whereas septic aged mice were less responsive. Ultimately, aged mice demonstrated decreased sepsis survival. Conventional sepsis treatment with norepinephrine resulted in a similar sepsis survival time in both age groups. cGMP production in aged lung and kidney did not increase. Notable aged myography findings included increased sensitivity to methacholine in mesenteric arteries and increased vasorelaxation 120 minutes post-sepsis induction.

Due to the mortality associated with sepsis, we aimed to discover a treatment that would be effective in young (Study 2) and aged (Study 3) septic mice. Mice were treated with saline or the soluble guanylate cyclase activator cinaciguat to increase cGMP production and improve vascular dysfunction in sepsis. Cinaciguat improved the survival of young septic mice, coinciding with improved hemodynamics. Cinaciguat increased cGMP production in young lung and kidney. Myography revealed an increased sensitivity of cinaciguat-treated young septic mesenteric vessels to methacholine and sodium nitroprusside. Hemodynamic improvements were also noted in septic aged mice treated with cinaciguat. Baseline aged systolic and diastolic blood pressure response to methacholine was increased while septic blood pressure was decreased. Aged sepsis

survival was also increased. However, no change in cGMP production was noted in aged lung or kidney. Myography demonstrated minimal vascular effect of cinaciguat.

In Study 4, both sexes of Oncostatin M receptor deficient mice displayed a distinct hemodynamic profile demonstrating systolic and diastolic hypertension as well as tachycardia. Septic response to methacholine was decreased in male but not female knockout mice (compared to controls). Oncostatin M deficiency did not improve survival for either sex. Oncostatin M receptor deficiency did demonstrate sex-specific myography effects associated with a decreased level of relaxation secondary to methacholine and sodium nitroprusside in both non-septic and septic male vessels. These changes were not seen in female vessels.

Summary and Conclusion:

This is one of the first studies to examine both *in-vivo* and *ex-vivo* vascular function in a young and aged septic murine model. The findings demonstrate that aged septic mice experience vascular dysfunction, which may contribute to mortality. The novel use of cinaciguat resulted in augmentation of vascular function in young mice and improvement in sepsis survival in both young and aged mice. Finally, Oncostatin M receptor deficiency did not improve sepsis survival but led to the development of distinct non-septic and septic hemodynamic phenotypes. Together, these results underscore the need to further delineate the mechanisms that underlie vascular dysfunction and septic circulatory collapse. This new information sets the stage for further testing of cinaciguat to understand the molecular mechanism(s) in improving septic outcomes.

Preface

I hereby certify that all of the work described within this thesis is the original work of the author and listed co-authors. Any published (or unpublished) ideas and/or techniques from the work of others are fully acknowledged in accordance with standard referencing practices.

Chapter 1 of this thesis is in preparation for submission for publication as Kimberly F. Macala, Ferrante S. Gragasin, Stephane L. Bourque, Rachel G. Khadaroo, "Review of Sepsis and Septic Vascular Dysfunction" KFM was involved in all aspects of the work included herein, including literature review and authoring/editing/submission of the manuscript. KFM did 100% of the writing. FSG, SLB, and RGK edited the manuscript.

Chapter 2 of this thesis is in preparation for submission for publication as Kimberly F. Macala, Sareh Panahi, Forough Jahandideh, Ferrante S. Gragasin, Stephane L. Bourque, Rachel G. Khadaroo, "A Comparison of Vascular Dysfunction and Survival in Young and Aged Septic Mice" KFM was involved in all aspects of the work included herein, including identification of the hypotheses to be tested, literature review, designing the research methodology, participation in the performance of all aspects of the experimental design, all data collection, analysis of collected data, and authoring/editing/submission of the manuscript. SP and FJ participated in conducting experiments while FSG, SLB, RGK edited the manuscript.

Chapter 3 of this thesis is in preparation for submission for publication as Kimberly F. Macala, Sareh Panahi, Forough Jahandideh, Ferrante S. Gragasin, Stephane L. Bourque, Rachel G. Khadaroo, "Cinaciguat Improves Blood Pressure and Survival in a Murine

Model of Sepsis" KFM was involved in all aspects of the work included herein, including identification of the hypotheses to be tested, literature review, designing the research methodology, participation in the performance of all aspects of the experimental design, all data collection, analysis of collected data, and authoring/editing/submission of the manuscript. SP and FJ participated in conducting experiments while FSG, SLB, RGK edited the manuscript.

Chapter 4 of this thesis is in preparation for submission for publication as Kimberly F. Macala, Sareh Panahi, Forough Jahandideh, Ferrante S. Gragasin, Stephane L. Bourque, Rachel G. Khadaroo, "Cinaciguat in Aged Septic Mice Improves Vascular Function and Survival" KFM was involved in all aspects of the work included herein, including identification of the hypotheses to be tested, literature review, designing the research methodology, participation in the performance of all aspects of the experimental design, all data collection, analysis of collected data, and authoring/editing/submission of the manuscript. SP and FJ participated in conducting experiments while FSG, SLB, RGK edited the manuscript.

Chapter 5 of this thesis is in preparation for submission for publication as Kimberly F. Macala, Sareh Panahi, Forough Jahandideh, Ferrante S. Gragasin, Stephane L. Bourque, Rachel G. Khadaroo, "Sex-Specific Vascular and Mortality Outcomes of Oncostatin M Receptor Deficiency During Sepsis in Mice" KFM was involved in all aspects of the work included herein, including identification of the hypotheses to be tested, literature review, designing the research methodology, participation in the performance of all aspects of the experimental design, all data collection, analysis of collected data, and

authoring/editing/submission of the manuscript. SP and FJ participated in conducting experiments while FSG, SLB, RGK edited the manuscript.

This work was completed with ethics approval by the University of Alberta research ethics board, Project Name "Perioperative Hypotension and Septic Shock," AUP00000987, 3/28/2014 – CURRENT.

(Kimberly Faye Macala)

(September 2022)

Acknowledgements

This work was supported by Fellowship funding for KFM from the Canadian Critical Care Trials Group (CCCTG) and Canadian Critical Care Translational Biology Group (CCCTBG) and with operational funding for KFM from the Royal Alexandra Hospital Foundation, Kingsway Intensivists, Dr. David Zygun, and Dr. Sean Bagshaw.

KFM and SLB also received operational funding from the Critical Care Strategic Clinical Network and the Canadian Foundation for Innovation.

SLB was supported by Canada Research Chair funding.

KFM and RGK also received operational funding from an Edmonton Civic Employees Research Award.

The work herein was conducted in the Women and Children's Health Research Institute laboratory space, University of Alberta, Edmonton, Canada.

Thank-you to my supervisory committee members: Dr. Ferrante S. Gragasin, Dr. Tom Churchill, Dr. Stephane L. Bourque, and Dr. Rachel G. Khadaroo, and to laboratory team members: Sareh Panahi and Dr. Forough Jahandideh.

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List of Abbreviations

abbMEDS: abbreviated mortality emergency department sepsis

ACE: angiotensin converting enzyme

ATP: adenosine triphosphate

ADH: antidiuretic hormone

AKI: acute kidney injury

ANOVA: analysis of variance

ANZICS: Australian and New Zealand Intensive Care Society

APC: activated protein C

ARDS: acute respiratory distress syndrome

ARRIVE: animal research: reporting of in vivo experiments

AT-II: angiotensin II

BH4: tetrahydrobiopterin

BPM: beats per minute

CaM: calmodulin

CARS: compensatory anti-inflammatory response syndrome

CASP: colon ascendens stent peritonitis

cAMP: cyclic adenosine monophosphate

cGMP: cyclic guanosine monophosphate

CLP: cecal ligation puncture

CO: cardiac output

CRP: C reactive protein

CSE: cystathionine-γ-lyase

CVP: central venous pressure

DAMPs: damage associated molecular patterns

DBP: diastolic blood pressure

DIC: disseminated intravascular coagulation

EC: endothelial cell

EC_{50}: half-maximal effective concentration

EDHF: endothelial-derived hyperpolarizing factor

EDTA: ethylenediaminetetraacetic acid

EET: epoxyeicosatrienoic acids

ELISA: enzyme-linked immunosorbent assay

eNOS: endothelial (constitutive) nitric oxide synthase

ESICM: European Society of Intensive Care Medicine

FAD: flavin adenine dinucleotide

FMN: flavin mononucleotide

FS: fecal slurry

G-CSF: granulocyte colony-stimulating factor

GM-CSF: granulocyte-macrophage colony-stimulating factor

GTP: guanosine triphosphate

HES: hydroxyethyl starch

HNOX: heme-NO/oxygen binding domain

HR: heart rate

IC_{100}: transmural pressure of 100 mmHg

ICAM-1: intercellular adhesion molecule-1

ICU: intensive care unit

iNOS: inducible nitric oxide synthase

IVIg: intravenous immune globulin

IL: interleukin

IP: intraperitoneal

IP_3: inositol triphosphate

IV: intravenous

KPSS: potassium physiologic salt solution

LNAME: L-N^G-Nitro arginine methyl ester

L-NMMA: N^G-Methyl-L-arginine

LPS: lipopolysaccharide

LPS:BP: lipopolysaccharide-binding protein

MCH: methacholine

MEWS: modified early warning system

MMP1: matrix metalloproteinase 1

MODS: multiple organ dysfunction syndrome

NADPH: reduced nicotinamide adenine dinucleotide phosphate

NE: norepinephrine

NEWS: national early warning system

nNOS: neuronal nitric oxide synthase

NO: nitric oxide

NOD-LRR: nucleotide-binding oligomerization domain-like receptors

NOS: nitric oxide synthase

NET: nuclear extracellular traps

NF-κB: nuclear factor kappa B

OSM: Oncostatin M

OSMR: Oncostatin M receptor

OSMR-/-: Oncostatin M receptor knockout

PAF: platelet-activating factor

PAI-1: plasminogen activator inhibitor-1

PAMPs: pathogen associated molecular patterns

PE: phenylephrine

PKG: protein kinase G

PLA2: phospholipase A2

PMN: polymorphonuclear cells

PPi: pyrophosphate

PRR: pattern recognition receptor

PSS: physiologic salt solution

qSOFA: quick sepsis-related organ failure assessment

ROS: reactive oxygen species

SBP: systolic blood pressure

SCCM: Society of Critical Care Medicine

$ScvO_2$: central venous oxygen saturation

SEM: standard error of the mean

SIRS: systemic inflammatory response syndrome

sGC: soluble guanylate cyclase

SNP: sodium nitroprusside

SOFA: sepsis-related organ failure assessment

TAFI: thrombin activatable fibrinolysis inhibitor

TF: tissue factor

TFPI: tissue pathway factor inhibitor

TIMP-1: tissue inhibitor of metalloproteinases

TXA_2: thromboxane

TNF: tumour necrosis factor

TLR: toll-like receptor

WHO: World Health Organization

VCAM-1: vascular cell adhesion molecule-1

Chapter 1

Review of Sepsis and Septic Vascular Dysfunction

1.1 Introduction

Sepsis, a life-threatening syndrome of dysregulated systemic inflammation secondary to an infection,[1] is a common, complex, and global medical condition responsible for significant healthcare expenditure. Sepsis can progress to an extreme state of circulatory shock that can affect any person worldwide. The outcomes can be severely disfiguring, debilitating, and deadly. Due to its high morbidity and mortality, sepsis awareness amongst the general public and physicians alike has been increasing.[2,3] As such, it was made a global health priority by the World Health Organization (WHO) in 2017.[4] Over the previous decades, extensive research has discovered much about multiple aspects of sepsis, including risk factors, pathophysiology, epidemiology, organ failure, mortality, cost of sepsis care, and quality of life outcomes. As research has progressed, our sepsis terminology has also evolved. Consensus groups have refined the definitions of the various sepsis syndromes to reflect improved knowledge concerning the pathophysiology of sepsis and the associated mortality risks.

Notwithstanding moving forward in the fight against sepsis, strategies for preventing sepsis, managing modifiable risk factors, early identification of sepsis (via the use of biomarkers[1] and severity scoring systems), and accurate prognostication of morbidity and mortality have not progressed in tandem with our increasing knowledge related to sepsis. Moreover, the development of targeted treatments for vulnerable patient

populations (elderly, intensive care unit (ICU) patients,[5] patients with bacteremia,[6,7] frailty,[8] obesity,[9] diabetes,[10] malignancy,[11,12] patients concurrently using opioids[13]) remain frustratingly elusive, despite substantial effort from the scientific community and industry. Treatment remains largely generic and supportive, including antibiotics, source control of infection, fluid resuscitation, vasopressors, inotropes, intubation and ventilation, and renal replacement therapies.[1]

Many investigational therapies targeting various aspects of the immune response in sepsis, though preclinically successful, have not improved patient outcomes.[14,15] However, the study of these unsuccessful therapies has invariably added to our understanding of sepsis pathophysiology and continues to inspire further research efforts. Here, we will provide a detailed review of sepsis. In latter sections, we will also focus specifically on how aging affects sepsis as the elderly population is recognized as a particularly vulnerable patient group disproportionately affected by sepsis.[16]

1.2 Terminology

Understanding current and prior sepsis terminology is essential to discuss sepsis research within the contextual timespan of specific studies. Before 2016, four major clinical sepsis syndromes existed to classify septic states. These clinical syndrome definitions were used worldwide and stemmed from the efforts of the Surviving Sepsis Campaign[17,18] and were endorsed by two of the world's largest critical care medicine societies, The American Society of Critical Care Medicine (SCCM) and the European Society of Intensive Care Medicine (ESICM). Sepsis definitions were first advanced in 1991[19] and revised in 2001[20] and 2012,[18] based on our improved understanding of sepsis pathophysiology.

The first syndrome stemming from these guidelines and used in the literature is the *Systemic Inflammatory Response Syndrome (SIRS)*. To be labelled as having SIRS, patients had to display a minimum of two out of four clinical criteria as listed in **Table 1**. The SIRS criteria remain an important triaging tool for identifying patients that will require further work-up and potential interventions. However, it is essential to note that the SIRS criteria are not specific for sepsis[21,22] and that patients with sepsis may not display SIRS criteria.[23] A non-infectious source such as pancreatitis, malignancy, pulmonary embolism, burns, or trauma may also result in a SIRS response,[24] making the diagnosis of sepsis clinically more difficult and may result in delayed treatment.

Table 1: Systemic Inflammatory Response Syndrome Criteria

The diagnosis of SIRS requires the presence of at least two of the below criteria. SIRS: Systemic Inflammatory Response Syndrome**.**

Systemic Inflammatory Response Syndrome Criteria[19]
Temperature of less than 36 or greater than 38 degrees Celsius
Heart rate greater than 90 beats per minute
Respiratory rate greater than 20 breaths per minute or a $PaCO_2$ of less than 32 mmHg
White blood cell count less than 4×10^9 or greater than 12×10^9 cells/L or greater than 10% band forms

The second syndrome from the pre-2016 classification system is *sepsis*. Sepsis was defined as the presence of SIRS with a source of suspected or documented infection.[25] Sepsis syndrome could deteriorate into *severe sepsis*, the third clinical syndrome. Severe sepsis consisted of sepsis combined with evidence of organ dysfunction, hypotension, or

hypoperfusion.[25] The fourth syndrome (and the most life-threatening) was *septic shock*. Septic shock was defined as the presence of severe sepsis with hypoperfusion despite adequate fluid resuscitation.[25] A fifth important term often used is Multiple Organ Dysfunction Syndrome (MODS). MODS has been defined as "the development of potentially reversible physiologic derangement involving two or more organ systems not involved in the disorder that resulted in ICU admission, and arising in the wake of a potentially life-threatening physiologic insult."[26] MODS has also been described as "the presence of altered organ function in acutely ill septic patients such that homeostasis is not maintainable without intervention."[19] Typically, MODS is the potential penultimate state a patient may achieve before death.

Over time, it was noted that the SIRS criteria were not inclusive of all warning symptoms and signs of sepsis. Some patients did not meet the SIRS criteria, yet still progressed to more severe clinical sepsis syndromes.[27] In fact, as many as one in eight patients with severe infection, organ failure, or mortality did not initially meet SIRS criteria.[23] To further complicate reliance on SIRS criteria, it is known that elderly patients (the largest population affected by sepsis[28]) commonly lack SIRS criteria,[29] resulting in delayed diagnosis and subsequent life-saving treatment (sepsis mortality increases with delayed treatment[30,31]). Due to the lack of specificity of the SIRS criteria, poor relation to patient morbidity or mortality, and confusion regarding what exactly constituted severe sepsis, updated definitions were necessary.

The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3) based on SCCM/ESCIM expert opinion were published in 2016.[1] Notable changes in

Sepsis-3 were the removal of SIRS criteria (for the reasons stated above) and elimination of the category of severe sepsis (as it was deemed redundant and confusing[23,25]) from the definitions. According to the revised 2016 guidelines, the first category was *early sepsis*. Although lacking a formal and specific definition, early sepsis included infections and bacteremia, which may progress to more severe states such as sepsis.[1] A vital component of the updated sepsis definitions was introducing the use of the quickSOFA (quick Sequential Organ Failure Assessment – qSOFA) score for early identification of patients who may progress to sepsis.[1] The qSOFA consists of three easily measured components, as listed in **Table 2**.

Table 2: Quick Sequential [Sepsis-related] Organ Failure Assessment

A score of ≥2 was associated with worse patient outcomes.

qSOFA[1]
Respiratory rate greater than or equal to 22 breaths per minute
Altered mentation
Systolic blood pressure less than or equal to 100 mmHg

As no biochemical or invasive tests were necessary to use the qSOFA, it could be widely applied. A score of greater than or equal to two was associated with worse patient outcomes, defined as greater than 10% in-hospital mortality or spending greater than or equal to three days in ICU.[1] The qSOFA was controversial as it had yet to be validated in several specific patient groups, including the elderly, and had demonstrated a low sensitivity (70% in a multi-centre European trial from 2017[32]). Subsequently, several trials examined the utility and benefit of qSOFA compared to using SIRS criteria.[33] The in-

hospital mortality prognostic accuracy was greater for qSOFA than the SIRS criteria in a multi-centre European study from 2017.[32] A 2018 Canadian meta-analysis (which included ICU, emergency department, and hospital ward patients) demonstrated that qSOFA was a less sensitive predictor for sepsis-related mortality compared to the SIRS criteria (61 versus 88%); however, qSOFA was more specific (26 versus 72%).[34] In 2017, the Australian and New Zealand Intensive Care Society (ANZICS) published a retrospective analysis of over 180,000 ICU patients demonstrating the inferiority of qSOFA compared to the full SOFA score for mortality prediction.[35] The SOFA score is a predictive mortality tool based on dysfunction severity in six organ systems. Like qSOFA, it is not used to diagnose sepsis, nor is it designed to direct medical treatment. A study published in 2020 demonstrated that qSOFA use at triage in American emergency departments performed poorly at predicting patient outcomes and was no better than using initial serum lactate measurement.[36] Due to the increased sensitivity of SIRS criteria compared to the qSOFA, many are reverting to using SIRS criteria.[37] Further to qSOFA and SIRS, there are other sepsis scoring systems for outcome predictions (NEWS: National Early Warning Score[38]).[39,40] However, no scoring system is infallible. It is important to remember that these scoring systems prompt the clinician's awareness of a condition and evidence-informed probable outcomes. The clinical use of the SOFA score is further discussed below.

The second category in the updated 2016 definitions was *sepsis*. According to Sepsis-3 guidelines, sepsis was defined as "life-threatening organ dysfunction caused by a dysregulated host response to infection."[1] The SCCM/ESICM task force specifically defined organ failure using the full SOFA score, not the qSOFA score. An increase in the

SOFA score of two or more points was required. SOFA was used instead of SIRS criteria for its superior predictive value for in-hospital mortality.[41] However, it is again noteworthy that the SOFA score is not diagnostic of organ dysfunction solely secondary to infectious causes.

Table 3: Sequential [Sepsis-related] Organ Failure Assessment

SOFA Score Components
PaO_2
FiO_2
Requiring Mechanical Ventilation
Glasgow Coma Scale
Mean Arterial Pressure/Requiring Vasopressor Support
Creatinine
Platelet Count
Bilirubin

Septic shock was the third and final category in the 2016 sepsis definitions. It was explicitly defined as "sepsis that has circulatory, cellular, and metabolic abnormalities that are associated with a greater risk of mortality than sepsis alone."[1] Patients with septic shock have failed resuscitation with fluid administration alone. They were dependent upon vasopressor treatment to attain and sustain a mean arterial pressure greater than or equal to 65 mmHg while developing a serum lactate concentration greater than two mmol/L.[1] Essentially, septic shock was the most severe form of sepsis. This definition was helpful

as it identified a group of patients that would experience higher mortality than patients with sepsis alone.[1]

In 2021, the most recent guidelines for treating sepsis were published.[42] While the definition of sepsis and septic shock remained the same, several noteworthy changes to the guidelines were instituted. Since the publication of the 2016 guidelines, several studies have examined the use of qSOFA and have demonstrated it was a poorly sensitive screening tool with contradictory results pertaining to outcomes.[42-45] While the guidelines still strongly recommend screening patients for sepsis, it is now recommended to use other screening tools such as the SIRS criteria, MEWS (Modified Early Warning System), or NEWS instead of qSOFA. A randomized control trial from 2017 and a meta-analysis from 2019 demonstrated the superiority of machine learning tools compared to conventional screening tools in predicting sepsis.[46-48] This suggests that future definitions and guidelines may rely less on conventional screening tools. The 2021 guidelines present, in total, 93 recommendations regarding various aspects of sepsis diagnosis and management, including the use of screening tools, hemodynamic goals, use of antibiotics, ventilation strategies, and use of additional therapies. In all, there are 21 updated recommendations compared to the 2016 guidelines. Notably, no new pharmacologic treatments for sepsis are recommended as previously promising investigational pharmacologic treatments (terlipressin and levosimendan) are recommended against due to lack of benefit and increased risk of harm.[49-51] There are also no age-targeted treatments recommended.

The terminology we use to discuss, research, and care for septic patients provides clinicians and researchers with a working frame of reference. Understanding the language

defining sepsis allows for consistency in research and improved communication and collaboration to increase knowledge translation. These terms must evolve with our understanding of sepsis and impart meaningful insights into care and outcomes. Herein, unless otherwise stated, we will use the term sepsis to refer to both sepsis and severe sepsis as, since 2016, they comprise the same category of clinical syndromes. However, in many cases, the outdated definitions must be employed when referencing studies published before the Sepsis-3 guidelines were introduced.

1.3 Epidemiology

Sepsis is a global health burden, and the incidence of sepsis has been generally reported as increasing.[52-58] The incidence of sepsis can be challenging to ascertain depending upon the origin of the data.[58-62] There exists no specific test for sepsis, and ultimately diagnosis is often based upon clinical judgment, which can be subject to inaccuracy and imprecision.[63] Incidence of a particular diagnosis is also subject to evolving definitions, lack of recognition, illness or death in the community, changing diagnostic and clinical practice coding, and what diagnosis is included on death certificates.[53,55,64] Diagnostic data can also be collected from retrospective chart reviews or prospective observational studies, which would improve the generalizability of the data. Incentivized reporting of illness and improved coding also can affect the recorded incidence of a disease.[65] The influence of increased recognition of sepsis and less restrictive coding in some countries is unknown.[66] The considerable variability in sepsis incidence reporting brings forward the need for a more uniformly consistent reporting method.[55] The increasing use of electronic health

records may improve incidence data,[66-68] but again, there is some subjectivity dependent upon clinician data input.

According to the most recent data from the Centers for Disease Control and Prevention, 1.7 million American adults per year develop sepsis, resulting in 270,000 deaths each year.[60] An American study published in 2017 reported that 6% of 2.9 million adults admitted to hospital between 2009 to 2014 had clinical indicators of sepsis with a mortality of 15%, a discharge to hospice rate of 6%, and described the incidence of sepsis during this period as stable.[60] However, data from mid and lower-income countries is scarce.[66] A 2016 international meta-analysis examining sepsis studies reported an incidence rate of 288 sepsis cases per 100,000 person-years and 148 severe sepsis cases per 100,000 person-years, with mortality rates of 17% and 26%, respectively.[62] A global study of estimated sepsis incidence from the Global Burden of Diseases, Injuries, and Risk Factors Study reported 48.9 million cases and 11 million sepsis-related deaths in 2017.[58] Although accurate incidence numbers are complex to ascertain due to inherent discrepancies in data collection, the overall trends demonstrate an increasing incidence. These estimates underscore the importance of the WHO global health sepsis initiative. Improving incidence and epidemiology surveillance is of utmost importance and should be prioritized given its vast implications for prevention, treatment, and improving outcomes.

Biological sex is an important factor when discussing the incidence and outcomes of sepsis.[69-71] Many studies have concluded that sex affects multiple aspects of sepsis, from cellular signalling pathways to clinical recognition of symptoms, treatment, and outcomes.[72-74] One Australian study from 2022 showed that older men were at more risk

of sepsis-related hospital admission, ICU admission, hospital readmission within one year, and death than were older women (average age 62.7 years).[75] Yet, the inclusion of females in sepsis studies and treatment recommendations lags behind that of males.[76] By examining sex-specific aspects of sepsis, we may add great value to our understanding of sepsis pathophysiology.[76]

1.4 Socioeconomic Burden of Sepsis

Care of patients suffering from sepsis has alarming financial implications. Over $38 billion USD[77] is spent yearly managing septic inpatient hospital care (the single most expensive condition treated in US hospitals and the second most reason for hospital admissions). The cost continues to increase from $24 billion USD in 2013.[78] Authors of a 2016 British systematic review estimate that the mean hospital cost of sepsis per patient is $32,421.[79] A 2018 study evaluated cost based on the severity of sepsis and demonstrated that the cost of care increases ($16,324 to $38,298 USD) when comparing sepsis to septic shock.[80] In Canada, the daily cost (up to 28 days) of non-survivors may be higher than those who survive.[81]

Caring for family members in the ICU and afterward places a financial burden on families (in the form of paying for care facilities, absenteeism from work and school, and early retirement from work) and can result in caregiver anxiety, depression, posttraumatic stress disorder, complicated grief disorder, and burnout.[82-84] This constellation of symptoms in patients and families is termed 'post-intensive care syndrome.'[85] Once sepsis survivors are discharged from the hospital, their cost of care typically remains elevated for at least three years.[86] Healthcare cost for Canadian survivors of severe sepsis in the first

year post-discharge is $20,859 CAD and remains elevated at $7,099 CAD in the third year post-discharge.[86] Diabetes is the comorbidity most likely to cause an increased cost of care post-discharge.[86] Evaluating these studies together reveals that aggressive intensive care has improved mortality at the expense of increased numbers of debilitated survivors. These survivors suffer recurrent infections, malnutrition, organ system insufficiencies, and decreased quality of life.[87,88]

With the increasing incidence of sepsis, improvements in recognition and timely treatment of sepsis, and acute mortality decreasing overall (long-term fatality has remained similar), survivorship of sepsis has increased, especially in the elderly.[89,90] Unfortunately, much morbidity (poor quality of life, cognitive and functional disabilities[91-93]) is associated with surviving sepsis and influences the cost of care not only with the initial admission but for years to come for both the patient and caregivers.[90]

1.5 Mortality

Mortality from the sepsis syndromes is related to age, presence of comorbidities, and severity of sepsis.[90,94] Overall mortality estimates of sepsis of all severities range from 15 to 56%.[95] To gain perspective on mortality in sepsis, many clinical sepsis studies use a follow-up time of only 28 days[96,97] compared to cancer or cardiac disease studies that evaluate survival in years. Mortality has been documented to increase in patients presenting with SIRS (7%), to sepsis (16%), to severe sepsis (20%), and to septic shock (46%).[98,99] Mortality appears to be low (less than 24%) in young (less than 44 years old) patients with no comorbidities,[100] and despite the general increase in the incidence of sepsis

over time, mortality in many studies has declined.[57,95] Despite improvements in mortality, it is estimated that as of 2020, globally, 20% of annual all-cause deaths (11 million people) are due to sepsis[58] (increased from an estimated 5.3 million in 2016[62]). This is likely secondary to an increased prevalence of resistant organisms, an increased number of invasive interventions, a growing elderly population, and higher use of immunosuppressive treatments.[101-103] The estimated mortality rate for sepsis in North American ICUs is 34%[95] while the ARISE study from Australia and New Zealand reported a reduction in mortality from 35% to 18% from 2000 to 2012.[104] This reduction in mortality may be attributable to the overall improvements in ICU care, (lung-protective ventilation, early mobility, venous thromboembolism prophylaxis, stress ulcer prophylaxis, delirium management) inclusion of less severe cases with less risk of death secondary to less restrictive coding, earlier intervention, and more aggressive management strategies.[105-110]

Mortality may vary according to the infection source. Intra-abdominal infection, especially when associated with ischemic bowel, has the highest in-hospital mortality.[111,112] Patients with sepsis have more comorbidities than other hospitalized patients, the most common of which (depending on geographic area) are diabetes mellitus and chronic renal disease.[113] The presence of comorbidities confers a significantly higher risk of death in sepsis.[114]

Even once a patient who has survived sepsis is discharged from the hospital, they have an increased risk of mortality, re-developing sepsis, and re-hospitalization.[115] Dying post-sepsis most often occurs within the first six months after discharge, but the risk remains elevated out to one year post-discharge.[116-120] As stated above, those who do not

die from sepsis face a decreased quality of life, cognitive impairment,[121] and a high admission rate to long-term care facilities in the first year post-hospitalization.[117,119,120,122] There are few longer-term follow-up studies of sepsis (five or more years post-sepsis), but it is likely that sepsis also confers lasting physical and cognitive dysfunction that may precipitate future health complications.[115,123-125]

1.6 Risk Factors

Sepsis can affect anyone, but several risk factors exist that put a person at increased risk. Previous hospital stays, especially if the patient has received prior antibiotics, result in changes to the patient's microbiome, placing them at a three-fold higher risk of developing severe sepsis in the following 90 days.[126] Prior admission to an ICU can increase the risk of developing sepsis, potentially secondary to exposure to resistant microorganisms, broad-spectrum antibiotics, and invasive instrumentation.[127] Other prevalent risks include the presence of bacteremia,[6,7] immunosuppression,[7] obesity,[128,129] diabetes,[10] malignancy,[12] community-acquired pneumonia[130] (confers a 48% chance of progressing to severe sepsis and a 4.5% chance of developing septic shock[130]), genetic factors,[131,132] and the extremes of age.[133]

1.7 Prognostic Factors

Several factors impact the outcome of sepsis. The *host's response* to infection is important. Poor outcomes are associated with hypothermia (temperature less than 35ºC),[134] leukopenia (white blood cell count less than 4 x 10^9 cells/L),[135] the presence of comorbidities (atrial fibrillation,[136] liver disease,[137] alcohol dependence,[138] immune

suppression[139,140]), and hypocoagulability.[141] The *site of infection* makes a difference in survival. Ischemic gut confers the highest mortality risk (78%) compared to sepsis from a urinary tract infection (26%).[112] Although positive blood cultures are found in increasing numbers as the severity of sepsis increases, positive blood cultures do not appear to correlate with mortality.[141,142] *Nosocomial infections* causing sepsis are associated with higher mortality than community-acquired infections.[143,144] *Delaying appropriate antibiotic treatment* substantially increases mortality by up to 50%.[135,141] Four specific factors, *older age, ventilator use in ICU, low weight,* and *hemato-oncologic disease*, have been shown to predict long-term survival of septic patients upon presentation at an emergency department in 2019 in a single centre in the Republic of Korea.[145]

1.8 Pathophysiology

1.8.1 Inflammation

To fully understand the clinical sepsis syndromes, a basic understanding of the underlying pathophysiology of sepsis is necessary. The following sections will review processes that are particularly affected in the elderly. The host must recognize invading pathogens as dangerous to be targeted for phagocytosis and destruction. The body has a normal response to infection. Sepsis results when the response to an invading pathogen is beyond that which occurs in 'straightforward infections,' and in turn, leads to damage or dysfunction to the host. The transition from normal host response to infection into sepsis is based somewhat upon the balance of pro-inflammatory and anti-inflammatory mediators. Historically, it was assumed that morbidity and mortality from sepsis resulted

from overzealous inflammation proceeding unchecked, causing a generalized host-wide response rather than a targeted reaction to an infection. It was later thought by Bone et al.[146] that inflammation eventually gave way to a compensatory anti-inflammatory response (CARS) (Figure 1). It is now known that infection triggers a complex reaction involving both pro-inflammatory and anti-inflammatory mediators simultaneously[147] (with the anti-inflammatory CARS stage potentially lasting longer than the SIRS stage in some patients) and that the response of each host is specific to the load and virulence of the pathogen and the baseline state of health. Pro-inflammatory reactions are beneficial for eradicating invading microbes, but an overly exuberant response will result in collateral damage to healthy tissue. The anti-inflammatory response may dampen the initial pro-inflammatory response, thereby limiting collateral damage. However, the anti-inflammatory response may quell the protective aspects of the pro-inflammatory response and is implicated in the increased occurrence of secondary infections.[148,149] Although it is known that the elderly display a heightened and prolonged anti-inflammatory response to sepsis,[150] the reasons behind such a response are unknown. Therefore, the protracted anti-inflammatory response is rarely considered in animal models or in preventing or treating sepsis in this group. There exists evidence of immune suppression in sepsis, and this anergy has been demonstrated *in-vivo*.[151]

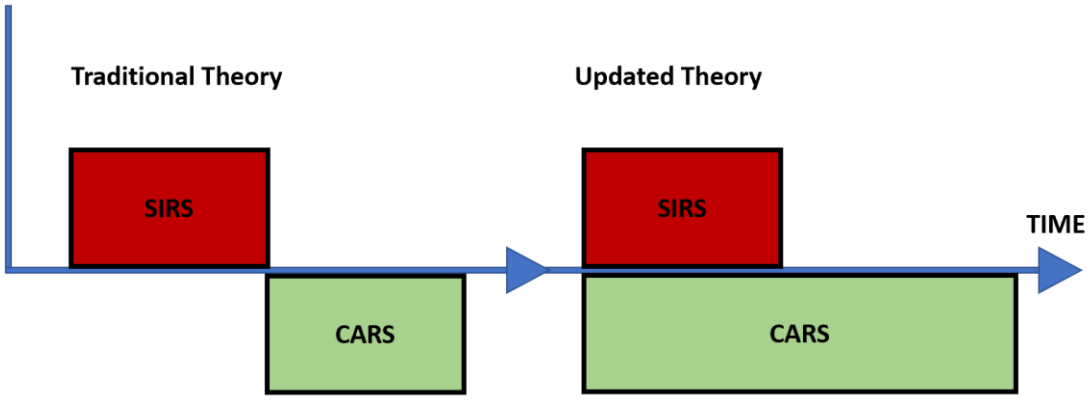


Figure 1-1: Pro-inflammatory and Anti-inflammatory Response in Sepsis Models

Traditionally the Systemic Inflammatory Response Syndrome (SIRS) was thought to occur separately from the Compensatory Anti-Inflammatory Response Syndrome (CARS). It is currently believed the two syndromes occur simultaneously.

1.8.2 Immune Function in Sepsis

Gram-positive organisms make up over half of sepsis-related infections, with gram-negative organisms,[152] fungi, and anaerobes contributing to the remaining infections. Whereas respiratory sources are the most common causes of infection in humans, intra-abdominal sepsis is the leading cause of death.[153] When pathogens encounter a normally sterile tissue field, antigens stimulate the initial mast cell release of histamine.[154] Invading pathogens must be recognized by the host as dangerous to stimulate histamine release and to be targeted for phagocytosis. Upon invasion of a pathogen, its pathogen-associated molecular patterns (PAMPs) are recognized by pattern recognition receptors (PRRs) of the sentinel cells (dendritic cells, mast cells).[155] PRRs include the toll-like receptors (TLR), nucleotide-binding oligomerization domain-like receptors (NOD-LRRs), C-type lectin receptors, and retinoic acid-inducible gene 1-like receptors expressed by the host's

innate immune system cells.[155] Recognition by these cells is a key initial pathologic step leading to the initiation of the clinical SIRS syndrome. The SIRS response can also be activated by damage-associated molecular patterns (DAMPs), which are endogenous and released under certain conditions of cellular stress, including ischemia, inflammation, and trauma, which may be related to the current septic state.[156] Bacteria can produce three types of exotoxins (PAMPs) that elicit an immune response.[157] Type I are superantigens and heat-stable enterotoxins.[158] These are surface-active toxins that damage the cell without entering it. Type II toxins destroy cell membranes and include hemolysins.[157] Type III toxins affect the internal cellular functions and include Shiga and cholera toxins.[157] Gram-negative bacteria are recognized by their lipopolysaccharide (LPS), also known as endotoxin. LPS attaches to LPS-binding protein (LPS-BP) in the circulation, and the resultant complex binds to the CD14 receptor present on macrophages, monocytes, and neutrophils.[159] CD14, through TLR-4 (TLR-4 is especially important in developing an innate immune inflammatory response for a gram-negative infection, while TLR-2 is important for generating an immune response to a gram-positive infection[160]) causes intracellular signalling that activates cytosolic NF-κB.[161] NF-κB then moves into the nucleus and induces gene transcription. This culminates in activating numerous pro-inflammatory cytokines, including tumour necrosis factor alpha (TNF-α) and interleukin-1 (IL-1), vascular cell adhesion molecule-1 (VCAM-1), chemokines including intercellular adhesion molecule-1 (ICAM-1), and nitric oxide (NO). Significant crossover between pathways exists (**Figure 1-2**).

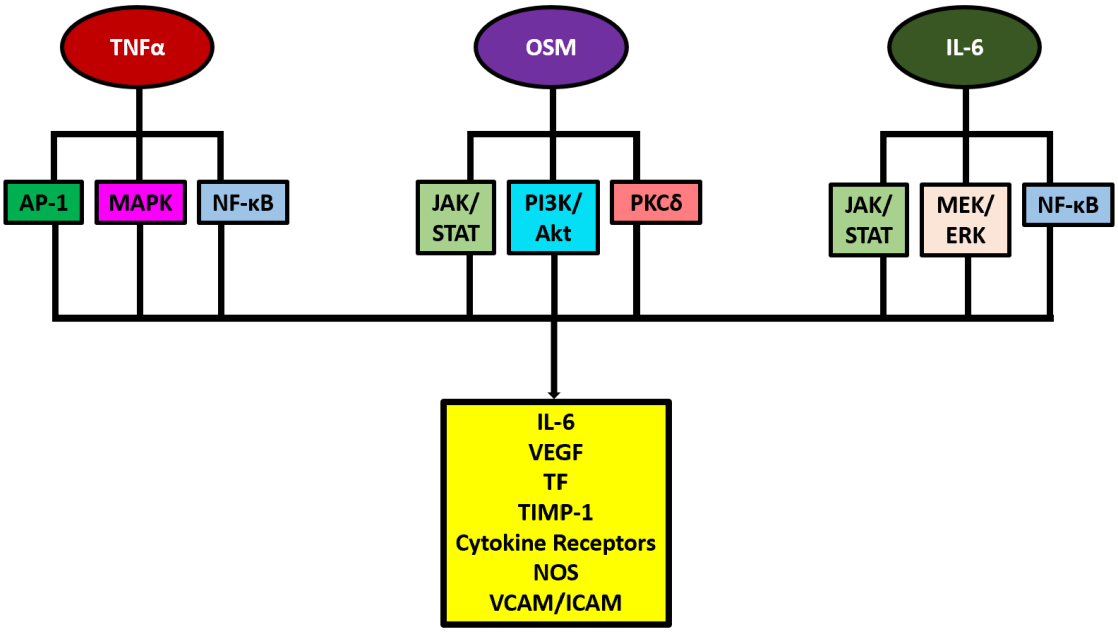


Figure 1-2: Cytokine Pathway Crossover

OSM: Activator protein-1; IL-6: interleukin-6; JAK/STAT: Janus-kinase/signal transducer and activator of transcription proteins; MAPK: mitogen-activated protein kinases; MEK/ERK: mitogen-activated protein kinase kinase/extracellular signal-regulated kinase; NF-κB: NOS: nitric oxide synthase; nuclear factor-kappa B; Oncostatin M; PI3K/Akt: phosphatidylinositol 3-kinase (PI3K)/protein kinase B; PKCδ: protein kinase C delta; TF: transcription factors; TIMP-1: issue inhibitor of metalloproteinases; VCAM/ICAM: vascular cell adhesion molecule/intercellular adhesion molecule; VEGF: Vascular endothelial growth factor

Pathogen recognition can also result in the production of bradykinins by converting kininogen to bradykinin by kallikrein.[162] Damage to the phospholipid membrane also stimulates phospholipase A2 (PLA2) to produce arachidonic acid.[163] Cyclooxygenase and lipoxygenase then convert arachidonic acid into prostaglandins and leukotrienes, respectively.[164] The release of bradykinin, prostaglandins, and leukotrienes results in the contraction of vascular endothelial cells coupled with the relaxation of vascular smooth muscle resulting in increased vascular permeability.[165] Histamines, leukotrienes, prostaglandins, and bradykinins also stimulate endothelial cell Weibel-

palade body release of p-selectins to facilitate polymorphonuclear leukocyte (neutrophil) and monocyte rolling and margination.[166] These neutrophils and monocytes then undergo ameboid diapedesis via interaction with PCAMS to enter the tissue. Histamines, leukotrienes, prostaglandins, and bradykinins also then provide a positive-chemotaxis signal directing the neutrophils and macrophages (monocytes that have left the circulation) to the site of infection.

Macrophages and neutrophils then attempt to remove invading bacteria through phagocytosis; the actin-myosin facilitated engulfment of pathogens.[167] The resultant phagosome of engulfed material fuses to a lysosome containing reactive oxygen species (ROS) or proteolytic enzymes. This degrades the invading pathogen.[167] The neutrophil can also undergo the process of NETosis, which is the expulsion of nuclear extracellular traps (NETs) made up of chromatin.[168] The histones then activate cathepsin-G to destroy the pathogen. Regardless of whether the macrophages and neutrophils can contain the infecting bacteria via phagocytosis, they stimulate a response from the immune system via the release of inflammatory mediators, including interleukin-1β (IL-1β), IL-6, and TNF-α. These inflammatory mediators then stimulate endothelial cells to recruit other innate immune phagocytic inflammatory cells (macrophages and neutrophils) along with adaptive immune response lymphocytes and induce the upregulation of adhesion molecules. More activated neutrophils and monocytes go through the process of rolling, adhesion, diapedesis, and chemotaxis to arrive at the site of infection. The neutrophils migrate into the vascular endothelium and adhere secondary to the increasing expression of adhesion molecules of the endothelium.

Neutrophils release mediators at the site of infection that are responsible for the clinical signs of infection (swelling, redness, pain, and warmth, via vasodilation and hyperemia) and the increase in vascular permeability which allows for the formation of protein-rich edema. Once released, the level of TNF-α increases through autocrine secretion (self-sustaining).[169] Non-TNF-α inflammatory mediators, including IL-1, IL-2, IL-6, IL-8, IL-10, and platelet-activating factor (PAF), participate in paracrine secretion, increasing the levels of other pro-inflammatory mediators.[169] Anti-inflammatory cytokines are simultaneously released, inhibiting TNF-α and IL-6 production. TNF-α is particularly important and is elevated to a higher degree in patients with septic shock than in shock states not related to infection.[170] When TNF-α is infused intravenously, it results in the same clinical picture as in patients suffering from septic shock.[171] Mice with anti-TNF-α antibodies were protected from dying via injection of lethal doses of TNF-α.[172] Some mediators (IL-6, IL-10) are very complicated, taking part in both pro-inflammatory and anti-inflammatory pathways, which enhance the development of cytotoxic T cells and B cell function. If the opposing mediator responses are balanced, infection will be overcome, and tissue will be repaired. If inflammation exceeds that which is necessary for containing an infection, pro-inflammatory mediators can spill into the circulation, resulting in sepsis. This process is even more complicated in elderly patients who are more likely to have pro-inflammatory comorbidities and receive medications with anti-inflammatory properties (discussed more in section 1.12). Oncostatin M (OSM) may be a useful inflammatory biomarker in sepsis. OSM belongs to the IL-6 class of cytokines,[173] released by many cell types active in sepsis,[174-176] has a receptor that is highly present on

the vascular endothelium,[176] and its function is related to the regulation of NO generation.[177-180] OSM level on the day of ICU admission has been shown to correlate with 28-day sepsis mortality and has a higher predictive value for mortality than the SOFA score.[173] High levels of OSM have also been associated with reductions in IL-6, IL-10, TNF-α, and IL-1β.[173] It has even been suggested that preoperative OSM levels can predict patients with a higher risk of post-operative infection following ventricular assist device insertion.[181] Systemic effects of cytokine released in sepsis can affect organ system function.[170]

1.9 Systemic Effects of Sepsis

Sepsis can impair the function of several organ systems as a result of tissue and cellular hypoxia (evident in autopsy studies).[182] This organ system dysfunction can be extensive and pervasive. Possible organ systems affected include the central nervous system, the cardiovascular system, the respiratory system, the gastrointestinal system, the renal system, and the coagulation system. These organ failure effects will be briefly discussed below. Though there are numerous biochemical mechanisms involved in septic organ dysfunction, it would be impossible to include a complete summation of all contributing mechanisms. Therefore, this review will focus on vascular mechanisms of organ failure and provide reference to other high-quality reviews pertaining to other biochemical mechanisms of sepsis-induced organ failure. As such, non-vascular aspects of sepsis will primarily be described in the context of what is known from a clinical perspective (rather than an in-depth description of all mechanisms known to date).

1.9.1 Systemic Effects of Sepsis: Cardiovascular System

Circulatory failure and hypotension with accompanying organ, tissue, and cell hypoperfusion are the consequences of septic shock. While dehydration, hormone irregularities, and septic cardiomyopathy are major components of this circulatory failure, vasoplegia can have a large impact. Much effort has been directed toward the diagnosis and management of septic vasoplegia.

Table 4: Cardiovascular Changes in Sepsis

A list of common cardiovascular changes seen in sepsis.

Cardiovascular Changes in Sepsis
Vasoplegia/Vascular endothelial dysfunction/increased permeability
Hypoperfusion
Microvascular stopped-flow
Septic myocardial dysfunction
Vasopressin deficiency
Relative adrenal insufficiency
Hypovolemia/dehydration

As previously discussed, sepsis is a clinical syndrome characterized by an intense systemic response in patients following infection. If unchecked, sepsis progresses to refractory hypotension, cardiovascular collapse, and multiple-organ dysfunction associated with vasoplegia. Vasoplegia is a condition characterized by low total peripheral systemic resistance and an unresponsive vasculature;[183] it is likely a critical

factor in septic shock progression, underlying irreversible hypotension and end-organ hypoperfusion. This can occur despite a compensatory increase in cardiac output and alongside fluid resuscitation with vasopressor treatment. Vasoplegia has been attributed to numerous factors acting in concert, including endothelial dysfunction/injury, the release of endothelial-derived vasodilatory and inflammatory factors,[184] and downregulation of vasopressor receptors.[185-187] Perturbed NO function secondary to ROS generation also greatly contributes to vasoplegia.[188,189] Multiple mediators of vascular tone under normal physiologic conditions can become greatly deranged in sepsis, contributing to not only vascular dysfunction but also promoting organ failure separate from their endothelial role.

Major mediators of vascular tone include the vasoconstrictors endothelin-1 (ET-1), thromboxane (TXA_2), and the renin-angiotensin-aldosterone system (RAS), and the vasodilators NO, prostaglandin I_2 (PGI_2), and endothelial-derived hyperpolarization factors (EDHF). There are three known endothelin isoforms, ET-1, ET-2, and ET-3, produced from prepropeptides by an endothelin converting enzyme. ET-1 is the most important concerning the cardiovascular system.[190,191] ET-1 secretion by vascular endothelial cells occurs in response to hypoxia,[192] presence of free radicals,[192] endotoxin,[193] angiotensin II (AT-II),[194] vasopressin,[195] low levels of shear stress,[196] cytokines (activated T-cells producing interferron-gamma (IFN-γ), and TNF-α[197]), thrombin,[192] and growth factors.[195] The release of ET-1 is inhibited by high shear stress,[198] NO,[192] PGI_2,[195] and atrial natriuretic peptide.[195] ET-1 has two receptors, the first of which is ETA, located on vascular smooth muscle cells. Secondly, the ETB, which are further

classified as ETB1 found on endothelial cells, and ETB2, which are also located on vascular smooth muscle cells. ETA G-protein coupled receptor stimulation results in increased inositol trisphosphate ($IP_{3)}$ levels. Effects of ET-1 through ETA include vasoconstriction and increased vascular permeability, as well as remodelling effects including hyperplasia, hypertrophy, and fibrosis.[199,200] ET-1 effects mediated by ETB1 include the release of vasodilatory PGI_2 and NO and inhibiting ET-1 production. ETB receptors present in the pulmonary system also can internalize and degrade approximately 50% of ET-1 from the circulation.[201] ET-1 plasma levels are increased in septic patients and may correlate to morbidity and mortality.[202] It has been well-shown that ET-1 can stimulate the production of ROS through actions mediated by both ETA and ETB,[203] potentially contributing to morbidity and mortality in sepsis.

Under normal circumstances, the RAS functions to maintain normotension. When blood pressure is reduced, as in sepsis, juxtaglomerular cells of the kidney release renin. Once in the liver, renin cleaves angiotensinogen to angiotensin-I, which then travels to the lungs and is converted to AT-II by the angiotensin-converting enzyme (ACE). AT-II has numerous effects on blood pressure control, including affecting the hypothalamus to result in the posterior pituitary release of antidiuretic hormone (ADH). ADH causes the insertion of aquaporin into the collecting duct of nephrons, allowing for water resorption, thereby increasing circulating blood volume and pressure.[204] AT-II also stimulates the zona glomerulosa of the adrenal cortex to release aldosterone, which increases distal collecting tubule resorption of water, also increasing circulating blood volume and blood pressure.[205] AT-II can directly cause peripheral arterial vasoconstriction by stimulating

the G-protein coupled AT1R, thereby increasing intracellular IP_3 and intracellular calcium. AT-II also results in glomerular efferent arteriole vasoconstriction and increased proximal convoluted tubule water reabsorption. All these effects work toward restoring normotension in settings of hypovolemia and hypotension.

TXA_2 is another potent vasoconstrictor produced by the metabolism of arachidonic acid by thromboxane synthase. In sepsis, TXA_2 levels are known to increase,[206] have been implicated in hepatic microcirculatory dysfunction,[207] functions as an important mediator of renal vasoconstriction in renal failure,[208] inhibits the expression of inducible nitric oxide synthase (iNOS),[209] and can augment platelet activation.[210] Altogether, TXA_2 effects in sepsis contribute to the development of multi-organ system failure.

Aside from vasoconstrictor dysfunction seen in sepsis, the roles of the vasodilators are vitally important. PGI_2 is derived from the metabolism of diacylglycerol or phospholipids.[211] Under normal health conditions, PGI_2 has a vasodilatory effect mediated by increased cyclic adenosine monophosphate (cAMP) and decreased ET-1.[212] The vasodilatory effect of PGI_2 has been shown to be compensatory when NO bioavailability is reduced,[213,214] as is the case in sepsis. However, it has also been shown that the endothelial dysfunction present in sepsis results in a reduced production of PGI_2 and increased production of TXA_2.[215] Reactive nitrogen species produced by sepsis have been demonstrated to block PGI_2 synthase by tyrosine nitration.[216,217] Though certain sepsis-induced inflammatory mediators may act to increase PGI_2 levels,[218,219] IL-6-mediated decreases in PGI_2 counteract these effects.[220] It has also been suggested that

during septic hepatic damage, synthesis of antithrombin is reduced and as such, the antithrombin-induced release of PGI_2 decreases.[221,222] It is evident that sepsis interferes with the vasodilatory effects and organ function effects of PGI_2. In aging, it is also evident that PGI_2 has vasoconstrictive actions by acting via smooth muscle thromboxane/endoperoxide receptors, thereby contributing to endothelial dysfunction.[223-225]

Another vital mediator of vasodilation is endothelial-derived hyperpolarization factor (EDHF). EDHF is a group of mediators whose actions result in vasodilation secondary to hyperpolarization of vascular smooth muscle cells and are distinct from NO and PGI_2.[226] EDHFs are different in different species as well as in different vascular beds from the same species. Thus far, EDHF can be categorized into electrical and chemical hyperpolarization signals. Electric hyperpolarization signals depend upon myoendothelial gap junctions for direct contact allowing for bidirectional signalling between the endothelium and vascular smooth muscle.[226] This permits the coordination of vasoconstriction and vasodilation to promote optimized blood flow.[226] Depending upon species, size, and location of the vessel, some chemical signals that have been identified include H_2O_2,[227] H_2S,[228] epoxyeicosatrienoic acids (EETs),[229] certain lipoxygenase metabolites,[230] and calcium-gated potassium channels (BKCa,[231] SKCa,[232] IKCa[232]). The influence of EDHF is greater in the smaller resistance vessels, whereas, in larger capacitance vessels, NO has been demonstrated to be of greater importance.[233,234]

The impact of sepsis on EDHF is complicated and involves mechanisms that are incompletely understood. Sepsis-induced ROS may result in oxidation and dysfunction

of certain EDHF pathways, such as oxidization of soluble guanylate cyclase, thereby impairing cyclic guanosine monophosphate (cGMP)/protein kinase G (PKG) signalling (or the oxidation of PKG itself[235]), and potentially decreasing cystathionine-γ-lyase-(CSE) induced H_2S formation.[236] However, many studies describe elevated levels of H_2S during sepsis as a consequence of iNOS-induced NO upregulation of CSE expression.[237,238] Higher levels of H_2S have been shown to be inversely correlated with blood pressure as well as correlating with higher ICU mortality.[239] In contrast, elevated H_2S has also been described as protective during sepsis.[240,241] Production of NO by iNOS stimulated by sepsis along with increased ROS generation may result in uncoupling of endothelial nitric oxide synthase (eNOS) and further production of ROS including H_2O_2. This may increase reliance on non-eNOS vasodilatory EDHF pathways, but also increase upregulation of eNOS expression to compensate.[242] NO produced by iNOS may also impair EDHF in certain vascular beds, but not others.[243] Increased bradykinin signalling may result in the increased metabolism of arachidonic acid to EETs.[244] Although not fully understood, the impact of sepsis on EDHF signalling and cross-talk with NO and PGI_2 pathways is likely to be a major contributor to septic vascular dysfunction.

NO is a vital mediator of vasodilation and is involved in a variety of other processes, including oxygen transport, maintaining microcirculatory integrity and tone, inhibiting platelet activation,[245] acting as a neurotransmitter (it is highly reactive and able to diffuse through membranes freely), and acting as a pro-oxidant (through its reactions to form peroxynitrite).[246] NO also modulates red blood cell[247] and white blood cell[248] deformation, which is essential for microcirculatory flow, regulates leukocyte adhesion

to the endothelium in the venules of the skeletal muscle and mesenteric system,[249,250] and importantly can enhance vascular permeability in vital organs such as the kidney, heart, and liver during sepsis.[251,252]

The formation of NO takes place in a multitude of cells such as macrophages, platelets, neutrophils, endothelial, parenchymal, and smooth muscle cells. There are three types of nitric oxide synthases (NOS) facilitating NO production. Type I, which is also known as a calcium-dependent neuronal NOS (nNOS), type II, also known as a calcium-independent iNOS (an important mediator in sepsis), and type III, which is also known as a calcium-dependent eNOS.

Soluble guanylate cyclase (sGC) is a heterodimeric enzyme consisting of an α-chain and a β-chain. For the purposes of this review, the sGC isoform discussed will be that of the α_1-chain/β_1-chain.[253] Once NO is bound, the prosthetic heme group detaches from the β_1-chain activating the unified catalytic domain to produce cGMP and pyrophosphate (PPi) from guanosine triphosphate (GTP). cGMP is then able to activate myosin light chain phosphatase, resulting in the removal of phosphate from the myosin light chain, thereby inhibiting myosin/actin interactions necessary for contraction. cGMP also activates PKG, including activation of CSE to produce H_2S as well as signalling through the Ras/Raf/MEK/Erk1/2 pathway to induce endothelial migration and proliferation, resulting in angiogenesis.[254]

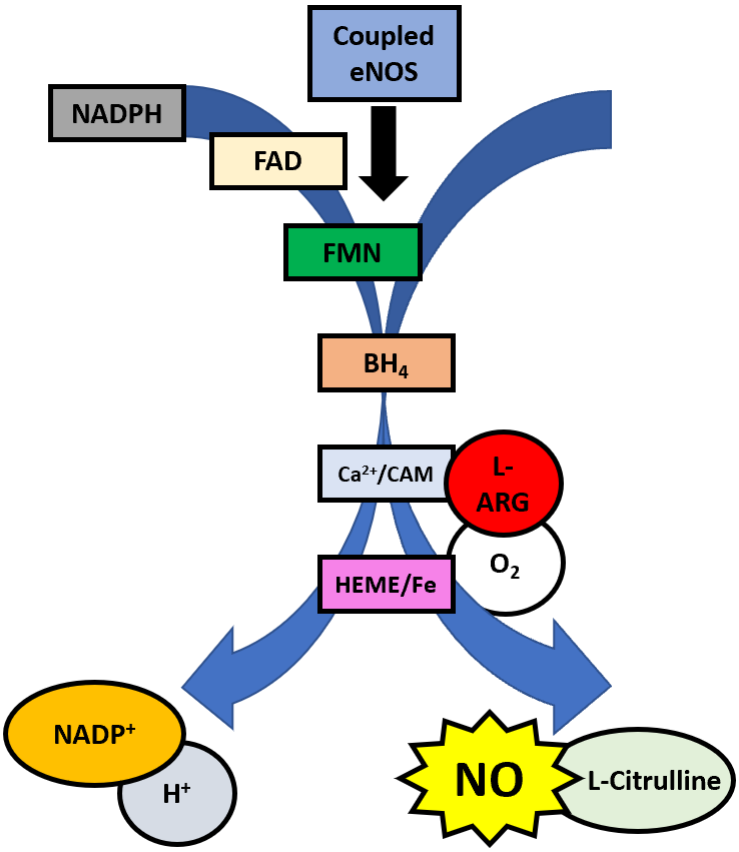


Figure 1-3: Functional Coupled eNOS Enzyme

In the presence of NADPH, FAD, FMN, Ca^{2+}-CaM, and BH4, L-arginine and O_2 can be converted to L-citrulline, NO, and H_20. (NADPH: reduced nicotinamide adenine dinucleotide; FAD: flavin adenine dinucleotide; FMN: flavin mononucleotide; CaM: calmodulin; BH4: tetrahydrobiopterin; NO: nitric oxide)

The elaborate NOS-NO-sGC-cGMP-PKG signalling system is interfered with at multiple levels during sepsis. The aforementioned LPS, TNF-α, and IL-1 produced during sepsis cause upregulation of iNOS, which in turn generates NO in quantities that are an order of magnitude higher than those generated by eNOS.[255] This has been demonstrated in various sepsis models in rodents, including direct injection of LPS and in a cecal ligation puncture model.[256-274] Elevated levels of NO result in the increased production of

ROS and reactive nitrogen species as NO can bind with superoxide anion to produce peroxynitrite. This is dangerous for the host as peroxynitrite can lead to lipid peroxidation and nitration with subsequent dysfunction of proteins.[275] Peroxynitrite is also capable of oxidizing tetrahydrobiopterin (BH4), reducing its ability to act as a co-factor for eNOS regulation of vascular tone.[276] The superoxide anion produced by sepsis can also be converted to H_2O_2 and hydroxyl radicals. This increase in oxidative stress can damage eNOS by oxidizing the ferrous heme of the oxidase domain to a ferric heme group. This produces uncoupling of the eNOS enzyme, and instead of producing NO, the uncoupled eNOS now produces superoxide anion.[277] The ferrous heme located on the β-chain of sGC also becomes oxidized and incapable of converting GTP to cGMP and PPi. Sepsis increases the amount of circulating neutrophils and neutrophils release arginase.[278] Arginase is capable of metabolizing L-arginine to ornithine and urea, thereby decreasing the amount of arginine available for use by eNOS.[278]

The overproduction of NO induced by iNOS stimulation during sepsis is therefore likely a critical factor in hypotension and septic shock. It seems, as such, that by inhibiting iNOS, refractory hypotension could be avoided. Moreover, because the progressive loss of NO signalling[279,280] secondary to the rise in ROS generation[281-285] and NOS uncoupling[286,287] are defining features of the aging and diseased vasculature, strategies to manipulate the NO system seem promising in aged patients.

Due to the involvement of NO in the cardiovascular decline associated with sepsis and septic shock, numerous studies have tested NOS inhibitors for treatment.[288] In 1990, Kilbourn et al.[265] demonstrated that non-specific inhibition of NOS reversed hypotension

in dogs with TNF-induced hypotension. Excited by the potential discovery of a treatment for septic shock, a flurry of animal studies followed[270,289,290] showing successful prevention and treatment of septic shock. A sheep model demonstrated that generalized and iNOS specific inhibition led to peripheral vasoconstriction that normalized systemic peripheral resistance and oxygen extraction.[269,271-273] Human clinical trials quickly followed.[264,266-269] Broccard et al. [264] demonstrated that using a non-specific NOS inhibitor increased mean arterial pressure allowing for the discontinuation of α-adrenergic support, along with decreasing cardiac output. However, despite initial success with phase I and phase II trials, a phase III non-specific NOS inhibitor trial was stopped early due to increased mortality from cardiovascular failure.[291] It is important to remember that normalizing number values (i.e., mean arterial pressure) in a severely physiologically deranged state counterintuitively may confer harm, as the deranged state may be the host's mechanism of surviving a condition that is inherently harmful (i.e., normalizing values may counteract adaptive responses). There exists a fine balance between vasoconstriction and vasodilation. Too much vasodilation will result in hypotension, tissue hypoperfusion, and organ failure, as above. Too much vasoconstriction will limit blood flow to tissues, resulting in tissue ischemia and organ failure.

In addition to the macrovasculature changes associated with sepsis, there are also important microvasculature changes. The microvasculature is comprised of three components; the *arterioles*, which feed red blood cells, often one at a time (to maximize the available surface area for oxygen-carbon dioxide exchange), into the *capillary network* to feed an organ and the *venules* that collect the red blood cells as they exit the

capillaries.[292] The alteration between constriction and dilation of the microvasculature with ultimately longer and longer periods of vasodilation eventually resulting in a state of complete vasodilation was described decades ago.[293,294] This vasodilation of both the macro and microvasculature can develop into a state of severe fluid volume depletion, especially in the setting of dehydration. This can result in the clinical situation of hypotension and hypoperfusion that occurs in sepsis and septic shock. On the contrary, when the vasodilated septic host enters a hyperdynamic state following fluid resuscitation, impaired oxygen delivery and carbon dioxide removal are seen following progressive arteriolar vasoconstriction in the small intestine.[295] Other organs and tissues (such as the diaphragm) display a combined response in which larger diameter vessels constrict and smaller diameter vessels dilate.[296] The endothelial lining of the microcirculation also plays a vital role through signal transduction of stress related to dilation or constriction. The overall importance of the integrated microcirculation is to maintain a balance between oxygen delivery and adequate carbon dioxide removal.

Another important concept concerning the septic microvasculature is that of stopped-flow[297] resulting in ischemia. Significant stopped-flow has been demonstrated in the liver, skeletal muscle,[298] diaphragm, intestinal villi, and the sublingual area.[299] This loss of capillary density causes hypoperfusion, as shown in liver sinusoids.[299] The exact mechanism underlying stopped-flow in sepsis is still unknown but is believed to be secondary to a multitude of factors. The activation of the inflammatory cascade and the coagulation cascade[300] likely play a large role in allowing for red blood cell deformation, adhesion, and aggregation, along with white blood cell deformation, and increased

adhesion.[301] Also seen are increased fibrin deposition and endothelial swelling, both of which can potentially occlude vessels and decrease perfusion. It appears sepsis with hypotension causes similar stopped-flow compared to non-septic capillaries experiencing a similar degree of hypotension.[302] In chronic sepsis models, capillary flow reversal in skeletal muscle has been demonstrated, likely impacting tissue perfusion.[303]

Septic patients often receive fluid resuscitation to restore perfusion to both the macro and microvasculature.[304] Despite the expanded fluid volume, a maldistribution of flow exists with an ongoing mismatch of blood supply and demand. Capillaries displaying stopped-flow with vasoconstricted arterioles do not actually receive the provided extra fluid. This extra fluid is supplied to the normal vasculature and over-perfuses these areas resulting in deleterious edema. Therefore capillaries that are stressed and supplying areas of high oxygen extraction are continually underfed.[305] In some tissue and organs, including the heart, gut, and liver, there are weak microvascular units that get bypassed altogether in sepsis.[299] Overall, these studies demonstrate that sepsis causes an inability to regulate oxygen supply and demand, especially in the microcirculation, resulting in organ dysfunction that may be severe enough to threaten life. It is unknown at what stage of septic circulatory dysfunction hypoperfusion-induced damage is occurring and how it affects morbidity and mortality. Tissue hypoperfusion culminates into *tissue ischemia*. In addition to increased oxygen demand coupled with poor oxygen delivery (ischemia), metabolic processing of whatever oxygen is available is limited due to *mitochondrial dysfunction*,[306] as the increased oxidative stress of sepsis leads to cytotoxicity. Studies have shown mitochondrial protein and membrane damage

functionally impair the electron transport chain, resulting in cytotoxicity following exposure to TNF-α and NO. [307,308] Other studies have demonstrated supranormal oxygen concentrations in suffering organs affected by sepsis, suggesting that mitochondrial oxygen use is impaired (cytopathic hypoxia) and that further increasing oxygen delivery is likely superfluous if the organ cannot make use of it.[309,310] In 28 ICU patients afflicted with septic shock, skeletal muscle biopsy showed lower levels of adenosine triphosphate (ATP) concentrations (demonstrating bioenergetic failure) in those who died from sepsis (P=0.0003) compared to sepsis survivors.[311] Mitochondrial perturbations may also be responsible for severe organ dysfunction without actual cellular demise.[312] However, cell death secondary to *apoptosis* is also greatly affected by sepsis. Sepsis can delay apoptosis of innate immune system cells such as macrophages, resulting in a prolonged inflammatory response, which can lead to the development of organ dysfunction. Sepsis also decreases the clearance of pathogens by inducing apoptosis of lymphocytes. Inhibiting apoptosis can protect a host from mortality and organ dysfunction,[313,314] but septic-induced apoptosis has also resulted in organ failure affecting the kidneys, heart, lung, liver, and brain.[315]

Clinically, tissue hypoxia can be manifested in a variety of ways. For example, hypoxia may present symptomatically with mottling of the extremities,[316] demonstrating poor oxygen delivery or even as confusion/altered level of consciousness.[317] Laboratory measurements may include a low $ScvO_2$ (indicating increased oxygen usage at the tissue level)[318] as well as supranormal $ScvO_2$ (indicating tissue inability to utilize the oxygen that is present).[309] As $ScvO_2$ requires central venous access, serum lactate is commonly used as

it requires only peripheral venous/arterial access. A high lactate (generally described as greater than 4 mmol/L in several studies[319,320]) may indicate increased reliance on anaerobic metabolism. It can be secondary to macro or microcirculatory dysfunction causing hypoperfusion or mitochondrial dysfunction.[321] It is important to note that not all increases in lactate are due to tissue hypoxia. As the liver metabolizes most lactate, liver dysfunction can result in elevated lactate levels. Also, some medications can be associated with elevated lactate levels (exogenous epinephrine stimulates the release of skeletal muscle lactate to be used for oxidation or gluconeogenesis,[322,323] toxic levels of metformin[324]) as well as increased pyruvate production.[325]

Aside from the aforementioned changes in the macro and microcirculation, other changes in sepsis affect the hemodynamic function of the patient. The circulating depressant factors/cytokines also decrease β-adrenergic receptor density and affinity.[326,327] Cardiac injury is evidenced by increases in troponin levels.[328] Hypotension in sepsis may also result from the combination of reduced vascular tone combined with the redistribution of intravascular fluid secondary to increased endothelial permeability.[329] Impaired vasopressin secretion is another factor involved in the development of vasodilation in sepsis. Plasma vasopressin levels in patients with septic shock are lower than in patients with cardiogenic shock despite similar blood pressures.[330,331] The addition of vasopressin therapy in septic patients also improves blood pressure and allows for lower doses of other vasopressors.[331] In the VASST trial[332] published in 2008, vasopressin treatment in addition to low dose norepinephrine (NE) was

comparable to using high-dose NE. In this trial, vasopressin also demonstrated some mortality benefit in less severely ill patients.

The SEPSISPAM study published in 2014 by Asfar et al.[333] examined a clinically important hemodynamic question of whether a higher mean arterial pressure would be beneficial in septic patients, especially those with a history of hypertension. Increasing the mean arterial pressure target to 75 mmHg in septic shock (via the increased use of vasopressors) as compared to the accepted standard of 65 mmHg did not decrease mortality (P=0.57) and did increase the occurrence of atrial fibrillation (P=0.02; likely secondary to the β_1-adrenergic receptor effects of the vasopressor use). The higher mean arterial pressure target did decrease the need for renal replacement therapy (P=0.046) in hypertensive patients but did not decrease mortality. This finding of reduced renal replacement therapy in hypertensive patients is likely secondary to the chronic rightward shift of their pressure autoregulation curve whereby a mean arterial pressure of 65 mmHg is relatively hypotensive.

1.9.2 Systemic Effects of Sepsis

In addition to the cardiovascular effects described in the preceding section, sepsis pathophysiology impacts multiple other organ systems. The central nervous system experiences several sepsis-related effects. Septic encephalopathy can be defined as "a diffuse cerebral dysfunction as a consequence of the systemic inflammatory response to an infection, with no direct central nervous system infection."[317] It is manifested as delirium or an altered level of consciousness. It is attributed to several pathophysiologic processes, including vagal nerve stimulation by peripheral cytokines altering cytokine synthesis

(known as the inflammatory reflex),[334] altered cell signalling resulting in enhanced production of cytokines and excitatory or inhibitory receptors in the brain and brainstem (known as neuroinflammation[335]), injury of the blood-brain-barrier allowing increased leukocyte entrance, direct TNF-α induced damage,[336] and mitochondrial dysfunction with reduction in oxidative phosphorylation ultimately leading to apoptosis.[337] Inflammation is also known to increase the blood-brain barrier's microvascular permeability, resulting in the inflammation of brain tissue.[338-340] Microthrombi formation coupled with endothelial dysfunction also results in microinfarcts of the brain tissue adding to brain dysfunction.[338] An altered level of consciousness is present in 23% of septic patients. It is unfortunately associated with a 49% mortality compared to only 26% of patients who do not present with neurologic findings.[341]

In the pulmonary system, lung endothelial injury results in pulmonary edema, ventilation-perfusion mismatch, and, most importantly, the development of acute respiratory distress syndrome (ARDS) – a life-threatening condition that may lead to the inability to ventilate or oxygenate a patient.[342] Alveolar capillaries and epithelial cells, as well as capillary endothelial cells, are injured by inflammatory cells resulting in diffuse alveolar damage. The outcome is increased vascular permeability and the development of pulmonary edema.[343] As alveoli fill with exudate,[344] hyaline membrane begins to form, thereby restricting oxygenation and carbon dioxide ventilation.[345]

Blood supply to the bowel may be impaired, resulting in bowel wall edema, increased permeability, and translocation of gut bacteria into the bloodstream. The combined microorganisms of the gastrointestinal tract, also known as the microbiome, also

play a vital role in sepsis. This microbiome has a diverse bacterial composition maintained in part by pro-inflammatory and anti-inflammatory signals.[346] When sepsis occurs, the signal is imbalanced and maladaptive bacterial survival is selected. This microbiome can also be interfered with by lack of enteral feeding and use of medications, including antibiotics and proton pump inhibitors that are commonly administered in septic patients.[346]

Acute kidney injury (AKI) is an ominous sign in septic patients and is well known to be associated with increased mortality.[347] AKI may be due to hypotension and hypoxemia producing acute tubular necrosis. However, renal vascular vasoconstriction and direct injury from cytokines are other possible causes of renal dysfunction. AKI may be severe enough to result in anuria, causing severe electrolyte and acid-base anomalies. This situation may be treated with the application of renal replacement therapies. There was interest in not only affecting the acid-base, electrolyte, and fluid status of a patient but also using renal replacement therapies membranes capable of removing toxic substances; however, the EUPHRATES trial has not shown benefit with this technique.[348] AKI may be manifested clinically as the development of oliguria, anuria, pulmonary edema, and peripheral edema.[347] Laboratory workup may reveal electrolyte abnormalities (hyperkalemia, hyponatremia), acidosis, and elevated creatinine.[347,349]

Sepsis can lead to the development of all elements of Virchow's triad (injury to the endothelium, abnormal blood flow, and hypercoagulability), leading to the development of a prothrombotic state (Figure 3). The acquired coagulopathy can range from mild decreases in platelet number and mild elevations in bleeding time to the

extreme with fulminant disseminated intravascular coagulation (DIC). Several investigations have demonstrated that DIC is an independent predictor of mortality and that as the severity of DIC increases, so does mortality.[350,351] As mentioned above, setting off the inflammatory cascade leads to the development of coagulopathy via the release of pro-inflammatory cytokines, including IL-1, IL-6, IL-12, and TNF-α, damaging the vascular endothelium. This ultimately decreases antithrombin levels (an inhibitor of thrombin) via decreased hepatic production, increased consumption by thrombin-antithrombin complexes, and neutrophil elastase degradation.

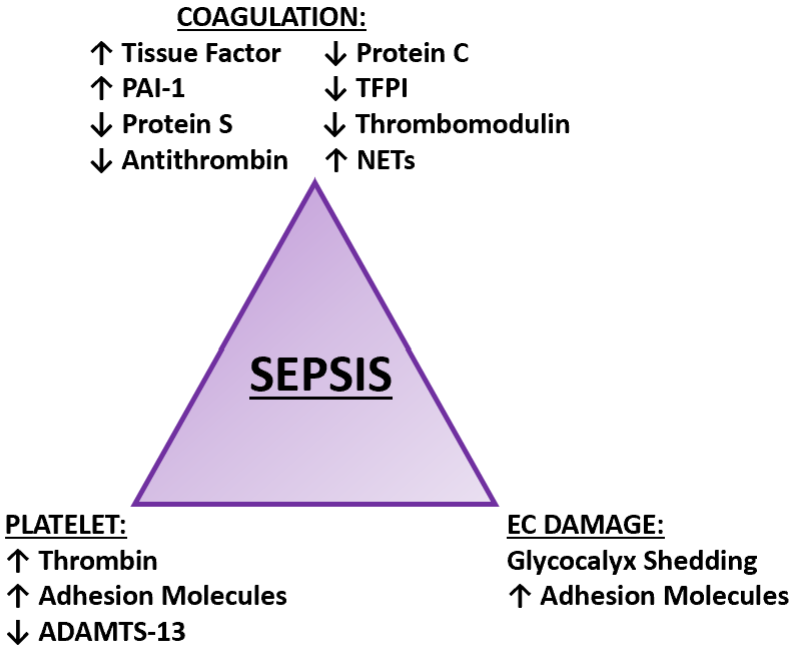


Figure 1-4: Virchow's Triad

Three main factors predispose to thrombosis. (ADAMTS-13: ADAM Metallopeptidase with Thrombospondin Type 1 Motif 13; EC: endothelial cell; NETs: neutrophil extracellular traps; PAI-1: plasminogen activator inhibitor-1; TFPI: tissue factor pathway inhibitor)

1.10 Treatment

Treatment regimens over the years have been introduced based on modifying the pathophysiologic development of sepsis. Unfortunately, investigational treatments have been unsuccessful and limited by our incomplete understanding of the pathophysiology of sepsis.[15] A limitation in preclinical and clinical research includes the tendency to classify all sepsis syndromes (perforated gut, pneumonia, urinary tract infections) as the same entity.[15] We have also limited our successes by considering and treating all patient populations (regardless of age, sex, comorbidities, environmental factors, and genetic factors) the same.[15] Treatments have failed as sepsis is an incredibly complex syndrome with multiple simultaneously involved pathways fueling mortality, rather than a singularly targeted mediator. Therefore, our current treatment armamentarium consists largely of general supportive measures with no currently approved specifically targeted pharmacologic therapies (see **Table 4**).[42] Pharmacologic treatment may have increased success if the deleterious progression to hemodynamic collapse is prevented while protecting vasodilation to infected tissues (allowing for increased delivery of sepsis mediators only where they are necessary). We also may need to personalize treatment strategies based on infection and host factors.[352,353] It is important to note, there are no age-specific guidelines for sepsis treatment.[42]

Table 5: Current Sepsis Treatments

Current sepsis treatment as per the 2021 Surviving Sepsis Guidelines.

Current Sepsis Treatments (SSC 2021)[354]	
Screening and Initial Resuscitation	• Hospitals should use a performance improvement program for sepsis • Do not use qSOFA as a sole screening tool • 30 mL/kg IV crystalloid • Resuscitation be guided by dynamic measures (serum lactate, capillary refill) • If need ICU, admit to ICU within 6 hours
Infection	• Appropriate antimicrobials as soon as possible (if sepsis highly probable, within 1 hour)
Hemodynamics	• Use of balanced crystalloids over normal saline • Norepinephrine as 1st-line vasopressor • May start vasopressor peripherally to avoid delay of starting central venous access
Ventilation	• Low-tidal volume/limited plateau pressure • Prone positioning as necessary • Recruitment maneuvers
Additional Therapies	• Consider steroid use • Do not use vitamin C
Goals of Care and Long-term Outcomes	• Screen for economic and social supports • Involve families in shared decision making • Medication reconciliation at ICU admission and hospital discharge • Critical Care transition program

Management of the septic patient mandates early recognition and treatment with priority placed on the support of the airway (potentially with intubation and lung-protective ventilation), ensuring adequate breathing (administering supplemental oxygen), and supporting the circulation (fluids, vasopressors, inotropes).[42] Administration of an appropriate spectrum of antibiotics early and the administration of fluid resuscitation are vitally important.[355,356]

Appropriate and timely use of antibiotics is essential. Numerous animal trials support the use of antibiotics in sepsis.[357] The Surviving Sepsis Campaign guidelines recommend that antibiotics be administered as early as possible[358] and preferably within the first hour of sepsis management. This goal is often not met in the aged due to atypical presentation and delayed sepsis recognition. It is beneficial to obtain cultures of potentially infected sources (blood, urine, sputum, cerebrospinal fluid, abscess aspirate) to correctly identify the pathogen prior to initiating antibiotic treatment.[359] However, obtaining cultures should not delay the administration of antibiotics. In 2006, Kumar et al. published a paper outlining the vital importance of early and correct antibiotic administration.[355] The overall mortality in this trial was 56.2%. This retrospective cohort of 2,731 Canadian and American patients from 1989 to 2004, showed that antibiotics were only administered a mean of 13.5 hours after the development of sepsis and that every hour delaying the administration of antibiotics resulted in an astounding 7.6% increase in mortality. Only a small percentage of patients received antibiotic treatment within the first hour (14.5%).[30] The relationship between the delay of antibiotic administration and mortality was maintained regardless of the source of infection, site of infection, and type of infection

(gram-positive, gram-negative, fungal).[355] It has also been shown that delays in first-dose administration, as well as second-dose administration, increase mortality.[360] Possible reasons for delay in the administration of antibiotics include failure to recognize infection as a potential cause of shock, waiting for the procurement of all cultures, failure to order antibiotics as an immediate/stat order, failure to administer antibiotics prior to the transfer of the patient to an ICU, no written order is present, or the written order is unclear.[361-365] Importantly, failure to recognize a host has risk factors for resistant organisms prevents appropriate spectrum antibiotic administration in a timely fashion and can be as harmful as not providing antibiotics at all.[366]

Inappropriate therapy is as harmful as providing no antibiotic coverage at all. A high index of suspicion is necessary when treating patients from long-term care facilities, nursing homes, diabetics, hemodialysis patients, or those with comorbidities associated with compromised immunity.[367-369] Appropriate treatment is often delayed in patients with fungemia. Garey et al.[370] demonstrated that delaying antifungal treatment until day three or four resulted in a 41% mortality rate compared to a mortality rate of 15% if antifungal therapy was given on day one. An important risk factor for clinicians to be aware of in the early course of management is the use of antibiotics in the preceding three months.[371] This specific risk factor is associated with increased mortality and increased occurrence of gram-negative septic shock.[372,373]

Using an appropriate time course of antibiotic treatment is essential for decreasing patient exposure and the risk of developing resistant and difficult to treat infections that may result in sepsis. In 2003, Chastre et al. showed that in patients with ventilator-

associated pneumonia, eight days of appropriate spectrum antibiotic was non-inferior in terms of mortality and pneumonia recurrence to a 15-day course in patients with recent antibiotic exposure.[374] However, in patients with certain non-fermenting gram-negative infections, a 15-day course may be more appropriate for preventing recurrence. The STOP-IT trial[375] published in 2015, showed that in patients with intra-abdominal infections who had undergone appropriate surgical source-controlling operations, four days of antibiotics had similar outcomes compared to those who received eight days of antibiotics.

Numerous clinical trials have also tested the efficacy of vasopressor treatment. In 2017 use of AT-II for the treatment of vasodilatory shock associated with sepsis was investigated the ATHOS-3 investigators.[376] This trial included 344 patients requiring vasopressor support for shock (almost all secondary to sepsis) and randomized them to treatment with angiotensin-II or placebo in addition to conventional treatment. Their primary endpoint was the improvement in mean arterial pressure by 10 mmHg or more or a mean arterial pressure exceeding 75 mmHg without requiring an increase in vasopressor, which was higher in the AT-II group than in the placebo group (69.9% versus 23.4%; $P<0.001$). Patients in the AT-II treatment group had higher heart rates than placebo patients. This study was not powered to examine mortality or length of stay. No clinical outcome differences were addressed. Several trials have also examined which is the best vasopressor to use to manage septic shock. VASST[332] examined whether adding vasopressin to patients already receiving NE at a dose of at least 5 μg/min versus additional NE would improve 28-day mortality. They found no mortality difference (additional NE 35.4% versus vasopressin 39.3%; $P=0.26$) but reported that patients on

low-dose NE 5-14 μg/min trended toward lower mortality than those with escalating doses of NE. It is important to note that this was an underpowered study with an expected mortality rate of 60%. The VANISH[377] trial in 2016 demonstrated a similar performance of vasopressin and NE for septic shock management. Together, these trials have led to the establishment of guidelines that recommend NE as the first-line vasopressor of choice.[358] Second-line recommended vasopressor choices are epinephrine and dopamine.[358] The addition of vasopressin should be considered when the mean arterial pressure is less than 65 mmHg despite NE treatment.[358] Terlipressin should be avoided.[358]

Another important tenant of care for the septic patient is early source control. The Surviving Sepsis Campaign guidelines recommend that source control of infection (elimination of infective source via drainage, debridement, removal of infected device/hardware, decompression, restoration of function) occur as early as possible and within six to twelve hours of presentation.[358] If an infection is the likely cause of the shock state and there is no obvious source, computed tomography is recommended to detect the source.[378,379] Surgical control of the infection is recommended early; however, some cases (infected pancreatic necrosis) may be best managed with minimal and delayed intervention.[380]

1.11 Clinical Failures of Successful Experimental Therapies for Sepsis

Following the experimentation of sepsis treatments in several animal models, many modalities have been tried in humans with poor success rates (see **Table 5** for a summary of ineffective sepsis therapies). One of the most well-known ineffective therapies is activated protein C (APC). The initial PROWESS trial[274] from Bernard et al. in 2001

demonstrated an improvement in septic shock 28-day mortality. However, multiple subsequent studies, including the PROWESS-SHOCK study[381] from Ranieri et al. showed no improvement in 28-day or 90-day mortality and led to the withdrawal of the drug in 2011.

Numerous trials failed to show benefit with high-dose steroids, but 2018 saw the publication of two large low-dose steroid-use in sepsis trials (ADRENAL, APROCCHSS), which demonstrated some benefits compared to high-dose steroid trials. The ADRENAL study by Venkatesh et al.[382] showed that 90-day mortality was unchanged (hydrocortisone 27.9% versus placebo 28.8%; P=0.5). Interestingly, this study did demonstrate a decreased duration of shock (3 versus 4 days; P<0.001), reduced duration of the first episode of mechanical ventilation (6 versus 7 days, P<0.001), and decreased initial ICU visit length of stay (10 versus 12 days; P<0.001). APROCCHSS,[383] published by Annane et al., demonstrated the use of intermittent hydrocortisone reduced 90-day mortality compared to placebo (43% versus 49.1%; P=0.03) and decreased amount of days vasopressor was in use (17 versus 15 days; P<0.001). It is difficult to determine why these two recent studies had different outcomes. Notably, both studies did show vasopressor-sparing benefit with little adverse effect. Current guidelines[358] recommend using low-dose steroid treatment with a patient in vasopressor-dependent shock.

Intensive insulin therapy, made popular by the 2002 van den Berghe et al.[384] trial, demonstrated that surgical ICU patients had improved mortality, reduced rates of renal impairment, and decreased bloodstream infections when their blood glucose was tightly maintained at 4.4-6.1 mmol/L. However, in 2009, the NICE-SUGAR[385] study was

published, demonstrating that intensive insulin therapy in critically ill patients (including those with sepsis) increased 90-day mortality. The COIITSS[386] trial in 2010 confirmed the finding that intensive insulin therapy did not improve mortality.

Colloid fluid resuscitation has been popular. As increasing edema is correlated with increased mortality, this seemed like an attractive fluid to use in septic patients.[387] However, several studies have demonstrated harm associated with colloid use. The VISEP[388] trial from 2008 showed hydroxyethyl starch (HES) for treatment in severe sepsis or septic shock led to renal impairment development. The CHEST[389] trial from 2012 showed that colloid increased the risk of renal failure. The 6S[390] trial published by Perner et al. in 2012 showed HES increased 90-day mortality, increased need for renal replacement therapy, and increased need for blood products.

Several other treatments for sepsis have been investigated. HA-1A was a human monoclonal antibody against LPS for the possible treatment of gram-negative sepsis. It had shown promise in reducing mortality in animal gram-negative sepsis models. It was marketed for sepsis treatment in 1992 to1993 following a demonstration of safety in a randomized, placebo-control trial involving 543 patients.[391] It was withdrawn[391] after a second randomized control trial (CHESS group[392]) failed to show mortality benefit. E5 was a murine monoclonal antibody against endotoxin that also had promising early results. However, a study published by Angus et al.[393] in 2000 demonstrated no survival benefit. Talactoferrin-α was an orally administered dendritic cell-mediated immunotherapy that had promising animal and phase I data demonstrating improved mortality, but a phase II/III[394] trial was stopped early secondary to increased mortality.

Clinical trials also failed to demonstrate the benefit of blocking the proinflammatory cytokines TNF-α[395,396] and IL-1.[397] Other mediator-directed treatments that failed to show benefit included blocking G-CSF and IFNγ.[398]

Inhibition of the NO pathway seemed attractive for stopping the hypotensive responses seen in septic shock. Early studies of a competitive non-selective NOS inhibitor (L-NMMA) were again promising in animals and phase I and II trials. However, a phase III trial[291] was stopped early due to increased mortality in the treatment groups. There continues to be interest surrounding NOS inhibition, specifically selective iNOS inhibition, which has shown benefit in several animal studies.[399]

Several animal studies[400] and human retrospective studies[401] showed promise that statins could modulate the pro-inflammatory state seen in sepsis and improve mortality. A meta-analysis of seven trials and 1,720 patients was published by Deshpande et al.[402] in 2015. The meta-analysis showed no improvement in in-hospital mortality or 28-day survival. Chen et al.'s meta-analysis in 2018 confirmed no treatment effect.[403]

Table 6: Ineffective Sepsis Therapies

Ineffective Sepsis Therapies	
Activated protein C	G-CSF/GM-CSF
High-dose steroid	INF-γ
Intensive insulin therapy	TNF-α antagonist
Colloid administration	IL-1 antagonist
HA-1A	L-NMMA
Talactoferrin-α	Statins
IVIg	TLR4 antagonist
Anti-Enterobacteriaceae common antigen monoclonal antibody	Anti-tumor necrosis factor monoclonal antibody
Antithrombin	Recombinant human tissue factor pathway inhibitor
N-acetylcysteine	Bradykinin antagonist
Levosimendan	Hypothermia
Polymyxin B hemoperfusion	Recombinant human soluble thrombomodulin
Selenium	High-dose vitamin D
High-dose vitamin C	Pentoxifylline
Naloxone	E5

1.12 Sepsis and Aging

Over time, healthcare interventions have led to steady growth in the elderly population worldwide. Consequently, more elderly people than ever are developing sepsis and requiring hospital and ICU level of care, leading to ever-increasing healthcare expenditure.[404,405] The processes of sepsis and aging have several aspects in common,

which lend to the elderly being at a particular disadvantage for developing sepsis (not just due to their growing population), progressing to septic shock, dying from sepsis, or surviving with poorer quality of life than the young.[16] Also, processes involved with aging can mask the symptoms and signs of sepsis, delay treatment, and render common treatment modalities less effective. Unfortunately, the elderly are often not included in preclinical or clinical sepsis trials, despite being disproportionately affected by sepsis.[406] Here, this review will provide a discussion focused on the impact of advanced age in sepsis.

1.12.1 Terminology and Aging

With the 2016 definitions of sepsis, there was also increased recognition that host factors, including age, added heterogeneity and complexity to our understanding of sepsis. It was acknowledged that further studies (transcriptomic, metabolomic, proteomic)[407,408] will lead to improved characterization of specific population subsets.[1] However, there currently exists no specific terminology for use in an elderly patient with sepsis. Also lacking is consensus regarding the terminology surrounding the definition of what defines an elderly person and how to differentiate younger elderly from the "oldest old."[409] As chronologic age can differ from biologic age, it has been suggested the terminology "healthspan" rather than "lifespan" be used.[410]

1.12.2 Epidemiology and Aging

It is increasingly evident when examining the incidence of sepsis that the age of patients should be taken into consideration. The American population aged 65 and older was

approximately 46 million in 2015.[411] It has been estimated that this number will double to over 98 million by 2060 (an increase from 15% to nearly 24%).[412] In Europe, 25% of people are over the age of 60 (expected to reach 35% by 2050, with the number of people over the age of 80 expected to triple).[412] In the Caribbean and Latin America, 12% of people were over the age of 60 in 2017, and this is expected to increase to 25% by 2050.[412] The incidence of sepsis is higher in the elderly and increases further in those aged 85 and above.[413,414]

A 2014 review reported the mean age of septic patients admitted to an ICU to be 69.[415] As patients over the age of 60 make up over 60% of all sepsis hospitalizations,[416] and the age of septic patients continues to rise,[412,417] this population is poised to place enormous and expanding strain on the healthcare system. The incidence of sepsis is also increasing faster in those aged 65 years or older (20.4% faster[418]) than those aged less than 65 years, and sepsis is the most common reason the elderly are admitted to ICUs.[419] Despite the preponderance of aged septic patients, it is estimated that **less than 1%** of all sepsis studies are performed in aged animals,[420] or include older adults in clinical studies.[421] Failing to include elderly people in research may partially explain the difficulty in improving septic mortality and functional outcomes[381,422] – we are not studying a group of people greatly affected by sepsis. A better understanding of the elderly patient's physiology and how they are affected by sepsis would be of immense value.

1.12.3 Socioeconomic Burden and Aging

The cost of caring for an elderly patient during and after a septic episode can be debilitating. A 2017 Canadian study showed the average cost of ICU admission supporting an elderly

patient over the age of 80 was $31,679 CAD ($48,744 CAD per survivor to discharge and $61,783 CAD per survivor at one year).[423] It is also important to acknowledge that the costs associated with sepsis are not simply those related to hospital care. Many patients who survive the initial septic insult are not discharged home and instead are transferred to another facility. Elderly patients in particular, are less likely to be discharged home and more likely to end up in long-term care (55% of sepsis patients over the age of 75 are discharged to a nursing home or other health-care site[424]).[120,425] Hospital readmission after surviving sepsis is 1.96 times more likely[426], with greater than 40% of elderly patients being re-hospitalized within three months[427] (with another septic event as the most common reason for admission[428]). Sepsis in the elderly is also more likely to leave survivors with functional deficits that require ongoing care either from a long-term care facility (45% of elderly patients are transferred to nursing homes, 11% are transferred to another hospital, with only 42% able to return home) or family members.[424] Recurrent infections, malnutrition, and poor quality of life can be especially severe in the frail elderly.[124] Therefore, providing an improved level of care to the elderly septic patient population could perhaps enable significant cost savings and improve the quality of life for both the survivors and their families.

1.12.4 Mortality and Aging

The elderly again seem to be particularly at risk with age as an independent predictor of mortality.[16,429] The elderly often experience frailty and poor pre-sepsis functional abilities, and their long-term prognosis could be highly influenced by pre-sepsis functional capability.[430-433] Some studies have demonstrated that the older the patient with sepsis, the

greater the risk of dying independent of comorbidities.[16,28,114,413] It has been reported that mortality in 2011 in those aged between 60 to 80 years old was as high as 60%, while mortality in those over the age of 80 was 78.9% in a tertiary care ICU.[414] A 2019 multi-centre trial from Spain also demonstrated that age over 80 years was an independent risk factor for mortality.[16] More recent Centers for Disease Control and Prevention data from 2019 also reported an increase in sepsis-related death rates with increasing age, with 150.7 per 100,000 deaths in patients 65 to 74 years old, 331.8 per 100,000 deaths in 75 to 84 years old, and up to 750.0 per 100,000 in those greater than 85 years old.[434]

Older patients also more rarely get admitted to an ICU[435,436] for aggressive management of sepsis and experience mortality earlier in the hospital course.[28] Mortality may be influenced by clinician bias. Physicians may limit ICU admission[437] for many reasons. Elderly patients tend to have lower pre-sepsis functional capabilities,[405] as well as a higher number[28,424] and increased severity of comorbidities (frailty,[438] congestive heart failure, peripheral vascular disease, dementia, and diabetes are associated with mortality[439]). The elderly also display higher mortality with increasing age, and a poor likelihood of returning to pre-sepsis baseline function post-discharge (only 26% of elderly patients with illness requiring ICU admission returned to baseline *physical* function in one Canadian study[440]) despite intense use of resources. In fact, authors of a 2017 multi-centre study reported that 29.6% of patients over the age of 80 were refused ICU treatment.[441] Even if admitted to an ICU, mechanical ventilation in the septic elderly is associated with a higher mortality post-discharge.[439,442] Elderly patients are at a notable disadvantage in that despite all the resources dedicated to their treatment, those who survive an ICU

admission for sepsis have an overall post-discharge mortality of 55% (30.6% one-year mortality rate, 43% two-year mortality rate).[439] Higher three-year mortality (39.5% versus 34.5%) compared to hospital matched controls[442] has also been reported. Increased cost and morbidity combined with decreased survival of the elderly is an important consideration, as age is frequently suggested as a criterion for rationing limited healthcare resources.[443] Given the likelihood of mortality in the elderly septic population, it is clear different tactics are required to manage their illness burden, potentially by modifying treatment or implementing strategies to affect modifiable risk factors.

1.12.5 Risk Factors and Aging

The elderly often have an increased number of comorbidities[424,444] which may result in immunosuppression, lead to institutionalization[445-448] (increased exposure to resistant pathogens and increased colonization), and result in an increased number of procedures and instrumentation (urinary catheters).[449] Malnutrition[450,451] is common secondary to decreased functional status, restricted diets, polypharmacy, poor dentition, dementia, and depression.[446,452] Older patients also demonstrate a reduced ability to tolerate sepsis-induced physiologic derangements[420] and may not display the typical SIRS criteria, resulting in delayed diagnosis and treatment of sepsis.[453] The presence of sepsis at the time of admission to the hospital is associated with increased rates of death in elderly patients.[419] Elderly patients (median age 75) with decreased skeletal muscle mass have demonstrated higher in-hospital mortality from sepsis ($P<0.001$).[454]

1.12.6 Prognostic Factors and Aging

Prognosticating survival and the morbidity burden of sepsis survivors in the elderly is extremely important given the abovementioned socioeconomic implications. One study showed that the presence of respiratory failure[455] and decreased muscle mass in those over 60 years predict mortality in the septic elderly.[454] A newer study from 2022 demonstrated vasopressor use, ventilator use, mean systolic blood pressure at 24 hours, 24-hour urine output, and lactate level as top predictors of 30-day mortality in a model using machine learning.[48] This study also included new prognostic factors, including red blood cell distribution width, presence of malignancy, presence of solid tumour, and type of ICU unit affected prognosis in the elderly.[48] A Brazilian study demonstrated several clinical prognostic factors in patients 65 years of age or older with sepsis or septic shock. Patients who had shock, elevated lactate, organ failure, or mechanical ventilation were at higher risk of dying.[455] More specific to the elderly, it has been demonstrated that in addition to the above factors, an elevated lactate and hypotension (systolic blood pressure less than 90 mmHg) at presentation to the emergency department and poor pre-sepsis functional capacity[405] are prognostic factors for increased short-term mortality at 30 days.[456] Age over 80 compared to those between the age of 65 and 79 years, demonstrated higher mortality.[16] A single-centre study from the Republic of Korea, examined whether ICU admission and mortality of septic elderly patients could be predicted.[457] They demonstrated that using SOFA and abbMEDS (abbreviated Mortality in Emergency Department Sepsis score) could predict ICU admission and mortality in the elderly.[457] They also examined specific

biomarkers and showed procalcitonin, IL-10, IL-6, and IL-5 could predict ICU admission but not death in the elderly.[457]

1.12.7 Pathophysiology and Aging

There exist several different pathophysiologic differences in sepsis between the young and the elderly. These differences often result in worse survival, increased severity and burden of morbidity post-sepsis, and sepsis-related mortality. Several age-related aspects will be reviewed below.

1.12.7.1 Inflammation, Immune Function, and Aging

Inflammaging is a chronic low level of inflammation without active infection in the elderly.[458] It is the result of chronic physiologic stimulation of the immune system and activation of immune receptors by self- and non-self-antigen.[459] This culminates in elevated baseline levels of inflammatory cytokines, including TNF-α, Il-6, and C reactive protein (CRP).[460,461] Many articles discuss the processes that have been implicated in inflammaging, including mitochondrial dysfunction, changes in microbiota, and oxidative stress beyond what is pertinent for this review.[462-465] Other contributors include exogenous environmental influences like industrial toxicant exposure or endogenous factors like DNA or telomere damage.[466] Several of these factors have been noted to increase with time, thereby playing more of a role as we age.[466] It has also been demonstrated that as people age, they develop decreased specific PRR activity (TLR1, TLR7/9[467,468]), resulting in heightened susceptibility to bacterial pneumonia and influenza, amongst several other pathogens.[469] In contrast, hyperactivation of other PRRs in the elderly (TLR3) have been

shown to result in increased inflammation and mortality.[470] The outcome of developing inflammaging may enhance susceptibility to infection and result in inadequate response to vaccination.[471] Inflammaging in the lung as demonstrated by increased levels of CRP, TNF-α, Il-6, and IL-1β results in the decreased immune response to pneumonia and increased risk of developing infection requiring hospital admission as well as increased mortality.[460,461,472-479] As a dysfunctional or maladaptive immunologic response to sepsis is a potential driver of post-critical care chronic illness, inflammaging may render the elderly more susceptible to these long-term outcomes.[480,481]

Aging of the immune system, a process known as immunosenescence,[101] can affect how an elderly person mounts an inflammatory response and fights infection. Elderly patients commonly experience chronic inflammation due to repeated infections (clinical and subclinical) or the presence of non-communicable disease (e.g., diabetes). Long-term exposure to antigens combined with an oxidative stress-induced alteration in the apoptotic function of immune cells differentiated from hemopoietic stem cells is also common. Both humoral[482] and cell-mediated (CD8+ more so than CD4+) adaptive[483] immune responses are decreased, and vaccinations are known to be less effective[484] secondary to impaired interactions between B and T cells. There are reduced numbers of naïve T cells (thymic involution) and B cells with increased memory and effector T and B cells.[484] Although originally thought to affect mainly adaptive immunity, evidence is accumulating demonstrating important alterations in innate immune function.[485-488] These changes include reduced cytokine production, decreased neutrophil function (reduced chemotaxis, phagocytosis,[489] superoxide production,[490] apoptosis[491]), decreased per-cell natural killer

cell cytotoxicity,[492,493] and a reduction in the function of dendritic antigen-presenting cells.[494] The consequences of immunosenescence include increased susceptibility to infection and increased occurrence of autoimmune disease[495] and cancer.[496]

1.12.8 Treatment and Aging

There exist no formal elderly-specific guidelines for sepsis management. However, some evidence exists to help direct treatment. The most recent Surviving Sepsis Guidelines note the lack of harm using a target mean arterial blood pressure of 60 to 65 mmHg in the elderly.[358] The elderly sub-group of patients from the SEPSISPAM trial demonstrated higher mortality when higher mean arterial pressure targets were used.[333] Judicious fluid resuscitation and arrhythmogenic medications such as dobutamine and NE must be used with care due to increased incidence of cardiac comorbidities, including diastolic dysfunction.[413,497] Early lactate guided therapy has been recommended in a Chinese study published in 2021 to reduce admission to ICU but did not impact mortality.[498] Bundled-care of elderly patients and use of ICU-goal direct therapy bundles also did not improve mortality in elderly septic Chinese patients in an article published in 2021.[499] Reviewing potentially inappropriate medications initiated in the ICU upon discharge has been recommended for the elderly.[500] There are no therapeutics directed at the elderly for sepsis treatment. We may have improved success in treating sepsis in the elderly if we can target therapies to the elderly age group.

1.13 Sepsis Models

1.13.1 Significance of the Model of Sepsis

Sepsis is a complex disease and not all aspects of evaluation are feasible in human studies. Therefore, animal models can provide replicable and controlled conditions to fill our knowledge gap. The human sepsis response can be variable compared to an animal model, and it is important not to base conclusions solely on animal studies.[501] Murine models are commonly used to study pathophysiologic changes of sepsis and to study potential therapeutic targets for sepsis treatment. Transgenic and knock-out mice have contributed extensively to our knowledge of sepsis.[501] The use of mice and rats is prevalent because they can be used and housed inexpensively and in large numbers. They are also widely available with a relatively short lifespan.[420] Rodents are, however, more difficult to instrument due to their small size making detailed hemodynamic observation sometimes undesirable. Also, their small blood volume makes serial blood sampling over a brief period of time impossible. Numerous differences exist between human and murine models making inferences from murine models often inapplicable.[502] This is demonstrated by the development of many sepsis treatments that though effective in murine models, do not clinically result in benefit.[503-505] However, despite their limitations, rodent models have provided unparalleled insight into the pathological mechanisms that underlie the transition from sepsis to septic shock.

1.13.2 Aging and Animal Models

The complex pathophysiologic changes of sepsis that alter hemodynamic parameters have been examined in animal models.[420] Much of this evidence focuses on the young or adult animal (approximating a human age of less than 20 years old,[420] a group not at high risk of developing sepsis or suffering complications secondary to sepsis) when as previously mentioned, much of the human population affected by sepsis is elderly. Few studies have examined the hemodynamic variability and subsequent mortality risk in the aged animal compared to adult or young animals. The hemodynamic changes associated with sepsis in the aged animal are even less well-defined than in the adult or young animals. As mortality rises with progression to a circulatory shock state, more knowledge of the compensatory hemodynamic changes in the aged may prove key to preventing mortality. A recent article by Dutta and Sengupta[506] explains that every human year is approximately equivalent to nine mouse days. However, determining an appropriate mouse age to represent an elderly human is more complicated than just counting days.[507] Each phase of life must be considered, including the weaning period, puberty, adulthood, reproductive senescence, and lastly, old age. As such, a two-year-old mouse approximates a 70 year-old human.[506] It is also important to consider how an aged mouse can reproduce or typify results expected in humans. For comparison to human non-elderly adults, it is appropriate to use mice that have stopped developing and have not yet begun demonstrating senescence.[507] As such, use of 12 week old C57BL/6 mice would be an example of a non-elderly adult control group. Aged animals used in studies may be of differing ages secondary to differing time-of-life onset of disease (i.e. hypertension) or desired effects of senescence (as may be

demonstrated by biomarkers).[507] Inbred strains also display different age-related phenotypes. C57BL/6J mice are known to have impaired glucose tolerance which may be an important outcome-defining variable depending on the study.[508] Aging studies that use female rodents must also consider the differences in reproductive senescence as mice do not enter menopause in a similar timeframe compared to humans.[509,510]

Invasiveness of the model may require more consideration in the aged animal as they may have poor physiologic tolerance to increased invasiveness and may have altered responses to the dose and length of the anesthetic required.[511-515] Although there is a paucity of information regarding sepsis in the aged rodent, we know some things about their physiology that may exacerbate morbidity and mortality outcomes.

1.13.3 Sepsis Induction Protocols

When examining the murine model of sepsis, the method of sepsis induction is important. In a 2014 Japanese study, intra-abdominal sepsis was the second-highest cause (21.3%) of sepsis behind pulmonary infections (41.8%).[415] However, the in-hospital mortality associated with intra-abdominal sepsis was proportionally higher at 31.8% versus 26.3% for pulmonary infection. There are currently four commonly used models to simulate intra-abdominal sepsis in rodents; these include (i) LPS injection; (ii) live bacterial injection; (iii) cecal ligation and perforation (CLP) – including the use of the colon ascendens stent peritonitis variation (CASP); and (iv) fecal-slurry induced peritonitis. The first model of intravenous or intraperitoneal injection of gram-negative endotoxin LPS results in a sterile SIRS response. This model is beneficial because it is dose-dependent, reproducible, and straightforward. However, it is essential to recognize that this technique produces a

chemical endotoxemia that stimulates a SIRS response rather than recapitulating the entirety of the sepsis response. As such, LPS models are not generally recommended by the Minimum Quality Threshold in Preclinical Sepsis Studies investigators.[516] The second technique of live bacteria injection can more closely approximate a sepsis response, but it can be challenging to maintain minimal bacterial load variation.[420] Bacterial and LPS injection also lack applicability because responses to inoculation tend to be immediate, and thus do not recapitulate the prolonged time courses that typically characterize sepsis in the clinical setting.

CLP is a third method in which the rodent has the cecum surgically ligated and subsequently perforated, allowing cecal content to leak into the intra-abdominal compartment.[517] CLP can provide controlled severity of sepsis based on the size of the puncture.[518] A variation of the CLP is the CASP model, which involves the continuous leaking of colonic contents into the abdominal cavity through a stent inserted in the ascending colon. The CLP and CASP models are easily reproducible and resemble the human response in that the source of sepsis can be removed with the removal of the stent, mimicking a surgical intervention.[519,520] However, there are some drawbacks. Notably, both CLP and CASP are surgical models that require laparotomy to induce sepsis and the use of anesthesia and analgesia. The invasiveness of the model increases the complexity of the experiment and may confound results. Rodents made septic with CLP are also likely to develop an intra-abdominal abscess rather than diffuse peritonitis due to small bowel coverage of the leak, and the number of punctures is not associated with a worsening clinical picture.[519] Rodents subjected to CLP have a minimal level of sustained

inflammation. This may be secondary to the holes in the cecum closing over and preventing continued leak of bowel contents. CASP, however, presents a clinical picture more consistent with that of true intra-abdominal sepsis and peritonitis with continuous bowel leakage and steadily increasing levels of cytokines up to 24 hours.[519]

Finally, the fecal-slurry induced peritonitis (FIP) model is a popular method as it easily produces the clinical sepsis picture and is highly reproducible. It is also minimally invasive, and the severity of the model can be easily titrated based on the dose of fecal slurry injected. Unlike the CLP model, changes in the microbiome (secondary to interventions of differences in facilities among institutions) are less likely to influence outcomes since large batches of fecal slurry can be made, aliquoted, and used over an extended period to ensure consistency amongst experiments.

1.14 Rationale for Proposed Studies

As explained above, sepsis in the elderly is a common, costly, and potentially life-threatening syndrome that is deserving of increased scientific attention. We believe the vascular function and resultant clinically observed hemodynamic function will reveal much about sepsis pathophysiology and simultaneously lend knowledge towards potentially useful treatments to improve morbidity and mortality. Initial investigations required use of animal models and we used the FIP model as it closely and non-invasively represents a picture similar to that of human intra-abdominal sepsis.

As impaired NO function is important in the development of vascular dysfunction leading to vasoplegia and organ dysfunction, we aimed to develop techniques to examine real-time bedside vascular dysfunction as a measurement of the progression of sepsis and

to determine whether sepsis-damaged sGC activation improves not only vascular function but also mortality in sepsis. This project had several innovative aspects, including an integrative approach to studying systemic hemodynamics and regional vascular dysfunction patterns as sepsis progresses; and a novel treatment strategy to improve regional and systemic circulatory function.

1.14.1 Integrative Approach

Due to the systemic nature of sepsis, there is great value in assessing the progression of circulatory decline via integrative approaches. Advanced stages of sepsis are characterized by regional heterogeneity in vascular function, which contributes to poor tissue perfusion and eventual failure of downstream organs. Thus, while the therapeutic goal of maintaining blood pressure within normal levels is important in sepsis, doing so without consideration of vascular dysfunction and altered blood oxygen and nutrient delivery may be misleading. Indeed, this point may explain why pharmacological interventions can improve hemodynamics in sepsis without improving overall mortality. A crucial aspect of improving blood pressure is preserving end-organ function. The following studies simultaneously monitored *in-vivo* systemic hemodynamics and vascular function (in response to various vasoactive medications) and combined this knowledge with *ex-vivo* vascular dysfunction confirmatory wire myography studies. This approach enabled us to determine whether our novel treatments improved vascular dysfunction. As our *in-vivo* approach was a novel approach, we used *ex-vivo* vascular function studies to confirm aspects of our *in-vivo* vascular function studies. However, it is important to note that our *in-vivo* studies examine the animal as a whole, while the *ex-vivo* studies depict separate

vascular beds and distinct vasoactive pathways. As such, the two vascular function study approaches may not always completely agree.

1.14.2 Novel Treatment Strategy

Excess ROS generation during sepsis damages numerous effectors, including oxidizing sGC, the downstream target of NO. Oxidizing sGC results in releasing the heme bound to the β_1 chain. The damaged form of sGC is then ubiquitinated and degraded, resulting in perturbed vascular function in regions affected by sepsis. Heme-free sGC produced in situations of increased ROS generation (aging, sepsis) can be rescued by a class of drugs known as sGC activators, which induce sGC function and production of cGMP in the absence of ferrous-heme. Cinaciguat (BAY 58-2667) is one such activator of sGC.

As cinaciguat increases cGMP production, inducing vasodilation and consequent hypotension, it may seem counterintuitive to administer it in sepsis, where excessive vasodilation secondary to systemic overproduction of NO from iNOS leads to cardiovascular collapse. However, studies demonstrate regional dysfunctional NO signalling in the microcirculation resulting in vasoplegia and heterogeneous tissue perfusion in septic patients and experimental models of the disease.[521] Consequently, restoring cGMP signalling with cinaciguat in septic shock is expected to restore endothelial function and vascular reactivity.[521] Consistent with this idea, Vandendriessche et al. recently showed cinaciguat improved survival in a sheep model of septic shock.[522]

The alteration in the pathophysiologic cascade of events needs to be examined in an aged model. This model would ideally be constructed in a mouse model of sepsis (in that this model would allow for more rapid aging than other animal groups, yet mice are

still large enough to provide blood samples for analysis) with an intra-abdominal type of sepsis as it has the proportionately highest risk of death. The method of sepsis induction should be one that would closely mimic the formation of sepsis in humans, ideally the FIP model, which is also the least invasive model. It would be important to gather hemodynamic variable information to better understand target hemodynamic goals in elderly patients. It would be ideal to test novel treatments to better define interventions in this age group to optimize survival and decrease morbidity.

Cinaciguat selectively binds and activates the damaged form of sGC, restoring sGC-cGMP signalling in these regions. Consequently, cinaciguat can selectively target damaged areas of the vasculature, and improve regional vasodilation and organ perfusion potentially without affecting healthy vascular beds. It is recognized that because cinaciguat has the potential to induce vasodilation, its use in sepsis seems counterintuitive; however, by recognizing that small doses of cinaciguat preferentially target damaged vascular tissues, we may improve regional blood flow without causing systemic vasodilation and hypotension.[86]

1.15 Hypotheses

It was hypothesized that:

i) Aged mice demonstrate a distinct septic hemodynamic phenotype with impaired *in-vivo* vascular reactivity compared to young mice and decreased *in-vivo* response to NE treatment resulting in decreased survival.

ii) Using an sGC activator (cinaciguat) will improve *in-vivo* hemodynamics and *ex-vivo* vascular reactivity in septic mice and improve sepsis survival.

iii) Using an sGC activator (cinaciguat) will improve *in-vivo* hemodynamics, *ex-vivo* vascular reactivity, and sepsis survival more so in the aged mice due to elevated baseline ROS levels.

iv) Oncostatin M (OSM: an inflammatory cytokine) is an important cytokine underlying vascular function in both non-septic and septic states, the loss of which (in a knockout model) will improve *in-vivo* hemodynamic and *ex-vivo* vascular function, as well as sepsis survival in a sex-specific fashion.

1.16 Specific Objectives

To investigate the above hypotheses, several specific objectives needed to be accomplished:

i) Characterize an *in-vivo* hemodynamic model of intra-abdominal polymicrobial sepsis in young and aged mice using the FIP method.

ii) Design an integrative model of novel methods for assessing *in-vivo* vascular reactivity with vasoactive medications methacholine (MCH), sodium nitroprusside (SNP), and phenylephrine (PE) combined with *ex-vivo* wire myography.

iii) Characterize an *in-vivo* sepsis treatment model using conventional therapies, including fluid maintenance and NE.

iv) Characterize the *in-vivo* hemodynamic and survival effects of the sGC activator cinaciguat in sepsis for young and aged mice.

v) Characterize the *in-vivo* hemodynamic and survival effects of OSM receptor (OSMR) deficiency in sepsis.

1.17 Summary

Sepsis results in pathophysiologic derangements, and despite aggressive treatment, it results in unacceptably high rates of morbidity and mortality. The aged are a particularly high risk and under-investigated group contributing to much of the socioeconomic burden of sepsis. Strategies that target the prevention of shock development and simultaneous maintenance of tissue perfusion hold promise. It is imperative that work continues to further define the vascular pathophysiology of sepsis and refine therapies in both animal and subsequently human studies considering both age and sex as major contributors to outcomes.

Chapter 2

A Comparison of Vascular Dysfunction and Survival in Young and Aged Septic Mice

2.1 Background

Sepsis is a dysregulated host response to infection that is life-threatening.[42] The elderly are disproportionately affected by sepsis,[414] potentially secondary to numerous physical and social factors including an increased number of comorbidities,[523] increased severity of comorbidities, poor functional status,[431] malnutrition,[524] increased exposure to the healthcare system (resulting in a higher number of invasive procedures[449] and more exposure to drug-resistant pathogens[525]), decreased consideration for admission to the high-intensity level of care settings (intensive care units (ICU), observation units),[526] polypharmacy,[527] and immunosenescence.[101] To further complicate matters, post-sepsis, the elderly are more often left with psychological and physical disabilities that more frequently necessitate transfer to long-term care facilities[445-448] or result in palliation.[528] Elderly sepsis survivors are also at risk of increased sepsis-associated morbidity and mortality.[101] Mortality has been estimated to be as high as 60% for people 60 to 80 years old and almost 80% for those over 80 years old.[414] It is challenging to know the exact epidemiologic information regarding sepsis in the elderly due to the vague definition of what age defines an elderly person;[529] however, most sources report the definition of elderly as 65 years or greater.[530] As the elderly are a fast-growing population worldwide (approximately 46 million in 2015 and projected to more than double to 98 million by 2060

in the USA[412]) and are currently the largest group of patients affected by sepsis (more than 60% of hospitalized patients with sepsis are over the age of 60),[416] they are positioned to place great strain on healthcare resources.

Vascular dysfunction is a significant contributor to the sepsis-related hypotension and organ-hypoperfusion that influence mortality.[531] A major regulator of vascular function is the constitutively expressed nitric oxide synthase (NOS) located on the vascular endothelium, aptly named endothelial NOS (eNOS).[532] The major function of eNOS is the production of nitric oxide (NO). In addition to the maintenance of endothelial-derived vasodilation, eNOS-produced NO plays several roles including the prevention of thrombosis[533] and leukocyte adhesion.[534] Numerous sepsis-related processes damage and impair the function of eNOS, while provoking the expression of inducible NOS (iNOS), which produces NO far in excess of what eNOS can produce.[329] While NO production in sepsis is useful to help defend against invading pathogens, excessive amounts of NO are deleterious, and contribute to the loss of vascular tone through various mechanisms including the generation of reactive oxygen species (ROS) and oxidation of crucial enzymes (NOS, soluble guanylate cyclase (sGC)), thereby compromising blood supply to oxygen-deprived tissues and organs potentially resulting in organ failure.

The elderly population is known to exhibit alterations in vascular function at baseline. This age-related vascular dysfunction is characterized, in part, by impaired NO production by eNOS and dysfunctional NO-sGC signalling.[535] Aging is also associated with increased levels of iNOS as a part of the senescence-associated secretome.[536] As eNOS and iNOS are critical players in developing vascular dysfunction that arises in sepsis,

we hypothesized that the septic elderly, who already experience baseline vascular dysfunction, would experience further diminished vascular responsiveness that would impair the effectiveness of vasopressor therapy and decrease survival compared to the young.

2.2 Methods

This study was conducted according to the Canadian Council on Animal Care guidelines, with approval from the Animal Care and Use Committee from the University of Alberta (Edmonton, Canada), and reported in adherence with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) Guidelines.

2.2.1 Animals and Preparation

Seventy-two male C57BL/6 mice (procedure- and drug-administration-naïve, with no genetic modifications) were purchased from Charles-River Inc. (Saint-Constant, QC). Young mice were 3-4 months of age; aged mice were housed until 22-24 months of age. Mice were housed five per cage in standard shoebox cages containing aspen-chip bedding, nesting material, and PVC tubing for environmental enrichment. Cages were located in the University of Alberta animal care facility, which maintained a 12h:12h light:dark cycle and an ambient temperature of 22±1ºC. Trained animal care personnel routinely performed welfare assessments before experimentation. Mice had *ad libitum* access to tap water and standard grain-based rodent chow (PicoLab 5LOD, LabDiet, St. Louis, MO).

Experiments were conducted in the same laboratory operating theatre during the day. Mice were anesthetized with inhaled isoflurane (induction: 5%, maintenance: 1.1-

1.5% in 100% O_2) and were kept spontaneously breathing via a nosecone. We used isoflurane as it is administered non-invasively, thereby avoiding the risk of puncture of the peritoneum with intraperitoneal (IP) or subcutaneous injections. Avoidance of peritoneal contamination was essential to this experiment as the mode of sepsis involved an IP injection. Isoflurane also provides a constant depth of anesthesia which is easily maintained for long durations. Following induction of anesthesia, mice were instrumented with a polyethylene catheter (PE10; inner diameter 0.58 mm, outer diameter 0.97 mm) in the left femoral vein for drug delivery and a fibreoptic pressure sensor (FISO 0.9 Fr/0.5 Fr; Harvard Apparatus, Harvard BioScience Inc.) in the right carotid artery for hemodynamic assessments. The femoral venous catheters contained heparinized sterile 0.9% saline (25 units/mL). Core body temperature was monitored via a rectal thermometer and mice were maintained on a warmed surgical platform connected to a water circulator for the duration of the experiment. Following instrumentation, mice were given 30 minutes to stabilize hemodynamic parameters. Mice were administered fluids (sterile saline; 2 mL/kg/hour IV) to maintain hydration. Arterial blood pressure waveforms generated from the indwelling FISO pressure sensor were monitored and recorded via a data acquisition system (Lab Chart Pro 8, ADInstruments, Colorado Springs, CO).

2.2.2 Study Protocol

After stabilization, fecal slurry (FS; 1.3 mg/g IP) was administered to induce sepsis. The dose was selected based on pilot studies that generated mortality in anesthetized mice within six hours of administration. *In-vivo* vascular reactivity was assessed via methacholine (MCH; 1 μg/kg IV – caused hypotension that resolved within 10 seconds

and was due to decreased systemic vascular resistance; the dose did not affect heart rate – **Figure 2-1**) injection every 30 minutes.

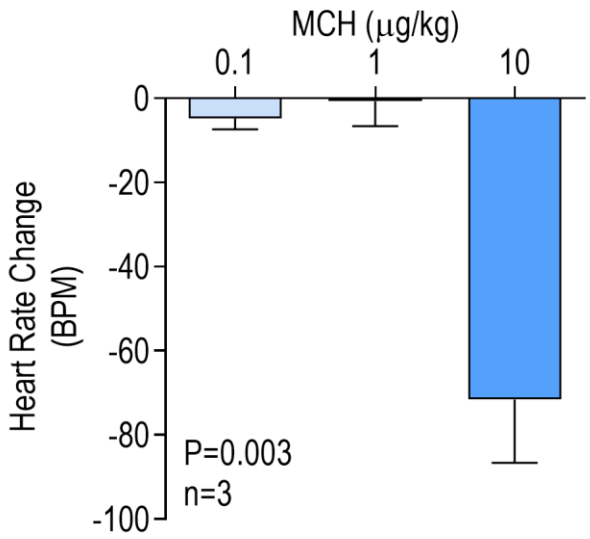
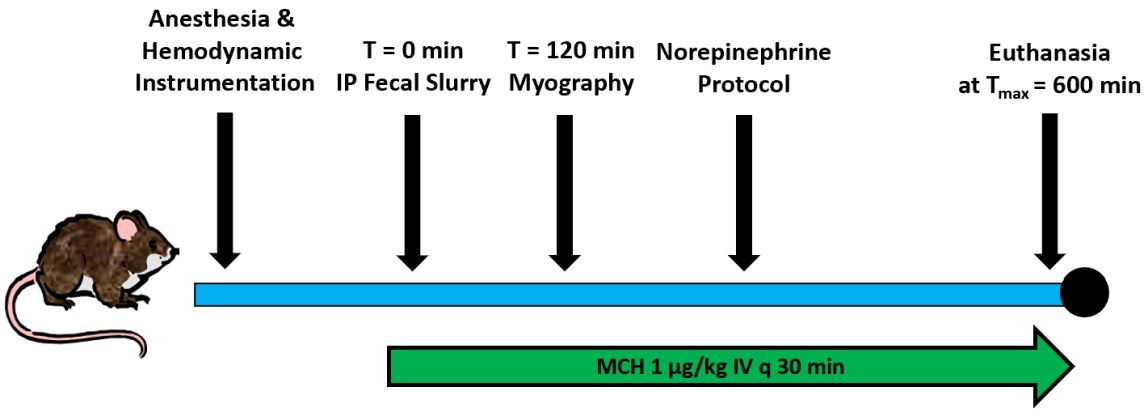


Figure 2-1: Methacholine Effect on Heart Rate

Methacholine (MCH) 1 µg/kg IV had minimal effect on heart rate beats per minute (BPM) compared to 0.1 µg/kg IV and 10 µg/kg IV.

In addition to these groups, a separate cohort of septic and non-septic mice were administered norepinephrine (NE) to assess the reversibility of cardiovascular collapse in the final stages of sepsis. NE infusion was initiated at 200 µg/kg/hour and advanced by 100 µg/kg/hour in five-minute intervals to a maximum of 600 µg/kg/hour until the mean arterial pressure was greater than 65 mmHg or systolic blood pressure was greater than 80 mmHg (**Figure 2-2**).



IP: intraperitoneal
MCH: methacholine
Norepinephrine Protocol: titrate norepinephrine 200-600 µg/kg/h IV when MAP < 65 mmHg or SBP < 80 mmHg
Young: 3-4 months; Aged: 22-24 months

Figure 2-2: Study Protocol

Schematic of basic protocol involving methacholine (MCH) and norepinephrine (NE) administration.

2.2.3 Wire Myography Protocol

Mice randomized to wire myography experiments were maintained under anesthesia for 120 minutes following induction of sepsis and subsequently euthanized by exsanguination and excision of the heart. Euthanization at 120 minutes was chosen as by this time point, septic mice of both age groups demonstrated *in-vivo* changes to MCH administration but were not so sick that vessels would be unresponsive despite escalating concentrations of vasoactive medications. Left common carotid arteries and second-order mesenteric arteries were rapidly removed and placed in ice-cold HEPES-buffered physiologic saline solution (PSS – composition: (in mM) HEPES 10, glucose 5.5, $CaCl_2$ 1.56, KCl 4.7, NaCl 142, $MgSO_4$ 1.17, KH_2PO_4 1.18, pH 7.45). Vessels were cleaned of extraneous perivascular

connective tissues, and small vessel sections (2 mm in length) were isolated and mounted in an isometric myograph system (DMT, Copenhagen, Denmark); 15 μm gold wire was used to mount mesenteric arteries, and 40 μm tungsten wire was used to mount the carotid arteries. Vessels were assessed at 37ºC and normalized to optimal resting tension (0.8 IC_{100}: the internal circumference equaling a transmural pressure of 100 mmHg) by small incremental increases in diameter. Vessels were then allowed 30 minutes to equilibrate to the optimal resting tension before initiating the experimental protocol. Vessels were also tested for viability (ability to elicit a robust constriction) by treatment with high potassium physiologic salt solution (KPSS). After rinsing, vessels were treated with phenylephrine (PE; 10 μmol/L) twice, with a rinse and return to baseline tension in between doses; following the second dose of PE, methacholine (MCH: 3 μmol/L) was administered to test the integrity of the endothelium. Following confirmation of endothelial integrity, vessels were rinsed and returned to baseline. Concentration-response curves were then generated for PE. A subset of vessels was incubated with non-selective nitric oxide synthase (NOS) inhibitor L-N^G-nitro-arginine-methyl ester (LNAME: 100 μmol/L) for 30 minutes prior to performing cumulative concentration-response curves with MCH and sodium nitroprusside (SNP). For concentration-response curves with vasodilators, vessels were first sub-maximally pre-constricted (80% maximum: EC_{80}) with PE. Finally, at the conclusion of each experiment, vessel integrity was verified by inducing constriction with KPSS was assessed; those vessels with a final KPSS constriction of less than 80% of the first KPSS constriction were excluded from analyses.

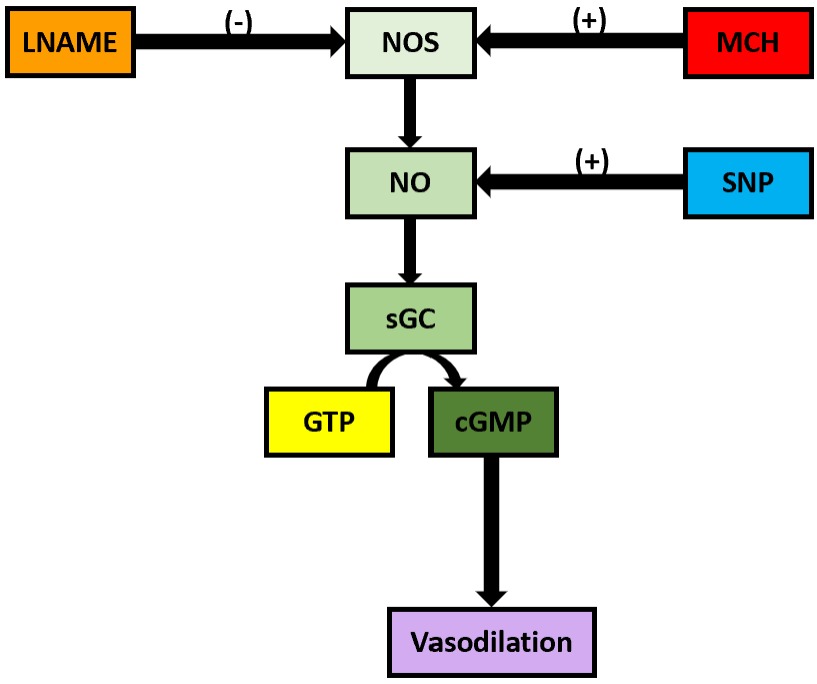


Figure 2-3: Vasoactive Medications Used for Wire Myography

Vasoactive medications used in wire myography. (cGMP: cyclic guanosine monophosphate; GTP: guanosine triphosphate; LNAME: L-N^G-nitro-arginine-methyl ester; MCH: methacholine; NO: nitric oxide; NOS: nitric oxide synthase; sGC: soluble guanylate cyclase; SNP: sodium nitroprusside)

2.2.4 Assessment of sGC Production

sGC activity was assessed indirectly by measuring cGMP production in two end-organs commonly acutely affected by sepsis, the lung and the kidney. Fresh left lungs and left kidneys were harvested from a subset of non-septic and septic mice 120 minutes post-vehicle or fecal slurry injection under isoflurane anesthetic. Lungs and kidneys were homogenized in EDTA, Tris-HCL, sucrose, dithiothreitol, protease inhibitor cocktail (Sigma, St. Louis, MO), and sodium orthovanadate. Homogenized tissue was centrifuged

(10,000 x *g*, 4°C, 20 minutes) and had total protein quantified using the bicinchoninic acid protein assay. Cytosolic protein was added to a reaction buffer mixture containing bovine serum albumin, benzamidine HCL, SNP, LNAME, 3-isobuyl-1-methylxanthine, L-cysteine, and GTP (Sigma, St Louis, MO). This mixture was incubated for 30 minutes at 37°C in a shaking water bath. The reaction was terminated with zinc acetate and sodium carbonate (Sigma, St Louis, MO). This mixture was then centrifuged (5,000 x *g*, 4°C, 10 minutes). cGMP was then quantified with the Cayman Chemical Cyclic GMP ELISA kit (Ann Arbor, MI, USA).

2.2.5 Reagents

MCH and NE (obtained from Sigma, St. Louis, MO) were dissolved in sterile saline (0.9% NaCl) for *in-vivo* experiments and subsequently diluted in PSS for final use in myography experiments. PE, SNP, and LNAME (Sigma, St. Louis, MO) were also dissolved in sterile saline and diluted in PSS for final use in myography experiments.

2.2.6 Statistical Analysis

Data are presented as mean±SEM. Patterns of cardiovascular collapse (as depicted by Kaplan-Meier curves) were analyzed by log-rank tests. Continuous variables, including arterial pressures, heart rates, and hemodynamic responsiveness to exogenous pharmacological agents were analyzed by repeated measures 2-way ANOVA for the overall effects of sepsis and time; subjects were matched within the time domain. The remaining data were analyzed by unpaired Student's *t*-test. For collection of septic systolic blood pressure, diastolic blood pressure, and heart rate, the most representative five-second

segment within each 30-second interval was captured from recorded parameters. Percent changes in hemodynamic variables in response to MCH administration were calculated as the minimum value compared to the baseline in the immediately preceding five-minute interval. For wire myography, concentration-response curves were fitted to the Hill equation using a variable slope, and pEC_{50} (mean effective concentration to produce a 50% response) and maximal responses were analyzed by unpaired *t*-test. cGMP production was analyzed with 2-way ANOVA and Tukey's multiple comparison test. Statistical outliers determined by Grubb's test were not included. Results were considered significant if $P<0.05$. GraphPad Prism 8 software (La Jolla, CA) was employed for statistical analysis.

2.3 Results

Baseline (i.e. prior to induction of sepsis) hemodynamic parameters (systolic blood pressure, diastolic blood pressure, and heart rate) were not different between the young and aged mice (**Figure 2-4: A-C).** Aged mice had greater declines in systolic (young: -10.88%, aged: -16.99%; difference: -6.11±2.59%; P=0.04) and diastolic (young: -10.87, aged: -19.59; difference: -8.72±2.68%; P=0.009) blood pressure in response to boluses doses of the endothelial-dependent vasodilator MCH (**Figure 2-4: E**). Heart rate response to MCH was not different in the aged compared to the young (**Figure 2-4: F**).

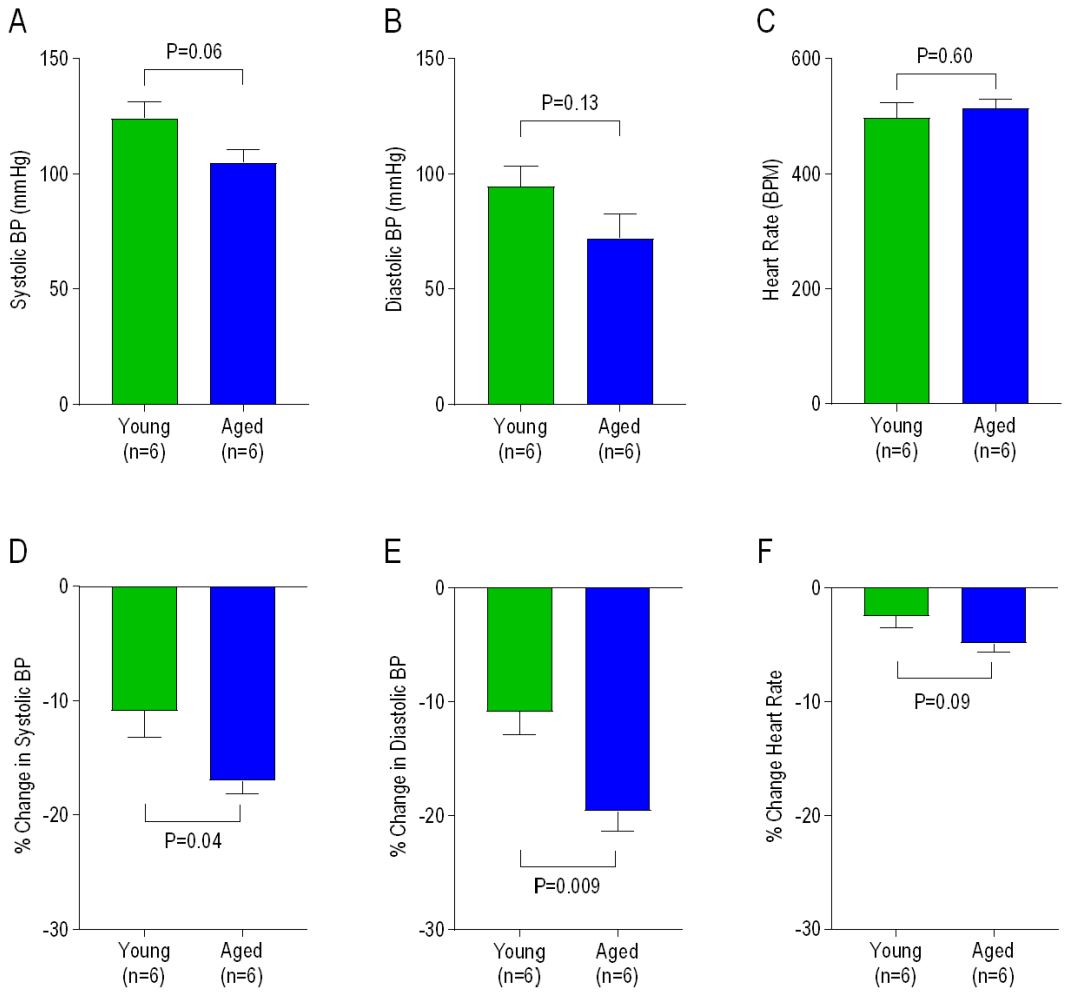


Figure 2-4: Baseline Hemodynamics

Baseline hemodynamics (A-C) and vascular responses to methacholine (MCH) (D-F) in control young male C57BL/6 and aged male mice.

Thirty minutes after induction of sepsis, aged mice had lower systolic blood pressure (P=0.04) and diastolic blood pressure (P=0.02), which persisted over the following 180 minutes. Heart rate was not different (P=0.79) between the age groups during this period (**Figure 2-5: A-C**). Aged mice also had reduced MCH-induced diastolic

blood pressure responses to the endothelial-dependent vasodilator MCH (-8±2%; (P=0.01) compared to young mice), albeit no difference between age groups was observed in systolic blood pressure or heart rate (**Figure 2-5: D-F**).

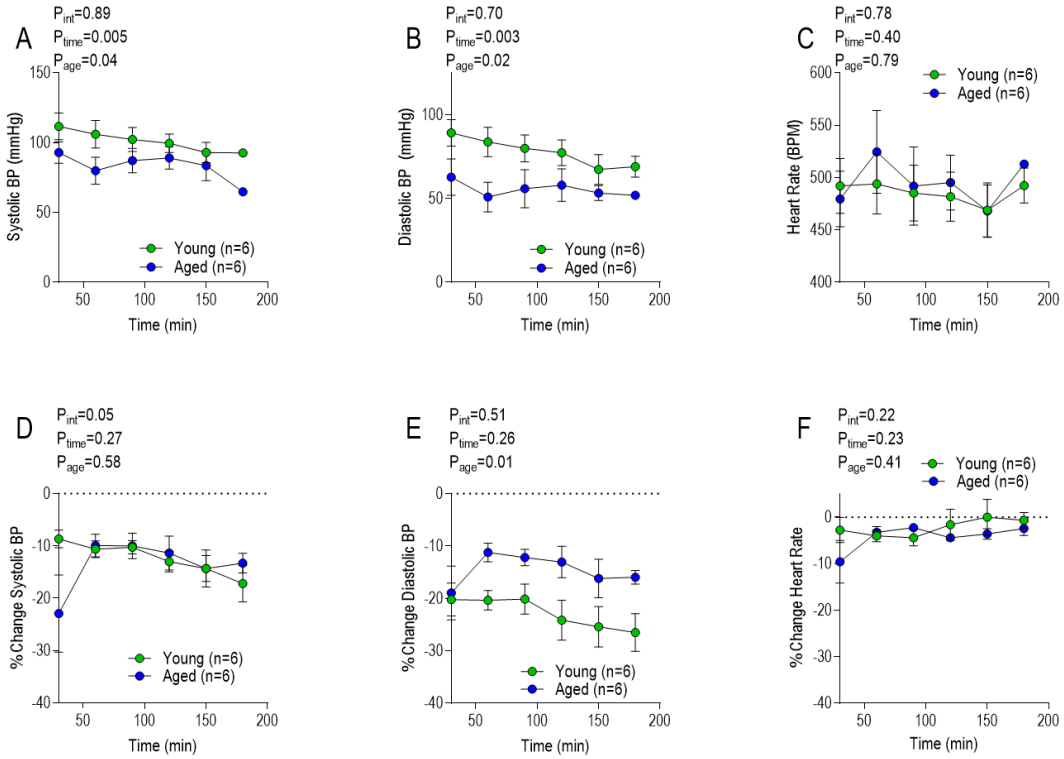


Figure 2-5: Hemodynamics

Hemodynamics (A-C) in C57BL/6 young and aged mice over time as sepsis progresses. Vascular responses (D-F) to methacholine (MCH: 1µg/kg IV) over time (Time=30 minutes: first methacholine measurement).

After 180 minutes, four of the six aged mice met predefined humane endpoints and therefore were euthanized. Probability of survival was higher for the young septic mice (P=0.01, hazard ratio: 6.924, 95% confidence interval: 1.538 to 31.17; **Figure 2-6: A**).

Based on predefined criteria for humane euthanasia, mean survival time post-induction of sepsis was markedly reduced in aged mice compared to young (**Figure 2-6: B**) (young: 283±67 min; aged: 115±24 min; P=0.04).

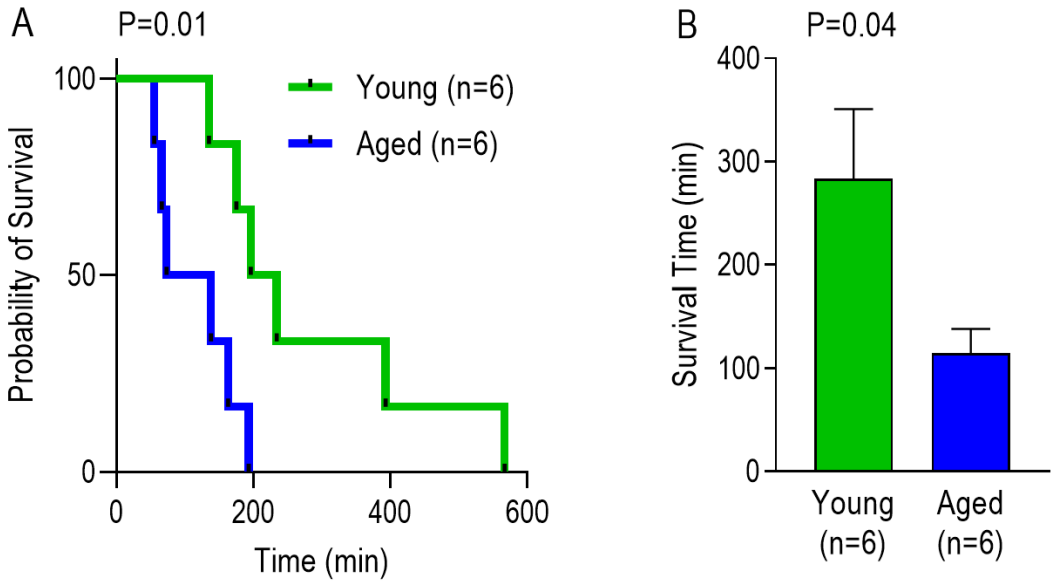


Figure 2-6: Survival for septic male young C57BL/6 and aged mice

Kaplan-Meier survival curve (A) for young and aged septic mice. (B) Mean survival times for young and aged septic mice.

As noted above, a separate cohort of mice were administered continuous infusions of NE at predefined hemodynamic thresholds to mimic clinical intervention with a vasopressor, and in turn assess the reversibility of cardiovascular collapse in the final stages of sepsis. In mice treated with NE, there was no difference in survival between the young and aged septic mice (P=0.79, hazard ratio: 1.732, 95% confidence interval: 0.5166 to 5.810, **Figure 2-7: A**; **Figure 2-7: B**: mean survival: young 232.8±24.46 minutes; aged 195.5±21.55 minutes; P=0.28). NE treatment in young septic mice compared to untreated young septic

mice did not improve survival (difference of 50.50±71.70; P=0.50), while the aged mice did experience an improvement in survival with the addition of NE (difference of 80.83±31.95 minutes; P=0.03). The aged mice received less total NE (**Figure 2-7: C**: young: 1,520±166.4 µg; aged: 710.6±242.1 µg; P=0.02) and spent less percentage of time with NE infusing (**Figure 2-7: D**: young: 74.50±4.703 minutes; aged: 45.33±8.99 minutes; P=0.02).

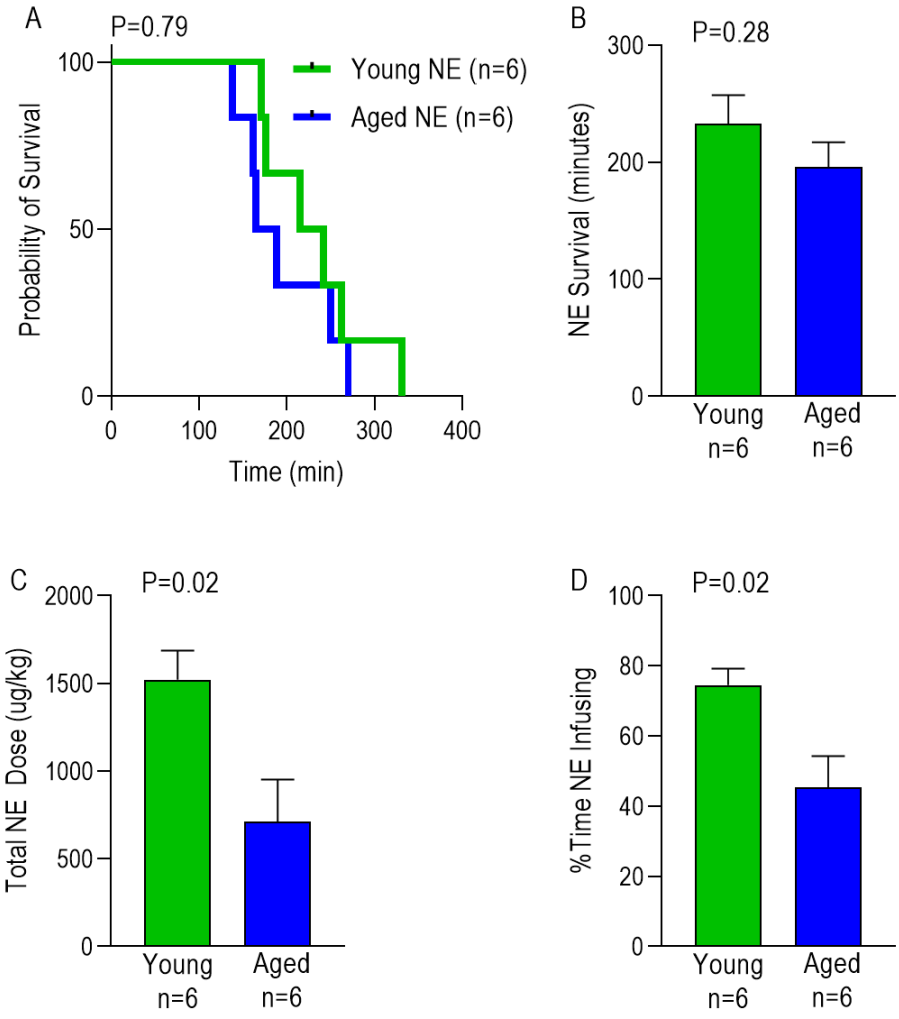
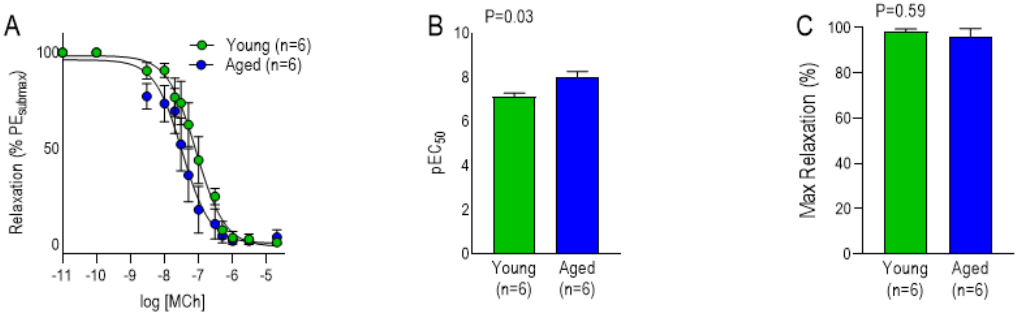


Figure 2-7: Norepinephrine Protocol

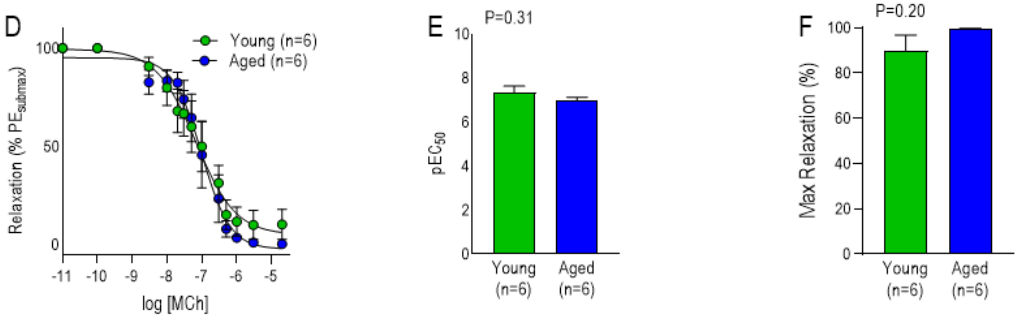
(A) Survival probability for male septic young C57BL/6 and aged mice treated with intravenous norepinephrine (NE) infusion. (B) Survival time in minutes for male septic young C57BL/6 and aged mice treated with intravenous norepinephrine (NE) infusion. (C) The total dose of NE infused in μg/kg for male septic young C57BL/6 and aged mice. (D) % of total survival time spent with NE infusing for male septic young C57BL/6 and aged mice.

Wire myography was used to examine *ex-vivo* vascular function in response to exogenously administered agents, including the vasoconstrictor PE, and the endothelial-dependent and -independent vasodilators MCH and SNP. Non-septic aged carotid arteries demonstrated an increased sensitivity to MCH (pEC_{50} young: 7.11±0.17; aged: 7.98±0.29; P=0.03) and SNP (pEC_{50} young: 7.52±0.15; aged: 8.45±0.30; P=0.02) compared to the young; however, there was no change in the percent maximal dilation achieved between age groups for either MCH (P=0.59) or SNP (P>0.99) (**Figure 2-8**). Carotid arteries from septic aged mice had no change in sensitivity to MCH compared to young septic mice (P=0.31), but had increased sensitivity to SNP (pEC_{50} young: 7.77±0.05; aged: 8.61±0.20; P=0.002) compared to their young septic counterparts. There was no change in the percent maximal dilation achieved between age groups for MCH (P=0.20) or SNP (P=0.30) (**Figure 2-8**).

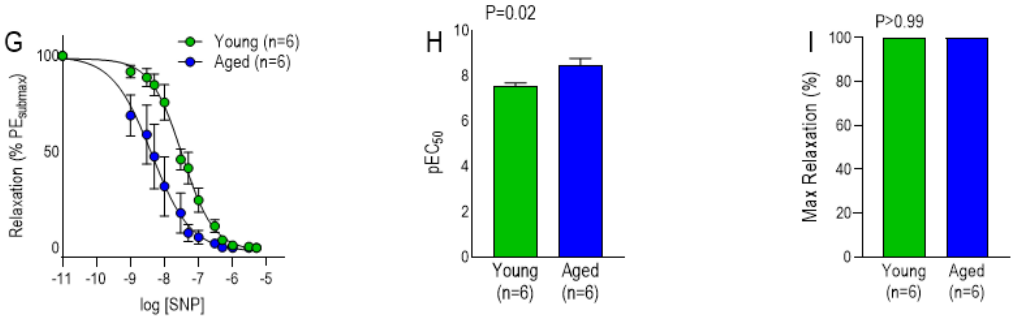
Control Carotid MCH



Septic Carotid MCH



Control Carotid SNP



Septic Carotid SNP

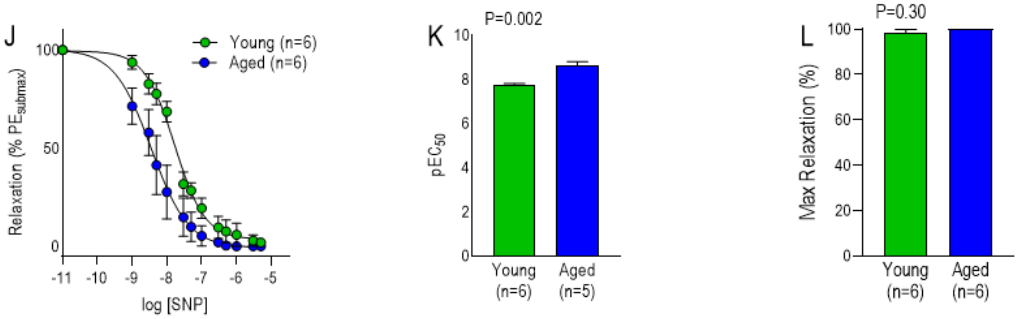
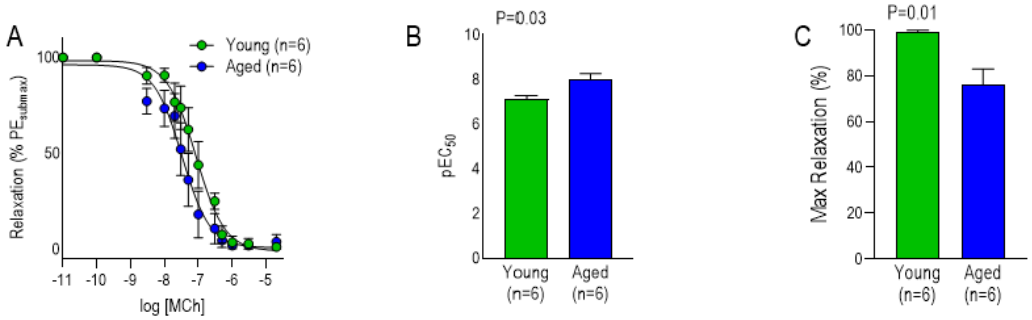


Figure 2-8: Wire Myography Carotid Arteries

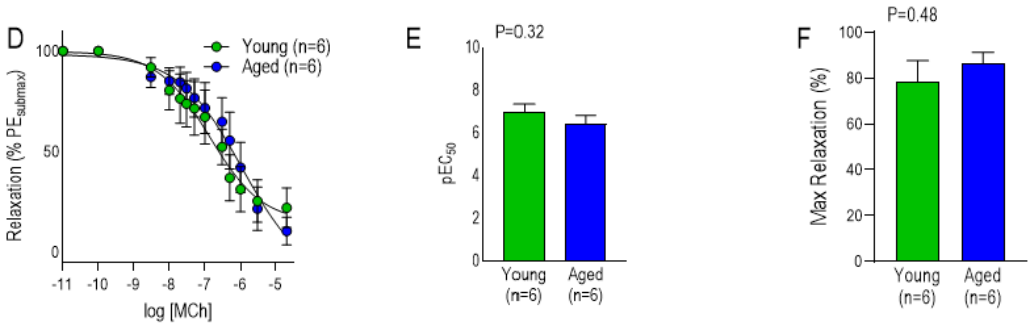
Control and septic *ex-vivo* wire myography for male carotid arteries depicting vasoactive parameters for young (green) and aged (blue) mice. (A, D, G, J) Carotid methacholine (MCH) and sodium nitroprusside (SNP) cumulative concentration-response curves. (B, E, H, K) pEC_{50} for carotid MCH and SNP cumulative concentration-response curves. (C, F, I, L) Maximum relaxation achieved by MCH and SNP administration.

Mesenteric arteries from non-septic aged mice had increased sensitivity to MCH (pEC_{50} young: 7.11±0.17; aged: 7.98±0.29; P=0.03) and decreased sensitivity to SNP (pEC_{50} young: 8.52±0.10; aged: 7.59±0.23; P=0.004) compared to the young. The percent maximal dilation achieved was less for MCH in the aged (young: 98.85±2.58; aged: 75.87±17.54; P=0.01); however, there was no change in the percent maximal dilation achieved between age groups for SNP (P=0.30) (**Figure 2-9**). Septic aged mesenteric arteries demonstrated no change in MCH or SNP sensitivities between the age groups (MCH P=0.32; SNP P=0.96) compared to the young. There was no change in the percent maximal dilation achieved between age groups for MCH (P=0.48) or SNP (P=0.30) (**Figure 2-9**).

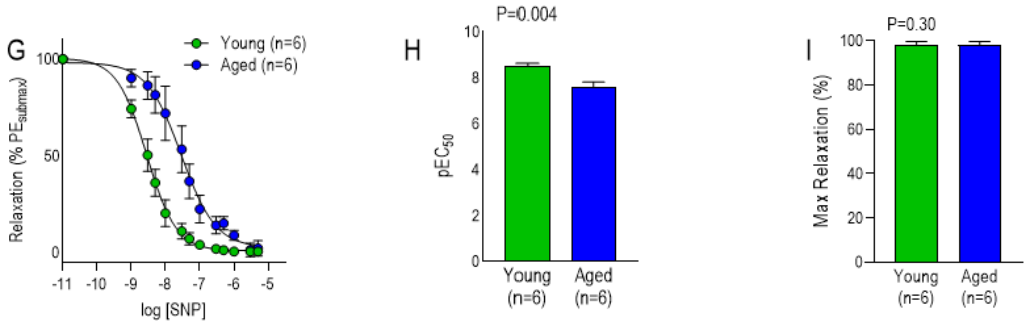
Control Mesentery MCH



Septic Mesentery MCH



Control Mesentery SNP



Septic Mesentery SNP

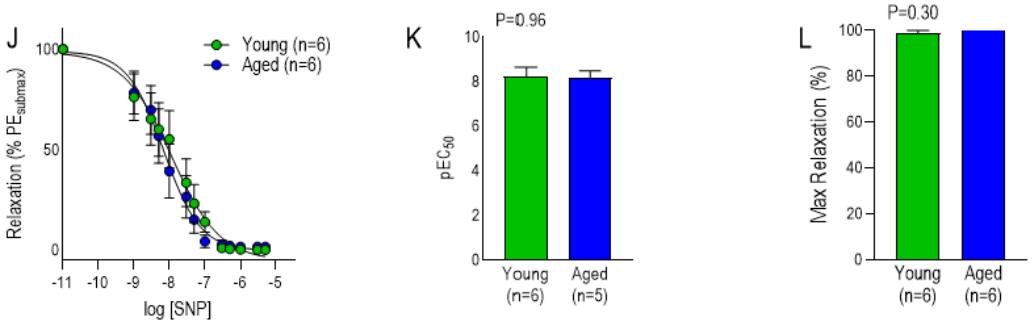
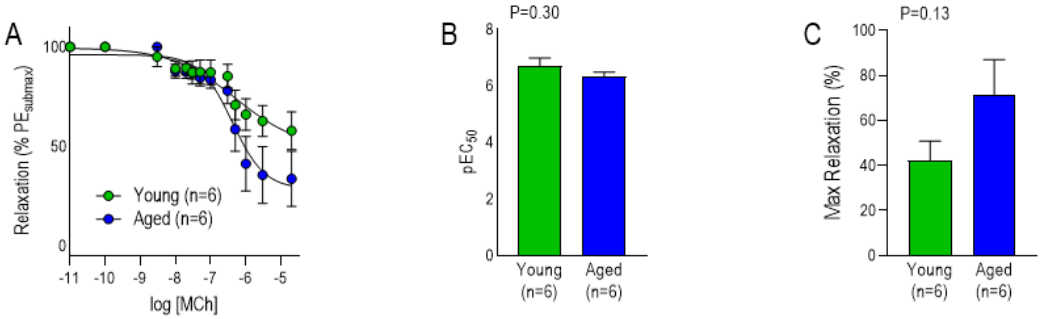


Figure 2-9: Wire Myography Mesenteric Arteries

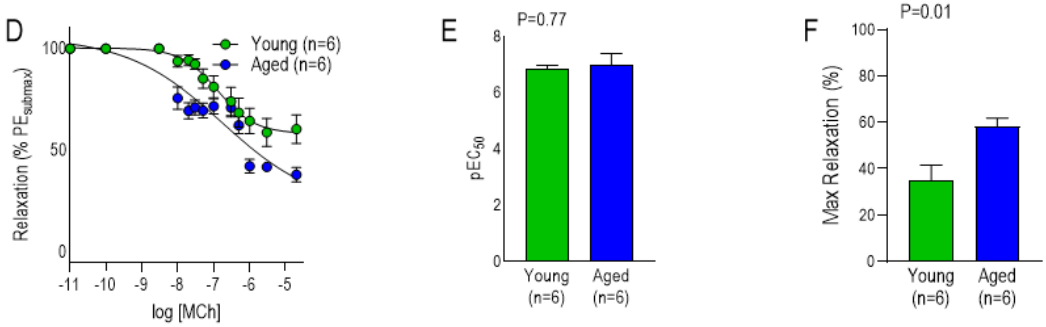
Control and septic *ex-vivo* wire myography for male mesenteric arteries depicting vasoactive parameters for young (green) and aged (blue) mice. (A, D, G, J) Mesentery methacholine (MCH) and sodium nitroprusside (SNP) cumulative concentration-response curves. (B, E, H, K) pEC_{50} for mesenteric MCH and SNP cumulative concentration-response curves. (C, F, I, L) Maximum relaxation achieved by MCH and SNP administration.

Incubation of isolated vessels with LNAME was used to examine *ex-vivo* septic vascular dysfunction in response to MCH- and SNP-induced vasodilation. Non-septic aged carotid arteries demonstrated an increased sensitivity to MCH (pEC_{50} young: 7.11±0.17; aged: 7.98±0.29; P=0.03) while SNP sensitivity (P=0.05) remained similar; however, there was no change in the percent maximal dilation achieved between age groups for either MCH (P=0.13) or SNP (P>0.99) (**Figure 2-10**). Septic aged carotid arteries demonstrated no change in MCH sensitivity between the age groups (P=0.77), but an increased SNP (pEC_{50} young: 8.17±0.13; aged: 8.68±0.08; P=0.008) sensitivity compared to the young. Percent maximal dilation was greater for MCH in the aged (young: 40.00±15.91, aged: 58.18±15.77, (P=0.01). There was no change in the percent maximal dilation achieved between age groups for SNP (P=0.30) (**Figure 2-10**).

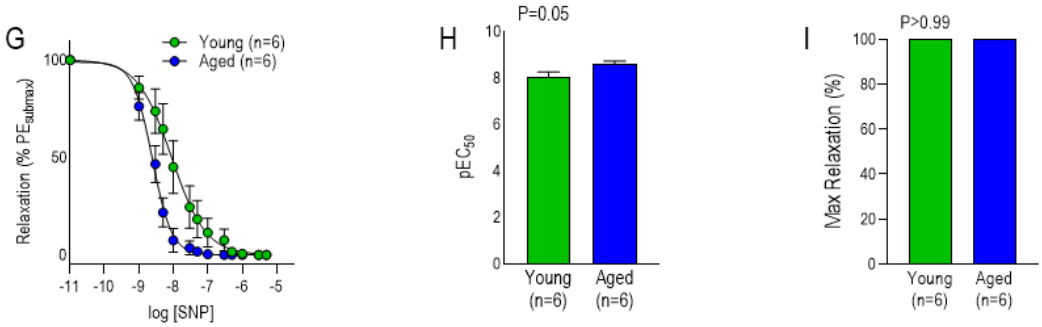
Control Carotid MCH



Septic Carotid MCH



Control Carotid SNP



Septic Carotid SNP

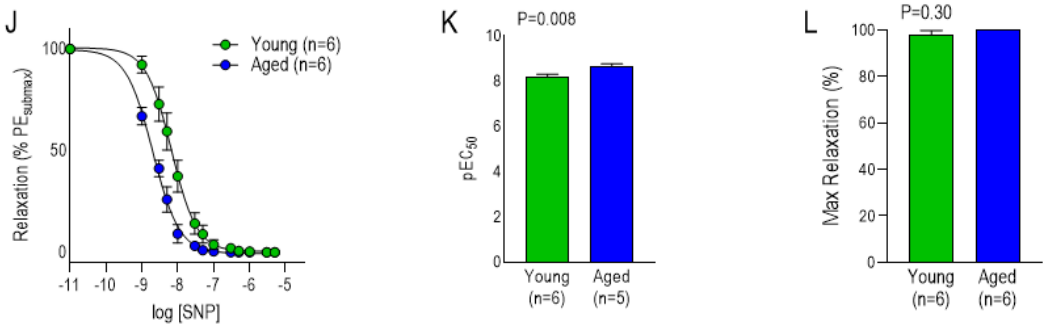
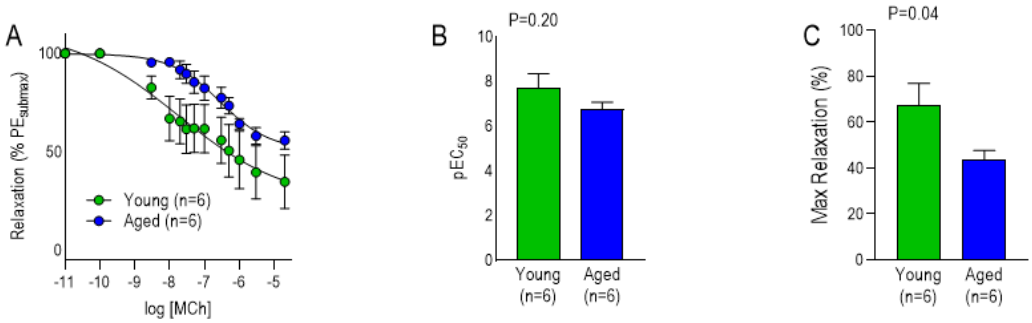


Figure 2-10: Wire Myography LNAME Carotid Arteries

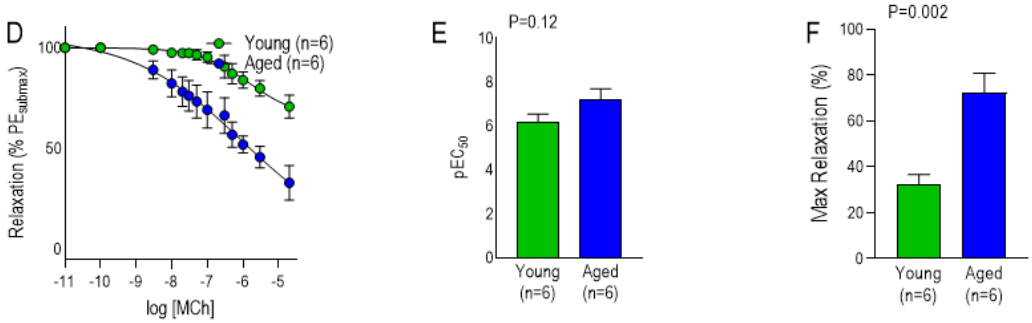
Control and septic *ex-vivo* wire myography for male carotid arteries incubated with L-NG-Nitro arginine methyl ester (LNAME) depicting vasoactive parameters for young (green) and aged (blue) mice. (A, D, G, J) carotid methacholine (MCH) and sodium nitroprusside (SNP) cumulative concentration-response curves. (B, E, H, K) pEC_{50} for MCH and SNP cumulative concentration-response curves. (C, F, I, L) Maximum relaxation achieved by MCH and SNP administration.

Non-septic aged mesenteric arteries incubated with LNAME demonstrated no change in MCH sensitivity (P=0.20) but had decreased sensitivity to SNP (pEC_{50} young: 8.53±0.13; aged: 7.87±0.26; P=0.04) compared to the young. The percent maximal dilation achieved was less for MCH in the aged (young: 67.64±22.56; aged: 43.46±10.42; P=0.04); however, there was no change in the percent maximal dilation achieved between age groups for SNP (P=0.13) (**Figure 2-11**). Septic aged mesenteric arteries demonstrated no difference in MCH sensitivity between the age groups (P=0.12) or SNP sensitivity (P=0.43) compared to the young. There was an increase in maximal dilation in the aged receiving MCH (young: 32.33±10.56, aged: 72.16±20.87, P=0.002) and no change in the percent maximal dilation achieved between age groups for SNP (P=0.64) (**Figure 2-11**).

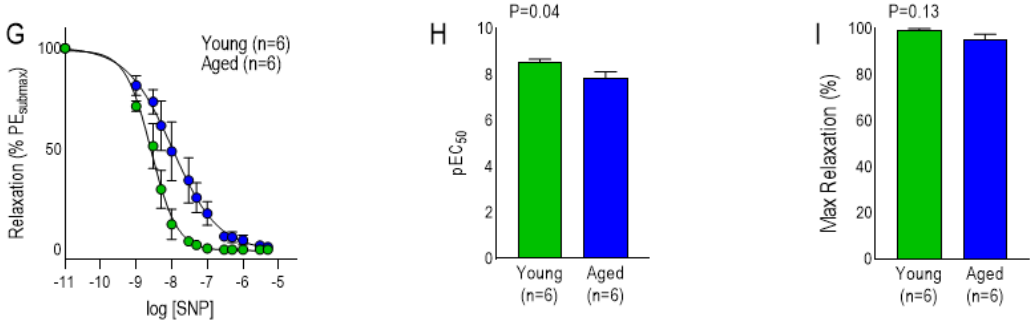
Control Mesentery MCH



Septic Mesentery MCH



Control Mesentery SNP



Septic Mesentery SNP

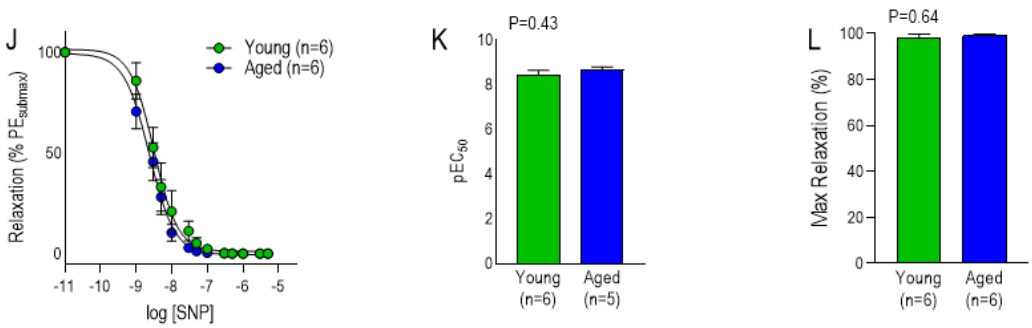
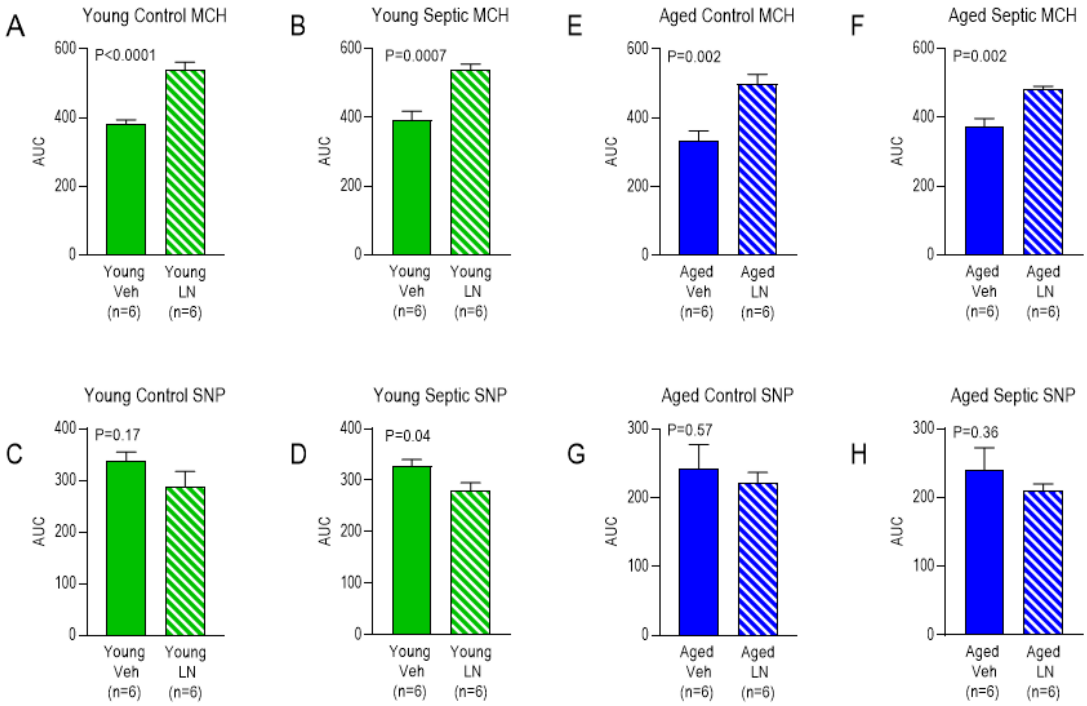


Figure 2-11: Wire Myography LNAME Mesenteric Arteries

Control and septic *ex-vivo* wire myography for male mesenteric arteries incubated with L-N^G-Nitro arginine methyl ester (LNAME) depicting vasoactive parameters for young (green) and aged (blue) mice. (A, D, G, J) Mesenteric methacholine (MCH) and sodium nitroprusside (SNP) cumulative concentration-response curves. (B, E, H, K) pEC_{50} for MCH and SNP cumulative concentration-response curves. (C, F, I, L) Maximum relaxation achieved by MCH and SNP administration.

Data demonstrating comparison between young control and LNAME treated vessels and aged control compared to aged LNAME treated vessels are presented in **Figure 2-12**.

Carotid



Mesentery

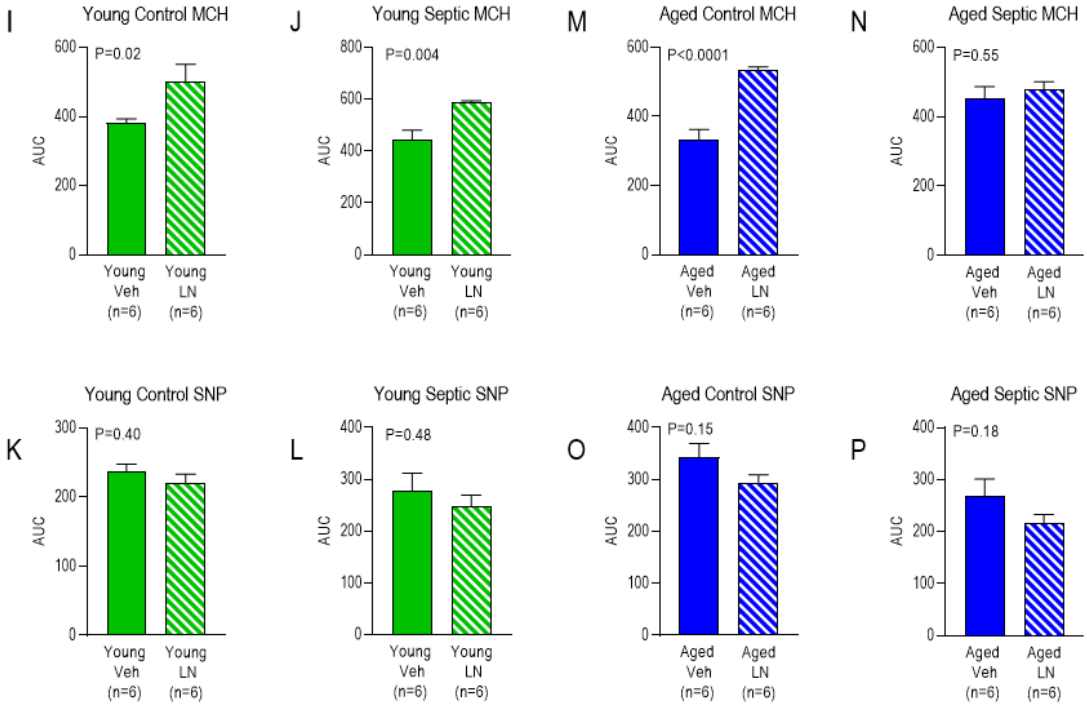


Figure 2-12: Control versus LNAME treated Vessels

Data depicting *ex-vivo* wire myography area under the curves (AUC) for age-wise comparisons for control and L-N^G-Nitro arginine methyl ester (LNAME) treated vessels incubated with methacholine (MCH) and sodium nitroprusside (SNP). A, B, C, and D represent young carotid arteries. E, F, G, and H represent aged carotid arteries. I, J, K, and L represent young mesenteric arteries. M, N, O, and P represent aged mesenteric arteries.

Non-septic aged carotid arteries demonstrated no change in sensitivity to PE (P=0.19) compared to the young, nor did septic aged carotid arteries (P=0.23). Non-septic and septic aged mesenteric arteries also demonstrated no difference in PE sensitivity between the age groups (P=0.32; P=0.10) (**Figure 2-13**).

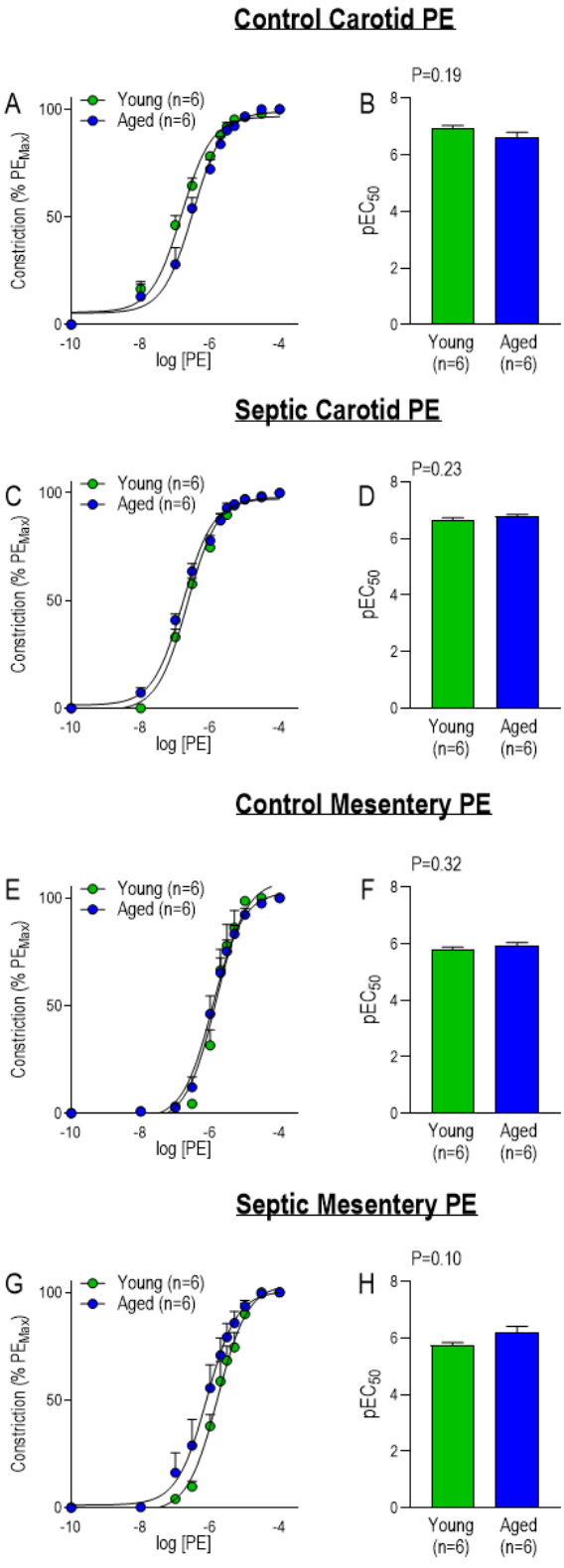

Control Carotid PE
A
Young (n=6)
Aged (n=6)
Constriction (% PE$_{Max}$)
log [PE]
B
P=0.19
pEC$_{50}$
Young (n=6)
Aged (n=6)
Septic Carotid PE
C
D
P=0.23
Control Mesentery PE
E
F
P=0.32
Septic Mesentery PE
G
H
P=0.10

Figure 2-13: Wire Myography Vasoconstriction

Control and septic *ex-vivo* wire myography for male carotid and mesenteric arteries depicting vasoactive parameters for young (green) and aged (blue) mice. (A, C, E, J) Phenylephrine (PE) cumulative concentration-response curves (B, D, F, H) pEC_{50} for PE cumulative concentration-response curves.

Organ cGMP production was assessed in the lung and kidney. In the lung, septic young mice produced an increased amount of cGMP compared to non-septic lung, as an indirect measurement of sGC activity (non-septic: 3.47±1.14 pmol of cGMP per mg of protein produced in 30 minutes; septic: 7.09±0.46 pmol of cGMP per mg of protein produced in 30 minutes; an increase of 3.61±1.14 pmol of cGMP per mg of protein produced in 30 minutes; P=0.01). In contrast, cGMP production in aged lung tissue was not changed in septic and non-septic mice (non-septic: 11.22±3.87 pmol of cGMP per mg of protein produced in 30 minutes; septic: 5.27±1.16 pmol of cGMP per mg protein produced in 30 min; a difference of -5.95±4.04 pmol of cGMP per mg of protein produced in 30 minutes; P=0.17) (**Figure 2-14: A**).

In the young kidney, cGMP production was unchanged between non-septic and septic conditions (non-septic: 7.03±1.88 pmol of cGMP per mg of protein produced in 30 minutes; septic: 6.54±1.47 pmol of cGMP per mg of protein produced in 30 minutes; P=0.84). cGMP production in aged kidney tissue did not demonstrate a change (non-septic: 8.00±3.34 pmol of cGMP per mg of protein produced in 30 minutes; septic: 2.60±0.69 pmol of cGMP per mg of protein produced in 30 minutes; P=0.21) (**Figure 2-14: B**).

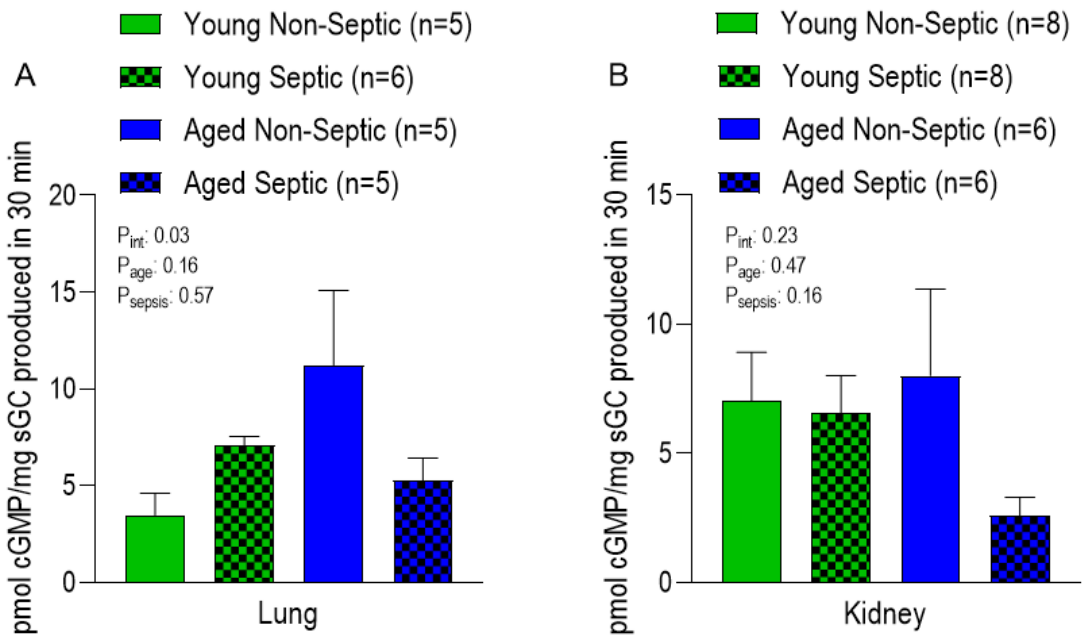


Figure 2-14: cGMP Production

Production of cyclic guanosine monophosphate (cGMP) (pmol) per mg of protein produced in 30 minutes as an indirect measurement of soluble guanylate cyclase (sGC) activity in (A) lung and (B) kidney in young and aged non-septic and septic mice.

Table 7: Summary of Non-Wire Myography Data

DBP: diastolic blood pressure; HR: heart rate; MCH: methacholine; NE: norepinephrine; SBP: systolic blood pressure; sGC: soluble guanylate cyclase

Summary	Effect of ↑ Age
SBP	Nil
DBP	Nil
HR	Nil
SBP-MCH	↑
DBP-MCH	↑
HR-MCH	Nil
SBP-septic	↓
DBP-septic	↓
HR-septic	Nil
SBP-septic MCH	Nil
DBP-septic MCH	↓
HR-septic MCH	Nil
Survival	↓
NE	↓ dose, time infusing
sGC Activity Lung	Nil
sGC Activity Kidney	Nil

Table 8: Summary of Wire Myography Data

MCH: methacholine; SNP: sodium nitroprusside

Effect of Age	
Carotid Arteries	**Mesenteric Arteries**
Non-Septic arteries: • ↑ sensitivity to MCH and SNP, no change for PE • No change in maximal vasorelaxation or vasoconstriction	Non-septic arteries: • ↑ sensitivity to MCH, no change for SNP or PE • ↑ maximal vasorelaxation to MCH, no change with SNP, no change in maximum vasoconstriction
Septic arteries: • ↑ sensitivity to SNP, no change to MCH or PE • No change in maximal vasorelaxation or vasoconstriction	Septic arteries: • No change in sensitivity to MCH, SNP, or PE • No change in maximal vasorelaxation or vasoconstriction
LNAME Non-Septic arteries: • ↑ sensitivity to MCH, no change to SNP or PE • No change in maximal vasorelaxation or vasoconstriction	LNAME Non-Septic arteries: • ↓ sensitivity to SNP, no change for MCH or PE • Less maximal vasorelaxation to MCH, no change for SNP, no change in vasoconstriction
LNAME Septic arteries: • ↑ sensitivity to SNP, no change to MCH or PE • No change in maximal vasorelaxation or vasoconstriction	LNAME Septic arteries: • No change in sensitivity to MCH, SNP, PE • No change in maximal vasorelaxation or vasoconstriction

2.4 Discussion

This study aimed to determine whether endothelial-dependent vasodilation is altered in aged mice resulting in harmful blood pressure and survival responses to sepsis. Before

sepsis induction, the young and aged mice had similar systolic blood pressure, diastolic blood pressure, and heart rate (**Figure 2-4: A-C**). The elderly have clinical hemodynamic changes, including increased systolic blood pressure, decreased diastolic blood pressure, and increased pulse pressure, resulting from a stiffened vasculature.[537,538] We expected the aged mice to demonstrate an increased baseline systolic blood pressure with reduced diastolic blood pressure. However, these baseline hemodynamics parameters were measured following induction of anesthesia with inhaled isoflurane, which may have a greater effect on aged cardiovascular function in both normal and pro-inflammatory disease states.[539-541]

In response to MCH administration, a greater decrease in systolic and diastolic blood pressure without a change in heart rate was observed in the aged mice (**Figure 2-4: D-F**). No change in the heart rate was meaningful as the heart rate can affect blood pressure as a contributor to cardiac output. With dysfunctional (uncoupled) eNOS combined with decreased substrate and cofactors for NO production, it may be expected that MCH would be less efficacious in in the elderly. However, there are two potential mechanisms that may contribute to increased responsiveness. First, reduced NO is associated with sensitization of sGC.[542] As such, after a prolonged state of relative depletion, endothelial-dependent activation with MCH may be exacerbated, even with reduced NO bioavailability. MCH may also stimulate vasodilation by activating endothelial-derived hyperpolarizing factors (EDHF) via the release of endothelial cell epoxyeicosatrienoic acid (EET - cytochrome P450 epoxygenase-induced products of arachidonic acid[540,543]) or activation of certain calcium-related K^+ channels[544] (resulting in hyperpolarization and relaxation of vascular

smooth muscle). Effects of EDHF in the aged may be upregulated due to the reduced reliance on a functional NO-sGC-cGMP signalling axis as has been shown in other disease states.[545] The aged may also respond with increased activity of prostaglandin-induced vasodilation.[546]

Despite similar baseline non-septic hemodynamic parameters as seen in **Figure 2-4**, aged mice demonstrated a greater decrease in both systolic and diastolic blood pressure following the induction of sepsis compared to the young mice, again, with no difference in heart rate (**Figure 2-5: A-C**). This hypotensive effect was observed to be maintained throughout the course of sepsis. This was an expected effect as elderly septic patients have been observed to develop more severe hypotension as a result of inability to produce and maintain sufficient levels of endogenous catecholamines.[547]

It is important to acknowledge that a comparison of hemodynamic parameters could only be carried out until 180 minutes after induction of sepsis, as 67% of the aged mice had met humane endpoints for euthanization (mean arterial pressure less than 20 mmHg) by this point. The observation that aged septic mice became less MCH-responsive over time while the young demonstrated some increase may be due to the young mice relying on multiple aspects of vasodilation, including functional NOS signalling, prostaglandins, and EDHF, as no one system was overly relied upon and potentially at a point of exhaustion. When NOS signalling became impaired by sepsis, other vasodilatory systems were able to compensate. In aged mice, which are more reliant on EDHF than NOS signalling under baseline conditions,[548] the EDHF system had potentially exhausted its

ability to upregulate further and provide increased vasodilation levels when sepsis further impaired NOS signalling.

Adding to the complexity of interpreting these *in-vivo* results is the presence of several other age-related vascular changes. As above, aging involves multiple signalling pathways (integrated with dysfunctional NO signalling and increased reliance on EDHF-related vasodilation on a molecular, cellular, and systemic level) that work in concert to facilitate vascular function, the precise roles of which, herein, are unclear.[549,550] Young septic mice were also observed to survive sepsis better than the aged mice (**Figure 2-6**). This was an expected outcome due to the aforementioned vascular changes that occur with aging combined with the pro-inflammatory milieu, harmful effects of excessive iNOS-induced NO production seen in sepsis, and decreased endogenous catecholamine production.[547]

To further examine the influence of catecholamines, the effect of NE, a standard treatment for patients experiencing septic shock, was examined. NE is known to have multiple cardiovascular effects, including stimulation of the α_1 receptors present on the vascular smooth muscle causing vasoconstriction, increase in heart rate due to the β_1 impacts on the pacemaker cells in the heart, increase in cardiac contractility via β_1 contractile myocardial cells, and stimulation of endothelin-1 (ET-1) release. NE did not improve sepsis survival in young mice, but did increase survival in the aged; however, the survival times of both age groups treated with NE was similar (**Figure 2-7: A**). Aged septic mice received less total NE than the young and had less percentage of total septic time with NE infusing despite having similar survival times when treated with NE (**Figure 2-7: B,**

C). Aging is associated with decreased levels of NE, possibly as a result of reduced synthesis from the adrenal gland.[551] During sepsis, the elderly may be more at risk of critical illness-related corticosteroid insufficiency and may experience a benefit from the repletion of deficient NE. In contrast, the administered NE in the young is likely surplus, resulting in potentially harmful vasoconstriction leading to compromised blood flow regulation in an already compromised system. The result of decreased NE required may have important consequences for the elderly who often have more cardiac comorbidity and may be increasingly intolerant of arrhythmia, a common side effect of NE.

In *ex-vivo* experiments and under non-septic conditions, the aged carotids demonstrated increased sensitivity to MCH and SNP to produce the same maximal dilation as in the young. As the carotids are larger capacitance vessels, they may rely more on NO than EDHF.[552] But it is also important to consider that maintaining blood flow to the brain under times of stress is critically important. Therefore, the influence of EDHF on carotid arteries may be greater than in other larger capacitance vessels[553,554] and aged carotid arteries have been shown to demonstrate increased reliance on EDHF.[555] As the endothelial-dependent vasorelaxation was more sensitive in the aged than the young, it could be inferred that the MCH induced a greater change than would generally be seen in the young, potentially secondary to increased MCH-EDHF stimulation. Chronic inflammation including the increased ROS seen in the aged may decrease reliance on the NOS signalling system (due to uncoupled eNOS and oxidized sGC) and upregulate the EDHF response. As the aged mice were deficient in bioavailable NO, a smaller dose of SNP would likely produce a larger reaction than in the young with a functional NO

signalling axis (**Figure 2-8**). When septic carotid arteries were examined *ex-vivo*, the same amount of MCH was required to produce a similar maximal vasorelaxation in both age groups. Although sepsis can impair EDHF[243] through the actions of NO produced by iNOS, ROS and bradykinin (present due to sepsis) can upregulate the EDHF system.[234,556,557] Therefore, increased MCH-EDHF stimulation may be seen in the young due to greater impairment of signalling through the sepsis-damaged NO signalling pathway, as is normally present in the aged.

Considering SNP administration, aged septic carotid arteries were observed to possess increased sensitivity to produce similar maximal dilation. In this case, the aged likely displayed increased sGC sensitivity to NO due to baseline decreases in bioavailable NO as well as having EDHF pathways that were likely highly functional to compensate for poor baseline NO signalling.

Mesenteric arteries are known as resistance vessels and have a greater reliance on EDHF mechanisms than NO signalling.[558] In our aged non-septic mesenterics, MCH produced a lesser amount of maximal vasorelaxation with a decreased concentration than observed in the young. This may be as above, where mesenteric arteries rely more heavily on EDHF. Therefore, in the aged, where the NO signalling is impaired at baseline, EDHF is of particular importance. Thus, the greater EDHF signal attempts to compensate for the more minor input from the NO signalling system secondary to MCH stimulation. In sepsis, the MCH stimulation should and did produce similar amounts of maximal vasorelaxation with similar MCH doses (**Figure 2-9**). This is likely related to sepsis-damaged NO signalling pathways as well as septic bradykinin stimulation resulting in increased reliance

of the young on EDHF pathways.[559,560] In non-septic mesenteric arteries, the aged required a similar SNP dose to produce similar maximal vasodilation. Sepsis was observed to similarly effect the young and aged mesenteric arteries such that no difference in SNP sensitivity or maximal vasorelaxation was noted (**Figure 2-10**).

We used LNAME (100 μmol/L) as a tool to examine how the absence of NOS-generated NO affects aged vascular function. We observed that NOS inhibition in non-septic carotid arteries resulted in no change in sensitivity to MCH or SNP dose; as well, no change in maximal relaxation was observed for either MCH or SNP. However, impairing NO bioavailability with NOS inhibition in mesenterics may have decreased sGC sensitivity as there was decreased NO available and sGC may have been oxidized by age-associated ROS. LNAME also equalized the maximal relaxation of both groups. NOS inhibition with LNAME resulted in the aged septic carotid and mesenteric arteries displaying increased maximal relaxation in response to similar doses of MCH. This was potentially secondary due to increased reliance on EDHF. Aged septic carotids were more sensitive to SNP without a change in maximal dilation, likely due to SNP-donated NO restoring some level of function in the highly damaged signalling system (**Figures 2-10, 2-11**).

There were no differences in the dose of PE required to produce a maximal vasoconstriction response between the young and aged in either carotid or mesenteric arteries. Sepsis also did not affect a change in PE dose between the young and aged (**Figure 2-12**). There is some evidence that α_1-adrenergic receptor sensitivity is decreased in the elderly.[561] However, aged vessels may be more reactive to α_1-adrenergic receptor activation,[562] potentially compensating for the reduced sensitivity. Sepsis can also lead to

desensitization of α_1-adrenergic receptors, but as the elderly remain more reactive to α_1-adrenergic receptor activation, the response to phenylephrine could be similar.

In the young, sepsis increased cGMP production in the lung as an indirect measurement of sGC activity. This was a likely outcome due to increased iNOS-induced NO production due to sepsis. This NO can then stimulate sGC to produce cGMP. The aged mice did not display an increase in cGMP production in the lung when exposed to sepsis. cGMP levels did not increase in either the young or aged kidneys. Although NO signalling is essential for renal function, sepsis decreases blood flow to the kidney as an 'expendable' organ to maintain perfusion to 'vital' organs such as the brain and the heart.[563,564] This data suggests that the sGC activity in tissues other than the vasculature may be what is impacting survival (**Figure 2-14**).

2.5 Limitations

This study used a model of septic shock with an intra-abdominal source. The fecal slurry dose (1.3 mg/g body weight) may have resulted in a septic shock phenotype too severe to elicit all changes in *in-vivo* hemodynamic and *ex-vivo* vascular dysfunction. Potentially a less acute model may demonstrate more subtle findings. Other models of sepsis may demonstrate different findings. Aged mice were 22-24 months old. Perhaps further survival and vascular findings would be evident with an older group; however, as above, this murine age approximates a human age when sepsis is common and therefore of interest to our group. This study omitted the use of antibiotics in these first series of experiments included herein as antibiotics may themselves impact vascular function[565] and the interest was in

observing the isolated effects of sepsis. Future studies will include use of antibiotics for comparison to these initial findings.

2.6 Conclusion

The elderly have increased morbidity and mortality in sepsis. The observation included herein have shown aged mice have a baseline exaggerated blood pressure response to MCH-induced vasodilation. When septic, the aged lose this response. This study demonstrated that aged mice had reduced septic survival compared to the young and improved survival when treated with NE. In wire myography experiments, the response to MCH and SNP were impacted by age, sepsis, and NOS inhibition by LNAME. Levels of cGMP increased in young septic lungs but did not increase in young septic kidneys. Aged lungs and kidneys did not produce more cGMP in response to sepsis. Further delineation of the contribution of NO and EDHF signalling pathways in the aged vascular response to sepsis will improve our understanding of why the aged septic population is at higher risk of organ failure and mortality.

Chapter 3

Cinaciguat Improves Blood Pressure and Survival in a Murine Model of Sepsis

3.1 Background

Nitric oxide (NO) is an important vasodilatory molecule with many functions including inhibition of smooth muscle proliferation, platelet aggregation, and leukocyte recruitment.[566] Importantly, NO can also affect blood vessel tone by stimulating the production of cyclic guanosine monophosphate (cGMP) by activation of the downstream enzyme soluble guanylate cyclase (sGC).[567] cGMP ultimately induces vasodilation.[567] However, in states of oxidative stress, dysfunctional sGC is increasingly recognized as a contributor to many disease states involving the cardiovascular system.[568] One such disease affecting sGC function is sepsis, a life-threatening condition of organ dysfunction secondary to infection.[1] In sepsis, high NO levels are produced secondary to increased inducible nitric oxide synthase (iNOS) function.[569] However, sepsis also produces high levels of reactive oxygen species (ROS) through NADPH-oxidases, which oxidize sGC to a heme-free NO-unresponsive form.[570-573] Septic ROS production is also associated with reduced sGC transcription and instability of sGC mRNA.[574] The overall result of disrupting the NO-sGC-cGMP signalling pathway is vascular endothelial dysfunction, a major contributing factor in developing refractory septic vasodilation known as vasoplegia.

Two classes of pharmacologic treatments to initiate sGC function have been brought to the forefront, the sGC stimulators and the sGC activators.[568] sGC stimulators have been examined for their role in improving symptoms in patients with pulmonary hypertension.[575] By binding to the heme-containing reduced form of sGC, sGC stimulators

can sensitize sGC to low NO levels.[576] Since sGC stimulators target all sGC molecules throughout the body, treatment can cause systemic hypotension in addition to reducing symptoms of pulmonary hypertension, and therefore is not well tolerated in all patients.[577] sGC activators act differently; they specifically bind and activate the heme-free oxidized sGC, thereby preventing is degradation. This restores cGMP signalling in vessels with high levels of oxidative stress. Because they preferentially target the heme-free oxidized sGC over the native form, sGC activators like cinaciguat may cause less systemic hypotension (although non-selective targeting of the native form can occur at high levels of drug[578]).

In sepsis, a mainstay of treatment involves ensuring hemodynamic stability (i.e. maintaining blood pressure above clinical thresholds). However, doing so without consideration of organ blood flow may be misleading. Indeed, many vasopressor medications used to maintain blood pressure levels do so by causing vasoconstriction, which may reduce end-organ blood flow, and thus compromise patient health and safety. Thus, prior to circulatory collapse (when vasopressor medications are needed), agents that selectively target damaged vasculature and restore blood flow may improve outcomes in sepsis. In this regard, sGC activators are an intriguing potential sepsis treatment to improve vascular function, organ function, and survival outcomes. Fully understanding the pathophysiologic contribution of dysfunctional sGC in sepsis is important as it provides a potential therapeutic target for a disease that remains the leading cause of intensive care unit (ICU) admission, morbidity, and death. We hypothesize that early sepsis treatment with an sGC activator (cinaciguat) will improve *in-vivo* and *ex-vivo* vascular function and improve sepsis survival without causing life-threatening hypotension.

3.2 Methods

This study was conducted according to the Canadian Council on Animal Care guidelines, with approval from the Animal Care and Use Committee from the University of Alberta (Edmonton, Canada), and reported in adherence with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) Guidelines.

3.2.1 Animals and Preparation

Ninety-six male C57BL/6 mice (procedure- and drug-administration-naïve, with no genetic modifications) aged 3-4 months were purchased from Charles-River Inc. (Saint-Constant, QC). Mice were multiply housed (five per cage) in standard shoebox cages containing aspen-chip bedding, nesting material, and PVC tubing for environmental enrichment. Cages were located in the University of Alberta animal care facility, where a 12h:12h light:dark cycle and an ambient temperature of 22±1ºC were maintained. Trained animal care personnel routinely performed welfare assessments before experimentation. Mice had *ad libitum* access to tap water and standard grain-based rodent chow (PicoLab 5LOD, LabDiet, St. Louis, MO).

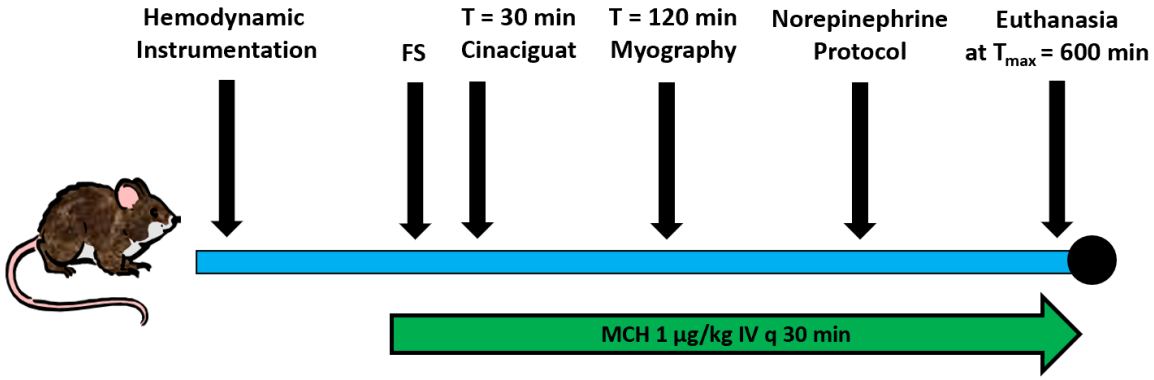
Experiments were conducted in the same laboratory operating theatre during the day. Mice were anesthetized with inhaled isoflurane (induction: 5%, maintenance: 1.1-1.5% in 100% O_2) and were kept spontaneously breathing via a nosecone. Throughout the experiment, mice were maintained on a warmed surgical table, and body temperature was monitored via a rectal thermometer. Following induction of anesthesia, mice were instrumented with a polyethylene catheter (PE10; inner diameter 0.58 mm, outer diameter

0.97 mm) in the left femoral vein for drug delivery and a glass fibreoptic transducer catheter (FISO 0.9 Fr/0.5 Fr; Harvard Apparatus, Harvard BioScience Inc.) in the right carotid artery for hemodynamic assessments. The femoral venous catheters contained heparinized sterile 0.9% saline (25 units/mL). Following hemodynamic instrumentation, mice were allowed 30 minutes to achieve stable baseline temperature and hemodynamic variables. The mice were administered hydration with sterile saline (2 mL/kg/hour IV). Arterial blood pressure and heart rate were monitored and recorded via the indwelling FISO catheter connected to a data acquisition system (Lab Chart Pro 8, ADInstruments, Colorado Springs, CO).

3.2.2 Study Protocol

Fecal slurry (1.3 mg/g IP) was administered to induce sepsis (dose determined in our pilot studies). Blood pressure, heart rate, and temperature were recorded. Mice received cinaciguat (15 µg/kg IV – the most effective dose in our pilot studies) 30 minutes after fecal slurry injection; age-matched controls received an equivalent volume of vehicle (60% phosphate-buffered saline, 20% diethylene glycol monoethyl ether, and 20% cremophor). Vascular reactivity was assessed via methacholine (MCH; 1 µg/kg IV – a dose that caused hypotension lasting under 10 seconds due to decreased systemic vascular resistance; this dose did not affect heart rate – see **Figure 2-1**) injection every 30 minutes. Subsets of septic mice were administered norepinephrine (NE) to assess the reversibility of cardiovascular collapse. NE infusion was initiated at 200 µg/kg/hour and advanced by 100 µg/kg/hour in five-minute intervals until the mean arterial pressure was greater than 65 mmHg or systolic

blood pressure greater than 80 mmHg was reached to a maximum of 600 µg/kg/hour (**Figure 3-1**).



Cinaciguat: 15µg/kg IV 30 minutes post-FS
FS: fecal slurry
MCH: methacholine
Norepinephrine Protocol: titrate norepinephrine 200-600 µg/kg/h IV when MAP < 65 mmHg or SBP < 80 mmHg

Figure 3-1: Study Protocol

Schematic of study protocol including methacholine MCH) and norepinephrine (NE) administration.

3.2.3 Wire Myography Protocol

Mice randomized to wire myography experiments were maintained under anesthesia for 120 minutes following induction of sepsis and subsequently euthanized by exsanguination and excision of the heart. Left common carotid arteries and second-order mesenteric arteries were rapidly removed and placed in ice-cold HEPES-buffered physiologic saline solution (PSS – composition: (in mM) HEPES 10, glucose 5.5, $CaCl_2$ 1.56, KCl 4.7, NaCl 142, $MgSO_4$ 1.17, KH_2PO_4 1.18, pH 7.45). Vessels were cleaned of extraneous perivascular connective tissues, and small vessel sections (2 mm in length) were isolated and mounted in an isometric myograph system (DMT, Copenhagen, Denmark); 15 µm gold wire was used for mesenteric arteries, and 40 µm tungsten wire was used for the

carotid arteries. Vessels were assessed at 37ºC and normalized to optimal resting tension (0.8 IC_{100}: the internal circumference equaling a transmural pressure of 100 mmHg) by small incremental increases in diameter. Vessels were then allowed 30 minutes to equilibrate to the optimal resting tension before initiating the experiment. Vessels were subsequently tested for viability by high potassium PSS (KPSS) treatment. After rinsing, vessels were treated with phenylephrine (PE: 10 µmol/L) twice, with a rinse and return to baseline tension in between doses; following the second dose of PE, MCH (3 µmol/L) was administered to test the integrity of the endothelium. Following endothelial assessment, vessels were rinsed and returned to baseline. Concentration-response curves for PE were performed. Next, a subset of vessels was incubated with non-selective nitric oxide synthase (NOS) inhibitor L-N^G-nitro-arginine-methyl ester (LNAME: 100 µmol/L) for 30 minutes before performing cumulative concentration-response curves with MCH and sodium nitroprusside (SNP); vessels were sub-maximally pre-constricted (80% maximum: EC_{80}) with PE. Finally, at the end of each experiment, vascular constriction to KPSS was assessed; those vessels with a final KPSS constriction of less than 80% of the first KPSS constriction were excluded from analyses.

3.2.4 Assessment of sGC Production

sGC activity was assessed indirectly by measuring cGMP production in two end-organs commonly acutely affected by sepsis, the lung and the kidney. Fresh left lungs and left kidneys were harvested from a subset of non-septic and septic mice 120 minutes post-vehicle or fecal slurry injection under isoflurane anesthetic. Lungs and kidneys were homogenized in EDTA, Tris-HCL, sucrose, dithiothreitol, protease inhibitor cocktail

(Sigma, St. Louis, MO), and sodium orthovanadate. Homogenized tissue was centrifuged (10,000 x *g*, 4°C, 20 minutes) and had total protein quantified using the bicinchoninic acid protein assay. Cytosolic protein was added to a reaction buffer mixture containing bovine serum albumin, benzamidine HCL, SNP, LNAME, 3-isobuyl-1-methylxanthine, L-cysteine, and GTP (Sigma, St. Louis, MO). This mixture was incubated for 30 minutes at 37°C in a shaking water bath. The reaction was terminated with zinc acetate and sodium carbonate (Sigma, St. Louis, MO). This mixture was then centrifuged (5,000 x *g*, 4°C, 10 minutes). cGMP was then quantified with the Cayman Chemical Cyclic GMP ELISA kit (Ann Arbor, MI, USA). Samples were assessed under three cinaciguat concentrations 0 µM, 10 µM, and 100 µM.

3.2.5 Reagents

Cinaciguat (Sigma, St. Louis, MO) was prepared in 60% phosphate-buffered saline, 20% diethylene glycol monoethyl ether, and 20% cremophor. MCH and NE (Sigma, St. Louis, MO) were dissolved in sterile saline (0.9% NaCl) for *in-vivo* and subsequently diluted in a PSS for final use in myography experiments. PE, SNP, and LNAME (Sigma, St. Louis, MO) were also dissolved in sterile saline and diluted in PSS for final use in myography experiments.

3.2.6 Statistical Analysis

Data are presented as mean±SEM. Patterns of cardiovascular collapse (as depicted by Kaplan-Meier curves) were analyzed by log-rank tests. Continuous variables, including arterial pressures, heart rates and hemodynamic responsiveness to exogenous

pharmacological agents were analyzed by repeated measures 2-way ANOVA for the overall effects of sepsis and time; subjects were matched within the time domain. The remaining data were analyzed by unpaired Student's *t*-test. For collection of septic systolic blood pressure, diastolic blood pressure, and heart rate, the most representative five-second segment within each 30-second interval was captured from recorded parameters. Percent changes in hemodynamic variables in response to MCH administration were calculated as the minimum value compared to the baseline in the immediately preceding five-minute interval. For wire myography, concentration-response curves were fitted to the Hill equation using a variable slope, and pEC_{50} (mean effective concentration to produce a 50% response) and maximal responses were analyzed by unpaired *t*-test. cGMP production was analyzed with 2-way ANOVA and Tukey's multiple comparison test. Statistical outliers determined by Grubb's test were not included. Results were considered significant if $P<0.05$. GraphPad Prism 8 software (La Jolla, CA) was employed for statistical analysis.

3.3 Results

There were no differences in baseline hemodynamic characteristics (systolic blood pressure, diastolic blood pressure, heart rate) between mice randomized to the cinaciguat treatment versus the control group (**Figure 3-2: A-C**). There were also no baseline differences (% change in systolic blood pressure, % change in diastolic blood pressure, % change in heart rate) between cinaciguat-treated and control groups in response to *in-vivo* administration of MCH (**Figure 3-2: D-F**).

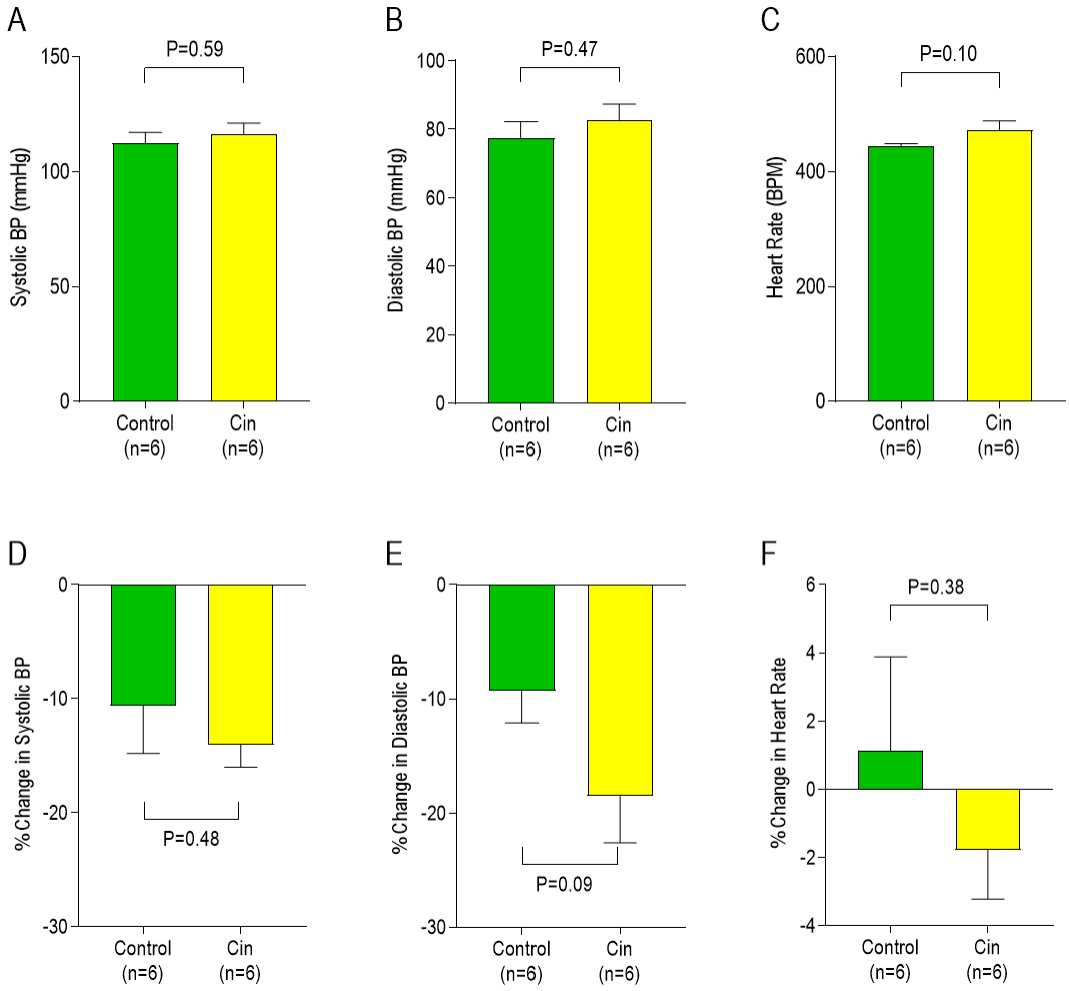


Figure 3-2: Baseline Characteristics

There were no differences in baseline hemodynamics (A-C) and vascular responses to methacholine (MCH) (D-F) in control C57BL/6 mice and those mice randomized to treatment with cinaciguat (cin: 15 µg/kg IV).

Following induction of sepsis, systolic blood pressure (P<0.0001), diastolic blood pressure (P<0.0001), and heart rate (P<0.0001) decreased in both cinaciguat-treated and control mice over time (**Figure 3-3: A-C**). However, all three parameters remained significantly elevated in the cinaciguat-treated group compared to the control (systolic

blood pressure P=0.01; diastolic blood pressure: P=0.04; heart rate: P=0.01). Over the time course of sepsis, systolic and diastolic blood pressure response to MCH in cinaciguat-treated and control mice decreased over time (P<0.0001), however heart rate responses remained similar (P=0.21). Mice treated with cinaciguat did not experience greater decrements in systolic (P=0.10) and diastolic blood pressure (P=0.09), but heart rate in response to MCH during sepsis increased (P=0.01) as compared to the control mice (**Figure 3-3: D-F**).

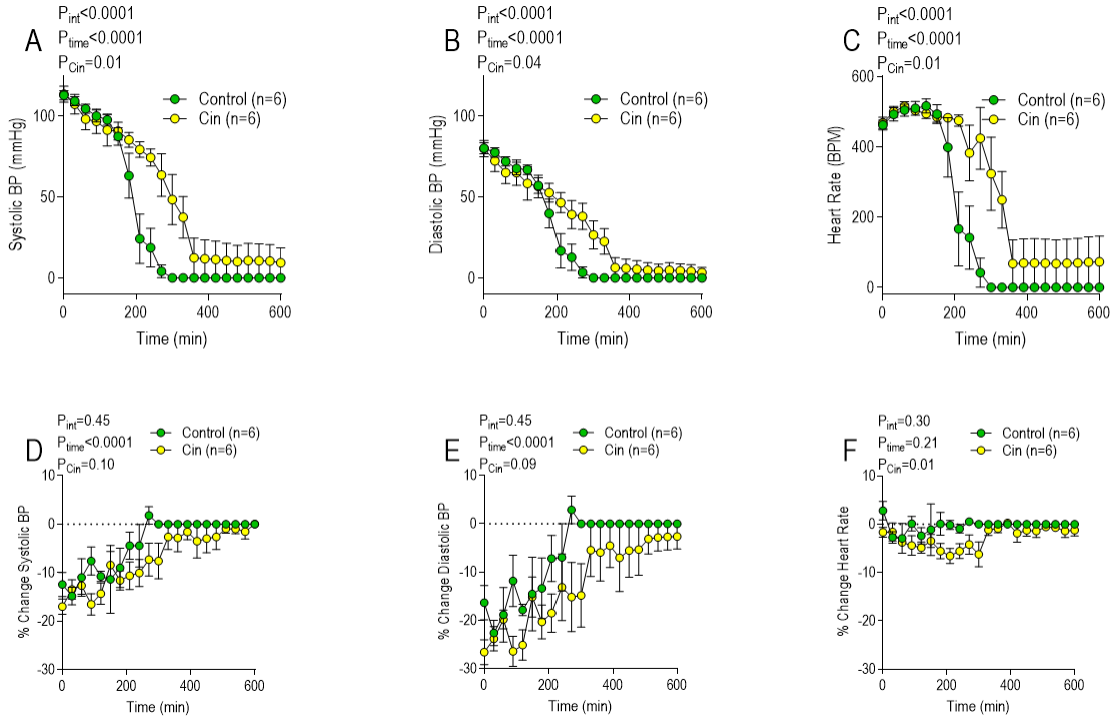


Figure 3-3: Septic Characteristics

Hemodynamics (A-C) in control (C57BL/6) young and cinaciguat (cin: 15 µg/kg IV) treated mice over time as sepsis progresses. Vascular responses (D-F) to methacholine (MCH: 1 µg/kg IV) over time (Time=0 minutes).

Cinaciguat treatment improved probability of sepsis survival (P=0.005; **Figure 3-4: A**) and septic survival time (P=0.03, hazard ratio: 0.1039, 95% confidence interval: 0.02173 to 0.4968; median survival time – control mice 237 minutes versus 345.5 minutes in cinaciguat-treated mice; **Figure 3-4: B**).

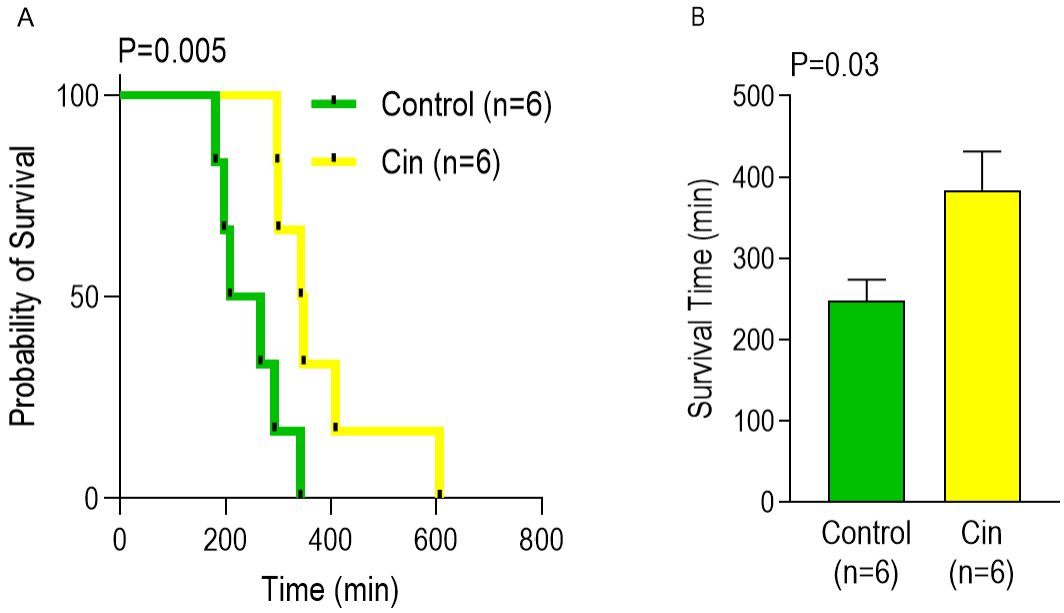


Figure 3-4: Survival

Survival probability (A) and time (B) depicted in minutes for septic C57BL/6 and cinaciguat (cin: 15 µg/kg IV) treated mice.

There was no difference in probability of survival between control and cinaciguat-treated mice administered NE (P=0.28; hazard ratio: 0.4963, 95% confidence interval: 0.1380 to 1.784 **Figure 3-5: A**). There was no difference in survival time in control mice who received NE (median survival: 228.5 minutes) versus cinaciguat-treated mice who received NE (median survival: 243.5 minutes) (P=0.35) (**Figure 3-5: B**). However, cinaciguat-treated mice required less total NE (control: 1551±178 µg; cinaciguat-treated:

368±112 µg; P=0.0002; **Figure 3-5: C)** and spent less time with NE infusing (control: 74±8minutes; cinaciguat-treated: 21±9 minutes; P=0.001; **Figure 3-5: D**).

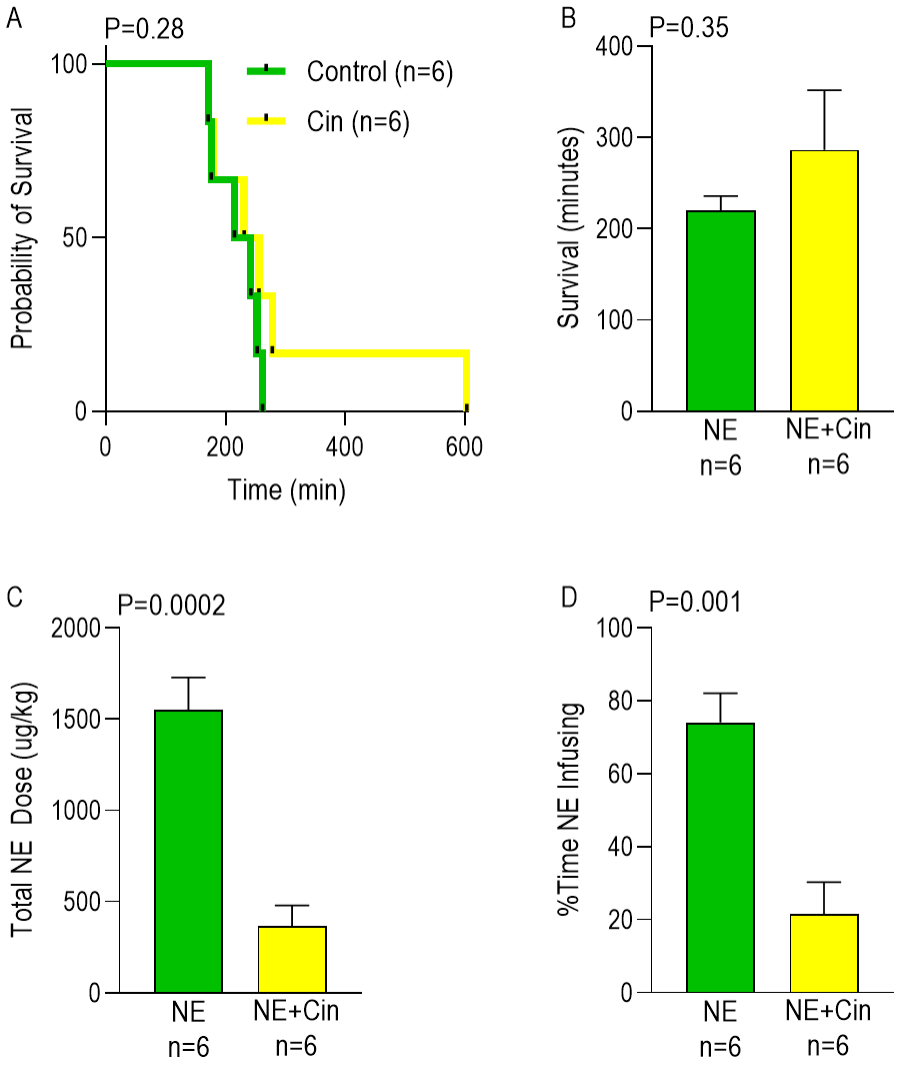


Figure 3-5: Effects of Norepinephrine

(A) Survival probability for septic C57BL/6 mice and septic C57BL/6 mice that received cinaciguat (cin: 15µg/kg IV) treated with intravenous infusion of norepinephrine (NE). (B) Survival time in minutes for septic C57BL/6 mice and septic C57BL/6 mice that received cinaciguat (cin: 15µg/kg IV) treated with intravenous infusion of norepinephrine (NE). (C) The total dose of norepinephrine (NE) infused in µg/kg for septic C57BL/6 mice and

cinaciguat-treated mice. (D) % of total survival time spent with norepinephrine (NE) infusing for septic C57BL/6 and cinaciguat-treated mice.

Wire myography was used to examine *ex-vivo* vascular dysfunction in response to MCH- and SNP-induced vasodilation and PE-induced vasoconstriction. Carotid arteries from septic mice had similar sensitivity to MCH (P=0.29) but increased sensitivity to SNP (pEC_{50} control 7.56±0.07; cinaciguat-treated 8.11±0.19; P=0.02). Similar maximal vasorelaxation in both control and cinaciguat-treated mice was observed for MCH (P=0.23) and SNP (P>0.99) (**Figure 3-6**).

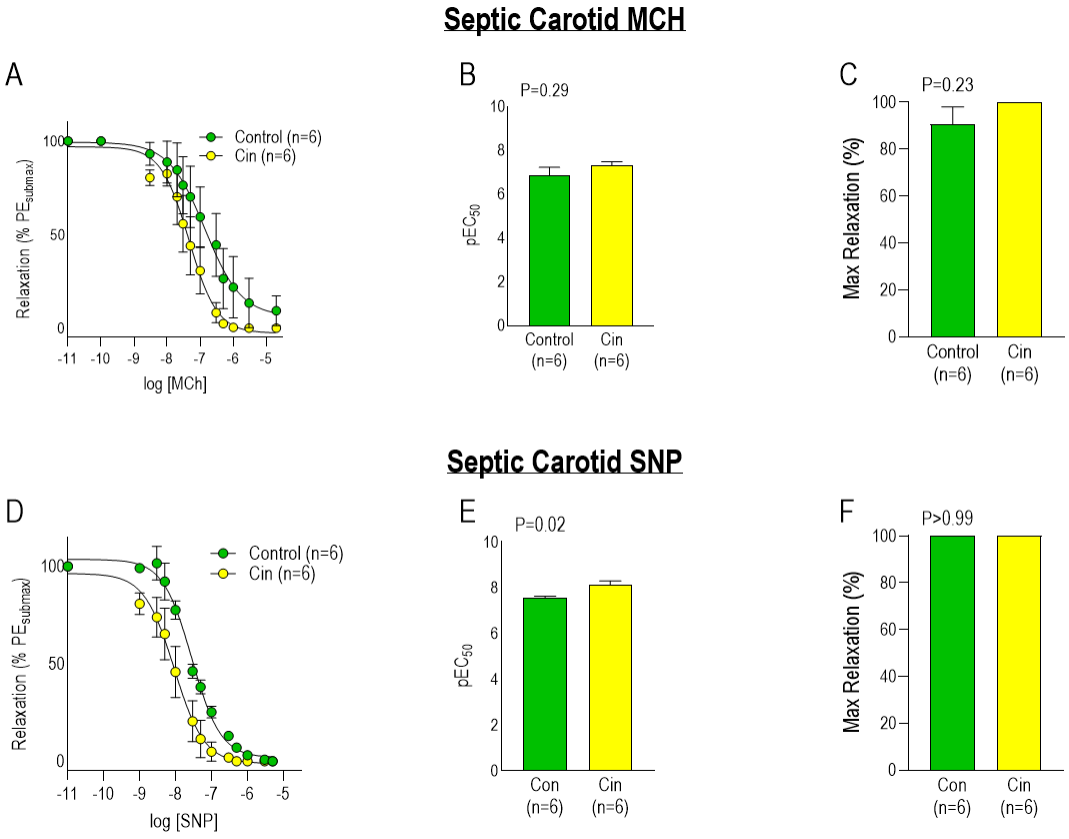


Figure 3-6: Wire Myography Carotid Arteries

Septic *ex-vivo* wire myography for carotid arteries depicting vasoactive parameters for control (green) and cinaciguat (cin: 15μg/kg IV) treated mice (yellow). (A, D) Carotid methacholine (MCH) and sodium nitroprusside (SNP) cumulative concentration-response curves. (B, E) pEC_{50} for carotid MCH and SNP cumulative concentration-response curves. (C, F) Maximum relaxation achieved by MCH and SNP administration.

In mesenteric arteries, differences were noted between cinaciguat-treated and control mice. Cinaciguat-treated mesenteric arteries displayed no change in sensitivity to MCH (P=0.50), but a left-ward shift of the concentration-response curve to SNP, corresponding to an increase in sensitivity (pEC_{50} SNP control: 8.67±0.10; cinaciguat-treated: 9.35±0.17; P=0.01) albeit but no differences in in maximal dilation were noted for either drug (MCH P=0.36; SNP=0.19) (**Figure 3-7**).

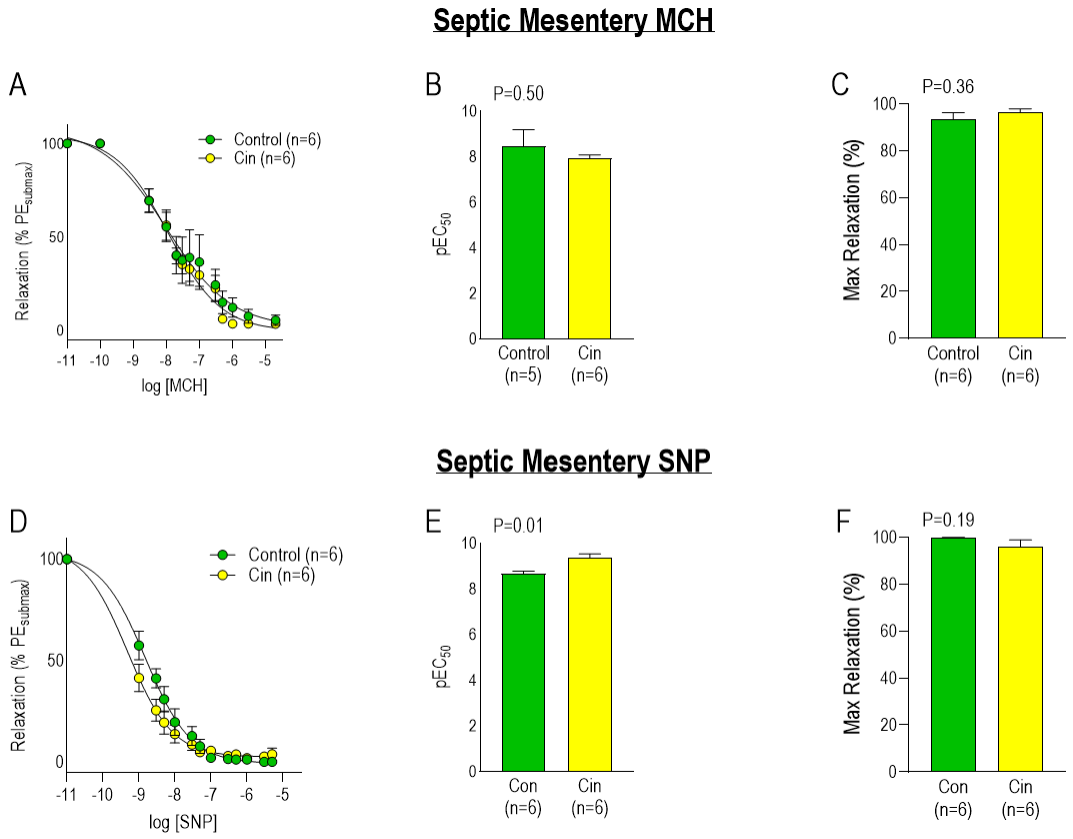


Figure 3-7: Wire Myography Mesenteric Arteries

Septic *ex-vivo* wire myography for mesenteric arteries depicting vasoactive parameters for control (green) and cinaciguat (cin: 15µg/kg IV) treated mice (yellow). (A, D) Mesentery methacholine (MCH) and sodium nitroprusside (SNP) cumulative concentration-response curves. (B, E) pEC_{50} for mesentery MCH and SNP cumulative concentration-response curves. (C, F) Maximum relaxation achieved by MCH and SNP administration.

In vessels pre-treated with LNAME, there were no differences in septic carotid artery pEC_{50} (MCH P=0.56) or maximal dilation achieved with MCH (P=0.91) or SNP (P>0.99) (**Figure 3-8**). Sensitivity for SNP in cinaciguat-treated septic carotid arteries was increased (pEC_{50} control: 8.04±0.11; cinaciguat-treated: 8.41±0.08). No change was observed in septic mesenteric arteries for MCH (P=0.05) or SNP (P=0.06). Maximal

dilation achieved with MCH (P=0.27) and SNP (P=0.12) (**Figure 3-9**) was similar between control and cinaciguat-treated vessels.

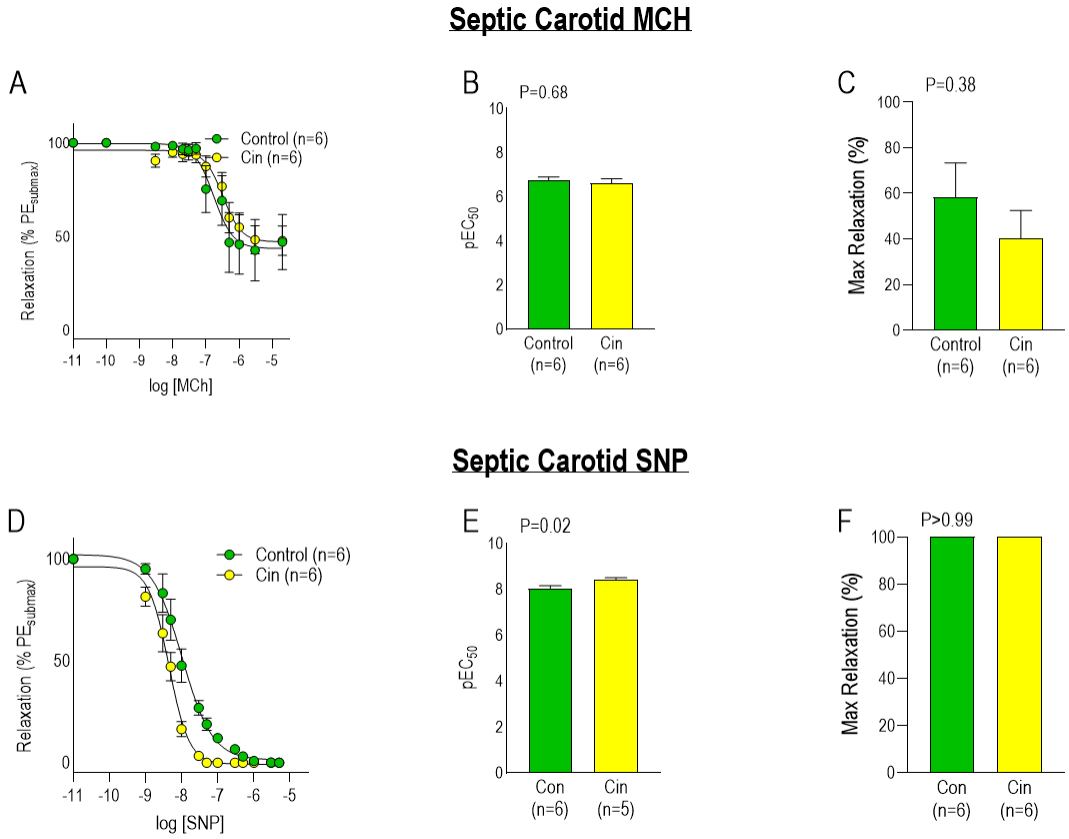


Figure 3-8: Wire Myography LNAME Carotid Arteries

Septic *ex-vivo* wire myography for carotid arteries incubated with L-N^G-Nitro arginine methyl ester (LNAME) depicting vasoactive parameters for control (green) and cinaciguat (cin: 15 µg/kg IV) treated mice (yellow). (A, D) Carotid methacholine (MCH) and sodium nitroprusside (SNP) cumulative concentration-response curves. (B, E) pEC_{50} for carotid MCH and SNP cumulative concentration-response curves. (C, F) Maximum relaxation achieved by MCH and SNP administration.

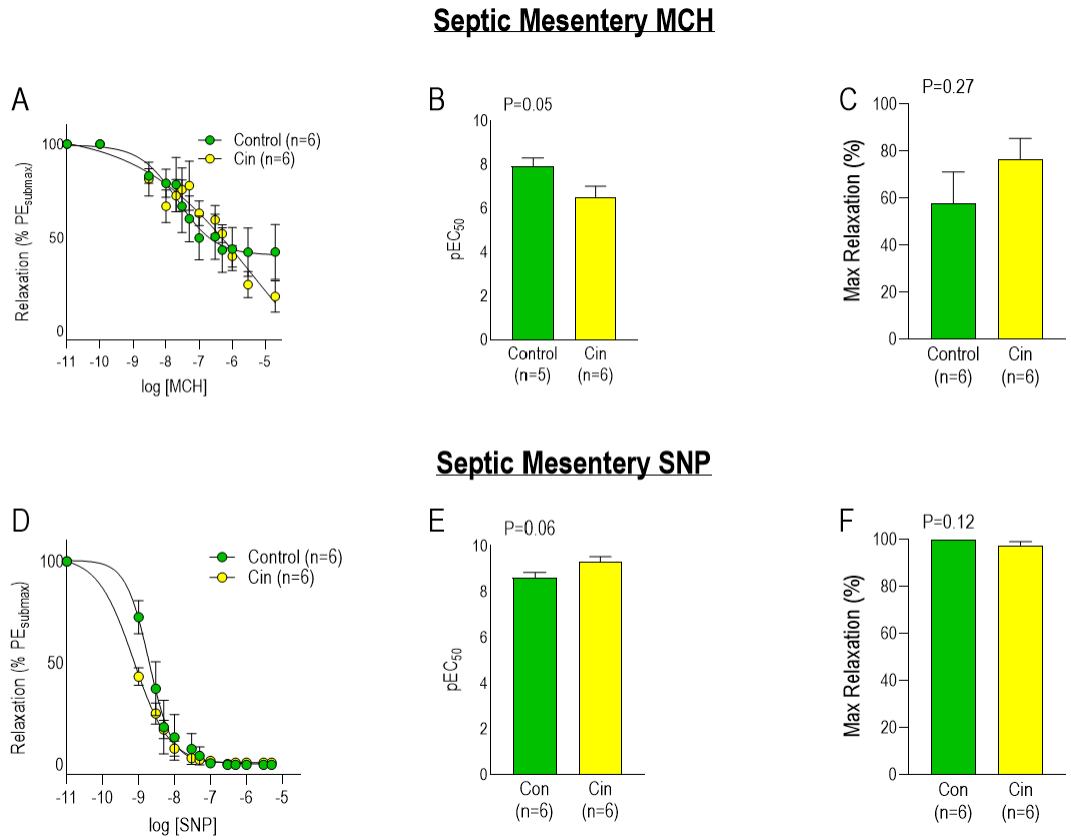


Figure 3-9: Wire Myography LNAME Mesenteric Arteries

Septic *ex-vivo* wire myography for mesenteric arteries incubated with L-N^G-Nitro arginine methyl ester (LNAME) depicting vasoactive parameters for control (green) and cinaciguat (cin: 15 µg/kg IV) treated mice (yellow). (A, D) Mesentery methacholine (MCH) and sodium nitroprusside (SNP) cumulative concentration-response curves. (B, E) pEC_{50} for mesentery MCH and SNP cumulative concentration-response curves. (C, F) Maximum relaxation achieved by MCH and SNP administration.

Data demonstrating comparison between control and LNAME treated vessels and cinaciguat control compared to aged LNAME treated vessels are presented in **Figure 3-10** as percent change in area under the curve.

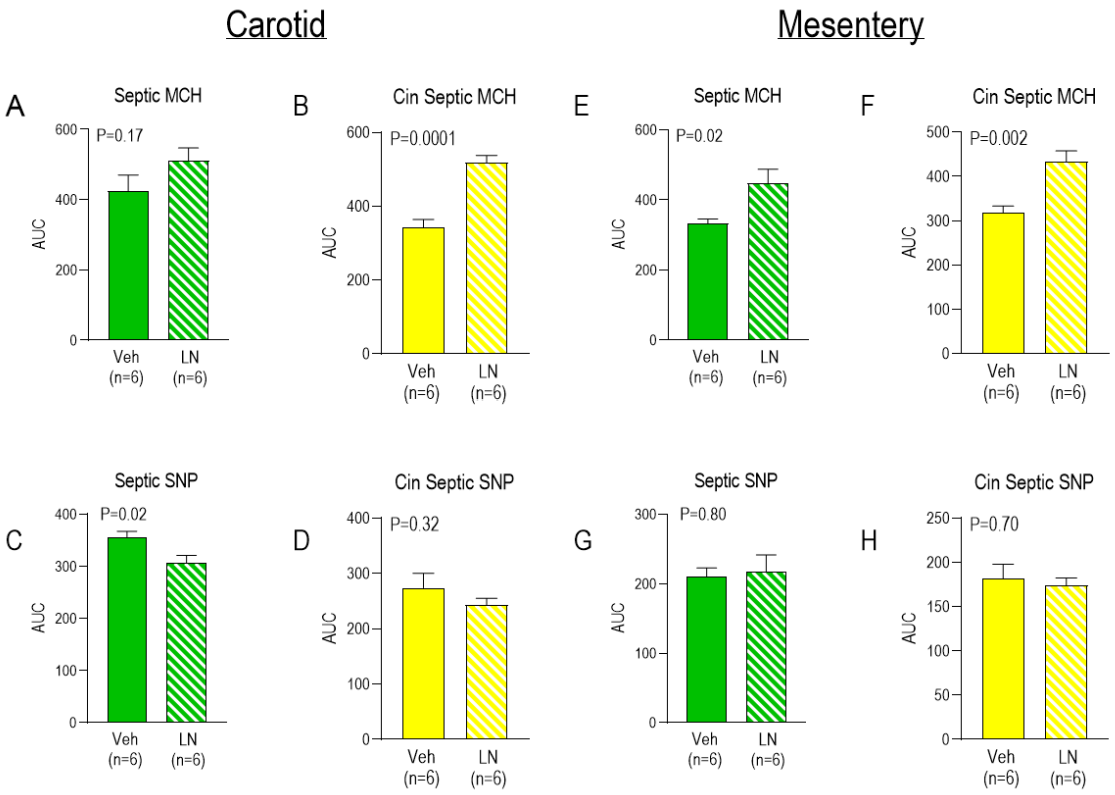


Figure 3-10: Control versus LNAME AUC %Change

Data depicting *ex-vivo* wire myography area under the curves (AUC) comparisons for control and L-N^G-Nitro arginine methyl ester (LNAME) treated vessels incubated with methacholine (MCH) and sodium nitroprusside (SNP) in vehicle and cinaciguat-treated (Cin) arteries. A, B, C, and D represent carotid arteries. E, F, G, and H represent mesenteric arteries.

There was no difference in vasoconstriction in response to PE between the control and cinaciguat-treated mice, without (carotid: P=0.14; mesentery: P=0.43) or with LNAME (carotid: P=0.82; mesentery: P=0.17).

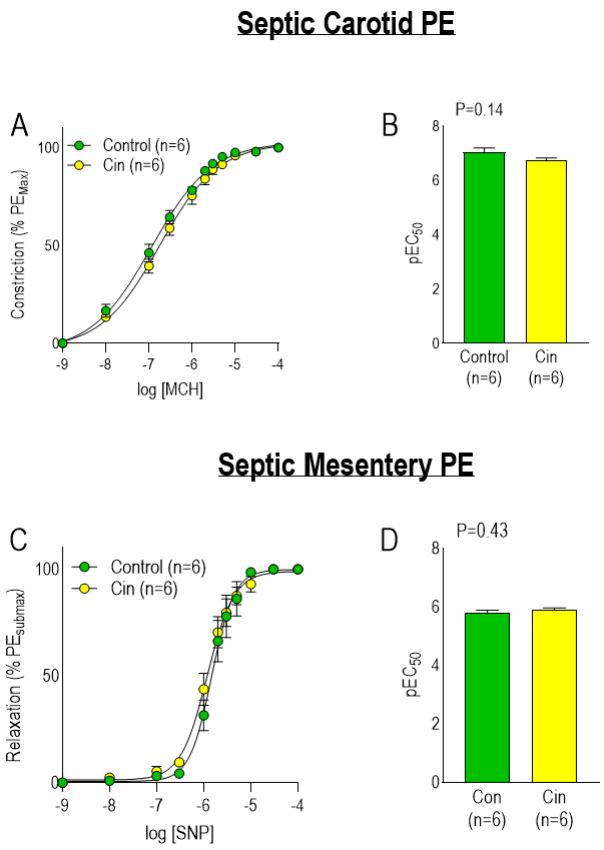


Figure 3-11: Wire Myography Vasoconstriction

Septic *ex-vivo* wire myography for aged carotid and mesenteric arteries depicting vasoactive parameters for control (green) and cinaciguat-treated (yellow) mice. (A, C) Phenylephrine (PE) cumulative concentration-response curves (B, D) pEC_{50} for PE cumulative concentration-response curves.

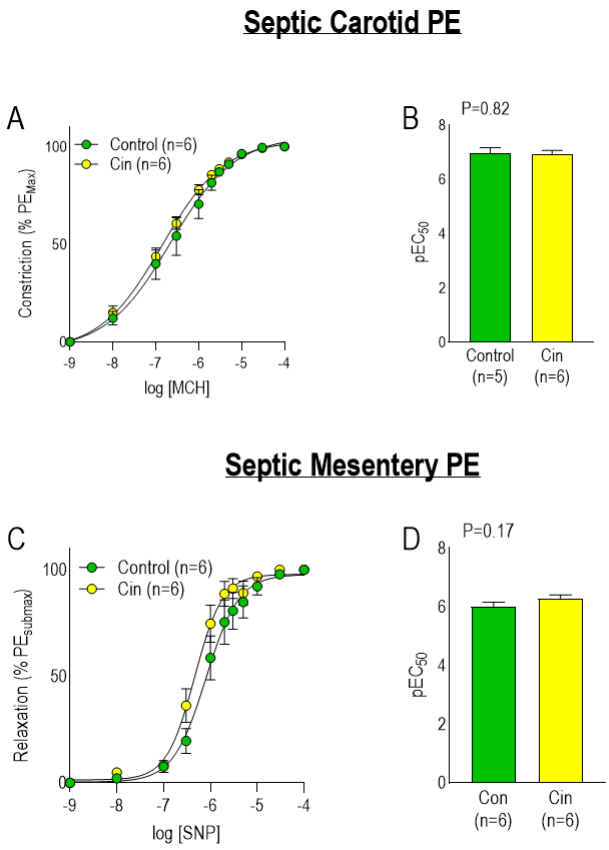


Figure 3-12: Wire Myography LNAME Vasoconstriction

Septic *ex-vivo* wire myography for aged carotid and mesenteric arteries incubated with L-NG-Nitro arginine methyl ester (LNAME) depicting vasoactive parameters for control (green) and cinaciguat (yellow) mice. (A, C) Phenylephrine (PE) cumulative concentration-response curves (B, D) pEC_{50} for PE cumulative concentration-response curves.

End-organ production of cGMP was assessed in lung and kidney. In lung, addition of cinaciguat to non-septic tissues failed to increase sGC activity (0 versus 10 µM: P=0.97; 0 versus 100 µM: P=0.97; 10 versus 100 µM: P>0.99; **Figure 3-13: A**). In lung tissue of septic mice, cinaciguat increased cGMP production (0 versus 10 µM: P=0.23; 0 versus 100 µM: P<0.0001; 10 versus 100 µM: P<0.0001; **Figure 3-13: A**). Kidney from non-septic

mice exhibited decreased cGMP production when exposed to cinaciguat (0 versus 10 µM: P=0.04; 0 versus 100 µM: P=0.049; 10 versus 100 µM: P>0.99; **Figure 3-13: B**), whereas kidneys from septic mice showed no increase in cGMP production when exposed to cinaciguat at low concentrations, but did so at higher concentrations (0 versus 10 µM: P=0.23; 0 versus 100 µM: P<0.0001; 10 versus 100 µM: P<0.0001; **Figure 3-13: B**).

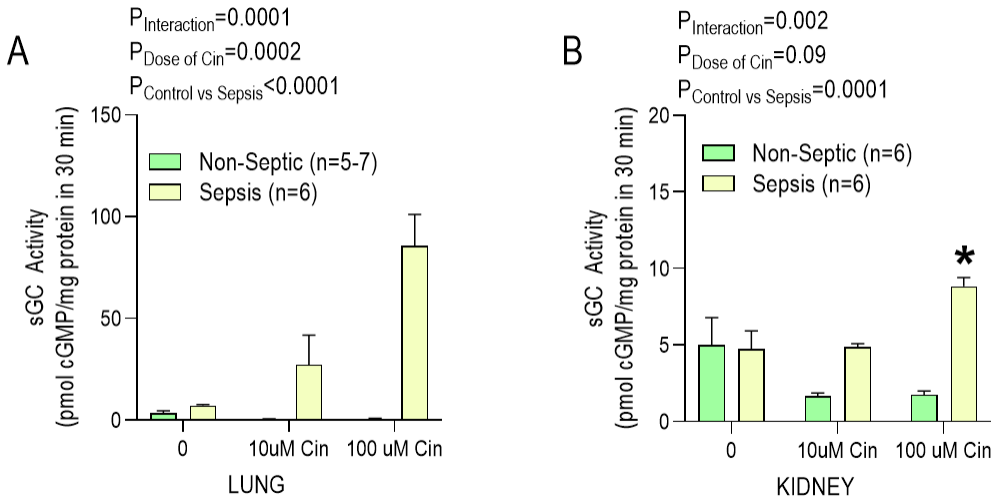


Figure 3-13: cGMP Production

Soluble guanylate cyclase (sGC) activity measured as pmol of cGMP produced per mg of protein in 30 minutes from (A) lung and (B) kidney in non-septic and septic C57BL/6 mice by ELISA in the presence of cinaciguat (cin) 0 µM, 10 µM, 100 µM.

Table 9: Summary of Non-Wire Myography Data

DBP: diastolic blood pressure; HR: heart rate; MCH: methacholine; NE: norepinephrine; SBP: systolic blood pressure; sGC: soluble guanylate cyclase

Summary	Effect of Cinaciguat
SBP-septic	↑
DBP-septic	↑
HR-septic	↑
SBP-septic MCH	Nil
DBP-septic MCH	Nil
HR-septic MCH	↑
Survival	↑
NE	Nil survival, ↓ dose, ↓ time infusing
sGC Activity Lung	↑
sGC Activity Kidney	↑

Table 10: Summary of Wire Myography Data

MCH: methacholine; SNP: sodium nitroprusside

Effect of Cinaciguat	
Carotid Arteries	**Mesenteric Arteries**
Baseline: • ↑ sensitivity to SNP • No change in sensitivity to MCH or PE	Baseline: • ↑ sensitivity to SNP • No change in sensitivity to MCH or PE
Baseline: • No change in maximal relaxation or vasoconstriction	Baseline: • No change in maximal relaxation or vasoconstriction
LNAME: • ↑ sensitivity to SNP • No change in sensitivity to MCH or PE	LNAME: • No change in sensitivity to MCH, SNP, or PE
LNAME: • No change in maximal vasorelaxation or vasoconstriction	LNAME: • No change in maximal vasorelaxation or vasoconstriction

3.4 Discussion

Herein, we tested the hypothesis that cinaciguat treatment would improve sGC-induced vascular dysfunction and thereby improve sepsis survival. We have newly demonstrated that intravenous cinaciguat treatment improves blood pressure and heart rate in an intraperitoneal murine sepsis model and results in a survival benefit (**Figure 3-4**). Improved *in-vivo* hemodynamic profiles in septic cinaciguat-treated mice may be secondary to restoration/prevention of septic vascular endothelial damage by re-establishing functional sGC signalling, producing necessary cGMP, and allowing for vasodilation as needed.

We chose to conduct our experiments in a model of intraperitoneal sepsis. It is the type of sepsis associated with the highest mortality and is the second-highest cause of sepsis in the adult human population.[579-581] While this approach will increase our findings' clinical relevance, we acknowledge that sepsis is a complex pathophysiologic syndrome that induces various effects on numerous signaling pathways, including the NO-sGC-cGMP signalling system, which may otherwise affect survival and hemodynamics. As such, our *in-vivo* findings may be influenced by other immune and inflammatory responses not seen with models of chemically activating the NO-sGC-cGMP signalling system with lipopolysaccharide or TLR-4 stimulation.

We used MCH in a novel fashion to induce a brief (less than 10 seconds) hypotensive response secondary to a decrease in systemic vascular response (heart rate remains unaffected at our dose developed in pilot studies – see **Figure 2-1**). MCH administration causes a transient hypotensive response secondary to activation of

muscarinic receptors on endothelium, causing the release of vasodilatory substances. Whereas in large vessels, this endothelial derived vasodilator tends to be NO, in smaller resistance vessels, a multitude of mediators, including NO and endothelial-derived hyperpolarization factor (EDHF) are thought to contribute. In control mice, it would be expected that as sepsis-induced endothelial damage progresses, loss of the brief hypotensive response would diminish over time with progressive endothelial damage. In cinaciguat treated mice, we expected sGC signalling to be restored and we expected the hypotensive response to MCH to be maintained to a greater degree than in control mice. However, neither systolic nor diastolic blood pressure response to MCH in cinaciguat-treated mice was maintained, as seen in **Figure 3-3**. This may be due to sepsis-damaged oxidized endothelial NOS (eNOS) being unresponsive to stimulus by MCH.

In sepsis, although eNOS is known to be compromised, thereby affecting vascular function, overall NO generation is increased, largely due to the actions of iNOS secondary to inflammation. This pronounced NO generation (iNOS produces NO at orders of magnitude larger than eNOS) during sepsis can result in profound hypotension, organ hypoperfusion and dysfunction, and ultimately death.[582] Sepsis survivors may suffer from long-term complications of organ hypoperfusion including cognitive changes (brain hypoperfusion), decreases in pulmonary function (requiring inhaled oxygen support or decreased functional tolerance of activity), need for hemodialysis (renal hypoperfusion), and inability to return to the workforce.[124,428] Paradoxically, despite the excessive systemic vasodilation, sepsis is also characterized by a loss of vasodilatory capacity in various vascular beds, resulting in inadequate perfusion and regional ischemia.[583] In this light,

therapeutic interventions to target the excess NO production, or promote vasodilation to relieve regional ischemia, are intriguing. Indeed, vasodilation, especially at injury sites, is important,[584] as it allows for increased blood flow for enhanced delivery of inflammatory mediators to help resolve an inciting infection. Therefore, maintenance of vasodilation during sepsis in certain vascular beds, activated by cinaciguat may be desirable if outcomes can be improved.

When septic hypotension is severe and unresponsive to fluid bolus, treatment includes vasopressor medications such as NE to induce vasoconstriction and improve cardiac output to improve blood pressure.[104,585,586] This clinical state is referred to as septic shock.[1] Improved blood pressure secondary to vasopressor support does not necessarily equate to improved outcome. Induced vasoconstriction can be severe and compromise blood flow, tissue and organ perfusion, and delivery of oxygen and nutrients. This restricted blood flow may contribute to organ injury, dysfunction, and death. Vasopressor therapy has other drawbacks. It may result in arrhythmias secondary to β_1-adrenergic receptor stimulation, which may be especially poorly tolerated in a septic patient population.[333,587] Vasopressors generally require placement of central venous access and have associated potential complications (pneumothorax, infection, bleeding, vascular injury).[588] Vasopressors are also typically administered in the highest level of care settings, usually an ICU, contributing to the high financial cost of care for septic patients.[589,590] Cinaciguat, which may be administered via peripheral intravenous access, may be provided in a lower-intensity care setting such as an observation/step-down unit or regular ward bed. It may also result in appropriate blood flow to septic tissues and organs, thereby restoring

nutrient and oxygen delivery, may improve organ dysfunction, mortality, and long-term septic outcomes, and may decrease costly intensive care unit admissions. It was also important to demonstrate the combined effect of NE and cinaciguat as peroxynitrite generated by NO scavenging during sepsis may deactivate NE.[591] We showed a reduction in the total amount of NE and the time spent with NE infusing in septic mice treated with cinaciguat (**Figure 3-5**). We theorize that if this finding is translatable to the human septic population, patients requiring only mild vasopressor support may improve with cinaciguat treatment received in the emergency department care setting and be hemodynamically stable enough for admission to a lower-intensity care setting.

Our results suggest improvements in vascular function may be specific to regionally separate vascular beds. We found no improvements in pEC_{50} or maximal vasodilation between controls and cinaciguat-treated mice with MCH in septic carotid arteries (**Figure 3-6**). However, cinaciguat-treated carotids demonstrated increased sensitivity to SNP while achieving similar maximal vasorelaxation. This increased sensitivity to SNP may be as a result of combined relaxation effect of both the SNP and cinaciguat. It was also observed that cinaciguat improved vascular sensitivity to SNP administration in septic mesenteric arteries. It may be that while cinaciguat increases cGMP production from heme-free sGC, SNP provides NO allowing for cGMP production from any reduced sGC available, thereby increasing the overall signal to produce cGMP.

We assessed our septic vessels 120 minutes post-sepsis induction as our *in-vivo* blood pressure changes were evident at this point. We aimed to avoid testing vessels so severely affected by sepsis that any test would be unlikely to result in a difference (as

demonstrated by the blunting of vasodilation induced with our *in-vivo* MCH administration after prolonged sepsis exposure in both control and cinaciguat-treated mice). We used MCH to compare endothelial-dependent relaxation, which would be expected to decrease over time, and vasodilation resultant from the nitric oxide donor SNP, which may not lose effectiveness over time. Cinaciguat treatment did not improve MCH- or SNP-induced *ex-vivo* maximal vasorelaxation in either carotid or mesenteric arteries. As cinaciguat-induced sGC production may have attenuated vasoconstriction, we tested control and cinaciguat-treated carotid and mesenteric vessels in response to PE (**Figure 3-11**). Cinaciguat did not affect sensitivity to PE-induced vasoconstriction in either carotid or mesenteric arteries.

We also examined septic carotid and mesenteric arteries incubated with the non-specific NOS inhibitor LNAME (**Figure 3-8**), which demonstrated almost no difference in sensitivity or maximal vasorelaxation and no difference in vasoconstriction (**Figure 3-12**). Only septic carotid and mesenteric arteries exhibited an increased sensitivity to SNP with no change in maximal vasorelaxation. This may provide further support towards to theory that cinaciguat in combination with SNP increases the overall cGMP production signal. Physiologically, it may be that the body prioritizes the preservation of blood flow to the brain over the gut during a septic hypotensive shock crisis, thereby explaining the regional variation in vasoactive control. Also, the septic insult of intraperitoneal fecal slurry to mimic the clinical situation of gut rupture places the septic source in proximity to the mesenteric vessels, potentially affecting them more severely than the more distant carotid arteries.

We also examined the amount of cGMP produced in two separate vascular beds that may be acutely affected by sepsis. Acute respiratory failure and acute kidney injury are common amongst septic patients. Therefore, we examined the lungs and kidneys. Non-septic lungs failed to produce increased cGMP levels as a measure of sGC activity, even in response to a high concentration of 100 μM. This demonstrates a low amount of oxidized sGC present in non-septic lungs. In sepsis, cinaciguat 100 μM was able to increase cGMP production. This showed that oxidized sGC was present in the septic lungs and was able to be activated by cinaciguat to improve the production of cGMP. In contrast, non-septic kidneys produced low cGMP levels at both cinaciguat concentrations. Potentially, the kidney experiences a baseline of oxidative stress allowing for the presence of oxidized sGC to be activated by cinaciguat.[592] During sepsis the kidney required higher doses of cinaciguat to produce cGMP (low concentration (10 μM) of cinaciguat did not increase cGMP production, a higher concentration of cinaciguat (100 μM) did increase cGMP production). Potentially as a larger amount of sGC became dysfunctional with sepsis, more cinaciguat was needed to activate the larger amounts of dysfunctional sGC.

3.5 Limitations

There are limitations to our study. Our fecal slurry dose may have resulted in a severely septic phenotype, potentially preventing us from demonstrating a more significant effect in less sick mice. Our young septic control mice in Chapter 2 displayed similar systolic blood pressure, however, the diastolic blood pressure was lower as compared to Chapter 2 septic young mice. This may be explained due to potential changes in microbiome as mice for the different studies were housed in separate rooms. However, all mice for each study

were housed in the same room decreasing risk of difference due to microbiome within each study.

Our dose of cinaciguat (15 µg/kg IV) may have been too high or too low to demonstrate a larger effect. However, we did initial pilot studies using doses ranging from 5-90 µg/kg IV specifically for examining survival. We demonstrated the greatest improvement in survival with 15 µg/kg IV and therefore this dose was used for experiments (**Figure 3-14**).

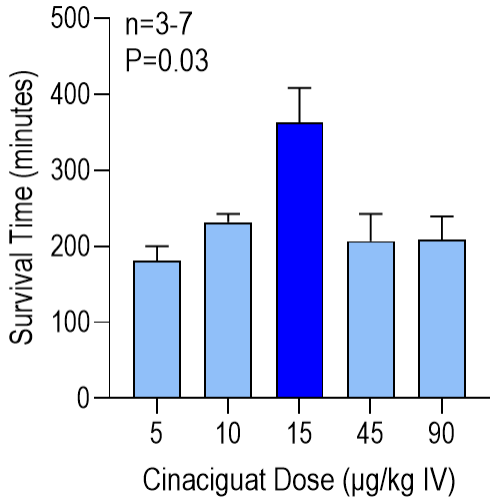


Figure 3-14: Cinaciguat Dose in Young Mice

Our NE protocol was based on clinical guidelines for human septic patients, which may not be optimal targets for septic mice. Therefore, although we showed reduced NE requirements, different blood pressure targets may have resulted in survival benefit.

We administered cinaciguat 30 minutes post-induction of sepsis with fecal slurry. We wanted to administer the drug following suspected sGC damage by sepsis-induced

ROS, but not delay treatment so long into the organ-damaging effects of sepsis, so as to negate any intervention being successful. We recognize that potentially later use of cinaciguat in the time course of sepsis could result in even more improved hemodynamics and survival as there could potentially be more oxidized sGC for cinaciguat to act upon.

3.6 Conclusion

Activating oxidized sGC with cinaciguat improves blood pressure and survival during intraperitoneal sepsis in mice. Treatment with cinaciguat also decreases dose requirement of NE to maintain hemodynamic stability. Taken together, these results suggest cinaciguat may be a useful therapeutic strategy to improve outcomes in sepsis. However, the effects of cinaciguat may be regionally specific to certain vascular beds depending upon reliance on NO for vascular function, and thus more detailed studies are required to elucidate its mechanism of action in various models of sepsis.

Chapter 4

Cinaciguat in Aged Septic Mice Improves Vascular Function and Survival

4.1 Background

Soluble guanylate cyclase (sGC) is the key heterodimeric enzyme involved in nitric oxide (NO) signalling. The sGC prosthetic heme group binds with NO and produces the second messenger cyclic guanosine monophosphate (cGMP).[593] cGMP is necessary to induce vasorelaxation and inhibit leukocyte recruitment, platelet aggregation, and smooth muscle proliferation.[569] As vasorelaxation plays a pivotal role in maintaining vascular function, its impairment can have far-reaching effects. sGC consists of an α-chain and a β-chain. The form of sGC in the vascular smooth muscle cell is the α1β1 cytoplasmic form.[594] Heme binds to the His^{105} of the β chain. NO is then able to bind to the ferrous iron of the heme group. Once NO is bound, the sGC catalytic site can now use guanosine triphosphate (GTP) to produce cGMP and pyrophosphate. When the ferrous iron of the heme group is oxidized to its ferric form, the heme group becomes desensitized to NO, binding NO with far weaker affinity.[595] This oxidized or heme-free form of sGC can undergo hydroxide-mediated reductive nitrosylation, inhibiting sGC activity.[596] Oxidized sGC is also targeted by ubiquitination for degradation.[597] Though not fully understood, cytochrome b5 reductase 3 (Cyb5r3) can reduce oxidized sGC and may act as a regulator of cGMP signalling.[598]

Aging is known to decrease the expression of sGC and the level of cGMP produced.[599-601,599,602] The low-grade chronic inflammation associated with aging also results in increased baseline oxidative stress and reactive oxygen species (ROS) generation (a part of the phenomenon known as inflammaging).[603] ROS may subsequently further

oxidize sGC, decreasing the sensitivity of the sGC enzyme for NO. In addition, levels of bioavailable NO in the elderly are decreased, thereby further limiting cGMP production.[604] Overall, the reduced availability of NO and the decreased sensitivity of sGC to NO combine to produce vascular dysfunction and impaired vasorelaxation.

Sepsis is associated with the excess production of harmful ROS,[605] which can lead to oxidation of the prosthetic heme group of sGC, rendering it inactive and targeted for destruction.[606] Sepsis-induced ROS also decreases the transcription and stability of sGC.[574] Therefore, it was hypothesized that the septic elderly would be at a particular disadvantage in maintaining blood pressure and flow and subsequent oxygen and nutrient delivery to tissues. Reducing vasodilation may seem beneficial in sepsis to prevent vasoplegia and septic shock. However, we know that blood flow to tissues is important for delivery of inflammatory mediators and removal of toxins. Therefore, elderly patients may have increased susceptibility to septic vascular dysfunction as, at baseline they possess a pool of dysfunctional, oxidized sGC.

There are two classes of drugs that can increase sGC production of cGMP. sGC stimulators directly stimulate sGC and stabilize the bond between sGC and NO by targeting binding sites in the β_1 subunit.[607] However, sGC stimulators require the sGC to be in the ferrous (reduced) form, and because they do not target the oxidized sGC, are not likely to improve vascular function in sepsis. In fact, systemic administration of these drugs are likely to cause widespread vasodilation and hypotension by activating functional sGC, which could have fatal consequences in a septic patients. In contrast, sGC activators, such as cinaciguat, bind to oxidized sGC and activate it.[608] As sGC activators function on the

heme-free form of sGC, they can be expected to produce vasodilation in a more targeted fashion (at the site of injury or infection).[609] We therefore hypothesized that administering cinaciguat to septic aged mice with a theoretically larger pool of heme-free sGC may improve survival by allowing for targeted vasodilation of vessels containing damaged sGC, thus improving blood flow to these injured sites.

4.2 Methods

This study was conducted according to the Canadian Council on Animal Care guidelines, with approval from the Animal Care and Use Committee from the University of Alberta (Edmonton, Canada), and reported in adherence with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) Guidelines.

4.2.1 Animals and Preparation

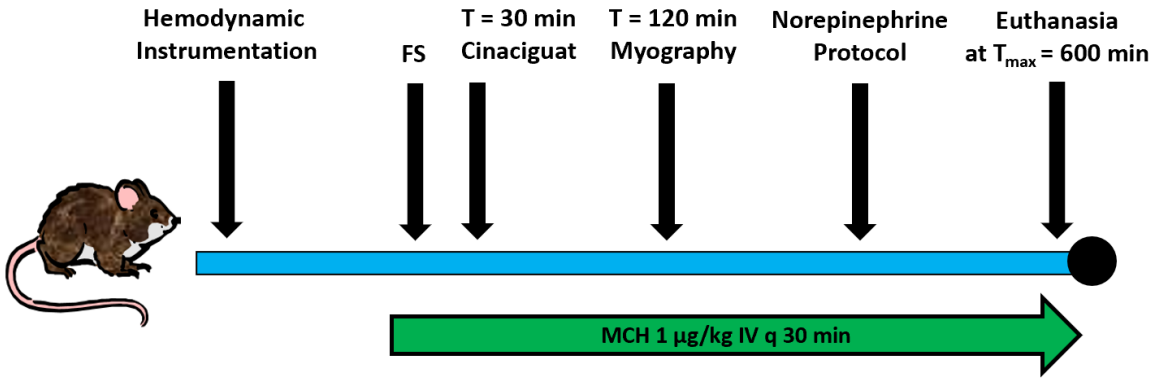
Ninety-six male C57BL/6 mice (procedure- and drug-administration-naïve, with no genetic modifications) were purchased from Charles-River Inc. (Saint-Constant, QC). Mice were aged 22-24 months. Mice were multiply housed (five per cage) in standard shoebox cages containing aspen-chip bedding, nesting material, and PVC tubing for environmental enrichment. Cages were located in the University of Alberta animal care facility, where a 12h:12h light:dark cycle and an ambient temperature of 22±1ºC were maintained. Trained animal care personnel routinely performed welfare assessments before experimentation. Mice had *ad libitum* access to tap water and standard grain-based rodent chow (PicoLab 5LOD, LabDiet, St. Louis, MO).

Experiments were conducted in the same laboratory operating theatre during the day. Mice were anesthetized with inhaled isoflurane (induction: 5%, maintenance: 1.1-1.5% in 100% O_2) and were kept spontaneously breathing via a nosecone. We used isoflurane as it is administered non-invasively, thereby avoiding the risk of puncture of the peritoneum with intraperitoneal (IP) or subcutaneous injections, and provides a constant depth of anesthesia for longer experiments. Following induction of anesthesia, mice were instrumented with a polyethylene catheter (PE10; inner diameter 0.58 mm, outer diameter 0.97 mm) in the left femoral vein for drug delivery and a glass fibreoptic transducer catheter (FISO 0.9 Fr/0.5 Fr; Harvard Apparatus, Harvard BioScience Inc.) in the right carotid artery for hemodynamic assessments. The femoral venous catheters contained heparinized sterile 0.9% saline (25 units/mL). Body temperature was monitored via a rectal thermometer and maintained with a warm water circulator. Following hemodynamic instrumentation, mice were allowed 30 minutes to achieve stable baseline temperature and hemodynamic variables. The mice were administered hydration with sterile saline (2 mL/kg/hour IV). Arterial blood pressure and heart rate were monitored and recorded via the indwelling FISO catheter connected to a data acquisition system (Lab Chart Pro 8, ADInstruments, Colorado Springs, CO).

4.2.2 Study Protocol

Fecal slurry (1.3 mg/g IP) was administered to induce sepsis (dose determined in our pilot studies). Blood pressure, heart rate, and temperature were recorded. Mice received cinaciguat (15 µg/kg IV – the most effective dose in our pilot studies) 30 minutes after fecal slurry injection; age-matched controls received vehicle treatments. *In-vivo* vascular

reactivity was assessed via methacholine (MCH; 1 µg/kg IV – this dose caused hypotension that resolved within 10 seconds and the hypotension was due to decreased systemic vascular resistance as the dose did not affect heart rate) injection every 30 minutes. Subsets of septic mice were administered norepinephrine (NE) to assess the reversibility of cardiovascular collapse. NE infusion was initiated at 200 µg/kg/hour and advanced by 100 µg/kg/hour in five-minute intervals to a maximum of 600 µg/kg/hour until the mean arterial pressure was greater than 65 mmHg or systolic blood pressure was greater than 80 mmHg.



Cinaciguat: 15µg/kg IV 30 minutes post-FS
FS: fecal slurry
MCH: methacholine
Norepinephrine Protocol: titrate norepinephrine 200-600 µg/kg/h IV when MAP < 65 mmHg or SBP < 80 mmHg

Figure 4-1: Study Protocol

Schematic of study protocol including methacholine (MCH) and norepinephrine (NE) administration.

4.2.3 Wire Myography Protocol

Mice randomized to wire myography experiments were maintained under anesthesia for 120 minutes following induction of sepsis and subsequently euthanized by exsanguination and excision of the heart. Left common carotid arteries and second-order mesenteric

arteries were rapidly removed and placed in ice-cold HEPES-buffered physiologic saline solution (PSS – composition: (in mM) HEPES 10, glucose 5.5, $CaCl_2$ 1.56, KCl 4.7, NaCl 142, $MgSO_4$ 1.17, KH_2PO_4 1.18, pH 7.45). Vessels were cleaned of extraneous perivascular connective tissues, and small vessel sections (2 mm in length) were isolated and mounted in an isometric myograph system (DMT, Copenhagen, Denmark); 15 μm gold wire was used for mesenteric arteries, and 40 μm tungsten wire was used for the carotid arteries. Vessels were assessed at 37ºC and normalized to optimal resting tension (0.8 IC_{100}: the internal circumference equalling a transmural pressure of 100 mmHg) by small incremental increases in diameter. Vessels were then allowed 30 minutes to equilibrate to the optimal resting tension before initiating the experiment. Vessels were subsequently tested for viability by high potassium physiologic salt solution (KPSS) treatment. After rinsing, vessels were treated with phenylephrine (PE: 10 μmol/L) twice, with a rinse and return to baseline tension in between doses; following the second dose of PE, MCH (3 μmol/L) was administered to test the integrity of the endothelium. Following endothelial assessment, vessels were rinsed and returned to baseline. Next, PE concentration-response curves were performed. A subset of vessels was then incubated with non-selective nitric oxide synthase (NOS) inhibitor L-N^G-nitro-arginine-methyl ester (LNAME: 100 μmol/L) for 30 minutes prior to performing cumulative concentration-response curves with MCH and sodium nitroprusside (SNP); vessels were sub-maximally pre-constricted (80% maximum: EC_{80}) with phenylephrine. Finally, at the end of each experiment, vascular constriction to KPSS was assessed; those vessels with a final KPSS constriction of less than 80% of the first KPSS constriction were excluded from analyses.

4.2.4 Assessment of sGC Production

sGC activity was assessed indirectly by measuring cGMP production in two end-organs commonly acutely affected by sepsis, the lung and the kidney. Fresh left lungs and left kidneys were harvested from a subset of non-septic and septic mice 120 minutes post-vehicle or fecal slurry injection under isoflurane anesthetic. Lungs and kidneys were homogenized in EDTA, Tris-HCL, sucrose, dithiothreitol, protease inhibitor cocktail (Sigma, St. Louis, MO), and sodium orthovanadate. Homogenized tissue was centrifuged (10,000 x *g*, 4°C, 20 minutes) and had total protein quantified using the bicinchoninic acid protein assay. Cytosolic protein was added to a reaction buffer mixture containing bovine serum albumin, benzamidine HCL, SNP, LNAME, 3-isobuyl-1-methylxanthine, L-cysteine, and GTP (Sigma, St. Louis, MO). This mixture was incubated for 30 minutes at 37°C in a shaking water bath. The reaction was terminated with zinc acetate and sodium carbonate (Sigma, St. Louis, MO). This mixture was then centrifuged (5,000 x *g*, 4°C, 10 minutes). cGMP was then quantified with the Cayman Chemical Cyclic GMP ELISA kit (Ann Arbor, MI, USA). Samples were assessed under cinaciguat concentrations of 0 μM and 10 μM.

4.2.5 Reagents

Cinaciguat (Sigma, St. Louis, MO) was prepared in 60% phosphate-buffered saline, 20% diethylene glycol monoethyl ether, and 20% cremophor. MCH, SNP, and NE (Sigma, St. Louis, MO) were dissolved in sterile saline (0.9% NaCl) for *in-vivo* experiments and subsequently diluted in PSS for final use in myography experiments. PE and LNAME

(Sigma, St. Louis, MO) were also dissolved in sterile saline and diluted in PSS for final use in myography experiments.

4.2.6 Statistical Analysis

Data are presented as mean±SEM. Patterns of cardiovascular collapse (as depicted by Kaplan-Meier curves) were analyzed by log-rank tests. Continuous variables, including arterial pressures, heart rates and hemodynamic responsiveness to exogenous pharmacological agents were analyzed by repeated measures 2-way ANOVA for the overall effects of sepsis and time; subjects were matched within the time domain. The remaining data were analyzed by unpaired Student's *t*-test. For collection of septic systolic blood pressure, diastolic blood pressure, and heart rate, the most representative five-second segment within each 30-second interval was captured from recorded parameters. Percent changes in hemodynamic variables in response to MCH administration were calculated as the minimum value compared to the baseline in the immediately preceding five-minute interval. For wire myography, concentration-response curves were fit to the Hill equation using a variable slope, and pEC_{50} (mean effective concentration to produce a 50% response) and maximal responses were analyzed by unpaired *t*-test. cGMP production was analyzed with a *t*-test. Statistical outliers determined by Grubb's test were not included. Results were considered significant if $P<0.05$. GraphPad Prism 8 software (La Jolla, CA) was employed for statistical analysis.

4.3 Results

The baseline hemodynamics parameters (systolic blood pressure, diastolic blood pressure, heart rate) were not different between aged mice randomized to the control group versus the cinaciguat group (**Figure 4-2: A-C**). There were also no baseline differences (% change in systolic blood pressure, % change in diastolic blood pressure, % change in heart rate) between cinaciguat-treated and control groups in response to *in-vivo* administration of MCH (**Figure 4-2: D-F**).

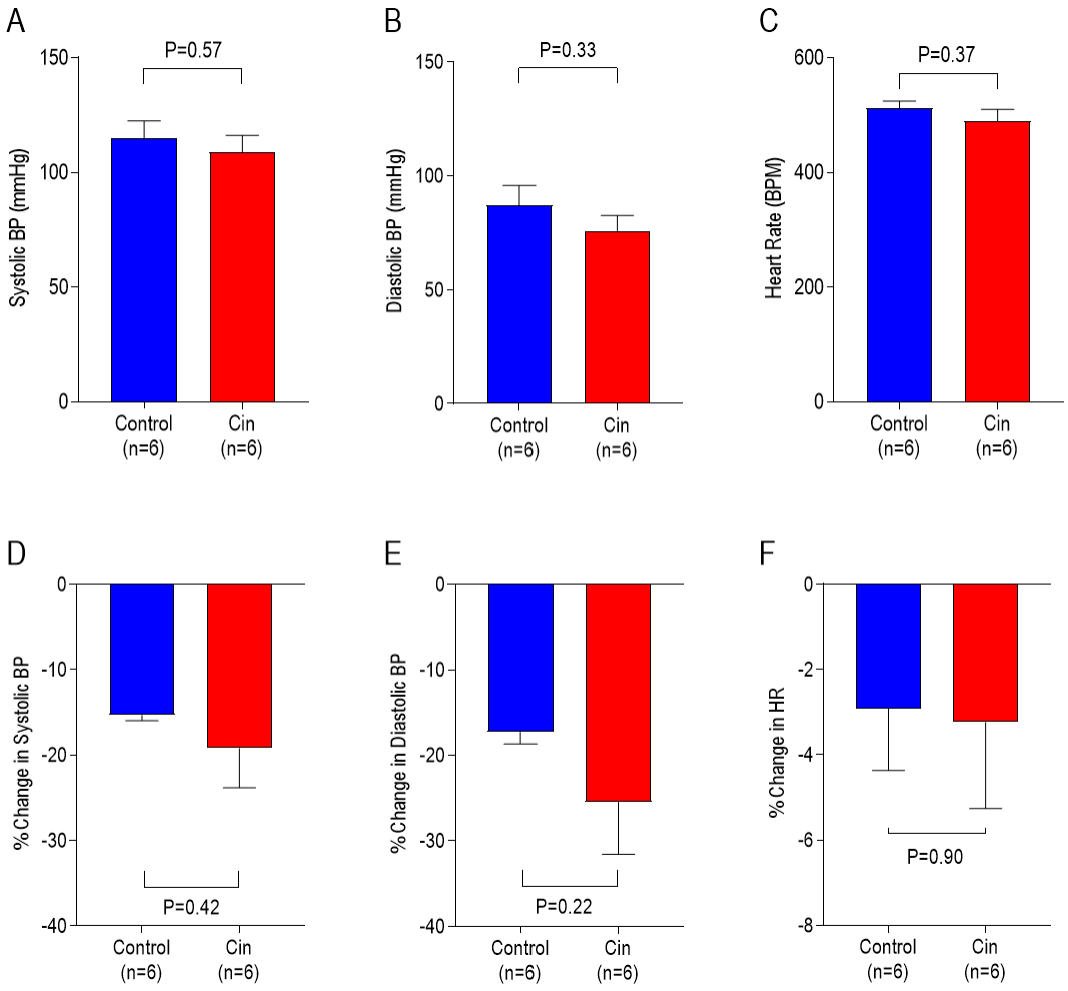


Figure 4-2: Baseline Characteristics

There were no differences in baseline hemodynamics (A-C) and vascular responses to MCH (D-F) in control aged C57BL/6 mice and aged mice to be treated with cinaciguat (cin: 15µg/kg IV).

After induction of sepsis by intraperitoneal injection of fecal slurry, systolic blood pressure ($P<0.0001$), diastolic blood pressure ($P<0.0001$), and heart rate ($P<0.0001$) decreased over time in both cinaciguat and vehicle-treated groups (**Figure 4-3: A-C**). There was no

improvement in systolic blood pressure (p=0.72), diastolic blood pressure (P=0.96), or heart rate (P=0.67) MCH response in the cinaciguat group (**Figure 4-3: D-F**).

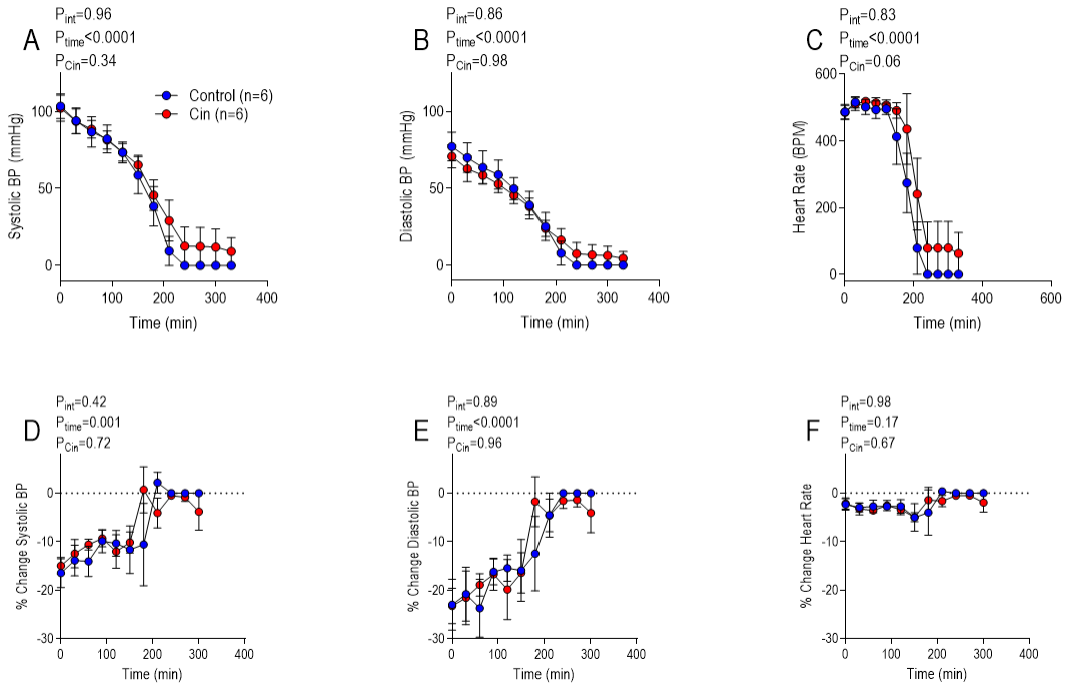


Figure 4-3: Septic Characteristics

Hemodynamics (A-C) in control (C57BL/6) aged and cinaciguat (cin: 15µg/kg IV) treated aged mice over time as sepsis progresses. Vascular responses (D-F) to methacholine (MCH: 1µg/kg IV) over time (Time=0 minutes).

Cinaciguat did increase the probability of survival in aged septic mice (P=0.01, hazard ratio: 0.1440, 95% confidence interval: 0.03071-0.6753; **Figure 4-4: A**). Cinaciguat treatment in aged mice also increased survival time compared to control, with a mean survival time for the untreated aged of 197 minutes and 258 minutes for the aged treated with cinaciguat (P=0.04; **Figure 4-4: B**).

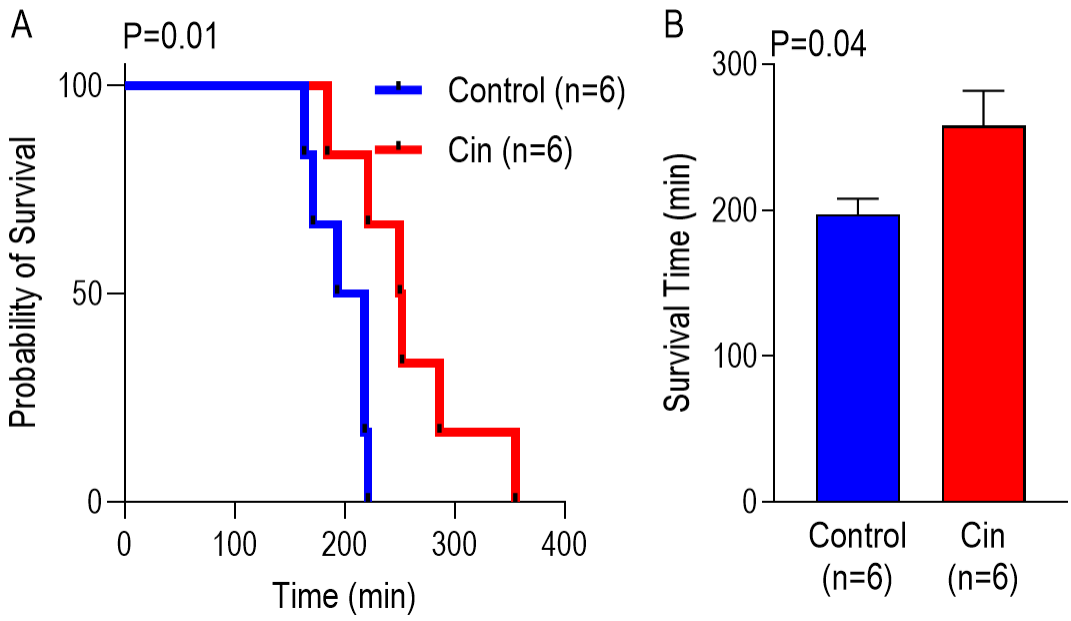


Figure 4-4: Survival

Survival probability (A) and time (B) depicted in minutes for aged septic C57BL/6 and aged cinaciguat (cin: 15µg/kg IV) treated mice.

Cinaciguat did not improve the probability of survival (P=0.28, hazard ratio: 3.349, 95% confidence interval: 0.8630-13.00; **Figure 4-5: A**) in aged septic mice also receiving NE treatment. Cinaciguat also did not increase mean survival time in addition to NE (P=0.10; **Figure 4-5: B**). However, cinaciguat did decrease the total dose of NE required (P=0.02; **Figure 4-5: C**) and the percent septic time with NE infusing (P=0.04; **Figure 4-5: D**).

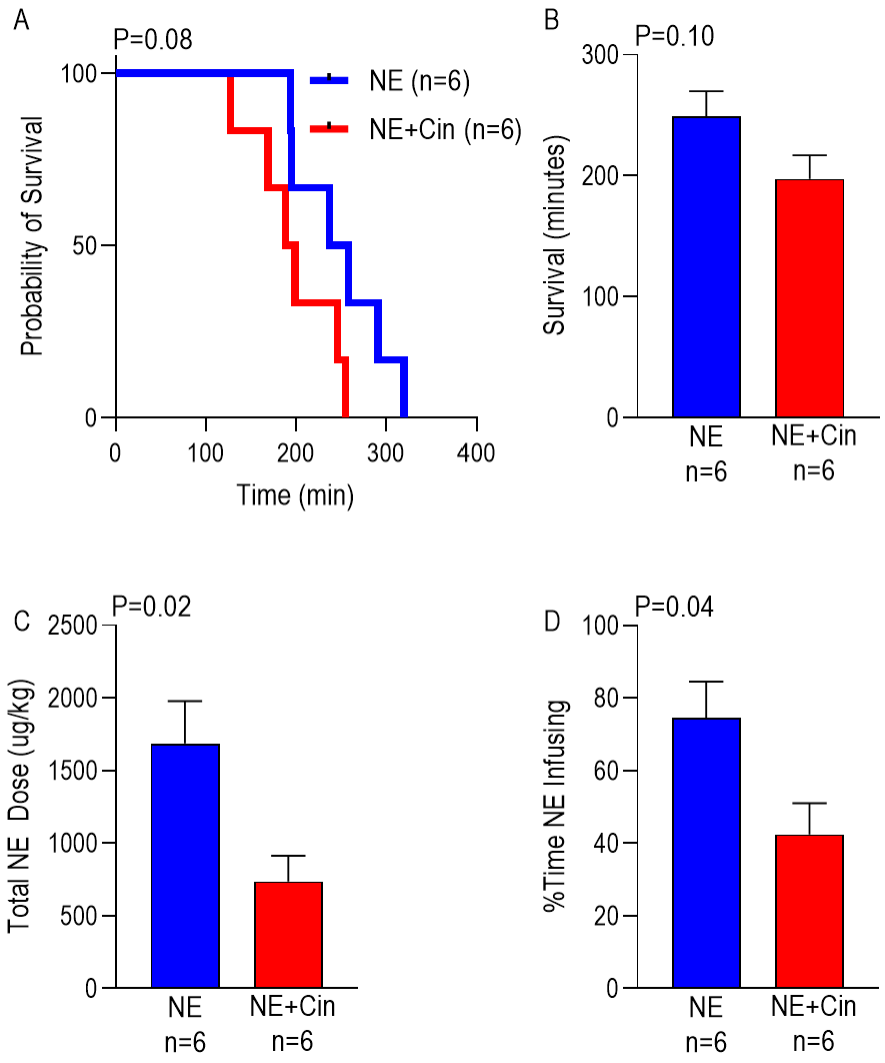


Figure 4-5: Effects of Norepinephrine

(A) Survival probability for aged septic C57BL/6 mice and aged septic C57BL/6 mice that received cinaciguat (cin: 15µg/kg IV) treated with intravenous infusion of norepinephrine (NE). (B) Survival time in minutes for aged septic C57BL/6 mice and aged septic C57BL/6 mice that received cinaciguat (cin: 15µg/kg IV) treated with intravenous infusion of norepinephrine (NE). (C) The total dose of NE infused in µg/kg for aged septic C57BL/6 mice and aged cinaciguat treated mice. (D) % of total survival time spent with NE infusing for aged septic C57BL/6 and aged cinaciguat treated mice.

In *ex-vivo* wire myography studies, there was no difference in the amount of MCH (P=0.06) or SNP (P=0.67) required to cause similar maximal vasorelaxation (MCH P=0.13; SNP P>0.99) in septic carotids (**Figure 4-6**).

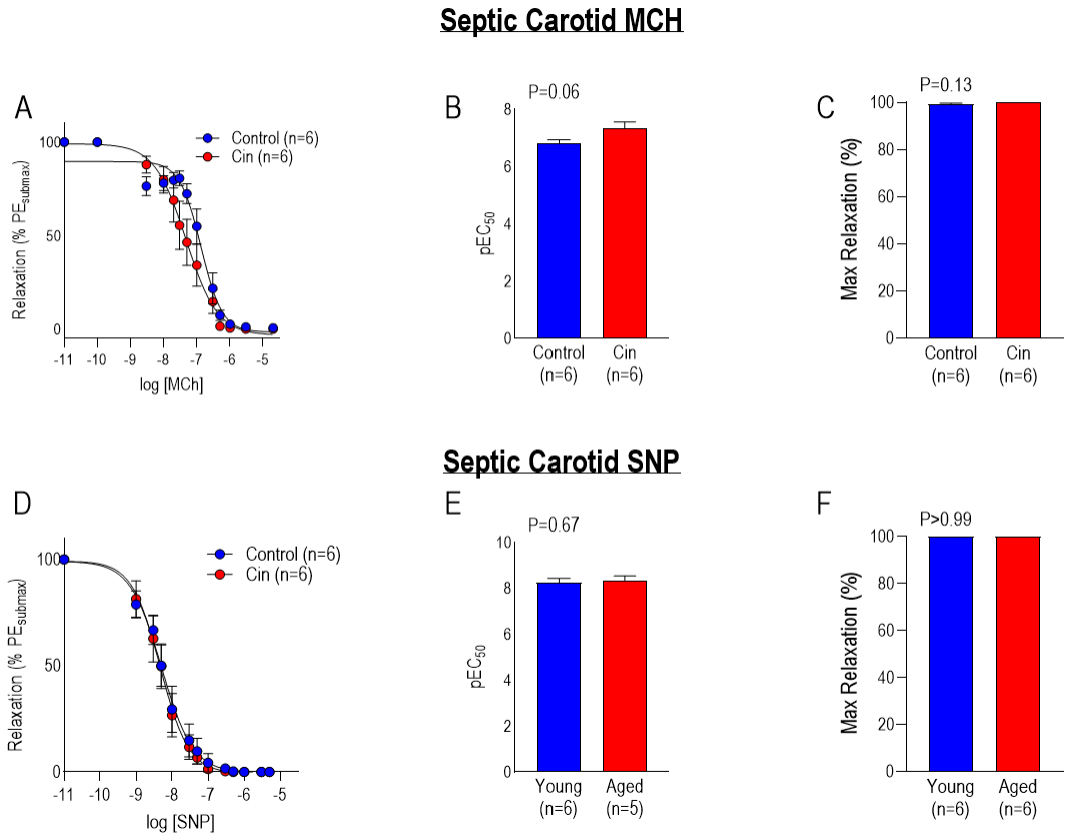


Figure 4-6: Wire Myography Carotid Arteries

Septic *ex-vivo* wire myography for aged carotid arteries depicting vasoactive parameters for control (blue) and cinaciguat (cin: 15µg/kg IV) treated mice (red). (A, D) Carotid methacholine (MCH) and sodium nitroprusside (SNP) cumulative concentration-response curves. (B, E) pEC$_{50}$ for carotid MCH and SNP cumulative concentration-response curves. (C, F) Maximum relaxation achieved by MCH and SNP administration.

There was also no difference in the amount of MCH (P=0.46) and SNP (P=0.54) required to produce a similar level of maximal vasorelaxation (MCH P=0.06; SNP P>0.99) in septic mesenteric arteries (**Figure 4-7**).

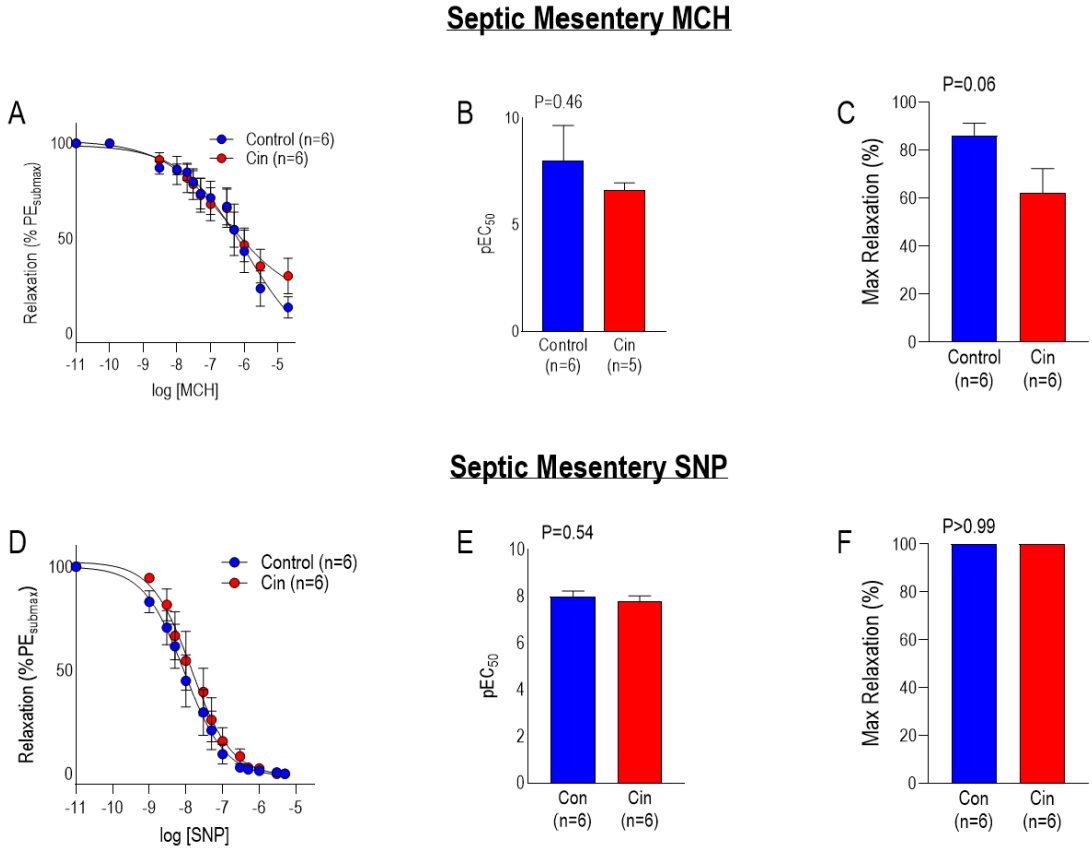


Figure 4-7: Wire Myography Mesenteric Arteries

Septic *ex-vivo* wire myography for aged mesenteric arteries depicting vasoactive parameters for control (blue) and cinaciguat (cin: 15µg/kg IV) treated mice (red). (A, D) Mesentery methacholine (MCH) and sodium nitroprusside (SNP) cumulative concentration-response curves. (B, E) pEC_{50} for mesentery MCH and SNP cumulative concentration-response curves. (C, F) Maximum relaxation achieved by MCH and SNP administration.

No difference was observed between septic control and cinaciguat-treated carotid arteries incubated with LNAME for MCH (P=0.59) or SNP (P=0.52) doses to produce similar (MCH P=0.44; SNP>0.99) levels of maximal vasorelaxation (**Figure 4-8**).

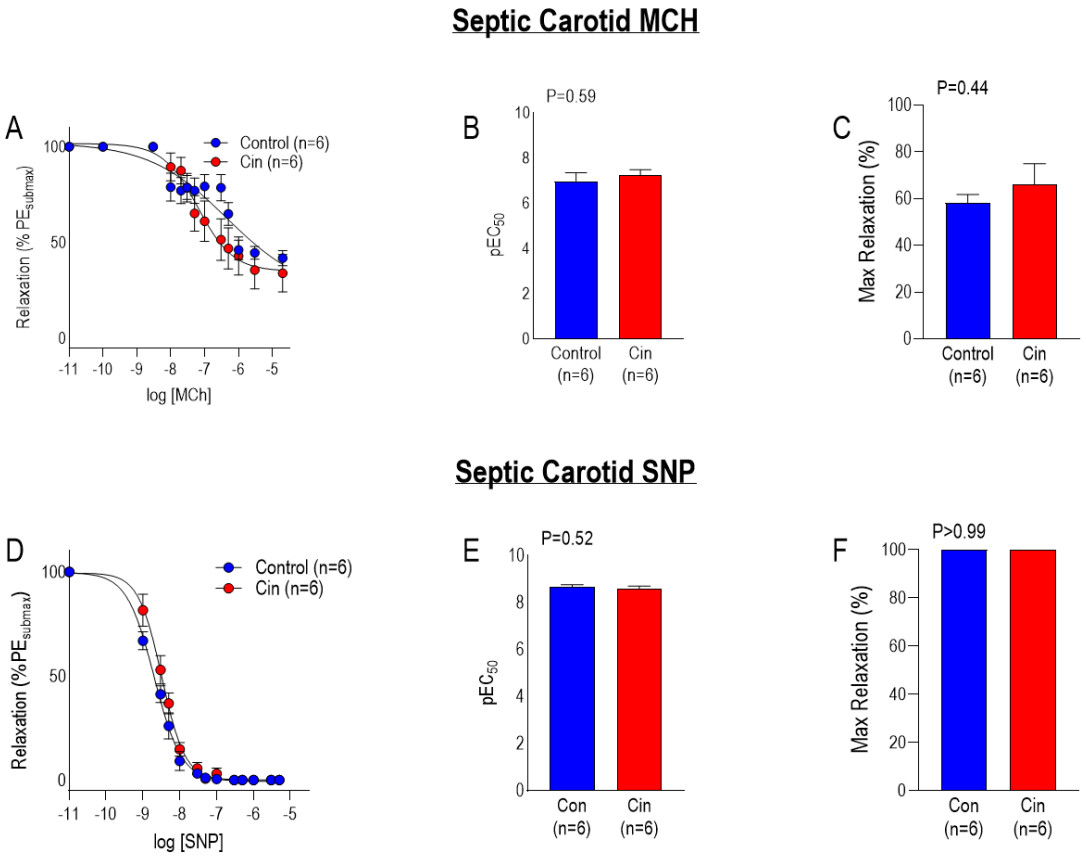


Figure 4-8: Wire Myography LNAME Carotid Arteries

Septic *ex-vivo* wire myography for aged carotid arteries incubated with L-NG-Nitro arginine methyl ester (LNAME) depicting vasoactive parameters for control (blue) and cinaciguat (cin: 15µg/kg IV) treated mice (red). (A, D) Carotid methacholine (MCH) and sodium nitroprusside (SNP) cumulative concentration-response curves. (B, E) pEC_{50} for carotid MCH and SNP cumulative concentration-response curves. (D, F) Maximum relaxation achieved by MCH and SNP administration.

No difference was observed between septic control and cinaciguat-treated mesenteric arteries incubated with LNAME for MCH (P=0.76) or SNP (P=0.06) doses to produce similar (MCH P=0.65; SNP P=0.30) levels of maximal vasorelaxation (**Figure 4-9**).

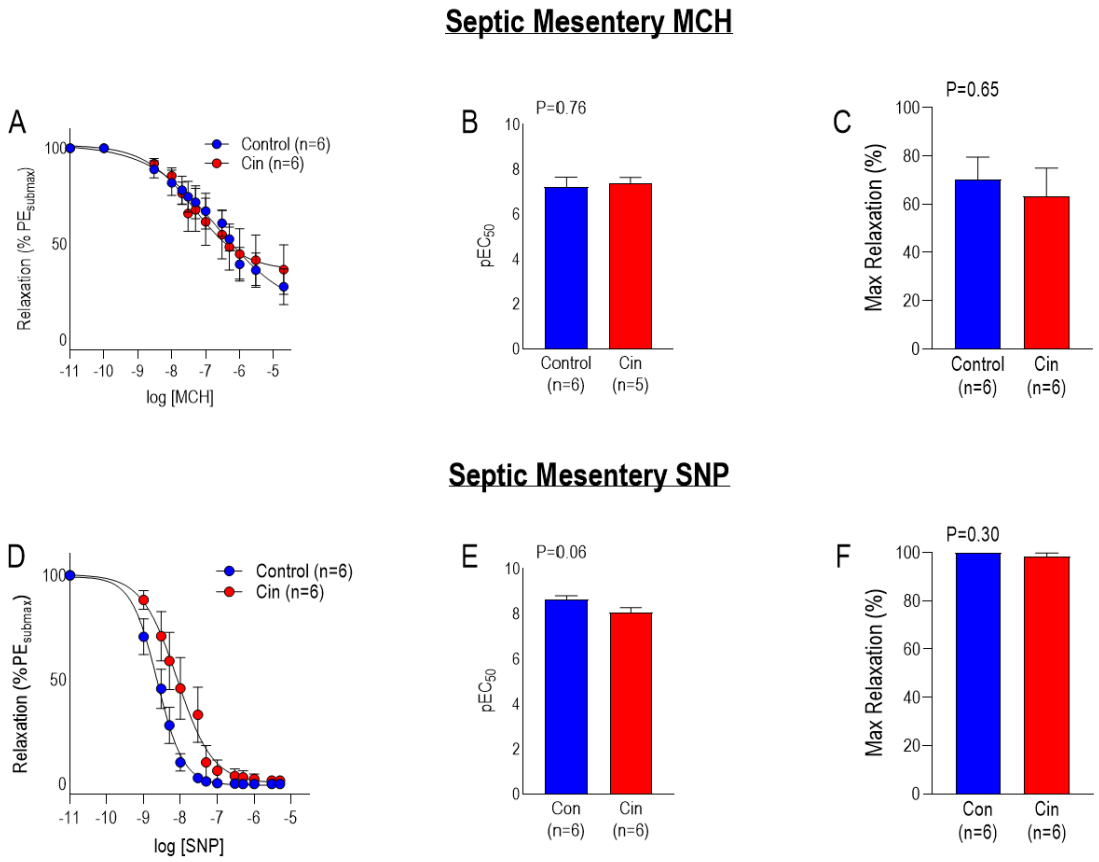


Figure 4-9: Wire Myography LNAME Mesenteric Arteries

Septic *ex-vivo* wire myography for aged mesenteric arteries incubated with L-NG-Nitro arginine methyl ester (LNAME) depicting vasoactive parameters for control (blue) and cinaciguat (cin: 15µg/kg IV) treated mice (red). (A, D) Mesentery methacholine (MCH) and sodium nitroprusside (SNP) cumulative concentration-response curves. (B, E) pEC_{50} for mesentery MCH and SNP cumulative concentration-response curves. (C, F) Maximum relaxation achieved by MCH and SNP administration.

Data demonstrating comparison between control and LNAME treated vessels and cinaciguat control compared to aged LNAME treated vessels are presented in **Figure 3-10** as percent change in area under the curve.

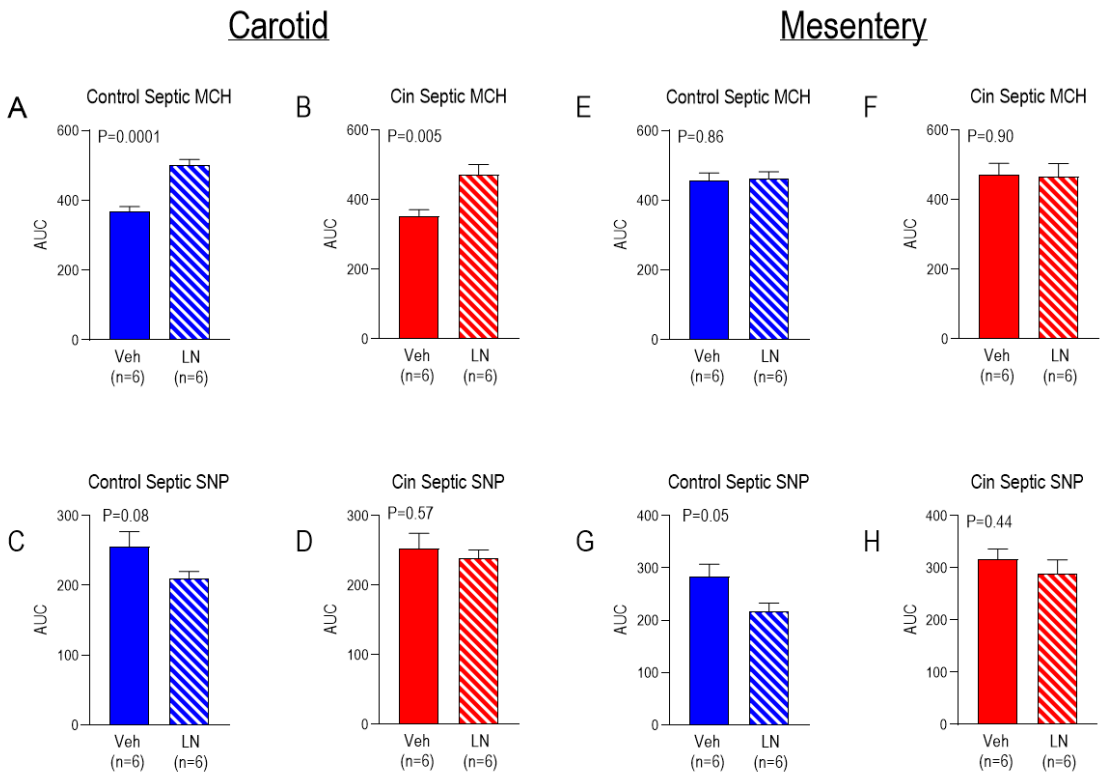


Figure 4-10: Control versus LNAME AUC %Change

Data depicting *ex-vivo* wire myography area under the curves (AUC) comparisons for control and L-N^G-Nitro arginine methyl ester (LNAME) treated vessels incubated with methacholine (MCH) and sodium nitroprusside (SNP) and cinaciguat-treated (Cin) arteries. A, B, C, and D represent carotid arteries. E, F, G, and H represent mesenteric arteries.

Vasoconstriction response to PE was not different in septic carotid (P=0.69) or mesenteric (P=0.08) arteries between control and cinaciguat-treated aged mice (**Figure 4-11**). With

LNAME, vessels were less sensitive to vasoconstriction with PE (pEC_{50} carotid control 6.93±0.05; cinaciguat-treated 6.26±0.18; P=0.006) mesenteric control 6.28±0.10; cinaciguat-treated 5.7 ±0.15; P=0.02) (**Figure 4-12**).

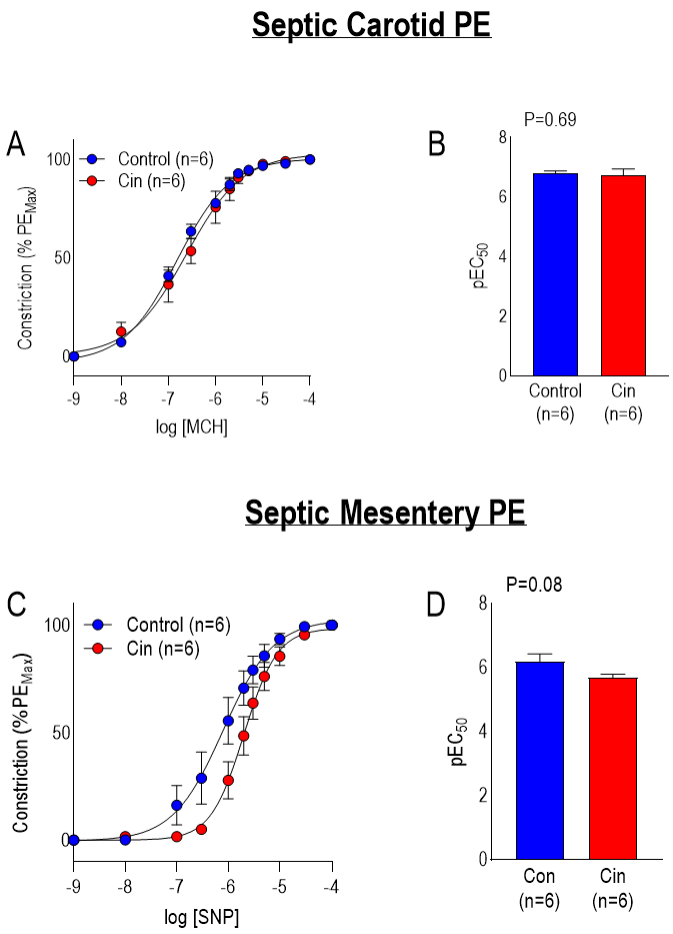


Figure 4-11: Wire Myography Vasoconstriction

Septic *ex-vivo* wire myography for aged carotid and mesenteric arteries depicting vasoactive parameters for control (blue) and cinaciguat-treated (red) mice. (A, C) Phenylephrine (PE) cumulative concentration-response curves (B, D) pEC_{50} for PE cumulative concentration-response curves.

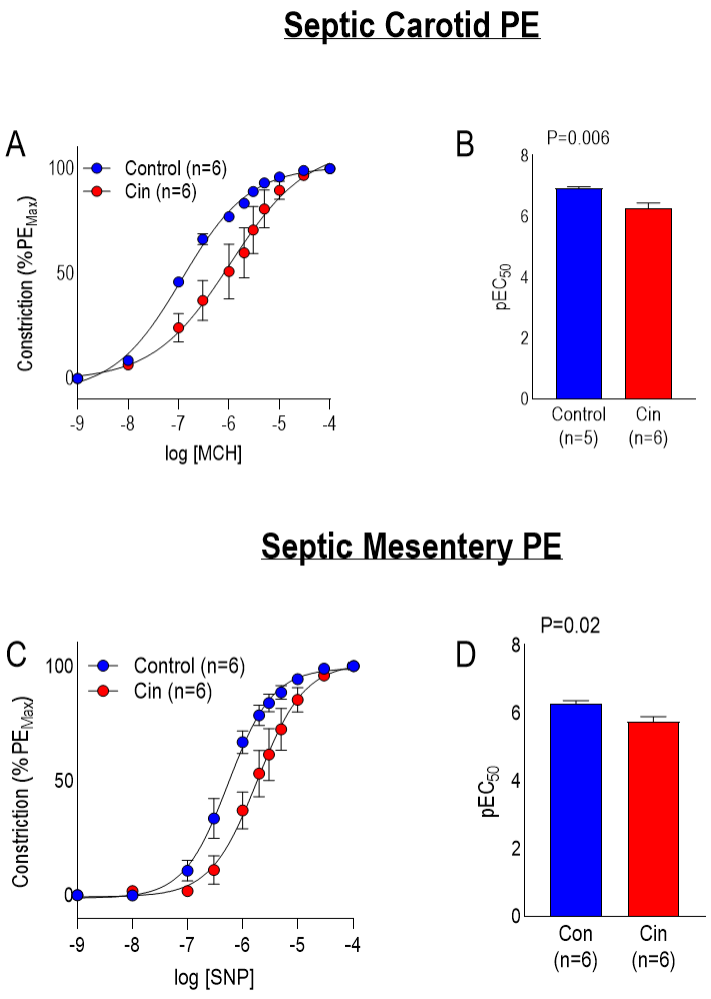


Figure 4-12: Wire Myography LNAME Vasoconstriction

Septic *ex-vivo* wire myography for aged carotid and mesenteric arteries incubated with L-NG-Nitro arginine methyl ester (LNAME) depicting vasoactive parameters for control (blue) and cinaciguat (red) mice. (A, C) Phenylephrine (PE) cumulative concentration-response curves (B, D) pEC_{50} for PE cumulative concentration-response curves.

In aged septic mice, the addition of cinaciguat increased the amount of cGMP (pmol) per mg of lung protein (P=0.02) and in the kidney (P=0.02) (**Figure 4-13**)

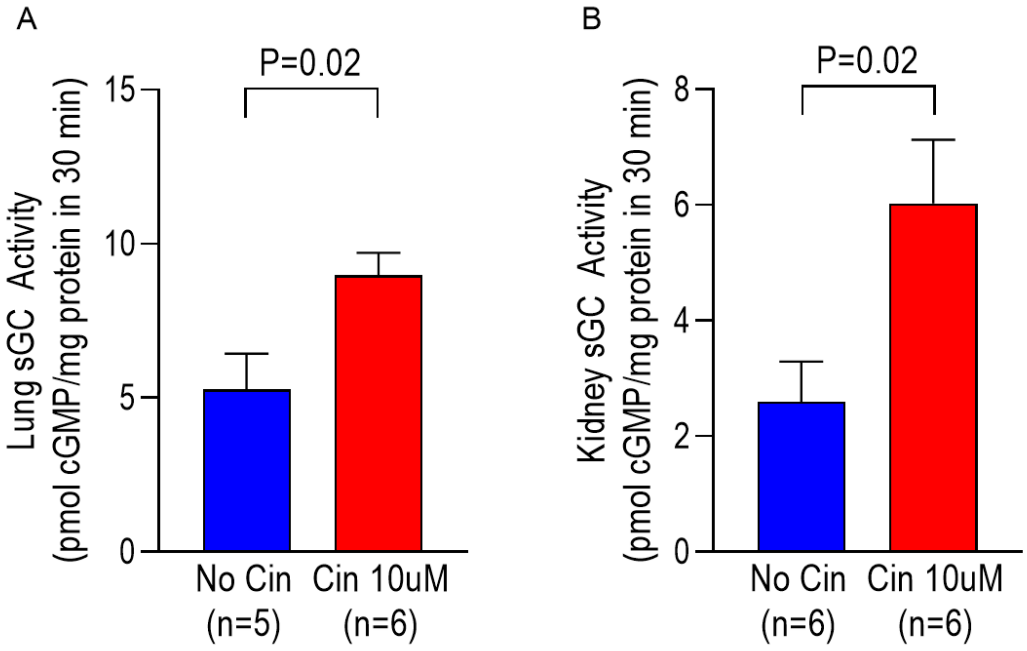


Figure 4-13: Assessment of sGC Activity

Soluble guanylate cyclase (sGC) activity measured as pmol of cGMP produced per mg of protein in 30 minutes from (A) lung and (B) kidney in non-septic and septic C57BL/6 mice by ELISA in the presence of cinaciguat (cin) 0 µM and 10 µM.

Table 11: Summary of Non-Wire Myography Data

DBP: diastolic blood pressure; HR: heart rate; MCH: methacholine; NE: norepinephrine; SBP: systolic blood pressure; sGC: soluble guanylate cyclase

Summary	Effect of Cinaciguat
SBP-septic	Nil
DBP-septic	Nil
HR-septic	Nil
SBP-septic MCH	Nil
DBP-septic MCH	Nil
HR-septic MCH	Nil
Survival	↑
NE	↓ dose, ↓ time infusing
sGC Activity Lung	↑
sGC Activity Kidney	↑

Table 12: Summary of Wire Myography Data

MCH: methacholine; SNP: sodium nitroprusside

Effect of Cinaciguat	
Carotid Arteries	**Mesenteric Arteries**
Baseline: • No change in sensitivity to MCH, SNP, or PE	Baseline: • No change in sensitivity to MCH, SNP, or PE
Baseline: • No change in maximal vasorelaxation or vasoconstriction	Baseline: • No change in maximal vaso relaxation or vasoconstriction
LNAME: • No change in sensitivity for MCH or SNP • ↑ sensitivity to PE	LNAME: • No change in sensitivity for MCH or SNP • ↑ sensitivity to PE
LNAME: • No change in maximal vasorelaxation or vasoconstriction	LNAME: • No change in maximal vasorelaxation or vasoconstriction

4.4 Discussion

This study examined whether treatment with the sGC activator cinaciguat in septic aged mice would improve vascular function and subsequently survival without increasing vasopressor requirements. Before sepsis induction, there were no differences between control and intervention groups regarding hemodynamic parameters and baseline responses to MCH (**Figure 4-2: A-F**). Septic systolic blood pressure and diastolic blood pressure remained the same (**Figure 4-3: A-B**) between control and cinaciguat-treated aged mice. We had hypothesized cinaciguat would improve sGC function in the absence of bioavailable NO and thereby improve vascular function. Improved vascular function was

then hypothesized to improve blood flow to damaged tissues, improve organ function, and subsequently improve blood pressure as we had previously seen in septic young mice (see Chapter 3). We speculate the lack of hemodynamic improvement observed herein with aged mice, was secondary to increased baseline levels of dysfunctional eNOS producing elevated amounts of ROS impacting non-sGC related mechanisms of maintenance of vascular tone. These latter effects would not be affected by cinaciguat treatment. Though not studied herein, it is also possible that increased cGMP in the aged may preferentially affect other vascular pathways upregulated by the chronic absence of bioavailable NO (i.e.: hydrogen sulfide – H_2S) resulting in increased and widespread vasodilation.[610] Response to MCH *in-vivo* was also similar between control and cinaciguat-treated septic aged mice (**Figure 4-3: D-E**). As MCH stimulates NO production from eNOS and the septic aged have dysfunctional eNOS, their endothelial-derived hyperpolarization factor (EDHF) pathways are likely upregulated and most MCH effect is not from increased NO generation by MCH stimulation.

Despite no observed change in systolic blood pressure or diastolic blood pressure, the aged septic mice did experience increased sepsis survival time (**Figure 4-4: A, B**). Improvement in survival may have to do with the non-vascular effects such as improved cardiac or renal function, potentially delaying organ failure.[611]

Clinically, septic patients with a mean arterial pressure of less than 65 mmHg are treated with vasopressor support. This vasopressor is commonly NE as it is a first-line recommended vasopressor.[42] Cinaciguat-treated mice may also display decreased blood pressure due to cGMP-stimulated vasodilation to improve blood flow to organ systems.

We suspected cinaciguat-treated mice might require more NE due to preserved cGMP-signalling to maintain blood pressure. Higher doses of NE may compromise blood flow, attenuating any cinaciguat-induced benefit in survival. Therefore, a subset of aged septic mice was treated with NE. The addition of NE to cinaciguat-treated septic aged mice did not decrease survival (**Figure 4-5: A, B**). However, the aged septic mice treated with cinaciguat required less total NE and spent a reduced time with NE infusing (**Figures 4-5: C, D**). cGMP activation of myosin light chain phosphatase (MLCP) blocks actin-myosin interaction leading to relaxation. As cinaciguat is expected to produce increased cGMP, it would seem that increased doses of NE would be required. However, cinaciguat has more than one effect. cGMP can also increase protein kinase G (PKG) mediated H_2S production.[612] Although H_2S can act as an EDHF in mesenteric arteries,[613-615] it has also been shown to be a vasoconstrictor in other vasculature, including the aorta.[254] This response can also be upregulated in vessels exposed to NE.[616] This may explain why the addition of NE to cinaciguat did not improve or decrease survival.

Ex-vivo myography studies did not demonstrate any effect of cinaciguat in response to SNP or MCH in aged septic carotid arteries. As MCH stimulates eNOS production of NO, due to the decreased substrate, cofactors, and increased presence of uncoupled eNOS as explained above, MCH was unlikely able to produce any change in cGMP production in either control or cinaciguat-treated vessels and vasodilation may have relied on similarly functional EDHF systems. As the elderly are known to have upregulated EDHF responses, the addition of SNP to cinaciguat-treated mice may also not have increased signalling

through the NO signalling pathway as much as through EDHF pathways, resulting in similar vasorelaxation in both groups (**Figure 4-6**).

As demonstrated in **Figure 4-7**, there were also no changes in response to MCH or SNP in the mesenteric arteries of either control or cinaciguat-treated septic aged mice. As EDHF pathways are more important in smaller resistance vessels like the mesenteric arteries, changes in response to cinaciguat administration were less likely than with the larger conduit carotid arteries. As LNAME blocks the function of NOS upstream from the site of cinaciguat function at the sGC, it was expected that LNAME would not demonstrate an effect, and there was no effect seen for either carotid or mesenteric arteries (**Figures 4-8, 4-9**). As cinaciguat-induced H_2S may affect vasoconstriction, we assessed for any difference in vasoconstriction response to PE. Both septic aged carotid and mesenteric arteries from the control and cinaciguat-treated groups possessed the same vasoconstrictive activity in response to PE (**Figures 4-11, 4-12**). In the presence of LNAME, cinaciguat-treated vessels were less sensitive to vasoconstricting effects of PE. Potentially when NOS is inhibited, H_2S production is favoured, resulting in vasoconstriction in control vessels; whereas, in the presence of cinaciguat, enough cGMP is produced to activate H_2S production and affect vasorelaxation through MLCP.

Lastly, to assess sGC activity in response to cinaciguat stimulation, pmol of cGMP produced per milligram of protein for lung and kidney using ELISA radioimmunoassay, were measured. We demonstrated that cinaciguat increased cGMP production in these two organs in aged septic mice. This observation supports the theory that cinaciguat increases cGMP, which may have more than vascular effects (as we showed minimal vascular effect)

to improve sepsis survival. These results differ from those in young septic mice (as seen in Chapter 3). Likely this is secondary to the young possessing "healthier" baseline systems of vascular function (including NO-sGC-cGMP signalling) with decreased baseline reliance on EDHF. Therefore, restoration of NO-sGC-cGMP signalling may improve vascular function and resultant hemodynamics, whereas in the elderly, EDHF and other-organ effects of cinaciguat-induced cGMP elevations may be at play.

4.5 Limitations

There are limitations to our study. A lower dose of fecal slurry used may have caused a less severe phenotype, which though less clinically relevant, may have demonstrated a more significant effect of cinaciguat. Cinaciguat 15 µg/kg IV may have been ineffective in establishing an effect. However, we completed concentration-response studies (5-90 µg/kg IV), with the greatest survival advantage demonstrated with 15 µg/kg IV (**Figure 4-14**). We used NE based on human sepsis clinical guidelines, as optimal septic targets for mice are unknown. Higher or lower doses may have affected survival. Inhaled anesthetic was used and is known to have effects on vascular function.[617] However, most anesthetics have various effects on vascular function, and isoflurane was chosen as it provides a stable, constant anesthetic level non-invasively and does not disrupt the integrity of the peritoneum.

Cinaciguat was administered 30 minutes post-induction of intraperitoneal sepsis with fecal slurry injection. Delaying treatment allows for increased development of increased levels of sepsis-induced ROS-damaged oxidized sGC. We previously had

administered cinaciguat at this time point in our similar studies involving young septic mice (see Chapter 3) with success in demonstrating improvement in several hemodynamic and vascular function parameters as well as improving survival. As the aged were hypothesized to possess more ROS-damaged sGC at baseline and increase this level as time while septic increased, this time point was thought to be appropriate in the aged as well. As we did not observe the same hemodynamic and vascular function improvements in the aged as we did in the young, it is possible that this time point may not be optimal for cinaciguat administration in the aged to fully capture all the benefits. Potentially as the aged have baseline elevations in ROS, earlier administration may have demonstrated more hemodynamic and vascular function effect.

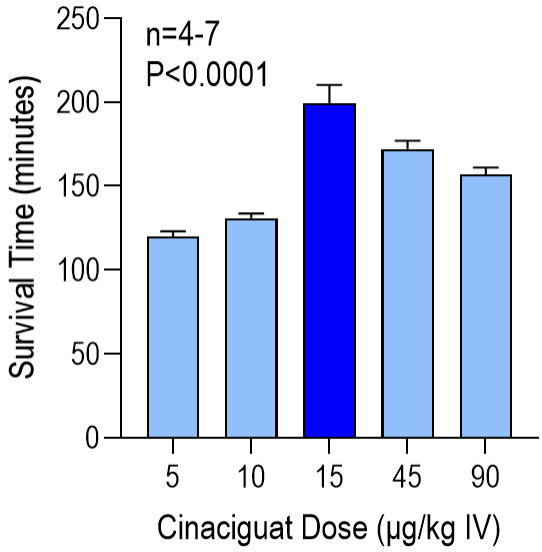


Figure 4-14: Cinaciguat Dose in Aged Mice

4.6 Conclusion

These studies have demonstrated that cinaciguat improves sepsis survival time in aged mice. This effect seems unlikely due to any vasoactive impact as we could not confirm *in-vivo* or *ex-vivo* vascular changes between control and cinaciguat-treated aged mice. As increased cGMP production in response to cinaciguat was observed, we speculate other organ effects of increased cGMP production are responsible for the improved survival.

Chapter 5

Sex-Specific Vascular and Mortality Outcomes of Oncostatin M Receptor Deficiency During Sepsis in Mice

5.1 Background

Sepsis is defined as life-threatening organ dysfunction due to a dysregulated response to infection.[1,618] Despite decades of research, translation of preclinical discoveries has been frustratingly elusive,[14,15] and the mortality of sepsis remains high.[58,62,619] Potentially contributing to our poor rate of preclinical success is the lack of inclusion of both biological sexes in many endeavours.[620,6218,9] Females have historically been under-represented,[620] resulting in an incomplete understanding of the pathophysiology of sepsis and potential bias regarding the relevance of specific cytokines in sepsis development.[622] The belief that being of the female sex is protective during sepsis and that being of the male sex is disadvantageous has been emphasized in numerous preclinical and clinical studies.[70,73,623] Interestingly, an early study showed female sex was an independent predictor of increased mortality in critically ill septic surgical patients.[624] Still, a more recent study showed no sex differences in short or late mortality for septic shock.[625] Several studies have also demonstrated altered cytokine levels between sexes during sepsis,[69] although detailed studies are lacking in this area.

Oncostatin M (OSM) belongs to the interleukin-6 cytokine family and is known to increase during sepsis.[173] It is released by many cell types associated with sepsis, including activated monocytes,[174] macrophages,[175] dendritic cells[174] stimulated by pathogen-associated molecular patterns, and neutrophils[176] activated by lipopolysaccharide. The sex-

specific vascular outcomes of OSM/OSMR (Oncostatin M Receptor) signalling in sepsis are unknown, as are the sex-specific effects on sepsis survival outcomes.

Oncostatin M could be of particular importance in sepsis due to a high presence of OSMR on the vascular endothelium.[626] Loss of vascular smooth muscle function significantly contributes to the distributive shock state developed in sepsis. It is associated with increased nitric oxide (NO) production due to upregulation of inducible nitric oxide synthase (iNOS) by OSM via Jak/STAT signalling and subsequent increased TNF-α as well as IL-6 production (**Figure 5-1**).[177-179] OSM/OSMR signalling can increase endothelial NOS (eNOS)-induced NO generation via PI3K/Akt-mediated phosphorylation.[180] Altogether, this demonstrates that by preventing OSM/OSMR signalling, blood vessels may display impaired vasodilation, which may prevent development of the life-threatening vasodilation present in septic shock.

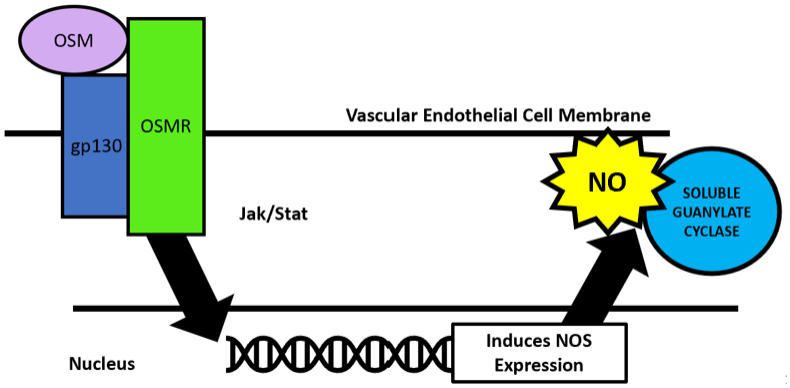


Figure 5-1:OSM Induces NOS Expression

NO: nitric oxide; NOS: nitric oxide synthase; OSM: Oncostatin M; OSMR: Oncostatin M Receptor

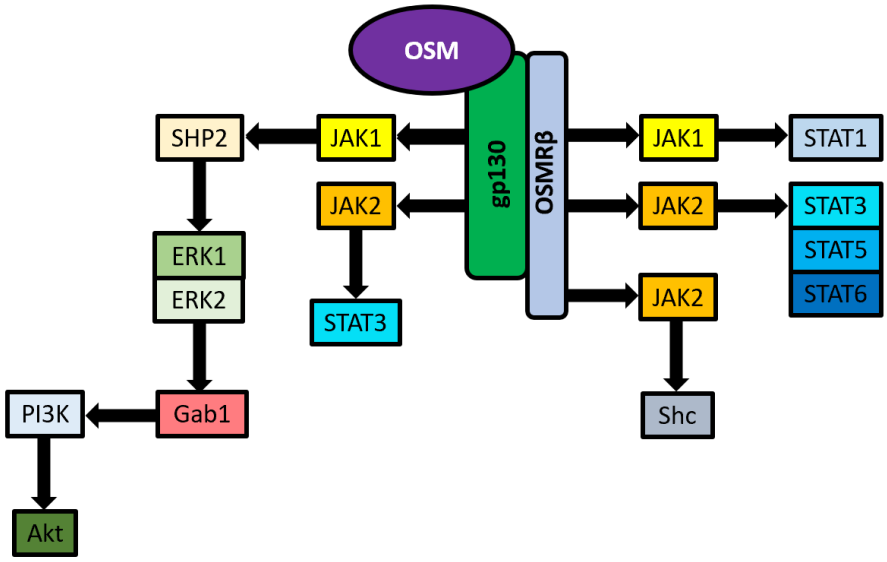


Figure 5-2: OSMR Signalling Pathway

Preliminary reports suggest OSMR deficiency may improve survival in males during sepsis[627] and that recombinant OSM administration to mice with polymicrobial intra-abdominal sepsis increased mortality.[173] Anti-OSM antibody has also been shown to improve survival.[173] Our prior work has demonstrated reduced mortality and lung injury in male OSMR deficient mice exposed to acute intestinal ischemia-reperfusion injury.[628] We hypothesized that by reducing OSM/OSMR signalling and thereby the production of NO, OSMR deficient mice may prevent the development of vascular collapse (also known as vasoplegia), resulting in improved survival. We expected OSMR deficiency might improve survival and mitigate the vascular dysfunction[173] to a greater extent in males than females as prior non-OSM cytokine studies have demonstrated a potential link between increased cytokine levels and male sex regarding mortality. Also, NO may be of more

importance in vasodilation in males,[629] whereas females may rely more heavily on endothelial-derived hyperpolarizing factors (EDHF).[630]

Vascular changes influenced by OSMR deficiency (and subsequent NO deficiency) as a mechanism for developing vasodilatory septic shock have not been rigorously evaluated, nor has its effect on survival been studied. The impact of sex on OSM/OSMR signalling has also not been assessed. In the present study, we aimed to investigate the sex-specific role of OSMR signalling related to vascular function through its effects on the nitric oxide synthase (NOS) system with both *in-vivo* and *ex-vivo* measurements of vascular function. We also sought to investigate whether impaired vasodilation (and subsequent elevation or stability of *in-vivo* blood pressure) would ultimately translate into a sex-specific survival benefit.

5.2 Methods

All studies were carried out in accordance with Canadian Council on Animal Care guidelines and the Animal Care and Use Committee at the University of Alberta (Edmonton, Canada), and reported as per Animal Research: Reporting of In Vivo Experiments (ARRIVE) Guidelines.[631]

5.2.1 Animals and Preparation

Forty-five female and 45 male C57BL/6 mice and 45 female and 45 male OSMR deficient mice, drug and procedure naïve, aged 3-4 months, were used. C57BL/6 mice were purchased from Charles-River Inc. (St-Constant, QC) and OSMR homozygous knockouts

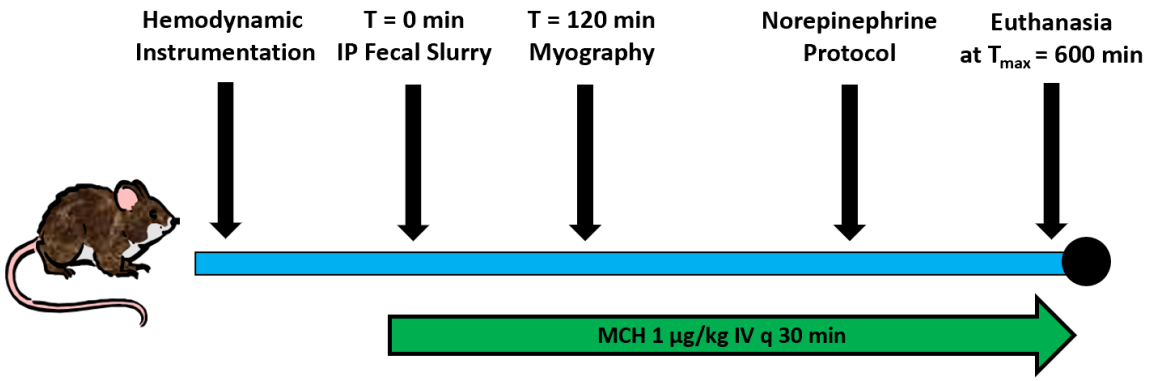
(OSMR-/-) were obtained from the line of B6.129S-Osmr$^{<tm1Mtan>}$ heterozygotes bred by Charles River Laboratories. Mice were housed in shoebox cages (five mice per cage – separated by sex) containing aspen-chip bedding, nesting material, and PVC tubing. Mice were located in the University of Alberta animal care facility, which maintained a 12h:12h light:dark cycle and an ambient temperature of 22±1ºC. Trained animal care personnel routinely performed welfare assessments before experimentation. Mice had *ad libitum* access to tap water and standard grain-based rodent chow (PicoLab 5LOD, LabDiet, St. Louis, MO).

Experiments were conducted in the same laboratory operating theatre setting during the day. Mice were anesthetized with inhaled isoflurane (induction: 5%, maintenance: 1.1-1.5% in 100% O_2) and were kept spontaneously breathing via a nosecone on a warmed surgical platform; body temperature was monitored via a rectal thermometer. Following induction of anesthesia, mice were instrumented with a fibreoptic pressure sensor (0.9Fr/0.5Fr; FISO Technologies Inc.) in the right carotid artery for hemodynamic assessments (blood pressure and heart rate) and a polyethylene catheter (PE10; inner diameter 0.58 mm, outer diameter 0.97 mm) in the left femoral vein for fluid and drug delivery. The mice were administered maintenance hydration with sterile saline (2 mL/kg/hour IV). Following instrumentation, mice were given 30 minutes to achieve stable baseline hemodynamic parameters. Arterial blood pressure and heart rate were monitored and recorded using Lab Chart Pro 8 (ADInstruments, Colorado Springs, CO). In non-septic and septic mice (see below), vascular reactivity was assessed via bolus administration of the endothelial-dependent vasodilator methacholine (MCH) (1 µg/kg IV); this dose of

MCH was selected as it causes measurable but transient drops in blood pressure without affecting heart rate (as per our preliminary studies – **Figure 2-1**).

5.2.2 Study Protocol

To induce polymicrobial peritonitis, mice were injected with fecal slurry (1.3 mg/g IP – dose determined in pilot studies); controls received sterile 0.9% saline as vehicle. Mice were allocated to one of three experimental protocols. In the first protocol, hemodynamic and vital parameters were assessed continuously via indwelling pressure sensors; in these mice, vascular reactivity was also evaluated using intravenous bolus doses of MCH every 30 minutes. In a second protocol, mice were also instrumented with pressure sensors for hemodynamic measurements, but underwent a treatment regimen with the vasopressor norepinephrine (NE) when they reached predefined endpoints (mean arterial pressure less than 65 mmHg or systolic blood pressure less than 80 mmHg). Intravenous NE infusion was initiated at 200 µg/kg/hour and advanced by 100 µg/kg/hour in five-minute intervals until the goal of mean arterial pressure greater than 65 mmHg or systolic blood pressure greater than 80 mmHg was reached to a maximum of 600 µg/kg/hour. This protocol aimed to mimic a clinical intervention with a vasopressor and evaluate survival as sepsis progresses to septic shock. In the third protocol, mice were intraperitoneally injected with fecal slurry or saline, euthanized after 120 min, and had vessels harvested to assess vascular function *ex-vivo* by wire myography.



MCH: methacholine
Norepinephrine Protocol: titrate norepinephrine 200-600 µg/kg/h IV when MAP < 65 mmHg or SBP < 80 mmHg

Figure 5-3: Study Protocol

Schematic of study protocol including methacholine (MCH) and norepinephrine (NE) administration.

5.2.3 Wire Myography Protocol

After induction of sepsis by injection of fecal slurry (1.3 mg/g body weight IP) or saline, mice were maintained under anesthesia for 120 minutes and subsequently euthanized by exsanguination and excision of the heart. Second-order mesenteric arteries were rapidly removed and placed in ice-cold HEPES-buffered physiologic saline solution (PSS – composition: (in mM) HEPES 10, glucose 5.5, $CaCl_2$ 1.56, KCl 4.7, NaCl 142, $MgSO_4$ 1.17, KH_2PO_4 1.18, pH 7.45). Vessels were cleaned of extraneous perivascular connective tissues. Small vessel sections (2 mm in length) were isolated and mounted in an isometric myograph system (DMT, Copenhagen, Denmark); 15 µm gold wire was used for mesenteric arteries. Vessels were bathed continuously in PSS at 37ºC for the experiments. After mounting, vessels were normalized to optimal resting tension (0.8 IC_{100}: the internal

circumference equaling a transmural pressure of 100 mmHg) by small incremental increases in diameter. Vessels were then allowed 30 minutes to equilibrate to the optimal resting tension before initiating the experiment. Before experimentation, vessel viability was assessed by exposing vessels to high potassium PSS (KPSS) treatment. After rinsing, vessels were twice treated with phenylephrine (10 μmol/L), with multiple rinses and a return to baseline tension between treatments; following the second treatment with phenylephrine (PE: 10 μmol/L), MCH (3 μmol/L) was administered to the baths to test the integrity of the endothelium. Following these viability assessments, vessels were rinsed multiple times and left to return to baseline tension. Next, concentration-response studies for PE were performed. Thereafter, a subset of vessels was then incubated with non-selective NOS inhibitor L-N^G-nitro-arginine-methyl ester (LNAME: 100 μmol/L) for 30 minutes before performing cumulative concentration-response curves with MCH; to do this, vessels were first sub-maximally pre-constricted (80% maximum: EC_{80}) with the vasoconstrictor PE. Finally, repeat vascular constriction to KPSS was assessed; those vessels with final KPSS constriction of less than 80% of the first KPSS constriction were excluded from analyses.

5.2.4 Reagents

MCH and NE (Sigma, St-Louis, MO) were dissolved in sterile saline (0.9% NaCl) for *in-vivo* experiments. MCH was diluted in PSS for use in myography experiments. PE and LNAME (Sigma, St-Louis, MO) were dissolved in sterile saline and diluted in PSS for use in myography experiments.

5.2.5 Statistical Analysis

Data are presented as mean±SEM. Patterns of cardiovascular collapse (as depicted by Kaplan Meier curves) were analyzed by log-rank tests. Continuous variables, including arterial pressures, heart rates and hemodynamic responsiveness to exogenous pharmacological agents were analyzed by repeated measures 2-way ANOVA for the overall effects of sepsis and time; subjects were matched within the time domain. The remaining data were analyzed by unpaired Student's t test. For collection of septic systolic blood pressure, diastolic blood pressure, and heart rate, the most representative five-second segment within each 30-second interval was captured from recorded parameters. Percent changes in hemodynamic variables in response to MCH administration were calculated as the minimum value compared to the baseline in the immediately preceding five-minute interval. For wire myography, concentration-response curves were fitted to the Hill equation using a variable slope, and EC_{50} (mean effective concentration to produce a 50% response) and maximal responses were analyzed by unpaired *t*-test. Statistical outliers determined by Grubb's test were not included. Results were considered significant if $P<0.05$. GraphPad Prism 8 software (La Jolla, CA) was employed for statistical analysis.

5.3 Results

Prior to the induction of sepsis, male OSMR deficient mice under isoflurane anesthetic displayed a distinct hemodynamic profile with elevated systolic blood pressure (OSMR-/- 138±8 mmHg; C57BL/6 113±5 mmHg; P=0.02) and diastolic blood pressure (OSMR-/- 100±7 mmHg; C57BL/6 78±5 mmHg; P=0.02) as compared to non-septic C57BL/6 mice

(**Figure 5-2: A, B**). The heart rate in male OSMR deficient mice was also elevated (OSMR-/- 491±20 beats per minute; C57BL/6 427±15 beats per minute; P=0.03) (**Figure 5-2: C**). The vasodilatory response to the non-selective muscarinic agonist MCH was compared in males of both genotypes under non-septic and septic conditions. Here, MCH was administered to test vascular reactivity. The dose used does not appreciably affect heart rate (tested in our pilot studies – see Chapter 2; **Figure 2-1**). Therefore, the changes in blood pressure largely reflect changes in systemic vascular resistance. Under non-septic conditions, MCH produced a sharp decrease in blood pressure which returned to baseline within 10 seconds. In male C57BL/6 mice, MCH (1 ug/kg) caused an approximately 15% reduction in systolic blood pressure, whereas this effect was mitigated in OSMR deficient mice (**Figure 5-2: D**). Similar outcomes were evident in diastolic blood pressure recordings (**Figure 5-2: E**). MCH also caused greater bradycardic responses in male OSMR deficient mice compared to C57BL/6 mice (**Figure 5-2: F**).

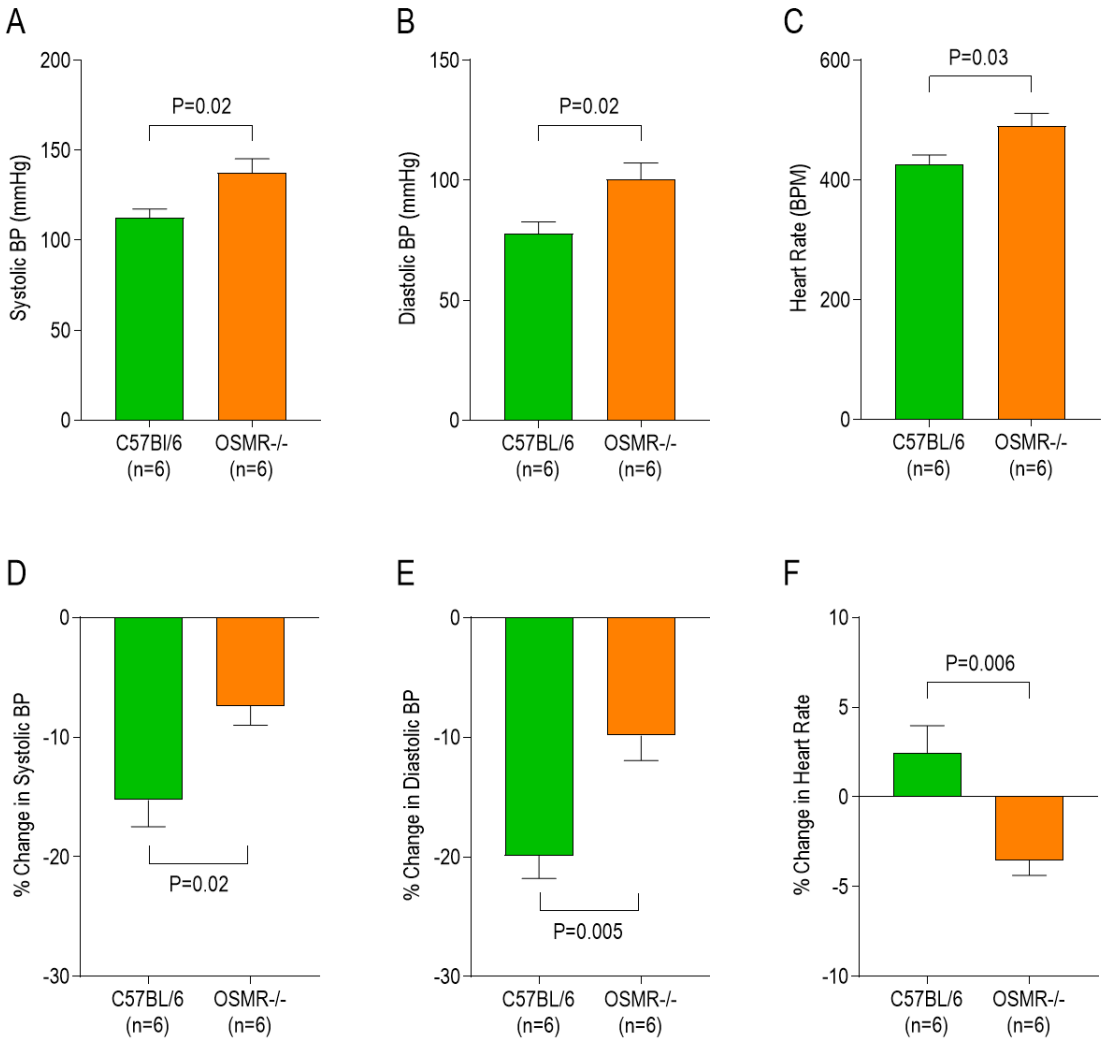


Figure 5-4: Baseline Male Characteristics

Baseline Hemodynamics (A-C) and vascular responses to methacholine (MCH) (D-F) in control male (C57BL/6) and OSMR deficient (OSMR-/-) mice.

Female OSMR deficient mice also displayed a distinct non-septic hemodynamic phenotype. Systolic blood pressure (in mmHg: OSMR-/-139±10; C57BL/6 98±3; P=0.002; **Figure 5-3: A**), diastolic blood pressure (in mmHg: OSMR-/- 112±10; C57BL/6 85±4; P=0.03; **Figure 5-3: B**), and heart rate (in beats per minute (BPM): OSMR-/- 509±10;

C57BL/6 440±3; P=0.03; **Figure 5-3: C**) were all elevated in female OSMR deficient mice as compared to female C57BL/6 mice. The MCH effect on systolic blood pressure was blunted in OSMR deficient mice compared to controls (OSMR-/- -21±10%; C57BL/6 -14±3%, with no effect on diastolic blood pressure (P=0.29) or heart rate (P=0.09).

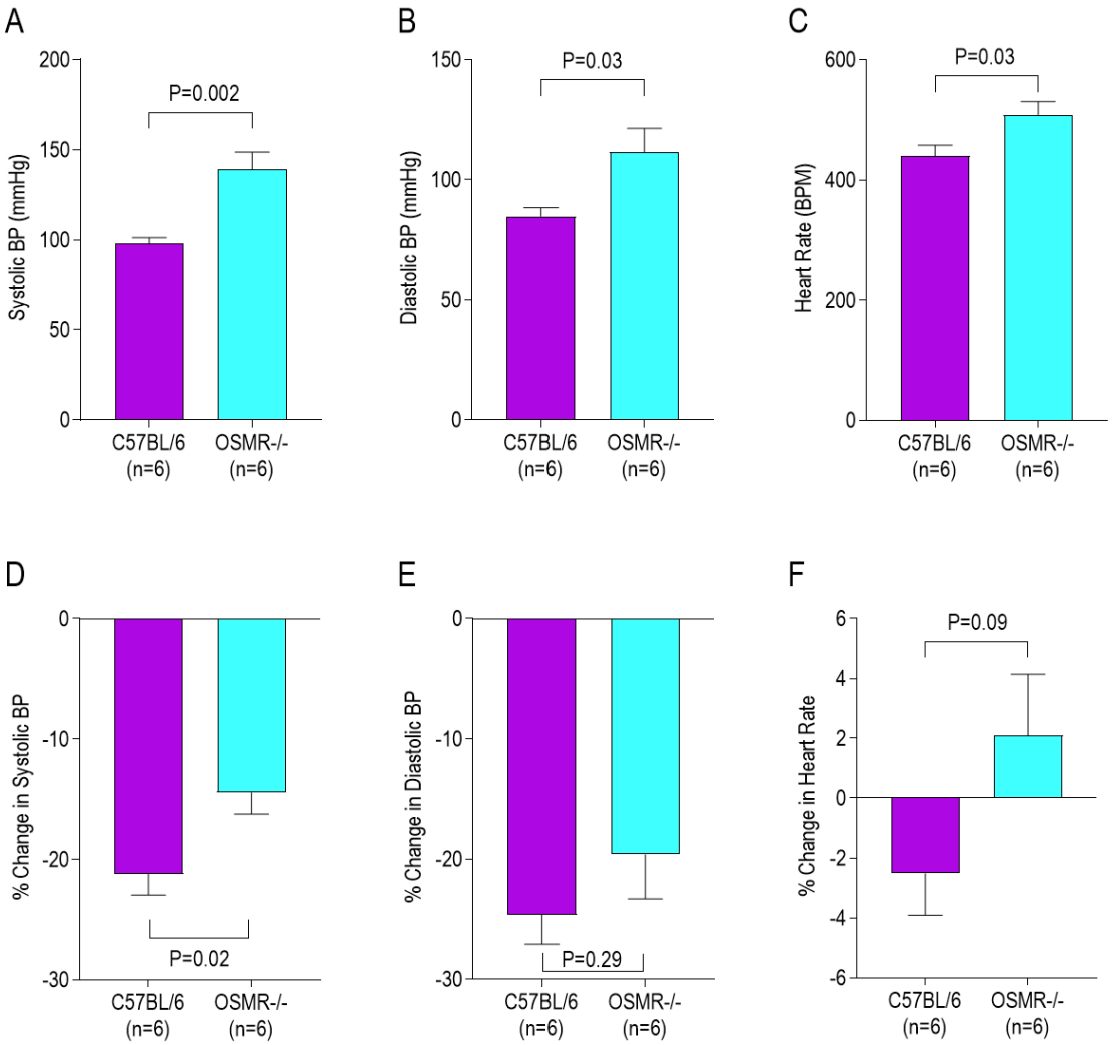


Figure 5-5: Female Baseline Characteristics

Baseline Hemodynamics (A-C) and vascular responses to methacholine (MCH) (D-F) in control female (C57BL/6) and OSMR deficient (OSMR-/-) mice.

Following the induction of sepsis in male mice, both genotypes exhibited gradual decreases in systolic blood pressure (P<0.0001; **Figure 5-4: A**) and diastolic blood pressure (P<0.0001; **Figure 5-4: B**) over time. However, the septic OSMR deficient mice displayed a higher systolic and diastolic blood pressure (systolic: P=0.003; diastolic: P=0.005; **Figure 5-4: A, B**) than the C57BL/6 mice throughout the entire septic time course. The heart rate of septic male C57BL/6 mice was similar to that of the OSMR deficient mice (P=0.05; **Figure 5-4: C**). After induction of sepsis, the systolic blood pressure responses to MCH decreased significantly in both male genotypes but to a greater extent in the OSMR deficient mice (P=0.02; **Figure 5-4: D**). Diastolic blood pressure response over time in septic male mice did not decrease over time in either genotype (p=0.66); however, the OSMR deficient response remained reduced compared to the C57BL/6 (P<0.0001; **Figure 5-4: E**). Heart rate response over time in septic male mice did not appreciably change over time in either genotype (P=0.44), and was not different between genotypes (P=0.11; **Figure 5-4: F**).

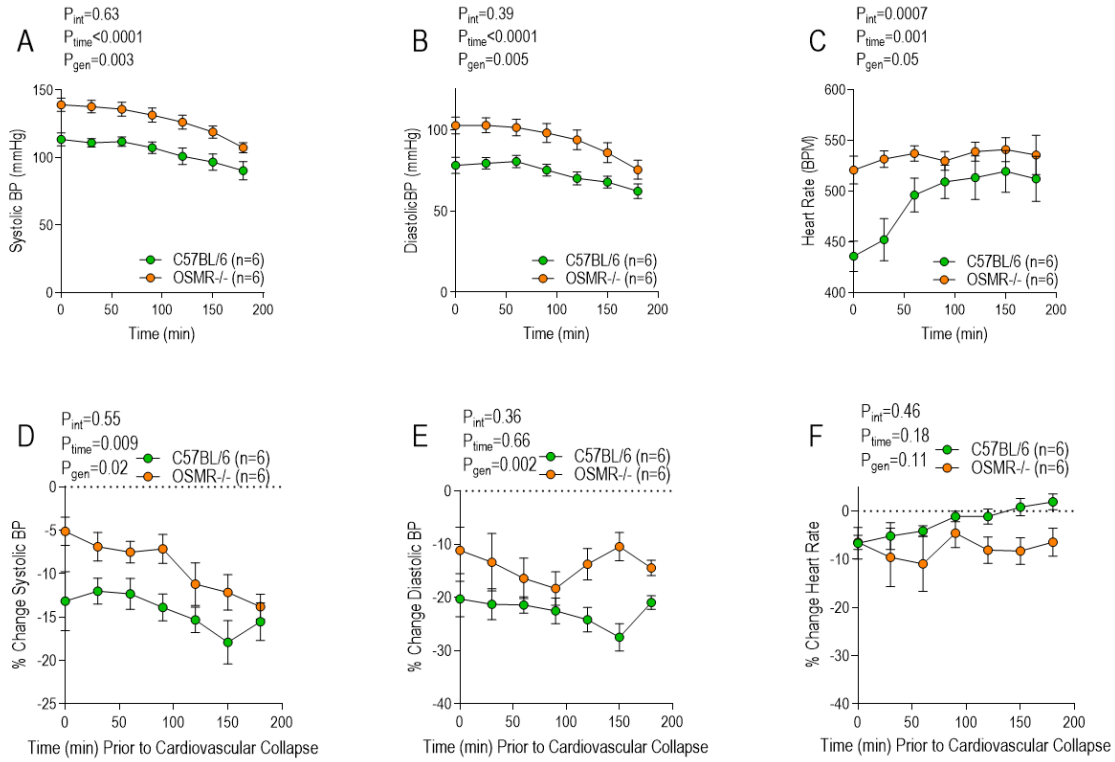


Figure 5-6: Septic Male Characteristics

Male hemodynamics (A-C) in control (C57BL/6) and OSMR deficient (OSMR-/-) mice over time as sepsis progresses. Male vascular responses (D-F) to methacholine (MCH: 1 µg/kg IV) beginning at cardiovascular collapse (Time=0 minutes).

In female septic mice, there were fewer genotype-specific differences. Although both the systolic (P=0.002; **Figure 5-5: A**) and diastolic blood pressure (P=0.003; **Figure 5-5: B**) decreased over time for both female genotypes, there was no change in heart rate (P=0.36; **Figure 5-5: C**). Female OSMR deficient mice had higher systolic blood pressure (P=0.04; **Figure 5-5: A**) and heart rate (P=0.0002; **Figure 5-:5 C**) than C57BL/6 mice. There was no difference in diastolic blood pressure between the genotypes (P=0.28; **Figure 5-5: B**). Similarly, MCH resulted in decreases in systolic (P<0.0001; **Figure 5-5: D**) and

diastolic blood pressure (P<0.0001; **Figure 5-5: E**) in both genotypes over time, and no change in heart rate for either genotype (P=0.50; **Figure 5-5: F**). There were no genotype-specific changes in systolic (P=0.47) or diastolic blood pressure (P=0.36) response to MCH, or with heart rate (P=0.26; **Figure 5-5: F**), consistent with results shown in **Figure 5-3: C**.

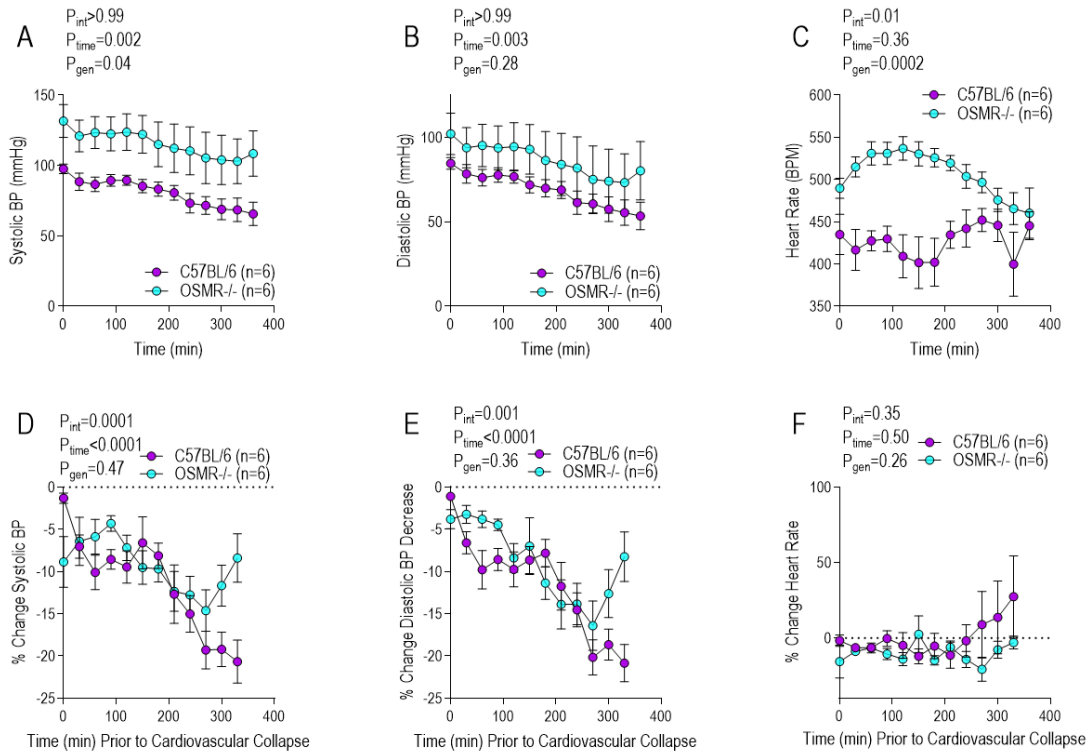


Figure 5-7: Septic Female Characteristics

Female hemodynamics (A-C) in control (C57BL/6) and OSMR deficient (OSMR-/-) mice over time as sepsis progresses. Vascular responses (D-F) to methacholine (MCH: 1 µg/kg IV) beginning at cardiovascular collapse (Time=0 minutes).

Despite the altered hemodynamic profile of the OSMR deficient mice, neither sex demonstrated a survival advantage associated with OSMR deficiency (**Figure 5-6: A-D**).

Male C57BL/6 mice had a median survival of 249 minutes and male OSMR deficient mice had a median survival of 227 minutes (P=0.36, hazard ratio 1.718, 95% confidence interval: 0.5110-5.774)). Female C57BL/6 mice had a median survival of 463 minutes and female OSMR deficient mice had a median survival of 479 minutes (P=0.52, hazard ratio: 0.6228, 95% confidence interval 0.1741-2.229).

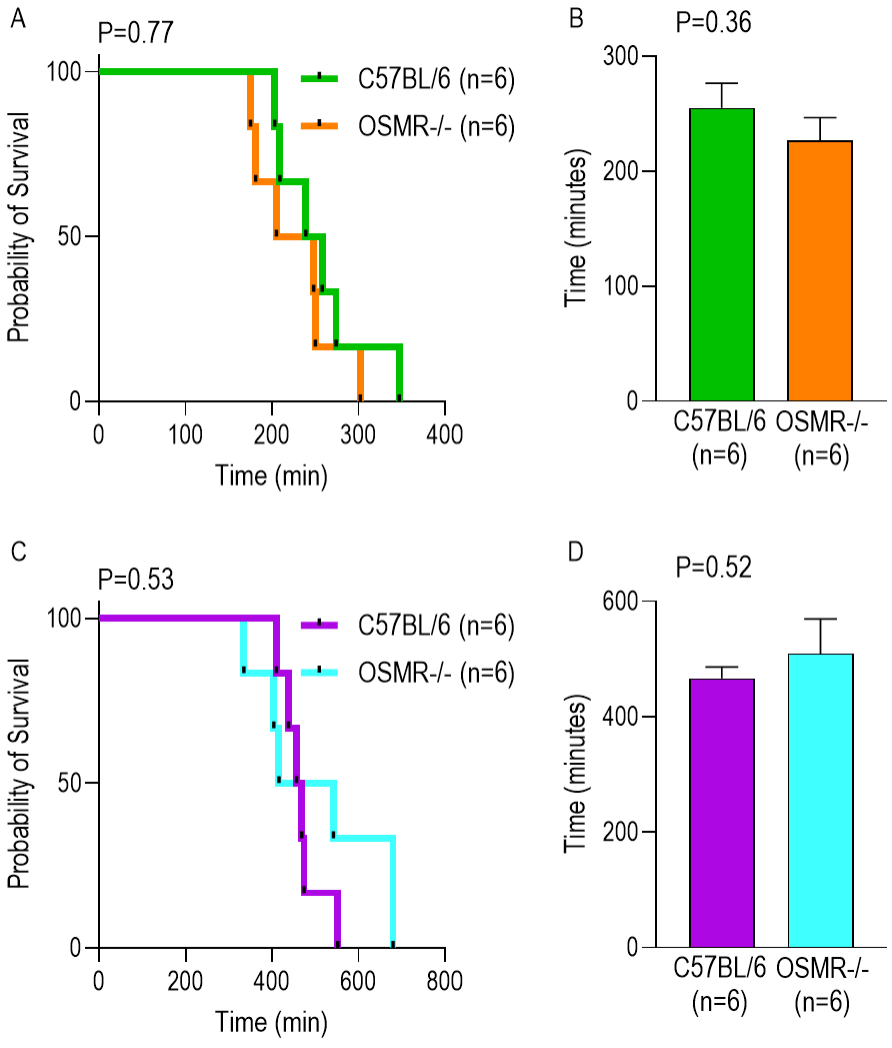


Figure 5-8: Survival

(A) Survival probability for septic male C57BL/6 and OSMR deficient (OSMR-/-) mice. (B) Survival time depicted in minutes for septic male C57BL/6 and OSMR deficient (OSMR-/-) mice. Survival probability for septic female C57BL/6 and OSMR deficient (OSMR-/-) mice. (D) Survival time depicted in minutes for septic female C57BL/6 and OSMR deficient (OSMR-/-) mice.

NE treatment was instituted to model the clinical management of sepsis and increase translational potential. NE did improve the probability of survival in male OSMR deficient mice (P=0.001; **Figure 5-7: A, B**). Male OSMR deficient mice demonstrated significantly improved survival when treated with NE (median survival time: C57BL/6 242 minutes, OSMR deficient 354 minutes; P=0.0003, hazard ratio: 0.07867, 95% confidence interval: 0.01717-0.3606), along with the decreased percent of survival time spent with NE infusing (median time spent with NE infusing: C57BL/6 83 minutes, OSMR deficient 44 minutes; P=0.04)) concomitant with a reduced total amount of NE required (P=0.03) (**Figure 5-7: C, D).** While NE treatment in females failed to improve survival time as observed in males (P=0.36, hazard ratio: 1.310, 95% confidence interval 0.3850-4.458; **Figure 5-8: A, B**), OSMR deficiency mice did reduce time spent with NE infusing (P=0.002) and a showed a reduction in the total amount of NE infused (P=0.0007) (**Figure 5-8: C, D**).

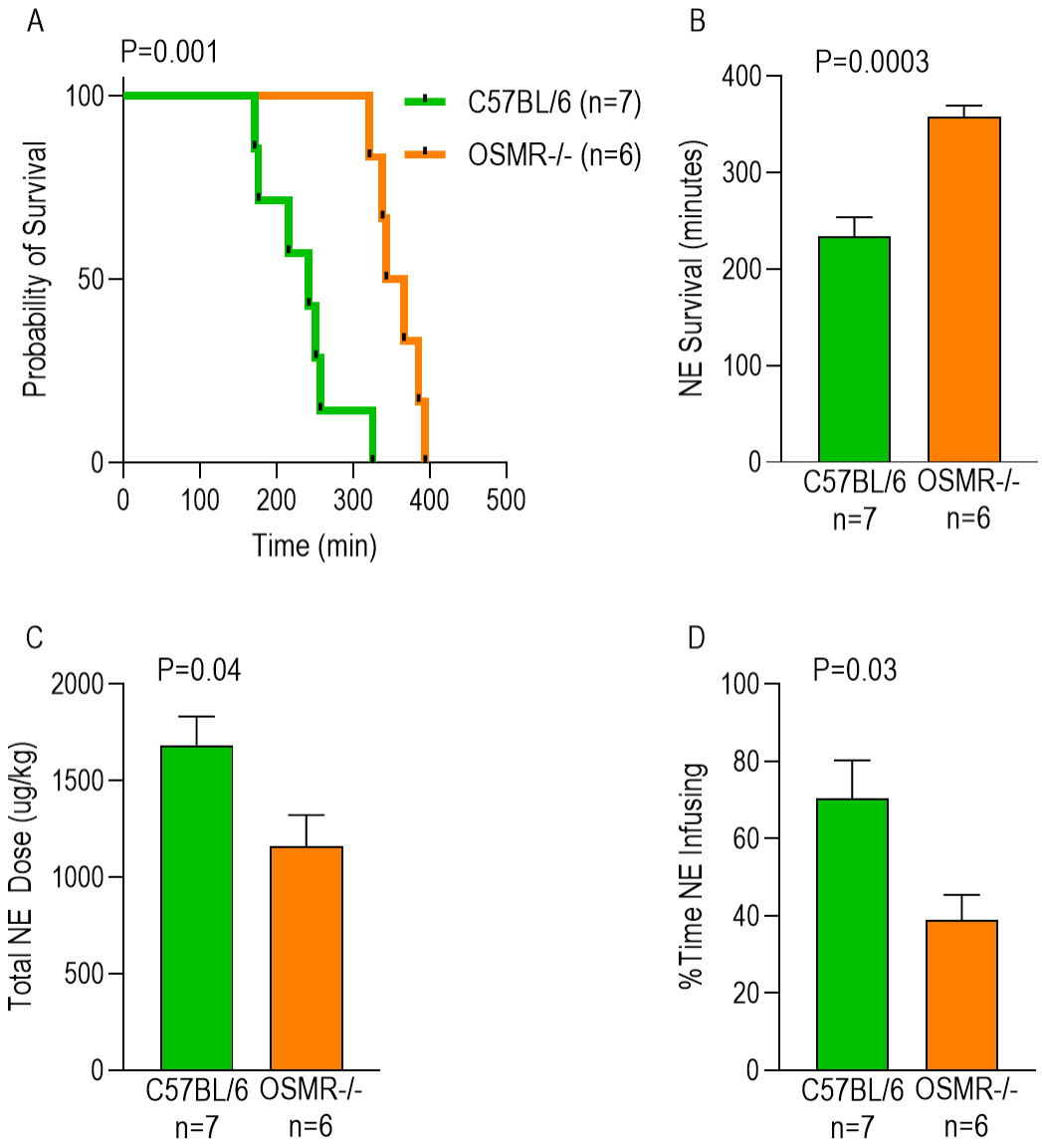


Figure 5-9: Effects of Norepinephrine in Males

(A) Survival probability for male septic C57BL/6 and OSMR deficient (OSMR-/-) mice treated with intravenous infusion of norepinephrine (NE). (B) Survival time in minutes for male septic C57BL/6 and OSMR deficient (OSMR-/-) mice treated with intravenous infusion of norepinephrine (NE). (C) The total dose of norepinephrine (NE) infused in µg/kg for male septic C57BL/6 and OSMR deficient (OSMR-/-) mice. (D) % of total survival time spent with norepinephrine (NE) infusing for male septic C57BL/6 and OSMR deficient (OSMR-/-) mice.

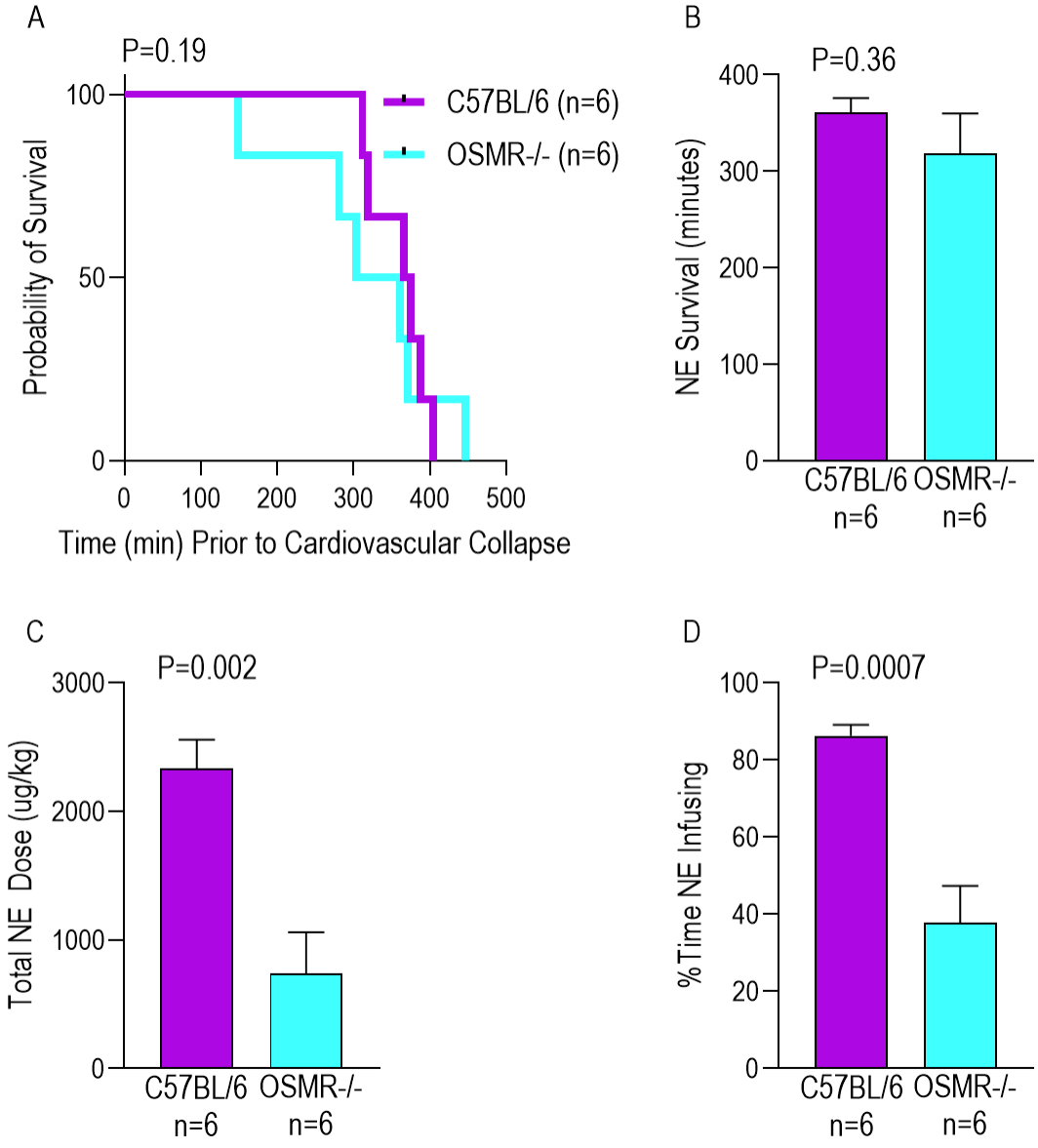


Figure 5-10: Effect of Norepinephrine in Females

(A) Survival probability for female septic C57BL/6 and OSMR deficient (OSMR-/-) mice treated with intravenous infusion of norepinephrine (NE). (B)Survival time in minutes for female septic C57BL/6 and OSMR deficient (OSMR-/-) mice treated with intravenous infusion of norepinephrine (NE). (C) The total norepinephrine (NE) dose infused in µg/kg for female septic C57BL/6 and OSMR deficient (OSMR-/-) mice. (D) % of total survival

time spent with norepinephrine (NE) infusing for female septic C57BL/6 and OSMR deficient (OSMR-/-) mice.

Isometric vascular function of isolated mesenteric arteries from control and OSMR deficient mice were studied by wire myography. Genotype did not affect the dose of MCH required to produce the half-maximal vasodilation (pEC_{50}) in non-septic (P=0.92; **Figure 5-9: A**) or septic (P=0.54; **Figure 5-9: C**) male mice. However, the maximal dilation achieved in non-septic (OSMR-/- 80.43±3.20; C57BL/6 91.96±1.79; P<0.0001; **Figure 5-9: B**) and septic (OSMR-/- 91.25±; C57BL/6 100.74±3.13; P=0.0003; **Figure 5-9: D**) male OSMR deficient arteries was significantly less than in the C57BL/6 arteries. Like males, female genotype did not affect the pEC_{50} of MCH in either non-septic (P=0.31; **Figure 5-9: E**) or septic (P=0.65; **Figure 5-9: G**) arteries. In contrast to male mice, Female OSMR deficient arteries also had no attenuation in maximal dilation achieved (P=0.74; P=0.70; **Figures 5-9: F, H**).

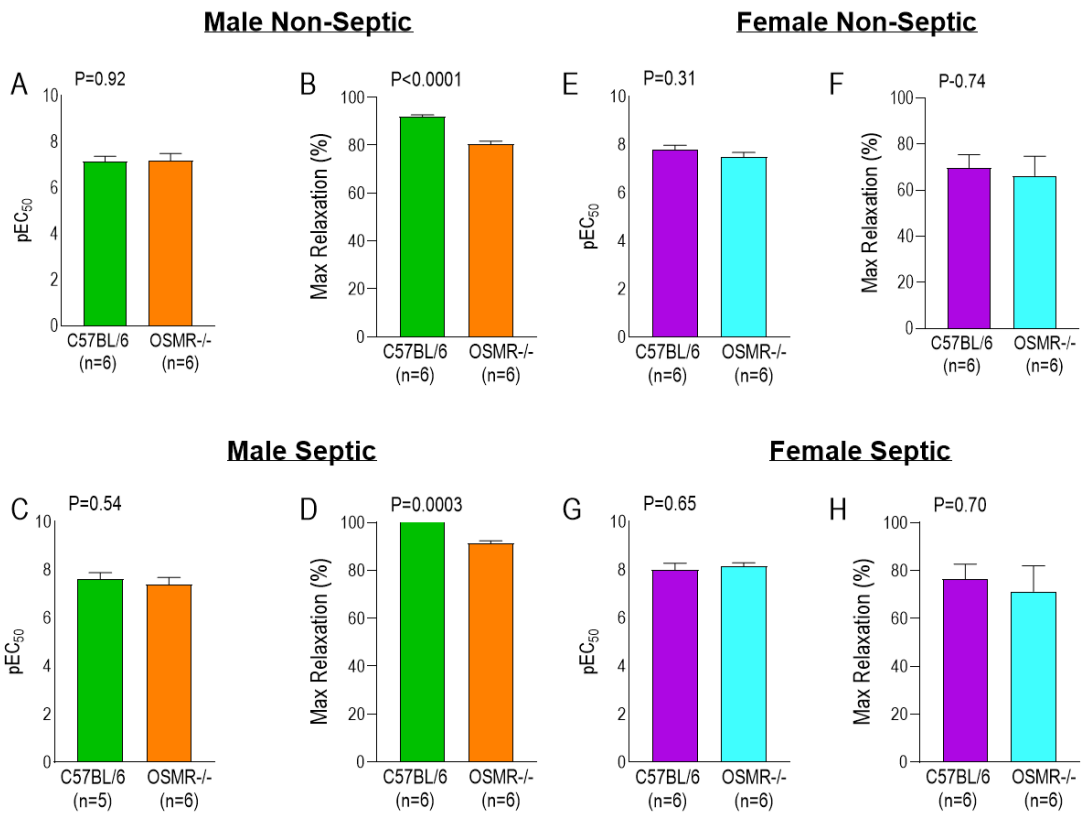


Figure 5-11: Wire Myography Male and Female

Non-septic and septic *ex-vivo* wire myography for male and female mesenteric arteries depicting vasoactive parameters for C57BL/6 (black) and OSMR deficient (OSMR-/-) (white) mice. (A, C) pEC_{50} for methacholine (MCH) cumulative concentration-response curves for males. (B, D) Maximum relaxation achieved by MCH administration for males. (E, G) pEC_{50} for MCH cumulative concentration-response curves for females. (F, H) Maximum relaxation achieved by MCH administration for females.

MCH-induced vasodilation was also assessed in the presence of the non-selective nitric oxide synthase inhibitor LNAME. In the absence of NO production, both male control and OSMR deficient mice exhibited similar MCH pEC_{50}s (P=0.30; **Figure 5-10: A, C**). Interestingly, exposure to LNAME under non-septic circumstances increased the

maximum dilation achieved by OSMR deficient mice (OSMR-/- 80.48±3.69; C57BL/6 69.01±3.31; P=0.0001; **Figure 5-10: B**) while decreasing the maximal dilation in sepsis (OSMR-/- 60.67±4.56; C57BL/6 74.75±5.24; P=0.0006; **Figure 5-10: D**). Female non-septic OSMR deficient mice had no change in pEC_{50} (P=0.25; **Figure 5-10: E**) or maximum dilation when treated with MCH compared to C57BL/6 mice (P=0.29; **Figure 5-10: F**). LNAME treatment in OSMR deficient septic mice increased the pEC_{50} (OSMR-/- 5.90±0.20; C57BL/6 8.05±0.21; P<0.0001; **Figure 5-10: G**) of MCH with no change in maximal dilation (P=0.05; **Figure 5-10: H**).

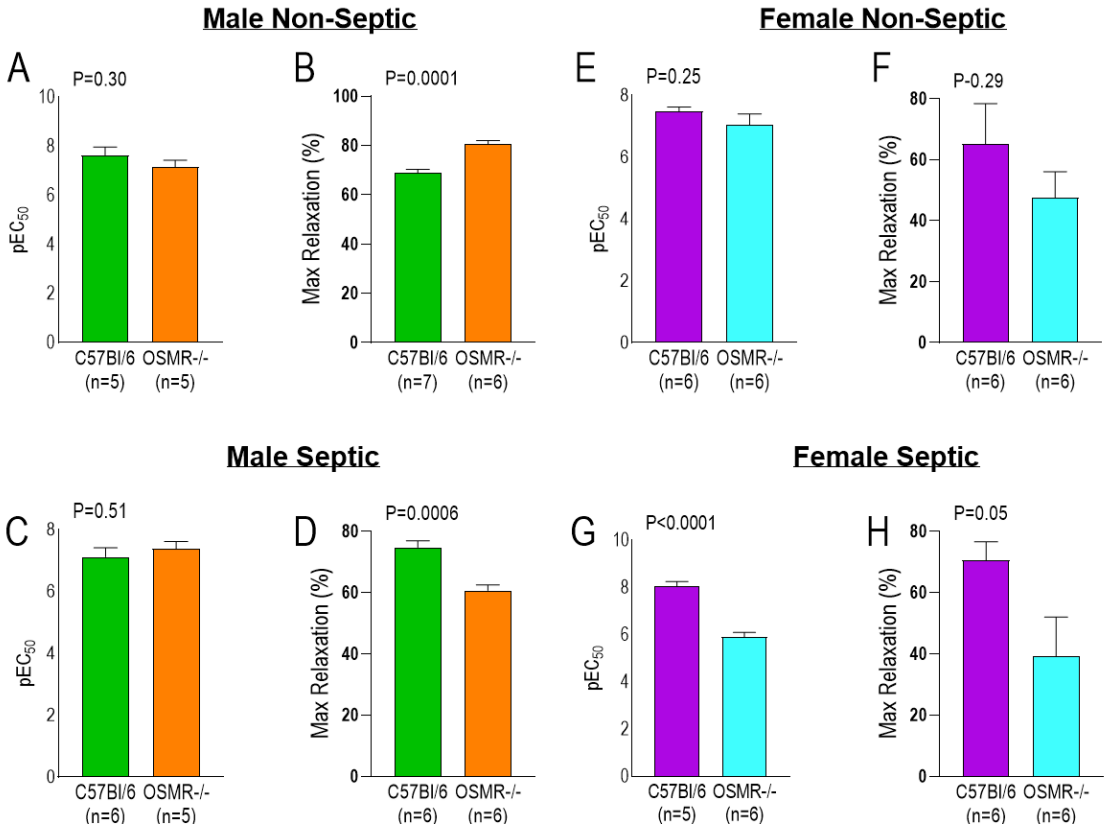


Figure 5-12: Wire Myography for Males and Females Incubated with LNAME

Non-septic and septic *ex-vivo* wire myography for male and female mesenteric arteries incubated with L-N^{G}-Nitro arginine methyl ester (LNAME) depicting vasoactive parameters for C57BL/6 (black) and OSMR deficient (OSMR-/-) (white) mice. (A, C) pEC_{50} for methacholine (MCH) cumulative concentration-response curves for males. (B, D) Maximum relaxation achieved by MCH administration for males. (E, G) pEC_{50} for MCH cumulative concentration-response curves for females. (F, H) Maximum relaxation achieved by MCH administration for females.

Sex- and genotype-specific data demonstrating comparison between control and LNAME treated vessels are presented in **Figure 5-13** as percent change in area under the curve.

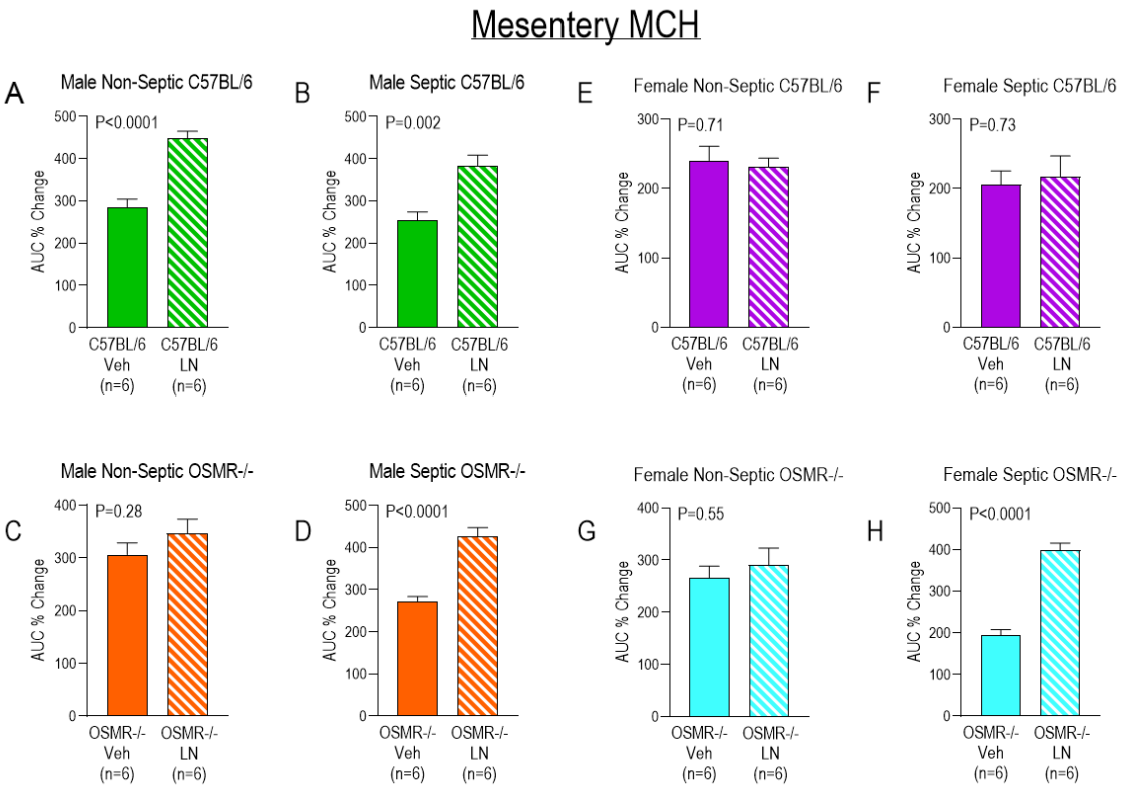


Figure 5-13: Control versus LNAME AUC %Change

Data depicting *ex-vivo* wire myography area under the curves (AUC) comparisons for control and L-N^G-Nitro arginine methyl ester (LNAME) treated vessels incubated with methacholine (MCH). A, B, C, and D represent male mesenteric arteries. E, F, G, and H represent female mesenteric arteries.

Table 13: Summary of Non-Wire Myography Data

DBP: diastolic blood pressure; HR: heart rate; MCH: methacholine; NE: norepinephrine; SBP: systolic blood pressure

Summary	Effect of OSMR-/- Male	Effect of OSMR-/- Female
SBP	↑	↑
DBP	↑	↑
HR	↑	↑
SBP-MCH	↓	↓
DBP-MCH	↓	Nil
HR-MCH	↓	Nil
SBP-septic	↑	↑
DBP-septic	↑	Nil
HR-septic	Nil	↑
SBP-septic MCH	↓	Nil
DBP-septic MCH	↓	Nil
HR-septic MCH	Nil	Nil
Survival	Nil	Nil
NE	↑ survival, ↓ dose, ↓ time infusing	Nil survival, ↓ dose, ↓ time infusing

Table 14: Summary of Wire Myography Data

MCH: methacholine; SNP: sodium nitroprusside

Male Mesenteric Arteries	Female Mesenteric Arteries
Non-septic: • No change in sensitivity to MCH • ↓ in maximal vasorelaxation	Non-septic: • No change in sensitivity to MCH • No change in maximal vasorelaxation
Septic: • No change in sensitivity to MCH • ↓ maximal vasorelaxation	Septic: • No change in sensitivity to MCH • No change in maximal vasorelaxation
LNAME Non-septic: • No change in sensitivity to MCH • ↑ maximal vasorelaxation	LNAME Non-septic: • No change in sensitivity to MCH • No change in maximal vasorelaxation
LNAME Septic: • No change in sensitivity to MCH • ↓ maximal vasorelaxation	LNAME Septic: • ↓ sensitivity to MCH • No change in maximal vasorelaxation

5.4 Discussion

Previous work has demonstrated the influence of sex on cytokine profiles in sepsis as they relate to morbidity and mortality.[73,632-635] OSM is a cytokine known to be elevated in sepsis.[173] This study assessed whether the absence of OSM/OSMR-induced production of NO[636] would confer a sex-specific survival advantage, as we showed previously in male mice.[627] In addition, it was investigated whether OSMR deficiency in both sexes would result in an *in-vivo* hemodynamically distinct phenotype and *in-vivo* and *ex-vivo* NO-related changes in vasodilation. We showed that non-septic male OSMR deficient mice have a distinct hemodynamic phenotype characterized systolic and diastolic hypertension

and tachycardia compared with C57BL/6 mice. OSMR deficiency was also associated with reduced vascular responsiveness to MCH and MCH-induced bradycardia *in-vivo*, accompanied by a lower amount of maximal vasorelaxation *ex-vivo* in mesenteric arteries. This hypertensive phenotype persisted in sepsis; however, heart rate responses were lost.

Female OSMR deficient non-septic mice displayed a similar phenotype compared to male OSMR deficient mice (elevated systolic and diastolic blood pressure and tachycardia). However, response to MCH resulted in no change in diastolic blood pressure and a relative tachycardia. In contrast to the males, the female OSMR deficient hemodynamic phenotype altered with sepsis, demonstrating no change in diastolic blood pressure and loss of the blunted diastolic blood pressure response to MCH. This was not accompanied by a lower amount of vasorelaxation achieved *ex-vivo*.

The hypothesis that the lack of a functional OSM/OSMR signalling pathway may result in decreased NO and a relatively hypertensive state was consistent with the findings herein, in both sexes. As blood pressure may be described as the product of systemic vascular resistance and cardiac output (with cardiac output itself being the product of stroke volume and heart rate), the elevated heart rate seen in both sexes may also partially contribute to elevations seen in blood pressure. However, the literature suggests that long-term inhibition of NO results in bradycardia.[637] Here, we demonstrated tachycardia in both sexes, suggesting the heart rate effect may not be solely attributed to NO deficiency. It is also important to note that long-term OSM/OSMR deficiency may have vascular structural effects that may impact vascular function. OSM/OSMR signalling can cause increased

tissue inhibitors of matrix metalloproteinases-1 (TIMP-1) levels, which is an inhibitor of matrix metalloproteinase 1 (MMP1), thereby favouring matrix accumulation.[638,639] Losing this ability may lead to morphological shape alterations in vascular smooth muscle, potentially limiting vasorelaxation.[640] The effects of altered TIMP-1 levels and MMP1 levels may have sex-specific effects which more severely effect male cardiovascular development.[641]

This study used MCH to examine the reliance of arterial vasodilation on NO availability as its administration results in an endothelial-dependent release of NO. MCH administration may partially replete a relative NO deficiency in OSMR deficient mice. This may result in some vasodilation, although not to the extent seen in C57BL/6 mice, where low baseline levels of OSM signalling and septic high-level OSM presence would increase NO concentration and induce vasodilation. OSMR deficient mice may also have increased reliance on non-NO mechanisms of vasodilation. The effect of prostaglandins (PG) on vasodilation may be inhibited as OSM is known to increase cyclooxygenase 2 (COX2) production,[640] thereby increasing PGI_2 levels and resulting in vasodilation. Decreased ability to activate NO and PG vasodilatory systems may result in OSMR deficient mice having upregulated reliance on other vasodilatory mechanisms such as endothelial-derived hyperpolarization factor (EDHF) as a physiologic adaptation. Indeed, it has been shown that there is cross-talk between the vasodilatory systems which may be sex-specific, with males relying more on NO-mediated vasodilation and females relying more so on EDHF.[642] Decreasing NO by OSMR deficiency may result in further sex-specific imbalance. Therefore, MCH administration in OSMR deficient females may induce less vasodilation

compared to OSMR deficient males. This is consistent with our finding that in males, MCH was unable to cause a similar amount of vasodilation in OSMR deficient mice compared to C57BL/6 mice (**Figures 5-2, 5-3**). In OSMR deficient females, while systolic blood pressure response to MCH-induced vasodilation was impaired, diastolic blood pressure response to MCH-induced vasodilation was similar. This finding is also consistent with the notion that females are less reliant on NO for vasodilation than males.

Sepsis partially induces endothelial dysfunction due to loss of NO-induced cGMP production secondary to dysfunctional oxidized soluble guanylate cyclase (sGC), a downstream NO target.[569] In septic C57BL/6 mice, loss of vasodilation in response to MCH would be expected as NO-sGC-cGMP signalling decreases.[643,644] OSM present in high concentrations in septic C57BL/6 mice would be expected to increase NO production. Initially during sepsis, an elevated NO level may cause vessel vasodilation, scavenging of NO, and eNOS uncoupling. Also early in sepsis, administration of MCH may cause a further release of NO and cause a maximal vasodilation represented *in-vivo* as hypotension. However, as sepsis progresses, reactive oxygen species will scavenge NO and oxidize sGC to an inactive form, resulting in reduced responsiveness. As oxidized sGC will not respond to NO to produce vasodilating cGMP, there is a resultant decreased or absent vasodilatory response (vasoplegia). Septic OSMR deficient mice would potentially be less sensitive to NO stimulated by MCH as they may be more reliant on other vasodilatory mechanisms that are less affected by sepsis (such as EDHF). As such, we hypothesized that OSMR deficient male mice would maintain their baseline blunted vasodilatory response to MCH

due to their higher reliance on NO. In contrast, female OSMR deficient mice would display a similar level of MCH-induced vasodilation due to a decreased reliance on NO.

Our work demonstrated key differences in vasodilation over time consistent with the above hypothesis that OSMR deficiency would have a sex-specific vascular function effect. Male OSMR deficient mice exhibited a decreased systolic and diastolic response to vasodilation stimulated by MCH throughout the septic time course. As septic vasoplegia developed over time, systolic blood pressure response to MCH was blunted to a greater degree in OSMR deficient males than in the C57BL/6 males. It could be argued that the OSMR-deficient males demonstrated end-stage vasoplegia (as above) and were, therefore, unable to further vasodilate from their maximally dilated state. However, this would not be congruent with the concurrent finding of relative hypertension compared to the C57BL/6 mice, though this may be attributable to vascular remodelling rather than dynamic vascular tone. Altogether, the increased reliance on NO and the impaired responsiveness to MCH in OSMR deficient mice suggests an important mechanistic effect of the elevated OSM levels seen in sepsis, especially in the males who rely more heavily on NO signalling for vasodilation.

While the female decline in systolic and diastolic blood pressure during sepsis was also evident, the lack of differences in response to MCH between genotypes was notable. However, as sepsis progressed, all females, irrespective of genotype, exhibited a clear and progressive decline in vascular responsiveness to MCH. This loss of responsiveness to MCH-induced vasorelaxation in both genotypes, while systolic blood pressure remained

elevated in the OSMR deficient females, potentially highlights the less prominent role of NO signalling in the maintenance of tonic vasodilation in sepsis in females (**Figure 5-5**).

Despite promising OSMR deficient hemodynamic profiles in septic males and females, in contrast to our previous study,[627] these differences did not translate into improved survival for either sex without vasopressor support (**Figure 5-6**). As numerous pathophysiologic derangements are present in sepsis, improved blood pressure coupled with general decreased vasodilatory capability seems unlikely to improve survival. In fact, the inability to vasodilate at injured sites may reduce delivery of necessary inflammatory mediators and overall may inflict increased damage. However, it should be noted that the model of sepsis used herein was severe. Specifically, the dose of the fecal slurry was titrated to ensure the development of septic circulatory shock within a shorter timeframe, to be conducive to experiments involving continuous blood pressure monitoring in anesthetized mice. It may be that the survival advantage was masked by the rapid decline in circulatory function. That is, perhaps the model is too severe to observe physiological benefit secondary to OSMR deficiency conferring a notable survival advantage. In this regard, and to demonstrate consistency with actual sepsis treatment in humans, we endeavoured to extend survival by implementing vasopressor support with NE.

As NE is part of standard care for the patient with septic shock, we were interested in how OSMR deficiency and the associated impaired vascular function would affect NE use and resultant survival in both sexes. This also increases our findings' generalizability and translational capability by mimicking a real clinical scenario. Interestingly, in males,

OSMR deficiency coupled with NE treatment improved survival while decreasing the total amount of NE used and the amount of time infusion was required (**Figure 5-7**). Survival was unaffected in OSMR deficient females but still resulted in a reduced amount of NE used and decreased the time NE was required (**Figure 5-8**). Potentially, as females may be demonstrating a lesser reliance on intact NO signalling for normal vascular function, and it has been reported that NO can deactivate NE functioning,[645] the survival benefit seen in males was not evident. It has also been reported that NE increases NO release,[646] which can subsequently increase COX2 signalling, PG production, and vasodilation. As we posit males are more susceptible to the decreased bioavailability of NO in sepsis, the production of NO induced by NE may improve outcomes to a greater extent in males.

The sex-specific effect of NE in OSMR deficiency is especially interesting in that it describes a new combined target of sepsis treatment to improve survival. Reducing exposure to NE has clinical relevance. The use of vasopressor support generally requires admission to a higher-level of care setting such as an observation unit or intensive care unit. Admission to these units often results in a higher cost of care. NE administration also requires the placement of a central venous catheter and the risk of associated complications including bleeding, infection, and pneumothorax.[647] NE is also associated with an increased occurrence of arrhythmias which may be poorly tolerated in a critically ill septic patient population.[333,648] Resultant vasoconstriction secondary to NE is regionally non-specific and may compromise blood flow to several areas of the body leading to tissue and organ ischemia and may impair oxygen and inflammatory mediator delivery to

injured/infected areas. Limiting the use of NE may therefore be beneficial, even if mortality is not improved.

We then compared our *in-vivo* results with *ex-vivo* examinations of vascular function to confirm sex-specific findings (**Figures 5-9, 5-10**). Using wire myography as described above, we showed no change in sensitivity to MCH between genotypes for both sexes under non-septic and septic conditions. In agreement with our non-septic and septic *in-vivo* findings, we demonstrated that OSMR deficiency resulted in a decreased maximal dilation attained in OSMR deficient males, whereas female OSMR deficient maximal dilation was similar. We also examined vasodilation in a subset of vessels treated with LNAME, a nitric oxide synthase inhibitor. It would be expected that LNAME would increase the amount of MCH necessary to produce the same extent of vasodilation under non-septic conditions within the same genotype. The sensitivity to MCH between genotypes did not differ under non-septic or septic conditions for males. In male vessels treated with LNAME, we were able to show an increase in maximal vasodilation in OSMR deficient non-septic vessels. We theorize that this *ex-vivo* result was observed as OSMR deficient vessels may be conditioned to vasodilate in response to other MCH-induced vasodilatory pathways (EDHF) and therefore NOS inhibition with LNAME may be better tolerated. In septic vessels, the rise in OSM may result in increased levels of NO present in C57BL/6 mice compared to OSMR deficient mice, such that lesser maximum dilation is achieved in OSMR deficient mice.

We demonstrated differences in the LNAME treated females, again potentially secondary due to a lesser reliance on available NO. Under non-septic conditions, females showed no change in sensitivity to MCH or resultant maximal dilation. When septic, OSMR deficient vessels had decreased sensitivity to MCH with similar maximal vasodilation under septic conditions. Again, we speculate these *ex-vivo* results are due to a lesser reliance on NO. Under non-septic conditions and inhibited by LNAME, even a small concentration of NO production induced by MCH may produce a similar maximal dilation in female vessels where NO is less critical. When septic and treated with LNAME, it is increasingly difficult for OSMR deficient vessels to produce large amounts of bioavailable NO due to inactivity of the OSM/OSMR signalling pathway, while OSM signalling may increase due to increased OSM production in C57BL/6 mice. As such, the sensitivity to MCH would be comparatively low in OSMR deficient female vessels if reliance on NO is greater than in males.

5.5 Limitations

There are limitations to this study. The dose of fecal slurry used in these experiments may have caused a severe septic phenotype, so minor improvements in survival may not have been observed, as discussed above. In the OSMR deficient mice, changes may not have been as evident as expected due to multiple receptor types. Our mice were deficient in the type of OSMR known as OSMR type II, comprised of gp130 and the OSMR complex. In these OSMR deficient mice, loxP sites exist on either side of the second exon of the OSMRβ gene. Therefore tissue expression of the gene is eliminated when this strain is

crossed with a Cre recombinase-expressing strain.[649] Our mice were homologous for OSMR type II knockout. Therefore, the OSM ligand is present while the OSMR type II is absent. The consensus is that mice only possess OSMR type II;[650] however, there potentially exist low-affinity interactions between OSM and gp130 and with the OSMR type I complex (gp130/LIFR) (Table 14). There is also evidence that pro-OSM (post-N-terminal cleavage and pre-C-terminal cleavage product) may be biologically active. It remains to be seen whether this pro-OSM acts on OSMR type I, II, or both. Some evidence exists that murine OSM is active in bone[651] which may be secondary to low affinity for OSMR type I. In addition, other cytokine receptors (i.e.: IL-31R) are known to heterodimerize with type II OSMR. Therefore, changes seen in OSMR deficient mice may not be solely related to lack of OSM signalling (and resultant vasodilation) but decreased signalling in other pathways. Finally, even with improved hemodynamic profiles in sepsis, OSMR deficiency may not result in enhanced survival secondary to other effects of OSM including decreased macrophage recruitment and decreased production of hepatic acute phase reactants.

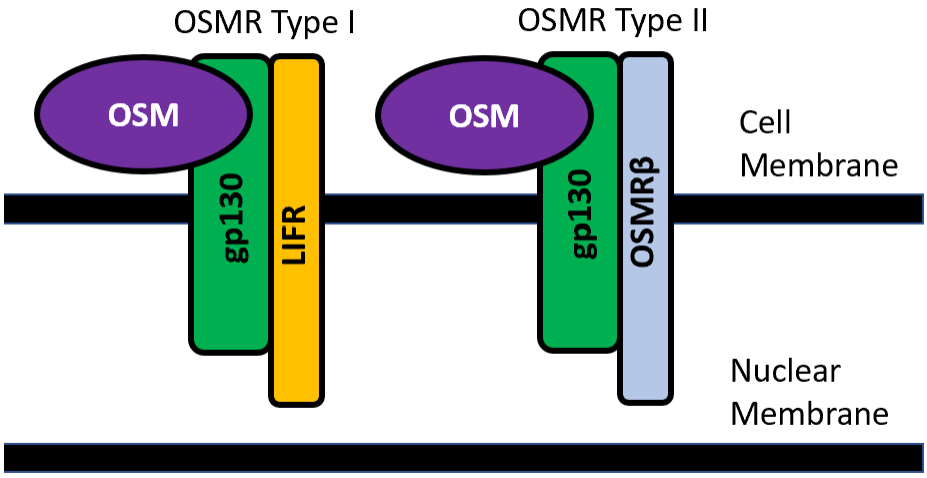


Figure 5-14: Two Types of Oncostatin M Receptors

OSMR Type I consists of gp130 and LIFR (leukemia inhibitor factor receptor) while OSMR Type II consists of gp130 and OSMRβ (Oncostatin M receptor beta).

Table 15: OSM Site of Action in Humans and Mice

OSM: Oncostatin M, OSMR: Oncostatin M Receptor

	Human OSMR Type I	Human OSMR Type II	Murine OSMR Type I	Murine OSMR Type II
Human OSM	+	+	+	
Mouse OSM				+

5.6 Conclusion

We have newly demonstrated that both male and female OSMR deficient mice have distinct hemodynamic profiles under non-septic and septic conditions. These distinct profiles extend to both non-septic and septic derangements in MCH-induced vasodilation.

Our results support a greater reliance on NO in vasodilation in males. OSMR deficiency demonstrates some benefit in reducing exposure to NE in both sexes and leads to improved sepsis survival, albeit this was only evident in male mice with vasopressor support.

Chapter 6
Summary and Future Directions

6.1 Summary

Herein, we have described several new findings contributing to the knowledge base surrounding vascular dysfunction in sepsis, particularly as it pertains to the elderly. Aged *in-vivo* vasodilation profiles were distinct from the young, showing increased vasodilation in response to MCH in non-septic aged mice, while septic aged mice displayed the opposite effect of decreased vasodilation in response to MCH. Myography findings supported these *in-vivo* results, such that mesenteric artery relaxation was greater in response to low doses of MCH in non-septic mice. This effect was ablated in aged septic mice, which is also in agreement with the *in-vivo* results. Septic aged mice produced similar amounts of cGMP in the lung and kidney as the young, implying sGC activity was impaired to a similar extent. Importantly, our results support the hypothesis that aging is associated with worse survival in sepsis. These findings lend support to our theory that ROS and functional sGC signalling, known to be particularly impaired with increased age, are important in the progression of sepsis to septic shock.

As the morbidity and mortality burden of sepsis is severe, and this burden is unequally shouldered by the aged, it was important to demonstrate the potential clinical relevance of these findings by attempting to improve hemodynamic function and thereby improve survival in both age groups. We used the sGC activator cinaciguat to restore sGC activity and cGMP production. We observed several improvements in septic hemodynamic and survival outcomes, suggesting cinaciguat activates the sepsis-damaged sGC-cGMP

signalling axis, a phenomenon we hypothesized would be exacerbated in the aged due to enhanced ROS production associated with aging. In young mice, cinaciguat improved multiple aspects of *in-vivo* hemodynamic function. Septic systolic and diastolic blood pressure, as well as heart rate, were improved. Responses to MCH were unchanged, potentially secondary to impaired eNOS function. Notably, survival was improved and sGC activity was increased in both the lung and the kidney. Using wire myography, we also demonstrated increased sensitivity of mesenteric arteries to MCH and SNP and increased sensitivity of carotid arteries to SNP, albeit vessel relaxation from all groups was ultimately similar. These findings may indicate that cinaciguat impacts sGC signalling as well as having other tissue/organ-specific results.

Aged mice also experienced a survival benefit from cinaciguat treatment, but without any observable *in-vivo* hemodynamic or *ex-vivo* vascular function changes. However, sGC activity was increased in both the kidney and the lung with cinaciguat treatment. These results, again, lend support to the notion that cinaciguat has other non-vascular targets that assist in improving mortality. Indeed, sGC is involved in numerous processes, ranging from unloading the heart resulting in improved regional blood flow to specific organs,[611] to preventing fibrosis and inflammation,[652] and in reducing ERK1/2 activity and TGF-ß expression thus influencing glomerular damage in the kidney.[653] Thus, treatment with cinaciguat, could, for example, improve renal function independent of its effect on renal blood flow. Given that real failure is a primary cause of mortality in sepsis, the specific mechanisms by which sGC may improve kidney function warrants future investigation.

Lastly, we examined whether inhibiting the function of OSM (increased expression in sepsis, with receptors prominently located on the vascular endothelium, and influences NO levels) would improve non-septic and septic hemodynamics in a sex-specific fashion. We were able to demonstrate that OSMR signalling is an important contributor in a sex-specific fashion to baseline hemodynamic regulation and function with both sexes displaying a distinct non-septic hemodynamic profile characterized by systolic and diastolic hypertension and tachycardia. However, males also displayed decreased systolic and diastolic blood pressure and heart rate responses to MCH while females displayed a decreased systolic blood pressure and increased heart rate response. We observed that OSMR signalling plays an important role in septic vascular hemodynamic and vascular functioning with sex-specific differences. Septic OSMR deficient mice of both sexes retained their hypertensive and tachycardic phenotype. The response to MCH still was decreased for both systolic and diastolic blood pressure but became increased for heart rate. Females lost any blood pressure response to MCH and maintained an elevated heart rate response. Wire myography supported the findings that OSMR signalling has sex-specific effects in sepsis. Although we were not able to demonstrate a survival advantage in OSMR deficient septic mice of either sex, our previous work with a less severe model of sepsis has shown a survival advantage in septic male mice, raising the possibility that this model was too severe to address subtle effects in less sick mice. However, in the context of these experiments examining septic shock (with a known higher severity and mortality than simple sepsis as in our previous study), this model was appropriate.

Altogether, our results support decreased survival for the aged, likely impacted by vascular dysfunction and other organ effects secondary to dysfunctional sGC signalling and impaired cGMP production. Treatments directed at restoring sGC function may ultimately be translatable to human sepsis treatment. Currently, there is interest in use of methylene blue, a medication that blocks sGC action and scavenges NO, instead of restoring dysfunctional sGC function as cinaciguat does, for treatment of distributive shock.[654] Much of the evidence surrounding methylene blue use stems from non-septic shock research (i.e.: post-cardiopulmonary bypass vasoplegia) with most studies failing to demonstrate mortality benefit in septic shock.[655,656] However, one study found improvement in mortality if administered to less sick patients who may have been treated earlier in the course of sepsis.[656] Our results demonstrate that cinaciguat has mortality benefit, even when no hemodynamic or vascular function changes are noted. Therefore, inhibiting sGC function, especially in the aged, with methylene blue may have detrimental effects. We also demonstrated that inflammatory cytokines may alter hemodynamic outcomes of sepsis in a sex-specific manner. It is evident from our observations that age and biological sex are important factors affecting sepsis hemodynamic and vascular function in addition to survival and further research is necessary.

6.2 Future Directions

We have defined a hemodynamic response to sepsis in the aged in a model of intraperitoneal sepsis. As we have also demonstrated that cytokines affect septic and non-septic hemodynamic and vascular function profiles in a sex-specific manner, we will need to further investigate the effect of age combined with the effect of biological sex by

repeating our initial experiments (from Chapters 2-4) in females. The current literature is inconclusive regarding the effects of sex on sepsis survival, with no information available about sex-specific hemodynamic or vascular function effects on survival.[70,657,658] It will also be important to assess how certain cytokines are affected by aging and the resultant influence on survival and short- and long-term hemodynamic and vascular function.

While intra-abdominal sepsis is the most lethal form of sepsis, we would like to confirm our findings in the most common type of sepsis, pulmonary sepsis.[153] To induce pulmonary sepsis and lung injury we aim to use the intratracheal injection of *streptococcus pneumoniae*, a common method.[659-661] It will also be important to demonstrate what the effect of cinaciguat is when combined with antibiotic therapy as it is a mainstay of sepsis management. We deliberately omitted antibiotic use in this first set of experiments to define the effects of sepsis more accurately as antibiotics may have effects on the vasculature, especially those with anti-inflammatory properties. However, we acknowledge that as clinical sepsis treatment necessitates the use of antibiotics, we aim to use antibiotics in our future studies (now that we have delineated the effects without them) to enhance the translational potential of our findings.

In our studies we used inhaled isoflurane anesthetic as a means to provide a consistent depth of anesthetic for a prolonged period of time. We acknowledge isoflurane itself may provide anti-inflammatory and pre-conditioning effects that could improve survival or hemodynamic and vascular function. Although all animals (non-septic, septic, treated) received isoflurane, the effect of isoflurane combined with cinaciguat may have affected our outcomes more so than cinaciguat alone. Therefore, it will be important to

demonstrate survival, hemodynamic, and vascular outcomes without isoflurane. However, due to the nature of these experiments some form of anesthetic is necessary.

As discussed extensively in Chapter 1, we know that although improving mortality in people with sepsis and septic shock is important, improving long-term septic outcomes would be of extreme importance. In all adults, not just the elderly, the organ injury and quality of life effects of sepsis can be long-lasting or permanent. So even though cinaciguat improved mortality and some aspects short-term vascular function during sepsis, it will be important to examine the molecular mechanism(s) of how cinaciguat improves mortality. Following determination of molecular mechanism(s), examining other longer-term septic outcomes including organ function, quality of life, and ability to live independently will be necessary. This will likely require multidisciplinary approaches involving quantitative and qualitative studies.

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