PATHOLOGY OF NEONATAL SEPSIS: DISCRIMINATING SURVIVAL AND IDENTIFYING NEW THERAPEUTIC TARGETS

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Abstract

The global mortality rate in the neonatal period is far greater than in any other time in childhood. Sepsis is one of the most significant causes of neonatal death worldwide and therefore represents a critical research target moving forward. There is an urgent need for pathogen-agnostic prophylactics and interventions to prevent and treat neonatal sepsis, but the development of these has been slow and ineffective due to a fundamental knowledge-gap surrounding the pathology of the disease. Here we first developed a method for classifying cecal slurry challenged mouse pups into likely survivors or likely non-survivors to identify why some pups survive the sepsis model and others do not. We then examined the transcriptomes of likely survivors and likely non-survivors and identified arachidonic acid metabolism as key point of differentiation between the two groups. We validated these findings by showing the administration of exogenous arachidonic acid prior to challenge significantly improved survival its protective effect was ultimately due to improvements in vascular endothelial integrity via interactions with the angiopoietin-TIE2 axis. Combinatorial treatment of exogenous angiopoietin-1 and L-Arginine significantly reduced mortality and represents a promising new intervention moving forward.

Lay Summary

Sepsis is the result of an out-of-control immune response brought on, generally, by an infection. Newborns suffer from sepsis at a much higher rate than adults for reasons not entirely understood. I set out to generate new treatments for newborn sepsis by using a mouse model wherein I looked at the differences between pups which were likely to die and which pups were likely to survive. I identified a few biological pathways as key points of differentiation between the groups, then I was able to use drugs to target the pathways and improve survival. I was also able to show these pathways were all connected and appear to be very important to the newborn immune response. In the end I came up with a treatment that improved sepsis survival in mice from 5% to 95%.

Preface

The introduction of this thesis contains excerpts from two previously published articles, both in *Frontiers in Immunology*:

- **Harbeson, D**., Ben-Othman, R., Amenyogbe, N. & Kollmann, T. R. Outgrowing the immaturity myth: The cost of defending from neonatal infectious disease. *Front. Immunol.* **9**, 1077 (2018).
- **Harbeson, D**., Francis, F., Bao, W., Amenyogbe, N. A. & Kollmann, T. R. Energy Demands of Early Life Drive a Disease Tolerant Phenotype and Dictate Outcome in Neonatal Bacterial Sepsis. *Front. Immunol.* **9**, (2018).

Specifically, section 1.2 was excerpted from "Outgrowing the immaturity myth: The cost of defending from neonatal infectious disease" and section 1.3 was excerpted from "Energy demands of early life drive a disease tolerant phenotype and dictate outcome in neonatal bacterial sepsis". These reviews were published under the guidance of Dr. Tobias Kollmann and with the help of Dr. Rym Ben-Othman, Dr. Nelly Amenyogbe, Freddy Francis, and Winnie Bao. In both reviews I was the primary author as I wrote and edited the text. All text excerpted and presented in this thesis was written by myself.

Chapter 2 was originally published as a standalone paper in PLOS ONE under the following citation:

- Brook, B*., **Harbeson, D***. *et al.* Robust health-score based survival prediction for a neonatal mouse model of polymicrobial sepsis. *PLoS One* **14**, (2019).

- *contributed equally

The work described in this this paper and therefore in chapter 2 was performed under the guidance and funding secured from Dr. Tobias Kollmann. I was listed as co-first author with my fellow doctoral student at the time Byron Brook. Byron and my other co-authors were responsible for data collection while I was responsible for analysis, writing, and making figures (though I also contributed to performing the experiments and collecting data). My collaborator Dr. Radhouane Aniba constructed the gradient boosting machine algorithm and wrote the appendices referenced within the chapter and included as appendices here. All animal work was performed under approval from the UBC animal care committee (protocol numbers A17-0110 and A14-0261).

Data collected in chapters 3 and 4 was also collected within Dr. Tobias Kollmann's laboratory with the assistance of Dr. Mario Fidanza. Dr. Mario Fidanza and I worked closely in data collection and experiment planning. The only analytical step which was not performed by me was the analysis of the fluorescence staining performed to measure reactive oxygen species described in Chapter 4-3 – this was done by Dr. Mario Fidanza. I performed all other analysis, constructed all figures, and wrote all the text within the chapters. This work was performed at Telethon Kids Institute in Perth, WA. Animal work was approved within Telethon Kids Institute (AEC 351, AEC 353, AEC 359, and AEC 363).

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List of Abbreviations

2DG	2-deoxyglucose
AMP	Adenosine Monophosphate
ANGPT1	Angiopoietin-1
ANGPT2	Angiopoietin-2
ARA	Arachidonic acid
ATP	Adenosine triphosphate
BH	Benjamani-Hochberg
CFU	Colony forming unit
CLP	Cecal ligation and puncture
CS	Cecal slurry
CSF	Cerebrospinal fluid
CYP1B1	Cytochrome P450 Family 1 Subfamily B Member 1
D5W	Dextrose 5% water
DE	Differentially expressed
DHE	Dihydroethidium
DMSO	Dimethyl sulfoxide
DOL	Day of life
DT	Disease tolerance
EET	Epoxyeicosatrenoic acid
ELISA	Enzyme-linked immunosorbent assay
EN	Enteral nutrition

eNOS	Endothelial nitric oxide synthase
EOS	Early onset sepsis
FTR	Fail to right
GBS	Group B Streptococcus
GO	Gene ontology
GSEA	Gene set enrichment analysis
HETE	Hydroxyeicosatetraenoic acids
HIF1α	Hypoxia-inducible factor 1 α
HPC	Hours post challenge
ICU	Intensive care unit
IL-2	Interleukin-2
IP	Intraperitoneal
L-Arg	L-Arginine
LCPUFA	Long-chain polyunsaturated fatty acid
LD	Lethal dose
L-NAME	N(gamma)-nitro-L-arginine methyl ester
LOS	Late onset sepsis
LPS	Lipopolysaccharide
MeSH	Medical subject headings
MyD88	Myeloid differentiation primary response 88
NEC	Necrotizing enterocolitis
NICU	Neonatal intensive care unit
NK	Natural killer

PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PCA	Principal component analysis
PGE2	Prostaglandin E2
PMN	Polymorphonuclear cell
PN	Parenteral nutrition
poly(I:C)	Polyinosinic:polycytidylic acid
PPARα	Peroxisome proliferator-activated receptor-α
PUFA	Polyunsaturated fatty acid
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SIRS	Systemic inflammatory response syndrome
sPLA2	Secretory Phospholipase A2
TCA	Tricarboxylic acid
TIE2	TEK tyrosine kinase
TLR	Toll-like receptor
TRIF	TIR-domain-containing adapter-inducing interferon-β
WHO	World Health Organization
WT	Wild type

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Thanks, Tobi.

Dedication

This thesis is dedicated to my loving partner, Francesca White. This process would have been infinitely harder without her.

Chapter 1: Introduction

Sections 1.2 and 1.3 are slightly modified versions of stand-alone review articles, both published in *Frontiers in Immunology* in 2018. Refer to the preface for more detail.

1.1 Epidemiology and defining neonatal sepsis

1.1.1 Incidence and causes of neonatal mortality

Despite massive reductions in global all-cause mortality over the last 30 years (an estimated 60% reduction since 1990), 7.4 million children died from treatable or preventable illnesses in 2019 alone.[1] Deaths in children under-five years old make up 70% of all deaths among those younger than 25, and nearly half of the deaths in the under-five age-group occur within the first month of life (the neonatal period).[1] The modest reduction of all-cause neonatal mortality over the last few decades has failed to match the pace of reduction in all-cause under-five mortality; deaths in the neonatal period therefore comprise a steadily growing proportion of all death in this period.[1–3] According to the 2020 UNICEF report on the levels and trends in child mortality, nearly 90% of countries in the sub-Saharan region of Africa are at risk of missing WHO targets for reducing neonatal death.[1] Deaths within this age range can be further summarized simply as: the closer a neonate is to their day of birth, the higher the risk of death.[4] While much of the death on the first day of life is indeed directly related to complications associated with the physical birthing process, the trend holds true for preventable or treatable illnesses as well.[1,4] The implications of this are massive and should not be ignored. If the goal of medical research is to minimize years of potential life lost, there is no better target then the first day or week of life.

According to the WHO, the "three major causes of neonatal deaths worldwide are infections (36%, which includes sepsis/pneumonia, tetanus and diarrhea), pre-term birth (28%), and birth asphyxia (23%)".[5] The broad classification of deaths into a 'pre-term' category means that pre-term deaths due to infectious disease are not included in the 36% statistic. One observational study across hospitals in Ethiopia found that within 1109 pre-term deaths captured by the study, 45% were attributed to respiratory distress syndrome and 30% the attributed to infection.[6] A similar observational study in India examined two independent cohorts containing 828 pre-term deaths and 514 pre-term deaths attributed sepsis as cause of death in 25% of cases and 41% respectively.[7] These two recent studies are included here in an attempt to illustrate the difficulty of capturing the true burden of neonatal infection. Often 'complications of pre-term birth' is listed as a cause of death in studies examining causes of neonatal mortality, but this compresses all the deaths in the pre-term period into a single category and pre-term deaths due to infection are lost in this calculus. This is not to say that infections in term and pre-term neonates are necessarily comparable in a biological sense (there are myriad differences between term and pre-term immunity), but rather that the true burden of neonatal infectious disease continues to be underestimated.

1.1.2 Defining neonatal sepsis

Sepsis is a dysregulation of the immune system which leads to multiple organ failure, classically believed to be the result of an over-exuberant inflammatory response following exposure to a pathogen of either bacterial, viral, or fungal origin.[8] The first consensus definition of sepsis

emerged out of a 1991 “American College of Chest Physicians / Society of Critical Care Medicine Consensus Conference” wherein clinicians first coined the phrase “systemic inflammatory response syndrome”, or SIRS. SIRS describes a massive inflammatory response which can exist independently from an infection, i.e. could be caused by trauma or burns. By defining SIRS as a condition which is not always caused by infection, these clinicians were able to write: “When SIRS is the result of a confirmed infectious process, it is termed *sepsis.* In this clinical circumstance, the term *sepsis* represents the systemic inflammatory response to the presence of infection.”[9] Thus, baked in to the original consensus definition of sepsis (now known as “sepsis-1”) was the requirement for a “confirmed infectious process” and a systemic inflammatory response. The latest consensus definition (“sepsis-3”) came in 2016 and recommended “sepsis be defined as life-threatening organ dysfunction caused by the dysregulation of the host response due to an infection”.[8,10] Important to note, culture positivity is not strictly required to meet the consensus definition of sepsis – all that is required is *suspected* infection. The movement away from focus on the systemic inflammatory response and towards organ dysfunction is the result of more recent data indicating that the anti-inflammatory or immunomodulatory components of sepsis are just as critical to determining outcomes as the pro-inflammatory state.[11–13] Sepsis appears to be a condition wherein pro-inflammatory and anti-inflammatory pathways are activated simultaneously and chaotically – hence, sepsis is a ‘dysregulation’ of the immune response.

This discussion of evolving definitions of sepsis has thus far been mostly restricted to the world of adult sepsis. As will be discussed in greater detail later, it is fallacious to project science built

around adult immunity onto that of the neonate. Only in the past decade has there been a true push to develop a consensus definition for neonatal sepsis in the same way that it exists for adults.[14,15] Despite this push, as of October 2020 no consensus definition for neonatal sepsis has emerged.[16] The most commonly used definition for neonatal sepsis still tracks most similarly to that described in the 1991 'sepsis-1' conference: neonatal sepsis is the systemic inflammatory response resulting from a pathogen, confirmed by isolation of said pathogen from a normally sterile bodily fluid (namely blood or CSF).[17] There are, of course, many symptoms which go into a clinical sepsis diagnosis in a neonate (i.e. fever, pallor, lethargy, tremors, tachycardia, hypotension, failure to thrive, poor feeding, etc.)[17] but the conceptual work of "what is neonatal sepsis" is still rooted in the sepsis-1 definition. This inadequate definition further hinders our ability to capture the true burden of global neonatal sepsis.

1.1.3 Etiology of neonatal infection

The gold standard for sepsis diagnosis has long been a positive blood or cerebrospinal fluid culture. However, 'culture-negative' sepsis is an extremely common phenomenon yet is poorly understood.[18] A recent major study (the "ANISA" study) which examined possible serious bacterial infections in a cohort of more than 63,000 infants across south Asia found that they were only able to attribute a causal pathogen in 28% of cases.[19] An older study which looked at newborns with 'unequivocal infection' confirmed at the time of autopsy found the premortem blood culture was negative in 14% of cases.[20] Even culture-positive sepsis can be broken down by pathogen – there is ample evidence that viral, fungal, and bacterial sepsis are far from interchangeable in both clinical manifestation and management[17,18,21]. When interpreting

aggregate epidemiological statistics about the incidence and burden of neonatal sepsis, one must consider that the definition of sepsis likely varies between different clinicians, different hospitals, and different researchers – a study which reports mortality due to culture-positive sepsis will have wildly different numbers than one which includes "likely sepsis" or culture-negative sepsis.

The question of culture-positivity aside, neonatal sepsis is generally classified as either early-onset, appearing within 72 hours after birth, or late-onset, appearing beyond three to seven days after birth.[17] It is generally believed that early-onset sepsis (EOS) is the result of an infection acquired before or during delivery (vertically) whereas late-onset sepsis (LOS) is the product of a hospital or community-acquired (horizontal) infection. The etiology of both EOS and LOS has varied widely across time and geography.[19,22–25] For instance, the aforementioned ANISA trial (which was looking explicitly for community-acquired infections) found that the leading causal pathogen to be respiratory syncytial virus at 5.4 episodes per 1000 livebirths, followed by *Ureaplasma* spp at 2.5 episodes per 1000 livebirths.[19] A 2017 review published in *The Lancet* described the most common pathogens responsible for LOS as GBS, *E. coli*, *L. monocytogenes*, *S. Aureus*, herpes simplex virus, and enterovirus infections.[17] Notice that neither of the leading causes of LOS in the ANISA trial were even identified as major contributors in a review published only a year prior. This is not to say that either of these publications are inaccurate in their description of the causes behind LOS, rather that there is tremendous diversity in the etiology of neonatal sepsis. This is the first of many factors that make neonatal sepsis a particularly difficult disease to treat and prevent, a concept that will be explored in greater detail below. This also demonstrates the importance of a host-focused response – developing a

pathogen-agnostic intervention for neonatal infectious disease should be a higher priority than focus on a single pathogen.

1.1.4 Summary and conclusions

The global mortality rate in the first few days of life is substantially higher than any other time in childhood, and sepsis is one of the most prominent causes of death in this period. Scientists and clinicians have iterated through multiple consensus definitions for adult sepsis which has moved from sole focus on inflammation towards more of a 'functional network' description, defining sepsis as a "dysregulated host response" to infection. The lack of consensus definitions for neonatal sepsis makes it difficult to accurately estimate the true burden of disease. There are a tremendous number of casual pathogens that are responsible for neonatal sepsis and these vary across time, geography, and laboratory: it is difficult to point to a single virus or bacterium as the primary cause of neonatal sepsis. Given this, as well as the disturbing rise in antibiotic resistance[26], I believe the only way to develop new interventions for neonatal sepsis must be pathogen-agnostic – they must focus on the host response rather than on the inciting agent. We must therefore understand the nature of neonatal immunity, which I discuss in the upcoming sections.

1.2 Neonatal immunity, immaturity, and disease tolerance

The disproportionately large burden of infectious disease in the neonatal period has classically been understood as a function of an 'immature' immune system.[27] This understanding has been challenged in the last decade due to 1) emergent evidence that neonatal immunity is tightly

controlled and well-regulated, i.e. not random or immature, and 2) a failure of the immaturity dogma to generate successful treatments or interventions for neonatal sepsis. The below section overviews the evidence that immaturity is an inadequate explanation for understanding the neonatal immune system.

1.2.1 Neonatal immune 'immaturity'

Portions from this section were excerpted from 'outgrowing the immaturity myth', frontiers 2018

Up until the last few decades newborns were widely considered to be simply immunodeficient "due, in part, to a limited capacity for IL-2 production and proliferation in neonatal T cells."[28] In the early 2000s the dogma shifted from simple immunodeficiency to saying newborns exhibited a more "Th2-skewed" response, based primarily on observations coming out of neonatal mouse models. Indeed, there are many differences between neonatal and adult immunity, many of which my co-authors and I summarized in my 2017 publication "Newborn susceptibility to infection vs. disease depends on complex in vivo interactions of host and pathogen" in *Seminars in Immunopathology*. Some pointing to an immature neonatal immune response include a higher proportion of naïve T-cells, lower counts of circulating neutrophils during infection, diminished functionality in neutrophils (lower phagocytic capacity, lower ROS and AMP production, etc), lower inflammatory cytokine production, and a reduced innate and killer T-cell cytotoxic capacity to name a few. However, most of this evidence comes from studies which used purified cell populations from cord blood *in vitro* to collect measurements about specific immune cell functionalities.[13]

Many studies have described deficiencies in the neonatal innate immune system that could be responsible for the decreased ability to clear invasive pathogens. For example, kinetics of pathogen clearance in animal models of neonatal infection show that neonates take longer to clear invasive bacteria than their adult counterparts.[29,30] A recent study comparing methicillin-resistant *Staphylococcus aureus* (MRSA) infection in neonatal and adult mice attributed delayed clearance in neonates to inefficient phagocytosis and limited neutrophil recruitment to the site of infection. Specifically, in neonates, neutrophil production dropped off despite the continued presence of bacteria, whereas in adult animals diminishing neutrophil production corresponded with bacterial clearance. Other studies have implicated impaired neutrophil recruitment as a potential explanation for the increased susceptibility to infection in early life.[31,32] While neonates have higher basal levels of circulating phagocytic cells than adults, as mentioned previously, they are generally considered to be less efficient phagocytes.[31,33–36]

It thus appears that neonatal immune cells are intrinsically worse than their adult counterparts, which would perhaps suggest that they are indeed simply immature. However, a 1976 study showed that the exposure of neonatal monocytes to adult plasma entirely restored the chemotactic bactericidal capacity of the neonatal cells to that of adult levels.[37] Another group examined phagocytic capacity and hydrogen peroxide production of neonatal polymorphonuclear cells (PMNs) in both neonatal and adult plasma, and concluded that both were "principally regulated by the plasma employed".[38] More recently, a thorough examination of neonatal T cells declared that "infants' T cells are unambiguously immunologically competent" – the diminished

IL-2 production of newborn T cells was entirely dependent on Ca^{2+} influx and availability of the environment they were in.[39] These three examples are to say that it appears to not be something intrinsic to the immune cells that are "worse" than that of adults, but rather the 'immunological environment' within the newborn is altering the functionality of the immune cells to behave in a way fundamentally different to that of adults.

Just as *in vitro* comparisons of neonatal and adult phagocytic cells have contributed to the theory that neonatal susceptibility to infection is a result of immaturity, so has the evidence accrued which describes diminished *in vitro* pro-inflammatory responses when comparing neonatal and adult cells.[27,40–42] However, animal models of neonatal sepsis using a variety of pathogens (both bacterial or viral) or TLR agonists have found neonates to generate an inflammatory response equal to or greater than that of adults.[13,30,43–46] Furthermore, exogenous supplementations of pro-inflammatory cytokines have been shown to greatly increase mortality in a polymicrobial model of sepsis in neonatal mice[47,48]. This increased mortality of neonatal sepsis does not relate to decreased bacterial clearance, as neonatal mice also suffer much greater mortality than adults when challenged with purified TLR agonists in the absence of an infection.[46,49] This has led to the realization that the inflammatory response itself is considered to be largely responsible for the higher mortality of infected newborns vs. adults.[47,48]

1.2.2 Regulation of the neonatal immune environment

This section is excerpted from 'outgrowing the immaturity myth', frontiers 2018

Given the higher risk of the newborn vs. adult to suffer from the immune response to an infection (or exposure to TLR agonist), newborns would benefit from mechanisms that would reduce the risk to unleash a harmful antimicrobial immune response. An *E. coli* model of neonatal sepsis found that neonatal TRIF$^{-/-}$ mice suffered higher mortality than WT or MyD88$^{-/-}$ strains with the opposite being true in young adults.[50] Neonatal prioritization of TRIF-dependent pathway activation when exposed to TLR agonists was then linked to a strong induction of type 1 interferon regulatory responses, as opposed to the adult MyD88-dependent pro-inflammatory response. A molecular explanation for these age dependent differences in defense strategy has recently been identified as the endogenous, heterodimeric complex of TLR4 ligands S100A8/A9: high levels of S100A8/A9 shift TLR signaling from MyD88- to TRIF-dependent pathways. S100A8/A9 alarmins are also known to be massively released at birth. This alarmin release is incongruous with the "immune immaturity" paradigm as it represents a purposeful shift away from MyD88 pathway activation, the preferred adult pathway. If neonatal death was driven by a simple lack of adult-like features one would expect that any external shift towards a more adult-like immune response would lead to better outcome. But the opposite is in fact the case, as *S100a9*$^{-/-}$ neonatal mice suffer much higher mortality than their WT counterparts when infected, implying the alarmin release at birth; i.e. the subsequent shift away from an adult-like response, is an important and necessary step to successfully mount a defense against an early-life infection.[13,51] The age-dependent production of S100A8/A9 thus represents an example of disease tolerance unique to neonates that has developed to avoid immunopathology from a MyD88-driven pro-inflammatory response at the potential cost of rapid bacterial clearance.

The importance of the TRIF-dependent newborn response is entirely incongruous with the "immune immaturity" paradigm. Yet, while this is true for pathogens that signal through TLR4, other mechanisms of disease tolerance to e.g. Gram-positive infections still need to be identified. For example, there are several other mechanisms in place in early life that commonly are described as immune suppressive, with the notion that these are remnants of the mechanisms that allow semiallogeneic mismatch *in utero* without rejection of maternal cells by the fetus.[52] However, these mechanisms persist far beyond the immediate perinatal period, and thus likely have other benefits in postnatal life, such as increasing disease tolerance by reducing immune mediated pathology ('immune cost") even if it comes at the cost of an increased bacterial burden.[53]

1.2.3 Implications and significance

The hypothesis that the full-term, neonatal immune system is simply underdeveloped does not explain the evidence which has emerged over the last few decades. While this paradigm may still hold some merit in the context of very preterm immunity, the concept of neonatal immaturity continues to drive research in term neonatal sepsis and it is crucial that we move on to a fact-based, data-driven understanding if we hope to develop new prophylactics and interventions in this space. The rejection of this hypothesis leaves an interesting gap in our knowledge which leads to the inevitable question: if the neonatal immune system is not immature, why are newborns so susceptible to infectious disease and sepsis? It was this question that led to the publication of section 1.3 as a standalone piece in *Frontiers in Immunology* and ultimately guided much of my work described later in this dissertation.

1.3 Disease tolerance and the cost of infection

To build the conceptual framework for reconcilation of the clinical observation (increased risk to suffer and die from sepsis in newborns vs. adults) with mechanistic insight regarding host defense in early and adult life, it is necessary to consider the range of host responses to infection that are available. Medzhitov *et al.* in 2012[53] outlined three distinct strategies of host defense to infection: disease avoidance, disease tolerance, and disease resistance. In disease avoidance, infection is avoided through behavioral adaptations (e.g. our evolved revulsion to the smell of rotting meat). Disease resistance focuses on the reduction of pathogen burden at the risk of host-inflicted damage (immunopathology). Disease tolerance (DT) strives to minimize immunopathological damage, or fitness cost to the host at the potential cost of unchecked pathogen proliferation (it is important to draw the distinction between "disease tolerance" and "immune tolerance", which describes regulatory T cell (Treg)-mediated unresponsiveness to potentially immune-activating agents)[53]. Animal models have demonstrated newborns to suffer increased mortality when infected with living bacteria[49], viruses[54,55], or purified inflammatory agonists.[32,46] DT is a well-established concept in biology, but not yet as readily accepted in the human realm. Specifically regarding DT in early life: newborns are able to withstand a circulating bacterial load 10-100 times greater than adults (less than 1 CFU per mL blood has been considered to be the clinical "low" threshold in adults, whereas less than 50 CFU per mL blood has been considered the "low" neonatal threshold; the same trends are observed in animal models).[56,57] The juxtaposition of increased sensitivity to infection with an enhanced ability to survive greater pathogen loads is the hallmark characteristic of a "disease tolerance" response.[53] Yet, as evidenced by the higher burden of infectious disease in newborns, a host defense strategy relying on DT is likely less effective than the adult focus on

disease resistance. Despite this clear clinical disadvantage, the newborn host as an organism appears programmed to more heavily rely on this DT strategy.[58,59]

1.3.1 Metabolism is fundamentally linked to immunity

Metabolic and immunological functions are intrinsically connected at a level beyond the former simply fueling the latter – metabolic substrates, enzymes, transcription factors, cell receptors and intermediates have all been shown to have a vast array of immunoregulatory properties. A recent surge in research into this phenomenon ('immunometabolism') has led to the publication of excellent reviews[60–65] which explore the regulatory role of different metabolic pathways on various leukocytes; with this in mind, we only present a brief overview to introduce the key themes of immunometabolic changes focused on bacterial sepsis.

It has long been known that changes in cellular metabolism occur during sepsis, although until recently these changes were considered to be a result of an oxygen-poor microenvironment due to inflammation-induced hypoperfusion.[66] However, there is a large body of evidence indicating that both metabolic shifts and tissue damage in sepsis occur independent of oxygen levels.[63,66–69] TLR activation in certain leukocytes has been shown to activate hypoxia-inducible factor 1α (HIF1α), which upregulates glycolytic pathways and downregulates oxidative phosphorylation – a process known as the Warburg effect.[65,70] This 'aerobic glycolysis' is critical to the inflammatory immune response (disease resistance) and represents the primary metabolic activity within immune effector cells (granulocytes, M1 macrophages, cytotoxic and helper T-cells, NK cells, etc.)[61,65]. While the purpose of switching to aerobic glycolysis in lieu of the more energy efficient process (i.e. ATP-

producing) of oxidative phosphorylation is still being debated, it is generally thought that effector cells rely on glycolysis because of one or all of the following reasons: (a) glycolytic intermediates are needed for rapid biosynthesis required for an inflammatory response, (b) glycolysis can be rapidly upregulated and thus can provide a burst of energy faster than oxidative phosphorylation, (c) reactive oxygen species (ROS) are produced during glycolysis which are used in an antimicrobial capacity, (d) glycolysis is better suited to hypoxic/normoxic conditions which may arise during inflammation, and/or (e) increased uptake of glucose minimizes the amount of energy available for invasive bacteria.[61,65,71] Whatever the reason, it is well established that aerobic glycolysis is a critical component of the disease resistance response.[65,72]

Where aerobic glycolysis is enhanced in effector cells involved in disease resistance pathways, the regulatory and longer lasting cells associated with DT (Tregs, M2 macrophages, memory T cells etc.) increase uptake of exogenous fatty acids and sustain high levels of β-oxidation and oxidative phosphorylation during infection and sepsis.[61,65] While lipids in excess have been shown to induce systemic inflammation (i.e. in obesity), many metabolic intermediates of lipid metabolism exert the opposite effect[73]. Circulating lipids which are generated through fatty acid metabolic pathways, namely high-density lipoproteins and very low density lipoproteins, are even capable of directly sequestering LPS and dampening the inflammatory response.[74,75] Lipids belonging to the group of omega 3 fatty acids inhibit the production of inflammatory cytokines and upregulate anti-inflammatory cytokines.[76] These lipids also act as precursors to a specialized family of lipids identified as “pro-resolving lipid mediators” (including lipoxins, resolvins, and protectins) which

are actively produced to tone down the inflammatory immune response produced at the site of infection.[77]

Fatty acid metabolism can therefore be considered to be a fundamental part of the DT response; not only do regulatory / immunosuppressive cells rely on exogenous fatty acids to enact their function, but the metabolites themselves reduce the inflammatory immune response. On the other side of the coin, aerobic glycolysis is a fundamental aspect of the disease resistance response. In addition to the aforementioned increase in glycolytic pathways in immune effector cells, multiple enzymes involved in glycolysis have been shown to either inhibit inflammatory pathways or activate immunosuppressive pathways.[65] When a TLR ligand induces high glycolytic flux, these enzymes are rendered incapable of maintaining these disease tolerant functions and the disease resistance response is enhanced.[65] To summarize, metabolic shifts in sepsis cannot be separated from inflammatory shifts – the two are fundamentally connected.

1.3.2 Metabolism in adult sepsis

Metabolic changes during sepsis in adults have been shown to not only be instrumental in diagnosing the disease, but also highly related to survival. A 2013 study[78] of adult patients with community-acquired sepsis examined changes in the plasma metabolome and proteome at time of enrolment and 24 hours later. Comparisons were made between survivors (split into three subgroups: uncomplicated sepsis, day 3 severe sepsis, and day 3 septic shock), non-survivors, and a control group of patients exhibiting symptoms but were later determined to have SIRS for non-infectious reasons (SIRS-positive controls). The plasma metabolome revealed four primary

findings: a) the profile of plasma metabolites during sepsis were distinct and reliably distinguishable from SIRS-positive controls, b) there were marked differences in plasma metabolites between sepsis survivors and non-survivors, c) there were no differences between the sepsis-survivor subgroups (varying degrees of severity), and d) there were no major differences between infections caused by *S pneumoniae, S. aureus, or E. coli.*[78] Plasma proteomics mirrored the trend – significant differences between sepsis vs SIRS-positive control, significant difference between survivors and non-survivors, minimal (only one) differences within the survivor subgroups, and no significant differences resulting from infections caused by different bacteria. Alterations in fatty acid metabolism largely separated sepsis survivors from non-survivors – the specific pattern of metabolites which were different "suggest a profound defect in β-oxidation in adult sepsis non-survivors that was absent in sepsis survivors".[78]

The authors indicate the above noted differences were not a result of organ dysfunction or hypoxia, but rather due to defects in the process which transports fatty acids from the cytoplasm into the mitochondrial membrane (the carnitine shuttle), which in turn may be attributed to a decrease in peroxisome proliferator-activated receptor-α (PPARα) expression during sepsis. PPARα is the primary transcription factor responsible for controlling host of genes associated ketone body synthesis (ketogenesis) and transport, a process which in adults is typically associated with prolonged fasting.[79] One explanation for the apparent requirement of ketone body production during sepsis is that ketone bodies act as the alternative to glucose for fueling brain metabolic activity, as they are one of the few energetic substrates which are able to cross the blood-brain barrier.[80,81] An animal model examining the impact of exogenous glucose and 2DG (an

unmetabolizable analog of glucose which inhibits glycolysis) on sepsis induced by *Listeria monocytogenes*, LPS, influenza virus, and poly(I:C) showed that 2DG's protective effect in bacterial sepsis was mediated through increase in ketogenic activity (PPARα-dependent), which reduced neuronal cell death independent of bacterial load.[21] Exogenous glucose alone worsened outcome acting through the same axis – ketone body production was inhibited, and neuronal cell death increased in bacterial sepsis. Curiously, these effects were reversed in the viral sepsis models (poly(I:C) and influenza) – 2DG caused 100% mortality and feeding / glucose caused 100% survival, indicating fundamental differences between metabolism during viral and bacterial sepsis.

Another recent study examining longitudinal changes in serum metabolite concentrations during sepsis in adults found non-survivors had elevated (and increasing) levels of TCA cycle metabolites as well as diminished (and declining) numbers of short and long-chain fatty acids[82] – the same trends have previously been described in animal models.[83,84] Though non-survivors in sepsis have diminished fatty acid levels relative to survivors, sepsis itself is generally associated with an increase in plasma lipids (including free fatty acids) when compared to healthy controls.[82,85] Not only do plasma lipids play a critical role in regulating inflammation and providing energy for the brain, but fatty acid metabolism and ketogenesis has also been shown to fuel metabolic activity in many vital organs during active infection.[86–88] An impaired capacity for β-oxidation and/or a depleted fatty acid supply will essentially turn off the disease tolerance pathways – death seems almost inevitable through either uncontrolled inflammation or uncontrolled energy expenditure, leaving vital organ functions without fuel. As with any homeostatic environment, poor outcomes are more likely if the balance tips too far to the either extreme.

Furthermore, there is mounting evidence that mortality in adult sepsis is less likely associated with excess inflammation, but rather an immunosuppressive or endotoxin tolerant phenotype (M2-macrophage polarization, anti-inflammatory cytokine production without impaired phagocytic capacity)[89–91]. One would expect that as organs begin to fail due to insufficient energy, the body would attempt to increase fatty acid metabolic activity (and inevitably anti-inflammatory activity) at all costs. A prolonged disease resistance response is energetically demanding and eventually it is necessary to revert towards DT by necessity. The heightened death observed in this period may therefore not necessarily be caused by DT, but rather the phenotypic switch to DT as a 'last-ditch' effort to adapt to an unsustainable metabolic demand. Perhaps it is time to consider these late-phase inflammatory changes in adult septic patients as a reflection of a different biological mechanism – a slow decrease in the energy available to power vital organ functions.

1.3.3 Energetic differences in neonates and implications for bacterial sepsis

The implications of the critical role metabolic pathways play in regulating inflammation and providing energy during infection are enormous for newborns, as the energetic demands of growth and development are intense. After adjusting for body weight, healthy newborns require on average three times as much protein (2.2 g/kg/day vs. 0.8 g/kg/day) and more than three times as much total energy (120 kcal/kg/day vs. 35 kcal/kg/day) as adults.[92] Newborns have a lower reservoir of energy, as demonstrated by the percent bodyweight made up of fat (14% vs. 18%) and protein 11% vs. 18%) in neonates and adults.[92] Sustaining a controlled immune response requires not only intense glycolytic flux to fuel the cellular proliferation and biosynthesis of disease

resistance, but it also requires substantial fatty acid metabolic flux to regulate the inflammation and provide energy to vital tissues. Adults are able to rely on fat and protein stores to provide enough energy to engage in a robust disease resistance response without pulling resources from critical processes, at least until later in infection (see above). This can be observed as up to a 150% increase in resting energy expenditure during bacterial infections in adults.[93] Neonates, however, show either no change or even a decrease in resting energy expenditure during sepsis.[94–96] An inability to increase energy expenditure relative to the resting state in neonates suggests that the energy to fuel the immune response must be redirected from processes elsewhere in the body. Clearly these processes (likely growth and development) are important enough to warrant maximum energy expenses at a basal state (part of the explanation for relying more heavily on DT than disease resistance). Adults are able to employ the "expensive" disease resistance response without seriously interfering with other vital survival processes; for neonates, any energy spent on immunity has to be 'borrowed' from somewhere else. The increased reliance on DT in the newborn allows for less glycolytic flux and thus a lower risk to incur organ failure through an energy deficit during septic episodes.

The first few postnatal days are likely the most energetically demanding period in all of life[97]. Immediately after birth, neonates must transition from reliance on maternal glucose to generating it themselves – this manifests as hormonal activation of both glycogenolytic and gluconeogenic pathways in order to rapidly ramp up glucose production to fuel developing organs, especially the brain.[97] Further, the newly born infant faces rapid heat loss in the transition from the warm uterine environment to the (relatively) colder external environment. Heat production and oxygen

consumption increase two to three-fold within minutes of birth, through both heightened cellular metabolism and non-shivering thermogenesis (metabolism of brown adipose tissue)[98]. The high mortality observed on the first day of life in particular may be related to this sudden inability to rely on maternal metabolic and thermoregulatory processes.[98,99] All of these pressures are likely even greater on the pre-term infant. The more energy siphoned towards mounting an immune response, the more sacrifices must be made to fuel the necessary cell proliferation and antimicrobial activities. One would anticipate evolutionary pressures to naturally equilibrate neonatal immunity towards a balance between immunity and development – hence a heightened reliance on DT in neonatal infection.

Metabolomics of the neonatal population have not been studied in nearly as much detail as adults, though what is available indicates that metabolism is a critical component of neonatal sepsis as well. A transcriptomic comparison of newborns with bacterial sepsis against healthy controls was used to construct a classifier that accurately identified septic neonates; inclusion of only genes which were associated with standard immune functions (inflammation, etc.) resulted in a classifier with 100% sensitivity but less than 30% specificity, but the inclusion of metabolic genes brought the specificity up to 100%.[100] Specifically, they showed that bacterial sepsis in neonates is associated with increased expression of genes related to glycolysis (glucose transporter GLUT3, glycolysis activator PFKFB3, and initiating hexokinase HK3), fatty acid metabolism and metabolic homeostasis (principally via regulatory STAT3 and receptor FFAR2). In the validation test set of the classifier, the three instances of viral sepsis clustered with the 6 controls; the viral patients did not show the distinct metabolic profile which was so visible in newborns with bacterial

sepsis, further indicating that uncontrolled viral proliferation has a profoundly different impact on the body than uncontrolled bacterial proliferation.[100]

As mentioned above, poor outcomes in adult sepsis correlate with an inhibited ability to produce ketone bodies via PPARα. While ketone body metabolism in the adult brain is typically reserved for a starvation response (which perhaps should be updated to "energetically demanding periods" such as sepsis), there is evidence from animal models that neonates rely on ketone bodies as an energy source in the brain independent of starvation.[81] Specifically, newborn rats rely on ketone bodies for up to 40% of the energy production in the brain[80] and newborn cynomolgus monkeys exhibited increased expression of blood-brain barrier ketone body transporter protein MCT1, with levels decreasing as a function of age (plateauing in adulthood).[101] The process of birth necessitates a series of metabolic adaptations from receiving nutrients via the placenta (high-carbohydrate, low-fat) to receiving nutrients via breastmilk (low-carbohydrate, high-fat).[102,103] One manifestation of these adaptations is the activation of PPARα immediately prior to birth, presumably in anticipation of the new, fat-rich diet[102]. Upon the switch to breastmilk, neonates rely on ketone bodies to fuel brain activity which allows glucose (broken down from lactose) to enter the pentose phosphate pathway, producing the nucleic acids and lipids necessary for cerebral growth.[81]

Similarly, a metabolomic analysis of urine from septic newborns indicated a substantial increase in acetone ketone bodies (and other byproducts of fatty acid oxidation) relative to healthy controls[104]. If more ketone bodies are found outside of the brain but there is little compensatory increase in ketone body production (as indicated by animal models[84], and neonates being "maxed-

out" in their energy expenditure at baseline), then one can assume that ketone bodies which are needed in the brain are being deployed elsewhere in the body, and hence the brain is running at an energetic deficit. This is just one example of the type of vital process which may be interrupted by mounting an immune response during bacterial sepsis.

1.3.4 Nutritional therapy and the microbiome

If mortality in bacterial sepsis can be attributed to an energetic deficiency, then one must be able to explain how nutritional supplementation (a standard practice in any ICU or NICU) does not represent the most effective sepsis treatment. As with everything else in sepsis, the efficacy of feeding as an intervention is limited by its ability to maintain homeostasis. The previously described study by Wang *et al.* where inhibition of glycolysis led to 100% survival (and feeding led to 100% mortality) in adult, LPS-challenged mice provides an excellent example of nutritional supplementation creating a homeostatic imbalance and leading to negative outcome. Both exogenous glucose and food gavage inhibited ketogenesis which led to an energetic imbalance (glucose being siphoned into the immune response with no ketone bodies to replace it) and an inflammatory imbalance (diminished anti-inflammatory lipid mediators). One also must consider the dangers of overfeeding - overfeeding has been shown to worsen sepsis outcomes in both animal models and human observational studies due to hyperglycemia, elevated inflammatory markers, dysregulated immune responses, and presumably enhanced nutrients for pathogen growth.[105–108] Further, this hypothesis poses that the metabolic risk comes from a shift in the proportion of energy expended towards disease resistance pathways over maintaining organ function – the danger is not only tied to the overall capacity, but the utilization of energy present. If a system has reached the

point where it is spending 100% of its resources on fighting infection, no amount of exogenous nutrients will make a difference (unless accompanied by a simultaneous change in resource allocation).

Early enteral nutrition (EN) in adult patients with prolonged sepsis has been shown to improve patient outcomes, reduce oxidative stress, improve gut epithelial integrity, and downregulate systemic immune responses[109]; correspondingly, negative energy balance has been shown to be associated with worse clinical outcomes.[110] EN not only addresses the caloric deficit which is inevitable in prolonged sepsis but seems to modulate immune functions through interfacing with the gut-associated lymphoid tissue and upregulating Th2 cell proliferation. Parenteral nutrition (PN) has been shown to be less effective than EN – a 2013 study by Elke *et al.* found that rate of death was significantly lower in adult ICU patients with sepsis which received EN rather than PN or EN + PN combined (26.7% vs. 41.3%); in addition to this mortality reduction, duration of mechanical ventilation and rate of secondary infection were also decreased in the EN-alone group[111]. Increased mortality from PN (relative to EN) is thought to be due to a heightening of the inflammatory response associated with hyperglycemia, an effect which exacerbated in PN due to bypassing metabolic regulatory axes associated with the GI tract[109]. This has interesting implications for neonates, where the nascent microbiome represents another axis which is distinct from adults[112]. The diet of neonates (lactate-heavy breastmilk) results in a colonization pattern of commensal bacteria which fascilitate nutrient absorption and produce a wide array of immunoregulatory metabolites[112,113]. The limited biodiversity present in the neonatal microbiome could result in an impaired ability to incorporate nutrients without altering inflammatory

homeostasis – any inability to control the potential energy flux of disease resistance represents another potential explanation for the neonatal reliance on DT.

1.3.5 Recontextualizing newborn immunity

The implications of this hypothesis are broad and may explain other aspects neonatal immunity. Newborns have been described as exhibiting an immunosuppressive phenotype, which has often been considered to be a vestige of time spent *in utero* where active fetal immunity could result in miscarriage[114,115]. This alternative hypothesis to DT fails to explain the persistance of many of these immunosuppressive actors well after the first few days of life. For example, neonatal myeloid-derived suppressor cells and anti-inflammatory $CD5^+$ B cells remain significantly higher than adult levels for more than six months and four months after birth, respectively.[115,116] Given the high burden of infectious disease in early life, one would anticipate evolutionary pressure to drive the time spend in this "anti-inflammatory phase" to as little as possible. If, however, neonatal immunity is limited by an availability of energy, then it would be critical to maintain some immunosuppressive cells to limit the magnitude of an inflammatory response until the body is able to better sustain it. While the 'fetal suppression' hypothesis may in part explain the susceptibility of term infants to bacterial sepsis[117], it seems unlikely that a biological liability of this magnitude (suppressed immune system) would exist and persist if it did not convey some sort of survival advantage (DT).

The extreme susceptibility to infection observed in preterm newborns may be in part due to the extreme energy demands associated with survival and rapid development, but it is more difficult

to discount alternative explanations such as immaturity and immune suppression to tolerate maternal antigens. As outlined in a recent review by Collins *et al.*, susceptibility of preterm newborns to infection can be in part attributed to "compromised [innate] barriers, inflammatory response elements, and cells".[118] Certainly there are many other factors at play in preterm immunity, some of which likely are indeed related to developmental immaturity (particularly for those born very preterm). More research is warranted to elucidate the role metabolic demands play in preterm immunity.[118]

1.4 Arachidonic acid and the vascular endothelium

The primary hypothesis presented in the previous section, that neonatal immunity is constrained by energy demands and depends on a more "disease tolerance" style of immune response, ended up not being the primary focus of this dissertation. However, this line of inquisition led to a heightened interest on metabolic pathways in neonatal sepsis, and particularly where metabolism and immunity directly interact with one another. This naturally brought me to arachidonic acid metabolism and eicosanoids – fatty acid metabolites which are major regulators of myriad immune processes. This section provides background on eicosanoids in neonatal sepsis as one of the most important downstream points controlled by eicosanoid metabolism: the vascular endothelium.

1.4.1 Eicosanoids in sepsis

Arachidonic acid (ARA) is a bioactive, polyunsaturated fatty acid which is abundant in the brain and produced by the breakdown of phospholipids, catalyzed by secretory Phospholipase A2

(sPLA2). Perhaps most importantly, oxidation and subsequent metabolism of ARA produces eicosanoids, a class of immunoregulatory signaling molecules which have been shown repeatedly to play an important role in infection and inflammation.[119,120] It is fallacious to attempt to classify eicosanoids as simply 'pro-inflammatory' or 'anti-inflammatory' – as with many immune signaling molecules, their production leads to cascading feedback loops with innumerable downstream effects. In truth, eicosanoid is the general term for any of the bioactive, 20-carbon lipids derived from arachidonic acid.[121] Eicosanoids have historically been sorted into three major categories – prostaglandins, leukotrienes, and lipoxins, but more recently discovered are eoxins, hepoloxins, resolvins, isoprostanes, epoxyeicosatrenoic acids (EETs), hydroxyeicosatetraenoic acids (HETEs) and more.[122] There is a notable asymmetry in what is known about the classical and non-classical eicosanoids – large bodies of research exist focused on prostaglandins and leukotrienes in inflammation and infectious disease, some on resolvins and lipoxins, and very little on EETs and HETEs. This winnows even further when looking specifically at how eicosanoids behave in newborns. The section below will summarize the known role eicosanoids play in sepsis.

Elevated and altered eicosanoid levels have been proposed as promising diagnostic biomarkers for adult sepsis, with different studies focused on different targets. A comparison of healthy and septic adults identified differences in arachidonic acid metabolites across the board, but most notably driven by PGE2, arachidonic acid itself, 11-HETE, and thromboxane B2.[123] This same group found that the *in vitro* inducibility (in response to an LPS stimulation) of some arachidonic acid metabolites was diminished by 80-90% in septic patients relative to controls. Another study

identified septic patients exhibit elevated levels of serum sPLA2, PGE2, and prostaglandin synthase. All three of these measurements were also directly correlated with the severity of bacterial sepsis.[124] This touches on the more compelling component of measuring arachidonic acid in sepsis – while there are plenty of sepsis studies which have identified a tremendous number of potential sepsis biomarkers for diagnostic purposes, fewer have directly associated biomarkers with severity and/or changes in survival. The focus of this thesis is not on diagnosing neonatal sepsis but rather better understanding the underlying pathology, and there appears to be strong evidence (at least in adult sepsis) that arachidonic acid metabolism plays a key role in differentiating survivors and non-survivors. For instance, a metabolomic comparison of adult sepsis survivors and non-survivors found significantly higher levels of both pro-inflammatory (leukotriene B_4, PGE2) and pro-resolving mediators (resolvin D1, resolving D2, protectin D1) in the non-surviving group.[119] A metanalysis wherein the authors built a tool to predict survival in adult sepsis using published metabolomic data identified 'Eicosanoid and Resolvin Metabolism' as the second most enriched term associated with sepsis death, behind only 'Vitamin B6 metabolism'. The authors concluded that the 'eicosanoid storm' was one of the key differentiators between survival and non-survival.[125]

1.4.2 Effects of exogenous arachidonic acid

Little has been published outlining the relationship between ARA or eicosanoids and neonatal sepsis – the research simply has not been done. There is, however, a decent body of work examining the role arachidonic acid plays in infant development and the significance of its presence in breastmilk. ARA is considered a nutritionally essential fatty acid and is always

present in human breastmilk at a markedly consistent level, comprising about 0.47% ± 0.13% of the milk by weight. By six months of age the mean daily ARA intake is estimated to be around 160 mg/day but this appears to vary widely across geography and age.[126] Over the first month of life, ARA rapidly accumulates in the brain and is important for early brain development.[127] This touches on an important consideration when discussing ARA metabolism and immunity – while ARA is broken down into eicosanoids which obviously play an important and direct role in immune regulation, ARA itself "mediates neuronal firing, signaling, and long-term potentiation. ARA also helps maintain membrane order and hippocampal plasticity, defends the brain against oxidative stress in the hippocampus by activating the peroxisome proliferator-activated receptor gamma, and aids in the synthesis of new protein in tissue".[126] ARA is also an important factor of cell-membranes, where it mediates signal transduction, gene expression, and acts as a source of substrate for many chemical intermediates. There is also evidence that ARA and its metabolites help regulate bone growth and formation in infancy[128]. This is far from a comprehensive account of the biological roles of ARA, but it is included here simply to highlight that ARA is an essential component of development irrespective of the immune system.

Most of the work examining the immunomodulatory effects of ARA supplementation have indicated that ARA itself, when administered as a diet supplement, has only a muted impact on the immune system, with some exceptions.[126] While much of the data surrounding the effects of ARA supplementation in animal models comes from tweaking diets to include more PUFAs in general, in one study authors fed weanling rats a ARA-enhanced diet and showed slight changes in leukocyte PGE2 production but no changes to lymphocyte proliferation, NK cell activity, or

cell-mediated immunity.[129] A different (adult) mouse study looking at a swath of PUFAs added to a safflower oil ester concluded that murine T-lymphocyte function and signal transduction were unaffected by exogenous ARA administration.[130] This appears to be in line with a human study wherein the diets of healthy, adult men were supplemented with 200 mg/day ARA (or placebo control) and PBMCs were collected regularly over the course of 130 days. There appeared to be no differences between the PBMCs from the ARA and control groups when examining the *in vitro* inflammatory response to a few different stimulations, but they did find the group receiving the high-ARA diet had significantly higher levels of circulating granulocytes and a fourfold higher "post-immunization proliferation in response to influenza vaccine".[131] One of the areas where ARA supplementation does seem to have a more dramatic effect is on the vascular endothelium – in a piglet model of the ischemia-injured ileum, formula containing 5% ARA significantly improved intestinal barrier function and exhibited enhanced recovery.[132] This has been somewhat mirrored in humans, where there is data that increased uptake of LCPUFAs improved recovery of infants affected by NEC.[126]

1.4.3 Vascular endothelium in neonatal sepsis

Arachidonic acid and its metabolites are known to be major regulators of vascular tone, gut epithelial integrity, blood pressure, endothelial cell proliferation, and essentially all things related to the vascular endothelium.[133–135] This is particularly relevant for sepsis as the vascular endothelium, the monolayer of endothelial cells which line arteries, veins, and capillaries, has repeatedly been shown to be a major player in the pathology of organ failure in sepsis.[136,137] Given that a) there is an enormous deficiency in host-focused interventions for sepsis and b) the

breakdown of the vascular endothelium is suspected to be one of the major contributors to death during sepsis, focus on the endothelium is a logical next step in the movement towards pathogen-agnostic approaches.

As with most things sepsis-related, only recently has the vascular endothelium fallen under scrutiny in neonatal sepsis. This was well outlined by in a 2019 review by Pietrasanta *et al.* which I will briefly summarize here. Neonates, particularly those born pre-term, have some key differences in the structure of the vascular endothelium in comparison to adults. Deficiencies in P-Selectin and overproduction of other adhesion molecules in response to LPS stimulation have been described and result in a diminished ability for neutrophil rolling, adhesion, and crawling.[137] There are also important differences in response to hyperglycemia - adult endothelial cells produce "higher levels of hydrogen peroxide and lower amounts of ROS-neutralizing enzymes", implying a systemic difference to an abnormal metabolic state. The relationship between ROS and endothelial stability is an important one. Balancing ROS and reactive nitrogen species (RNS) is critical to the regulation and maintenance of the vascular endothelium.[138] Any differences between neonatal and adult endothelial cells and their relationship to ROS and RNS could explain broader differences in barrier function, and potentially even the heightened susceptibility to sepsis in neonates.

The importance of endothelial cells in neonatal sepsis cannot be understated – despite high concentrations of inflammatory cytokines in septic neonates, correspondingly high levels of mRNA for the same cytokines were not found in leukocytes isolated from the same neonates.[139]

These cytokines must originate from somewhere, and more recently there is data suggesting that endothelial cells may be the culprit.[137] This is sensible as the expression of endothelium-derived cytokines helps to guide neutrophils and monocytes into tissues where they are needed, however, this process appears to be attenuated in newborns compared to adults (and even more so in those born pre-term). As with most everything else, the vascular endothelium in neonates is fundamentally distinct from that of adults.

1.4.3.1 Angoipoietin-TIE2 axis

One of the major regulatory points of vascular integrity is the angiopoietin-TIE2 axis, which involves angiopoietin-1 (Angpt1) and angiopoietin-2 (Angpt2) directly competing to bind the TIE2 receptor. While it is known that eicosanoids are also major regulators of vascular integrity and angiogenesis, a direct link between arachidonic acid and the angiopoietin-TIE2 axis has yet to be made. However, there is data showing that mutations in both the Cyp1b1 gene, one of the regulators of the Cyp450 pathway of arachidonic acid metabolism, and Angpt-1 both lead to a rare type of open-angle glaucoma, indicating a functional connection.[140,141] In healthy, steady state conditions, Angpt1 stays bound to TIE2 and initiates a signaling cascade which broadly maintains tight vascular integrity. Angpt2 is produced by and stored within endothelial cells; when released, Angpt2 outcompetes Angpt1 for the binding site in the TIE2 receptor and leads to neovascularization and vascular instability.[142] Inflammatory or oxidative stress leads to Angpt2 release and therefore creates vascular instability. In sepsis, the ratio of Angpt1:Angpt2 swings far to the side of Angpt2 and has been associated with worse outcomes.[142,143] This has been recapitulated in neonatal sepsis as well – a 2017 study in Suriname found the ratio of

Angpt1:Angpt2 was directly associated with incidence of EOS.[144] Multiple studies looking for neonatal sepsis biomarkers or markers of disease severity have found that low levels of serum Angpt1 and high levels of serum Angpt2 are associated with worse outcomes or death.[145,146] At least one group has shown that prophylactic, intravenous administration of Angpt-1 was protective against LPS challenge in adult mice, but this has not been repeated in a neonatal model.[147]

1.4.3.2 Nitric oxide in sepsis

Nitric oxide (NO) is a gaseous signaling molecule which has been long studied in sepsis research due to its significance in vasodilation and inducing angiogenesis.[136,148] NO is synthesized by nitric oxide synthase (NOS) starting from L-Arginine as a substrate. NO has been shown to be released by endothelial NOS (eNOS) and has been suspected as a contributing factor to "hypotension, cardiodepression and vascular hyporeactivity in septic shock".[149] This logically led researchers to attempt to suppress NO production in sepsis, but this was found to have a detrimental impact on outcomes.[148] Research then shifted in the other direction, hypothesizing that NO bioavailability is an important feature in maintaining vascular stability and actually improving sepsis outcomes. Exogenous L-Arginine supplementation (with the express purpose of increasing NO availability) has ben shown to improve microvascular function and organ perfusion in animal models, though these benefits have not been consistently repeated in human studies. In another trial comprised of 50 sepsis patients, researchers found no benefit to direct administration of inhaled NO but they were able to show it resulted in an increase systemic availability.[148]

Some connections have been made between arachidonic acid and nitric oxide release. For instances, arachidonic administration *in vitro* was shown to promote nitric oxide release and proliferation in human endothelial cells.[150] This proliferative effect was diminished by administration of eNOS inhibitor NG-nitro-L-Arginine methyl ester (L-NAME) and was Ca^{2+} dependent. This research is one of the few papers which have looked directly at the relationship between arachidonic acid and nitric oxide – given the significance of both entities in sepsis, more mechanistic work is warranted. Finally, therapeutic inhaled nitric oxide has also been used as an intervention in preterm neonates with severe pulmonary hypertension. The authors concluded there was a "positive response to rescue [inhaled] NO in preterm infants" which was "associated with survival benefit".[151] While not directly related to sepsis treatment, this indicates that there may be some utility to NO administration in the context of neonatal sepsis.

1.5 Cecal slurry model of neonatal sepsis

Animal models represent an important tool which enables researchers to dissect sepsis and infectious disease in a manner which cannot be done *in vitro* or *in silica*. The model relied upon throughout this dissertation is a modified version of cecal ligation and puncture (CLP) procedure commonly performed on adult mice.[152,153] While far from a perfect representation of human sepsis, CLP has been widely accepted as the "gold standard" of animal sepsis models as it has been reported to "recreate human sepsis progression with similar hemodynamic and metabolic phases and the presence of both hyper- and hypoinflammatory phases".[154] CLP also uses "the complete spectrum of host enteric bacteria" and exhibits a "prolonged and lower elevation of cytokine release, as in humans". CLP, however, is a surgical procedure wherein the cecum is

ligated and perforated. This is simply not a viable option for mouse pups: it is technically difficult due to their size and gut friability, and there is a heightened risk of maternal cannibalization after any surgical procedure.[49]

The need for a neonatal "equivalent" to CLP drove researchers to develop the cecal slurry (CS) model of neonatal murine sepsis. Briefly, cecal contents were collected from adult mice, filtered, and injected intraperitoneally into mouse pups about 7 days old. The CS model appears to behave biologically similarly to the CLP model and has been shown to reproduce "several of the classic temporal responses to sepsis, characterized by an initial hyperdynamic response followed by decompensation, organ failure, and death".[49] Administration of CS in young adult animals produced changes to splenocytes which looked nearly identical to the phenotype observed following CLP. This is not to say that the two are identical, however – another group found that, in young adult mice, two hours after sepsis the CS model resulted in "greater magnitude of early inflammatory gene expression" whereas CLP had "more significant expression of genes associated with IL-10 signaling pathways".[155] Bacterial colonization in the blood was detected in neonates within two hours of CS administration. CS challenge caused significant spikes in plasma cytokines (IL-1β, IL-6, IL-10, TNF-α, and IFN-γ among others) which at least somewhat mirror those seen in human neonatal sepsis.[49] For these reasons, the CS model has become increasingly popular among neonatal sepsis researchers and is widely accepted as standard in neonatal mouse work.[156–159]

This is of course not to say that CS injection perfectly mimics human neonatal sepsis – there have been many valid criticisms of mouse sepsis models for a variety of reasons. There are questions of whether it is better to approach sepsis from a single pathogen model wherein researchers directly administer a known pathogen to induce the immune response, but this inevitably prompts further criticism of the relevance and generalizability of the pathogen chosen. A CS model certainly does not avoid this criticism but there is a line of thought that perhaps an intervention which is effective in a polymicrobial sepsis model may be more generalizable than in a single pathogen model: this is ultimately just conjecture. Further, some bacteria have been shown to infect mice at greater or diminished efficiency than they infect humans.[156,160] The route of administration (intravenous, intraperitoneal or subcutaneous) of a pathogen leads to differences in murine immune responses and it is generally unclear which best maps to humans. The most striking criticism was published in a 2013 paper titled "Genomic responses in mouse models poorly mimic human inflammatory diseases" wherein the authors argued that the differences between inflammatory processes in mice and humans were so great that future research into inflammatory diseases should avoid mouse models altogether.[161] The findings in this paper were challenged by another group in a 2015 paper titled "Genomic responses in mouse models greatly mimic human inflammatory diseases".[162]

The CS model relied upon in this thesis is far from a perfect picture of human neonatal sepsis. To argue anything else would be a failure to acknowledge the obvious reality that humans and mice are fundamentally different creatures. This is not to say that the data generated from mouse models (and more specifically, from mouse sepsis models) is not useful – if a prophylactic or

therapeutic intervention was able to significantly and substantially improve survival a mouse model, it warrants further exploration and has the potential to translate to success in humans.

Another model used briefly within this dissertation is a 'sterile' model of inflammation wherein lipopolysaccharide (LPS) is injected IP to induce an inflammatory response without any actual living bacteria present. LPS is one of the most frequently used animal models to induce inflammatory responses[163] and has been used in direct concert with cecal slurry in the context of neonatal sepsis to attempt to parse the impact of the immune response itself *vs.* the actual presence of bacteria.[164] LPS appears to elicit an inflammatory response more rapidly and of greater magnitude than that of CS, but there is still utility in the ability to remove any living pathogen from the equation.[163]

1.6 Experimental approach and objectives

Sepsis is responsible for a substantial proportion of all neonatal mortality, particularly in the developing world. One of the major difficulties in capturing the true burden of neonatal sepsis comes from a lack of a consensus definition, which in turn may reflect a gap in our understanding of what sepsis is. The hypothesis that the unique construction of the neonatal immune system is a function of immaturity should be rejected and has failed to generate effective treatments for neonatal sepsis. We desperately need a new approach to tackling neonatal sepsis, built on facts and not built upon the false dogma of immaturity. I therefore decided to take a data-driven approach wherein I generated new hypotheses by comparing likely survivors and non-survivors in a mouse model of neonatal sepsis (explained in greater detail in

Chapter 2). Within this model I focused specifically on the differences between survivors and non-survivors, operating under the hypothesis that the biological pathways which best discriminated survival represented the best targets for novel treatments. My interpretation of these results was loosely informed by the underlying assumption that a strict focus on inflammatory pathways was less likely to generate impactful results, as that is where much of the field has been over the last few decades.

My approach was split into three primary objectives, reflected in the next three chapters:

1) Develop a way to reliably classify likely survivors and likely non-survivors at time of experimental sacrifice;
2) Use gene expression data in organs and whole blood to identify pathways which differentiate likely survivors and likely non-survivors and generate leads to target in further investigations;
3) Directly administer drugs or metabolites to modify the critical pathways identified in the dry-lab portion to see if the improve survival.

Through accomplishing each of these objectives I was able to generate extremely promising treatments, some of which I found to be effective in improving murine survival when administered both prior to and after cecal slurry challenge, i.e. prophylactics or therapeutics.

Chapter 2: Laying the groundwork: Robust health-score based survival prediction for a neonatal mouse model of polymicrobial sepsis

This chapter has been previously published[165], please refer to the preface for more information. The explicit goal of this research was to develop a tool which would enable us to classify neonatal mice into likely survivors or non-survivors which forms the basis for the research presented in Chapter 3. In pursuit of this we were able to develop and validate a health scoring system for neonatal pups in a widely used model of sepsis – this became the focus of this publication as it filled an important hole in the literature.

2.1 Introduction

An estimated 3.0 million cases of newborn infection every year result in nearly 350,000 deaths, making infection one of the most lethal threats in early life.[3] Many deaths due to infection can be attributed to sepsis, a difficult to define disease characterized by failures of regulatory immune system components resulting in hyper- or hypo-inflammation.[14,89] Sepsis may be caused by an invasive bacteria or virus, leakage of commensal microbes from the gut, exposure to maternal vaginal flora or any number of inflammatory stimuli.[23,166] It is widely known that the neonatal immune system is fundamentally distinct from that of adults, indicating that data generated from adult animal models should not be assumed to apply to newborns. [52,59,117,167] While neonatal mouse models offer several practical benefits (availability, existing infrastructure, well-understood biology, etc.), working with neonatal mice presents some unique challenges which have not yet been effectively addressed. In particular, researchers are limited by the size of the mice; day of life (DOL) 7 mice (considered to be immunologically closest to human term

newborns at birth)[28] are still far too small to collect meaningful volumes of blood without sacrificing experimental animals. In adult mice, about 7 to 8 μL of blood can ethically be collected per gram of mouse bodyweight without sacrifice or fluid replacement – this translates to a maximum single collectible blood volume from neonatal mice of about 20 μL.[168] Serial sampling (every 24 hours) would only allow for collection of 5 μL of blood or less in each draw.[169] This limitation (in addition to the practical difficulties of drawing blood from such small animals) only allows terminal methods of bleeds to collect samples of adequate volume, which fundamentally separates biological findings from true 'clinical' outcomes (i.e. survival). This inability to serially sample neonatal mice without sacrifice also forces researchers to rely heavily on biomarkers as a stand-in for outcome when exploring a potential treatment of interest, assuming causality between e.g. pathogen load or inflammatory cytokine production and mortality.

Health scores for sepsis are widely used in human clinical settings as they are critical for capturing disease progression and can inform potential interventions.[170–174] The ability to assess disease severity in a quantitative way is also extremely useful in animal models as it can help investigators avoid an overreliance on assumptions about the relevance of a given biomarker. For example, one group developed a series of symptom scores for adult mice in a similar model of sepsis (fecal suspension injected IP) which relied on, among other things: activity, response to stimulus, eyes (open or shut), level of consciousness, and appearance.[175] This study illustrated that when grouping adult mice by these health scores, TNFα and IL-1β levels did not correlate with outcome despite elevated levels in comparison to unchallenged controls – these cytokines

went up following challenge but did not appear different in survivors and non-survivors. This demonstrates the capacity for health scores to narrow the focus of investigators onto biomarkers which are functionally relevant to a clinical outcome of interest – a capacity even more critical when working in a neonatal population from which even blood samples are extremely difficult to collect without harming them. While the quantitative observations used in this model may be strong markers for adult animals, none of these characteristics are readily applicable to a neonatal population (i.e. no fur to be ruffled, eyes are always closed, minimal basal activity levels, etc.).

To address this need, we developed a data-driven and simple scoring system directly related to outcome (humane endpoint) in neonatal mice. The addition of our health scores to neonatal mouse experiments provides: (a) an improved ability to track disease progression which can inform time of sacrifice for sample collection and (b) a quantitative method to prospectively differentiate survivors from non-survivors. We here correlate this health scoring system with mortality in a polymicrobial model of neonatal sepsis. This model confirms that clinical outcome was highly correlated with bacterial load. It took less than 20 seconds to determine these composite health scores per animal. The prospective validity of our health score in assigning outcome provides the much-needed metric to begin assessing causality of biomarkers, which in turn will allow assignment of mechanistic cause-effect relationships.

2.2 Materials and methods

2.2.1 Mice

Male and female C57BL/6 mice aged 6 weeks (Jackson Labs, catalogue #664) were purchased from The Jackson Laboratory and used for breeding purposes; all mice used in this study were first or second-generation progeny of Jackson mice bred in-house. This study was carried out in strict accordance with animal ethics guidelines outlined by University of British Columbia and was approved by the Animal Care Committee (protocol numbers A17-0110 and A14-0261). Animals were housed in OptiMice cages, with food and water *ad libitum*. Breeding mice were fed a high fat diet (Tekland Cat 2919) while others were fed a low-fat diet (Tekland Cat 2918). The pellet base was cellulose ¼" Performance Bedding (Lab Animal Supplies INC, Cat L0108), and environmental enrichment included a red hut, nesting material of Envirodri (Sic.) (Jameson, B501) and Nestlets (Ancare, Cat CABFM00088). Mice underwent a controlled 12 hour light cycle, with temperature maintained at 23 °C Mice were anesthetized with isoflurane prior to euthanasia through CO_2 exposure, confirmed by decapitation. Humane endpoint was defined as the lack of an attempt to right themselves when placed on their back on both sides, explained in greater detail below.

2.2.2 Cecal slurry mouse model

Polymicrobial sepsis was induced using a modified version of the model first described by Wynn *et al.* in 2007 and recently outlined on video in the Journal of Visualized Experiments.[49,157] Briefly, cecal material was extruded from adult male mouse ceca (aged 6-10 weeks), diluted in dextrose 5% water (D5W) to 160 mg slurry / mL D5W, filtered through a 70 μm sterile strainer,

pooled, aliquoted, and frozen at -80 ºC. Aliquots were thawed at room temperature, diluted further in D5W to the desired weight-adjusted dose, and kept on ice prior to intraperitoneal (IP) injection. The challenge dose was titrated to achieve a lethal dose equal to 50% (LD50) per litter – in this instance, multiple different slurries were used to generate the large amount of data. No differences between cecal slurries were found. Neonatal mice were injected IP with approximately 100 µL cecal slurry (0.8 – 1.1 mg/g mouse) on day of life (DOL) 7 or 8 to induce polymicrobial sepsis, with slight variations in volume due to weight adjustment. Litters were monitored for morbidity (i.e. lethargy, inability to right) up to 96 hours post challenge (HPC). In these survival experiments, mice were monitored either until full recovery was observed (gaining weight, alert / active), or until humane endpoint was reached at which point the mice would be euthanized. A separate cohort of pups were sacrificed at approximately 24 HPC in order for the collection of blood and tissue samples. Bacterial loads in liver, lungs, spleen, and blood were subsequently calculated by serially diluting the organ homogenates in PBS, drop-plating on 5% sheep's blood agar, and the counting colony forming units (CFU) after 24 hours of incubation at 37 ºC.

2.2.3 Monitoring and health scores

Monitoring commenced 12 HPC (i.e. prior to the onset of expected mortality) and continued 2-3 times daily (07:00 – 09:00, 12:00-14:00, 16:00-18:00, spanning both light and dark cycles) through to experimental endpoint. All mice were challenged in the evening (17:00 – 18:00) so monitoring schedules were not adjusted based on time of challenge. During mouse scoring the neonatal mice were separated into a secondary cage to avoid stressing the dam. Nesting material

was rubbed in hands to transfer that litter's smell to the gloves, and then each mouse was individually scruffed and placed on their back as visualized in Brook *et al.* 2019.[157] Health scores arose naturally out of a need to define a humane endpoint for extremely ill neonatal pups. Placing a pup on its back and recording whether it was able to right itself was a simple and effective discriminator of outcome, but alone it failed to capture the obvious difference between mice which were vigorously attempting to right and those which barely moved. Thus, we began recording qualitative observations of vigor to pair with righting reflex data, which eventually became quantitative descriptors of mobility (i.e. angle of hip movement when attempting to right). Righting reflex measurements were further standardized by adding a four-second cutoff, determined by the observation that more time was unnecessary, and less time risked inappropriately assigning 'fail to right' to a healthy pup. Previous work has shown minimal variance in score assignments between trained individuals.[157] Scores were always assigned in a manner wherein the challenge dose was unknown to the scorer. For simplicity of recording and interpretation, these observations were transformed into ranked scores from 0 - 5, as presented in Table 2-1. Scores were recorded at each monitoring time point twice, once with the mouse placed on its back-right and once on the back-left side. In addition to scoring, mice were weighed at each time point using a top-loading balance. All mice used were either 7 or 8 days old and weighed an average of 3.7 g (2.25 g - 6.06 g). Pups were identified by markings with an ethanol proof pen on the top or bottom of the tail. Monitoring sheets for each litter were used to record scores and weights. A total of 424 mice were used in this study: 266 with an endpoint of mortality or recovery, 125 sacrificed at 24 hours for a readout of bacterial load, 33 used in follow-up experiments. Most of these animals were untreated controls used in other experiments

not described here – all animals which had monitoring data at two time points were included in the study. In survival experiments, all mice were allowed to proceed to humane endpoint or recovery (gaining weight four days post-challenge with scores of 4-5), whereas in bacterial load experiments all mice were sacrificed at 24 HPC regardless of perceived illness.

2.2.4 Classifier

The ability to confidently predict outcome (survival or non-survival) at time of sacrifice would greatly help limit the number of assumptions required by investigators. We interpreted survival vs. non-survival as a binary classification problem and constructed multiple models aimed at classifying mice into one of these two groups at 24 HPC. In order to identify the best model, we tested our dataset in 6 different algorithms split across two primary classes: baseline learning algorithms / simple regression (Logistic Regression, K Nearest Neighbors and Decision Tree) and ensemble learning algorithms (Random Forest, Gradient Boosting and XGBoost). Feature selection began from all available data collected for each mouse: sex, date of birth, time of challenge, weight at challenge, weight at each monitoring time point, precise HPC each monitoring time occurred, righting reflex, mobility at each monitoring time point, and the change in any of the above from one point to another. Pearson correlation coefficients were used to avoid including highly co-correlated and irrelevant features, with a correlation threshold of 0.2 (with outcome) acting as the minimum cutoff for inclusion. This threshold and all other parameters were optimized iteratively using GridSearch, which examines all possible combinations of values for each parameter and tunes each parameter to maximize classification strength. For specific parameters used during classifier construction, see the source code linked

in the appendices (Appendix A.1). The righting reflex and mobility were treated separately (rather than combined as scores) to maximize the amount of information available for the classification problem and minimize the potential for biasing what should be an unsupervised process with our own assumptions about what should be associated with mortality. A total of 222 mice were split into a training set (148 mice) and a test set (74 mice) and the different approaches were evaluated by accuracy (total number of correct classifications over total number of instances) and area under the receiver operating characteristic curve (AUC), which quantified sensitivity and specificity and represented a good metric of the quality of a classifier (Appendix A.2). Evaluation based on these performance metrics led us to select Gradient Boosted Machine learning as the strongest classification algorithm and was applied to an external data set of 21 mice for further validation.

2.2.5 Statistics

Comparisons of survival curves were evaluated using the log-rank test, with statistical significance assigned when $p < 0.05$. All tests comparing score and survival excluded the assigned score of 0, as this was the defined humane endpoint and thus could not be treated as independent. Percent mortality was analyzed using two-sided Fischer's exact tests with Bonferroni adjustment where appropriate. Scores were only treated as numerical quantities for the purpose of examining change over time – all other analyses treated scores as categorical. Any statistical test applied identically to blood, spleen, liver, and lung was adjusted using the Bonferroni method. Bacterial load data did not exhibit a normal distribution (per Shapiro-Wilk normality test) and were compared using the Wilcoxon rank-sum test. When analyzing

independent variables with multiple levels, data were first tested with the non-parametric Kruskal-Wallis test and only if significant ($p < 0.05$) were post-hoc, pairwise comparisons made using the Wilcoxon rank-sum test (Benjamani-Hochberg adjusted). All statistical analyses were performed in R (version 3.5.2).

2.3 Results

2.3.1 Health scores and outcome

At all monitoring time points, righting was associated with survival and failure to right (FTR) was associated with non-survival (Fig 2-1A). As 24 HPC was as far into disease progression as one could wait to sacrifice mice without incurring heavy survivor bias (Fig 2-1B), this timepoint became the time of sacrifice for the alternative cohort of mice assessed for bacterial load. It was therefore critical to be able to distinguish survivors and non-survivors prior to sacrifice and no later than 24 HPC. Mice which failed to right at 24 HPC were significantly more likely to die than those which successfully righted (log-rank test, $p < 0.001$) and had a statistically significant, 100-fold higher bacterial load across all compartments (two-sided Wilcoxon rank-sum tests with Bonferroni correction, $p < 0.001$) (Fig 2-1C). Righting reflex alone was strongly correlated with survival and bacterial load, especially after 24 HPC. We also captured three different levels of mobility: non-mobile, lethargic, and mobile (Table 2-1). One of the strengths of a scoring system should be the ability to provide a metric predictive of future outcome. To assess the predictive value of this righting / mobility combination metric, we assigned scores from 0 (fail to right non-mobile, humane endpoint) to 5 (rights mobile, perfectly healthy) (Table 2-1). The “mobility”

component of these scores emerged from an attempt to quantify the qualitative observation that some pups appeared sicker than others – some pups which failed to right did so with vigor and were expending all of their energy for the entire monitoring duration, while others barely moved upon being placed on their backs. Similarly, some pups which were able to right immediately took off running while others stood still and appeared to be shaking. Unless otherwise indicated, the reported score is the lower of the two recorded for each mouse.

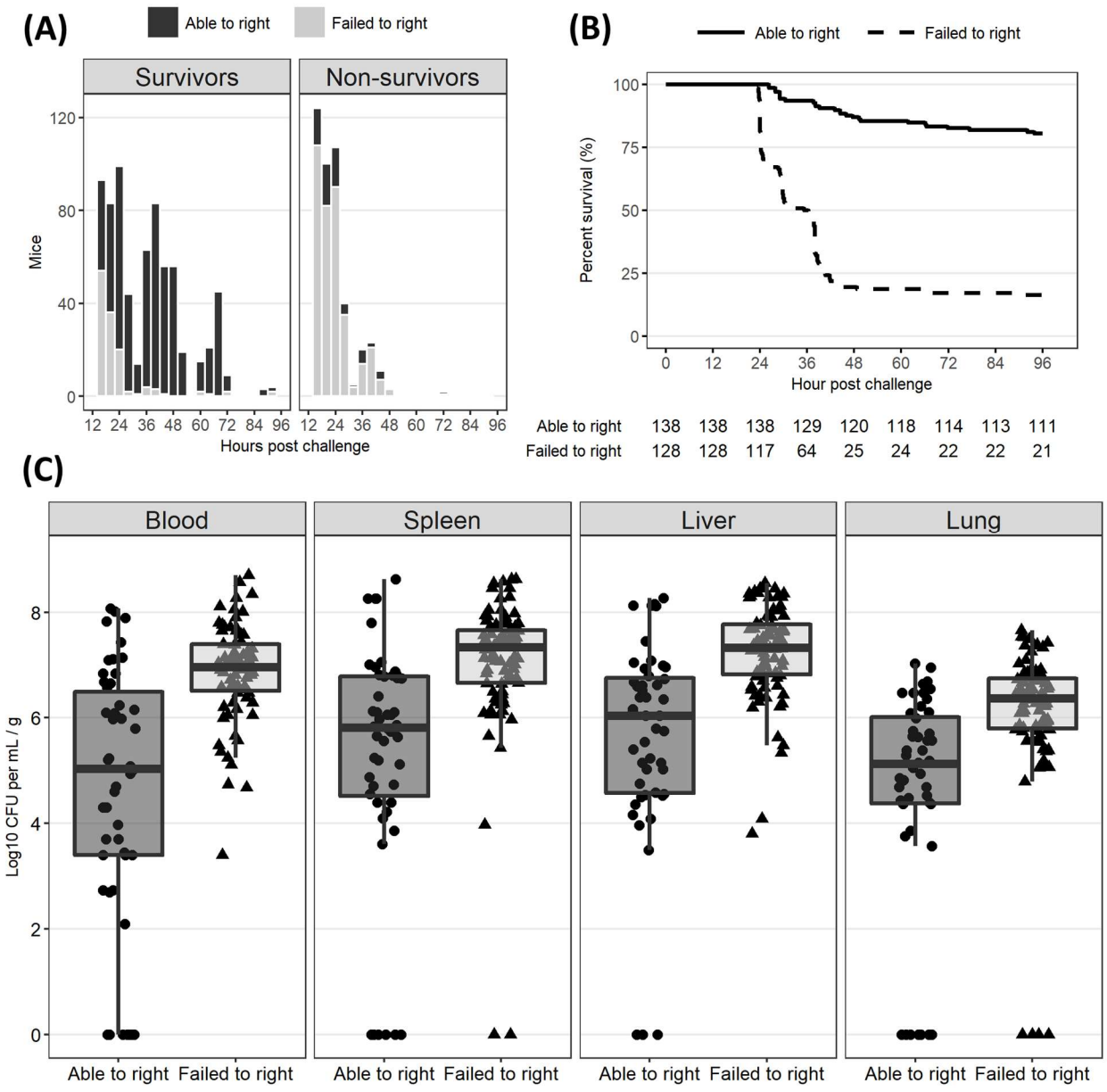

Figure 2-1. Righting reflex at 24 HPC is an excellent discriminator of survival and bacterial load. Righting reflex of neonatal mice (DOL 7-8) challenged IP with cecal slurry at an LD50. (A) Ability to right at different hours post challenge (HPC) in survivors and non-survivors. (B) Survival curve of mice split by righting reflex at 24 HPC. Mice which were able to right themselves at 24 hours were significantly more likely to survive the cecal slurry challenge than mice which failed to right (log-rank test, $p < 0.001$). (C) Bacterial load split by righting reflex at time

of sacrifice, 24 HPC. Mice which were able to right had significantly lower bacterial load in blood and all measured organ tissues (two-sided Wilcoxon rank-sum tests with Bonferroni correction, $p < 0.001$).

Table 2-1. Assigning numerical health scores to quantitative observations in neonatal mice

Numerical Score	Righting Reflex*	Mobility	Description
5	Rights	Mobile	• Multiple steps in a row, responsive to tail tap • Alert, may be shaky but moving with energy • Might fall over while walking
4	Rights	Lethargic	• Takes at least one step, but stops between steps • Steps are slower, may be very shaky, may fall over
3	Rights	Nonmobile	• Stationary after righting • May take a half-step after tail tap but largely or entirely unresponsive
2	Fails to right	Mobile	• Vigorous hip movement, swinging side to side at an angle > 90° from horizontal at least once during the observation period (4 seconds)
1	Fails to right	Lethargic	• Slower hip movement, < 90° angle • Halting attempts to right over the observation period, may be some pauses but always starting again
0	Fails to right	Nonmobile	• Zero or extremely weak hip movement • Gives up attempting to right or never starts, may be uncontrollably shaky • limbs may bend at the knee and extend but hips will not rock side to side

*Righting reflex measured by placing mice gently on their back and observing their ability to right within 4 seconds. All scores were taken in duplicate with the lower of the two reported here.

2.3.2 Health scores are strongly associated with survival and bacterial load

Combining mobility with righting reflex allowed us to generate six categories which were assigned to numerical scores for ease of recording and visualizing. As the score of zero was not independent from mortality (score of zero was the defined humane endpoint and necessitated euthanasia), it was excluded from statistical analyses. Mice which received sham challenges with either phosphate buffered saline (PBS) or dextrose 5% water (D5W) exhibited no mortality and had significantly higher scores than those which received cecal slurry, indicating that the drop in score post-challenge was not a reflection of injury due to the injection itself but rather was in response to disease. Comparing the categorical scores at 18 and 24 HPC indicated a significant relationship between score and survival (Fisher's exact test, $p < 0.001$). A simple linear regression model examining percent overall survival against assigned score at 24 HPC (mice with a score of 0 were excluded) was found to be significant ($R^2 = 0.946$, $p = 0.0035$, $n = 210$) with each increase in score equating to a 22% ± 8% improved chance of survival (Fig 2-2A). Visualizing the changes in score over the first 48 hours of disease (capturing 93% of deaths) showed a clear separation between survivors and non-survivors beginning around 18 HPC, with higher scores associated with recovery and survival (Fig 2-2B).

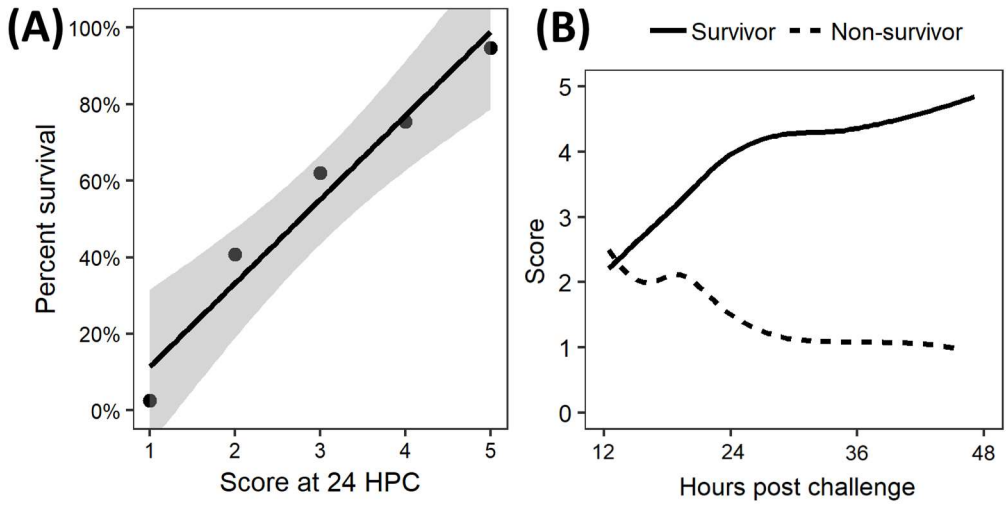

Figure 2-2. Health scores were directly associated with outcome in a polymicrobial model of sepsis in neonatal mice. (A) Scores recorded at 24 hours post challenge (HPC) plotted against the percent of mice which survived the IP cecal slurry challenge. Scores of zero were excluded as they were the pre-determined humane endpoint. A significant linear regression was found ($R^2 = 0.946$, $p = 0.0035$, $n = 210$) with mean percent survival increasing by 22 ± 8% for each single point increase in score. Shaded area represents standard error. (B) Visual representation of change in score defined numerically over time following IP cecal slurry challenge.

The inability to distinguish survivors through health scores at 12 HPC (Fig 2-2B) was consistent with qualitative observations that most mice appeared similar during this time period irrespective of final outcome. At 24 HPC, the scores were well distributed with the least frequent score (3) assigned to 10.9% of mice (29/266) and the most frequent, the healthiest score of 5, assigned to 21.0% (56/266) of mice (Appendix A.3). At 18 HPC, many of the mice appeared similarly ill, with only 6.9% at the healthiest score of 5 and 46.8% at the middle score of 2. Thus, health scores at 24 HPC were most useful in their ability to capture a range of sickness in neonatal mice.

To test the validity of our scoring system, a separate cohort of 125 mice generated under the same experimental conditions were sacrificed at 24 HPC and samples of whole-blood, spleen, liver, and lung tissue were plated on sheep's blood agar and the colony forming units (CFU) growth was assessed 24 hours later. Two separate, non-parametric Kruskal-Wallis tests were performed to assess the relationship between categorical scores at 18 and 24 HPC and bacterial load (at 24 HPC) across all four biological samples. A significant effect was attained both at 18 HPC (Bonferroni-adjusted p-values < 0.001) and 24 HPC (Bonferroni-adjusted p-values < 0.001) (Fig 2-3). The distribution of scores broadened as disease progressed towards an outcome – nearly all mice scored between 1-3 at 12 HPC, most fell within that same range at 18 HPC, but an even distribution was present at 24 HPC (Appendix A.3). Our health scores were strongly associated with both survival and bacterial load at 24 HPC.

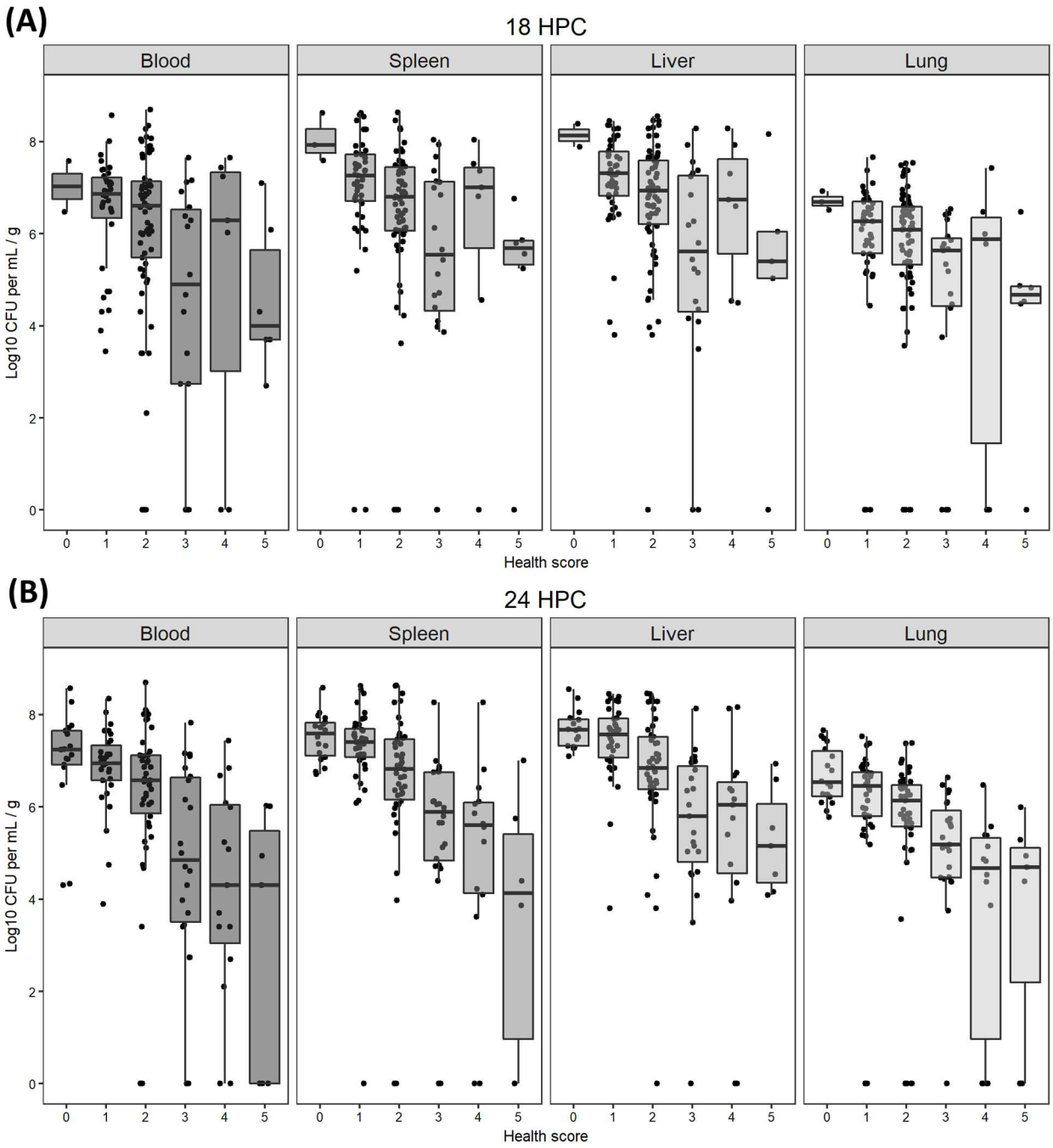

Figure 2-3. Health scores at 18 and 24 HPC are related to bacterial load at 24 HPC. Health scores at 18 and 24 HPC are related to bacterial load at 24 HPC. (A) A Kruskal-Wallis comparison of score 18 hours post challenge (HPC) with bacterial load in whole-blood and tissues showed a significant effect (Bonferroni-adjusted p-values < 0.001) indicating score at 18 HPC was related to the bacterial load measured in tissues of the same animals

collected 6 hours later (24 HPC). (B) A significant effect was also attained at 24 HPC (Bonferroni-adjusted p-values < 0.001) demonstrating the relationship between health scores and bacterial load across all biological compartments.

2.3.3 Righting reflex and mobility are predictive of outcome prior to sacrifice

We used a machine learning approach to classify mice as survivors or non-survivors based primarily on righting reflex, mobility, and weight, specifically looking at how each metric changed from 18 to 24 HPC. Only 12% (28 / 222) of mice were at humane endpoint at 24 HPC, so while the association between score components and outcome may be influenced by the inclusion of these mice (mice which were FTR non-mobile on both sides are at humane endpoint and must be sacrificed), they did not represent a large enough proportion to call the results into question. Pearson correlation proved the most effective method of feature selection and identified attributes most associated with death: a mobility score of "non-mobile", a change from able to right at 18 HPC to failed to right at 24 HPC, and weight loss between 18 and 24 HPC (Appendix A.4). The ability to right was strongly correlated with survival, especially the consistent ability to right at both 18 and 24 HPC.

We used 10-fold cross validation training on a combination of six algorithms and three methods of feature selection in order to identify the most effective method of classification (Appendix A.2). For the final predictions, we used Gradient Boosting Machine as our classifier, combined with features selected by Pearson correlation coefficients. This approach had a cross validation average accuracy of 0.91 with a low standard deviation of 0.06, indicating little risk of over or underfitting. With the hyperparameters selected for our Gradient Boosting model, we built a

model on the training data subset with known outcome and applied it to 33% of the original data set not used during the training phase. With a total of 74 cases to classify as "survivor" or "non-survivor", the Gradient Boosting model performed well with an accuracy score of 0.85 and an AUC of 0.93 (Fig 2-4A), with 33 true positive cases classified as "non-survivor" and 30 cases classified as "survivor" (Table 2-2). To further validate our model, we generated an additional dataset with the same data collection process as well as the same data cleaning and standardization pipeline. With a total of 21 new data points, our model accurately classified 18 with an average score of 85% accuracy (Appendix A.5). Righting reflex and mobility at 24 hours together with change in weight were the features most strongly correlated with outcome (Appendix A.4). Finally, we applied our classifier to a different set of pups sacrificed at 24 HPC and identified a clear relationship between survival and bacterial load (Fig 2-4B).

Table 2-2. Confusion matrix showing accuracy of Gradient Boosting Machine model when applied to test set of 74 mice. Using monitoring data collected at 18 and 24 HPC (weight, righting reflex and mobility), a Gradient Boosted machine learning model was able to distinguish survivors and non-survivors with an accuracy score of 0.85; 33/39 survivors and 30/35 non-survivors were correctly classified.

True class

	Survivor	Non-Survivor
Survivor	**33**	6
Non-survivor	5	**30**

Predicted class

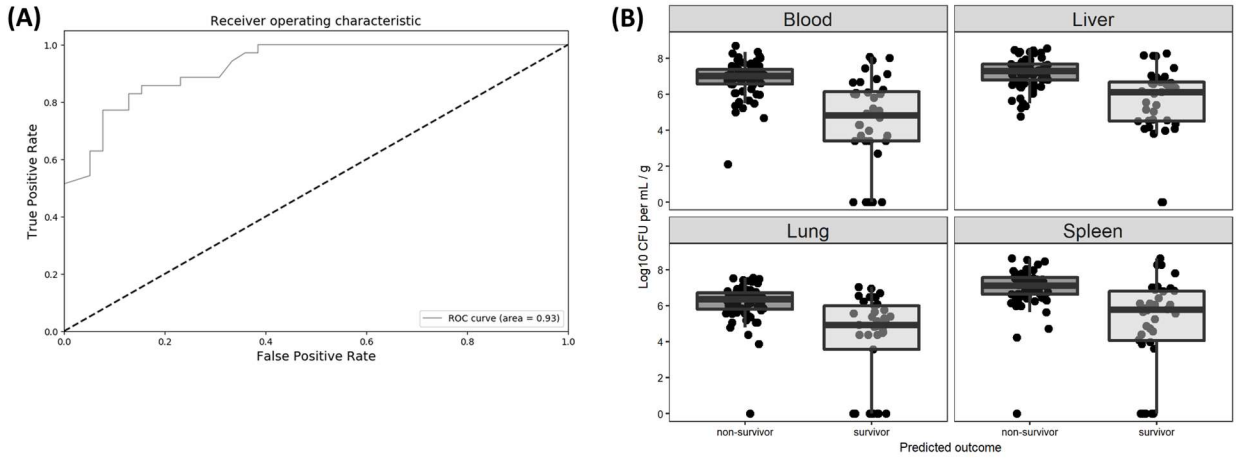

Figure 2-4. Survival or non-survival of neonatal mice was predicted at 24 HPC and was strongly associated with bacterial load. (A) Receiver Operating Characteristic curve illustrating the sensitivity and specificity of a Gradient Boosting Machine model trained on 148 mice and tested here on 74 mice. The model was primarily driven by weight change, righting reflex and mobility at 18 and 24 HPC. (B) Neonatal mice injected IP with cecal slurry were sacrificed 24 hours post challenge, with tissue and blood samples plated on sheep's blood agar at time of sacrifice. Mice were predicted to survive or not survive based on righting reflex, mobility, and other biometric data. Mice which were predicted to survive had a significantly lower bacterial load in all measured biological compartments than predicted non-survivors (two-sided Wilcoxon rank-sum tests with Bonferroni correction, $p < 0.001$). All mice with zero bacterial load in the whole-blood (n=9) were predicted to survive.

2.4 Discussion

Here we present a system of health scores for neonatal mice which were used to confidently predict mortality 24 HPC in an established model of neonatal sepsis.[49] The significance of this is twofold: 1) the classifier enables investigators to include "clinical" outcome (e.g. mortality) as a covariate of interest when all animals are sacrificed, and 2) the scoring system can represent a new standard in recording and reporting neonatal mouse health which has implications beyond this specific model. Given the tremendous number of biological differences between neonates

and adults which have been revealed in the last few decades[13,59,100,176–179], it is no longer tenable to assume data generated from adult animal studies would be relevant to neonatal infectious disease. In order to study neonatal diseases, it is critical to work with neonatal animals, though this presents a unique set of challenges which may act as a barrier towards investigators who may consider working with neonatal mice.[180] The inability to ethically collect a meaningful volume of blood (>2 μL) without sacrifice precludes investigators from pairing survival data directly with data generated from any biological sample. Thus, the significance of this work is three-fold: (1) investigators may use this classification method to include mortality as a covariate of interest when all animals are sacrificed, (2) the scoring system can represent a new standard in recording and reporting neonatal mouse health which can standardize neonatal mouse work across different laboratories, and (3) animal suffering for future work can be minimized with a uniform, quantitatively established humane endpoint.

We also demonstrated that survival can still be assigned even for neonatal mice sacrificed 24 HPC; without such an approach, one is forced to rely on biomarkers (i.e. inflammatory cytokines, cell mobilization, etc.) as a proxy outcome. The decision to separate scores into their base components (righting reflex / mobility on each side) was made to maximize the amount of information being input into the classifier. Where human interpretation of data requires simplicity, i.e. a single score, machine learning approaches have the capacity to parse much more complex data and identify meaningful relationships. Given that it is increasingly unclear whether death from sepsis is associated with an inflammatory or an anti-inflammatory response [12,13,40,59,89], it is critical to minimize reliance on biomarkers, as most lack rigorous evidence of

their relevance to survival. Our choice of bacterial load as an outcome of interest served a dual purpose, it: 1) validated the legitimacy of the scoring system and classifier, as a correlation between outcome and bacterial load is often assumed[170,181], and 2) provided evidence that there truly is a link between bacterial load and outcome in this model of polymicrobial sepsis. Thus, already at this stage we were able to remove one assumption which underlies neonatal mouse work. While a link between outcome and bacterial load may seem unsurprising, this demonstrates a proof of concept for situations wherein one must rely on a less obvious biomarker as a proxy outcome (i.e. sterile inflammatory models, inactivated bacteria, etc.). These health scores and classification approach provide a direct mechanism for assigning survival and non-survival rather than solely observing inflammatory markers and assuming a relationship which may or may not exist.[175]

We were surprised to see that righting reflex alone was strongly correlated with bacterial load across all measured biological compartments, indicating it was a robust metric of neonatal mouse health (Fig 2-1). The significance of righting reflex was also apparent in the process of feature selection during classifier construction – righting reflex was the single most correlated feature with survival (Appendix A.4). Given the simplicity of collecting, these data suggest righting reflex should be recorded whenever neonatal mouse well-being is in question. Righting reflex in neonatal mice has previously been used as a tool to study behavioral development but has never been used to measure disease progression nor assess overall health in the context of an infection.[182] These data represent an important contribution towards describing neonatal mouse health which can help to inform ethical decisions around humane endpoints.

A potential limitation of this study is that mice which were non-mobile and failed to right on both sides (score 0) were sacrificed for ethical concerns, potentially violating the assumption of independence between score and survival. We addressed this by excluding mice who received a score of 0 from statistical analyses when survival was the outcome of interest. Further, the high bacterial load observed in whole-blood and the various organs independently validated the biological relevance of the scoring system presented. The use of righting reflex as a decision point in humane endpoint can help to standardize mouse models and reduce unnecessary suffering for newborn mice. In fact, the extremely low survival rate (<10%) of mice which failed to right and were lethargic 24 HPC suggests that this should be used as the humane endpoint for future studies. This approach could be further improved by rigorously selecting the times at which monitoring data were collected in order to maximize the potential difference between survivors and non-survivors whilst minimizing the potential cost of survivor bias. Finally, there are also some questions surrounding the use of righting reflex as a metric in younger mice which may be unable to right even at a healthy state – further research is warranted prior to assigning these health scores to mice prior to DOL 7.

Further research would be required to generalize this gradient boosting machine model to other applications in other laboratories. The model required one time point prior to the onset of mortality and an additional timepoint approximately at the onset of mortality – it is not clear if this would be generalizable to another model where the exact timing of these (i.e. 6 hours apart) was different than the one trained here. If this model was tested on a broader variety of datasets it

could potentially be pickled and made available for use as a free tool for any researchers working in the space of neonatal infectious disease models.

The availability of a robust scoring system, with the additional proof that it can be predictive of outcome, should allow for a stratification of experimental groups beyond treated / untreated, or challenged / unchallenged, but also treated survivors / treated non-survivors, etc. The latter comparison will provide mechanistic insight into responders and non-responders of novel treatments – precisely what one hopes to attain from working with an animal model. Neonatal mouse models provide a mechanism to explore neonatal infectious disease in a manner that is unparalleled in humans. The ability to sacrifice and examine neonatal mice while not losing the potential associations with survival has the potential to break open the mysteries of neonatal sepsis. Here we demonstrated that bacterial load across all measured biological compartments was strongly correlated with our predictive health scores and therefore final outcome in neonatal polymicrobial sepsis. This study represents a blueprint for how to report neonatal mouse health in infectious disease models, and how to use that data to examine survival in mice which had to be sacrificed for sample collection.

Chapter 3: Hypothesis generation: Identifying and testing critical pathways in differentiating likely survivors and likely non-survivors

3.1 Introduction

Despite the massive burden of neonatal infectious disease and sepsis, surprisingly little is known about what the precise cause of death truly is. For instance, though a positive blood culture is still considered the "gold-standard" for sepsis diagnosis, a causal pathogen can only be identified in less than 30% of cases.[19] It is therefore unlikely that pathogen virulence is the direct cause of death in most instances. Much of the research into neonatal sepsis has been focused on the identification of biomarkers to be used as diagnostic aids – in some instances, researchers have also correlated disease severity with said biomarkers. These studies built on correlations are virtually all we have to understand what is happening on a biological level that is leading to death in neonatal sepsis. How can we possibly generate effective interventions without understanding why neonates are dying from infection? So many attempts at novel interventions have failed to significantly reduce mortality in human neonatal sepsis: granulocyte transfusion, exogenous GM-CSF / G-CSF, monoclonal IVIG, activated protein C, glutamine, and anti-endotoxin antibodies, just to name a few.[183] We cannot and should not design large-scale clinical intervention trials based on observed correlations between biomarkers and disease severity – i.e. just because a septic newborn has high levels of TNF-α, we can't assume that a drug targeted at lowering TNF-α levels will improve survival. Of course this approach is still a valid way to generate and test hypotheses, but relying on an unbiased, data-driven approach to pinpoint causal pathways and then determining if we can reliably and effectively modify these pathways

represents a more promising way to target sepsis. One way to start on this journey is using an animal model, though this comes with its own issues as discussed in section 1.5.

In this chapter, I relied upon the gradient boosting machine algorithm constructed in Chapter 2 to classify cecal slurry-challenged pups into likely survivors or likely non-survivors. By extracting RNA and comparing the transcriptomes from whole-blood, liver, and spleen, I was able to identify key pathways that distinguished likely survivors and likely non-survivors of murine neonatal sepsis. Specifically, I identified arachidonic acid metabolism in the liver, eicosanoid metabolism in the blood, and angiogenesis / regulation of the vascular endothelium in the spleen as the most promising signals upon which to focus. Of note, as I will show subsequently, these pathways functionally link to each other.

3.2 Materials and methods

3.2.1 Sample preparation and RNA extraction

Transcriptomic data was generated using the same cecal slurry model of neonatal sepsis described in Chapter 2. Briefly, pups aged 7-8 days old were injected IP with either cecal slurry (CS, 1.1 mg/g mouse in D5W) or vehicle control. Pups were sacrificed 24 hours post challenge (HPC) to collect whole blood, spleen, and liver samples. RNA was extracted using a Qiagen RNeasy Plus Micro kit from whole blood, spleen, and liver samples from 12 CS-challenged likely survivors, 13 CS challenged likely non-survivors, and 10 healthy controls. Library preparation and data pre-processing was performed by the Bob Hancock lab, spearheaded by my

collaborator Dr. Amy Lee. Samples which had less than 1 million unique reads after sequencing and removal of globin were excluded from analysis.

3.2.2 DE, pathway and gene set enrichment analysis

Normalization and generation of DE gene lists was done using DESeq2 in R version 4.0.2. Differential expression was defined as BH-adjusted p-value of < 0.05 and minimum fold change of 1.5 (absolute log2 FC > 0.585). PCAs were created manually using tidyverse v1.3.0 and heatmaps were generated from the pheatmap v1.0.12. Gene set enrichment analysis was performed using the msigdbr package (v7.1.1) in R which relies upon datasets provided by GSEA and MSigDB. The "hallmark" and "canonical pathways" gene sets were selected in order to broadly categorize DE genes. Reactome, MeSH, and GO term enrichment analysis was ran using the clusterProfiler package (v3.16.0).[184] In all instances terms or pathways were considered significantly enriched if they had a BH-adjusted p-value of less than 0.05.

3.3 Results

3.3.1 DE gene analysis between likely survivors and non-survivors

Prior to any DE analysis I first looked to see how the gene expression data grouped when combined across all organs. Unsurprisingly, there were three distinct clusters which represented blood, spleen, and liver (Appendix B.1). The first analytical step was to perform a series of principal component analyses (PCAs) for each biological compartment. These PCAs examined all genes which remained after the normalization and data cleaning steps and thus represent an

unsupervised examination of the transcriptome (Fig 3-1A). As the pups were classified into likely survivors or likely non-survivors through the gradient-boosting machine learning algorithm developed and described in Chapter 2, I anticipated approximately 15% of pups should be misclassified. This may have been reflected in the PCA performed on the blood transcriptome wherein there was some clustering between challenged and non-challenged pups but little differentiating the likely survivors and likely non-survivors within the challenged group. However, in both the spleen and liver there were not only clear and obvious separations between healthy and septic pups along PC1 (as anticipated), but within the challenged subset the likely survivors and likely non-survivors could be easily differentiated.

The lack of obvious clustering by outcome in the blood was also reflected when simply looking at the total number of differentially expressed (DE) genes between likely survivors and likely non-survivors – less than 200 in blood, compared to >800 in both liver and spleen (Table 3-1). This was perhaps a reflection of sequencing depth and the impact of globin reads. Globin comprised a substantially larger portion of reads in the whole blood, yet all compartments were sequenced to approximately the same depth. Regardless, there were enough DE genes to move forward with more complex enrichment and pathway analyses in all biological compartments.

Table 3-1. Differentially expressed genes between likely survivors and likely non-survivors. Lower number of DE genes in the blood was likely due to globin removal shrinking the library size.

Compartment	Up in likely non-survivors	Up in likely survivors	Total
blood	60	127	**187**
liver	429	620	**995**
spleen	365	530	**850**

Within the liver PCA (Fig 3-1A) I observed that the CS-challenged, likely survivors appeared to be more like healthy controls along PC1. This pattern also emerged after construction of a heatmap of all genes differentially expressed between likely survivors and likely non-survivors (Fig 3-1B). Within the dendrograms, across all biological compartments the gene expression patterns of likely survivors appear closer to healthy controls than pups likely to die. These heatmaps also showed clear but imperfect separation of the likely survivors and likely non-survivors, approximately in-line with the anticipated accuracy of the classification algorithm. To identify potential gene targets for later manipulation, I first looked for genes which were differentially expressed in both likely survivors and likely non-survivors but moving in opposite directions relative to healthy controls, i.e. expression increasing in survivors and decreasing in non-survivors. There were virtually no instances of this occurring. However, plotting the relative differences against healthy controls of each DE gene between likely survivors and non-survivors revealed a clear and obvious pattern that further enforces the notion that the likely survivors are more similar to controls than likely non-survivors (Fig 3-1C). Regardless of the directionality of the change, whether the cecal slurry challenge increased or decreased its overall expression, the magnitude of that difference was generally greater in likely non-survivors. This general pattern occurred in >85% of instances across all measured biological compartments.

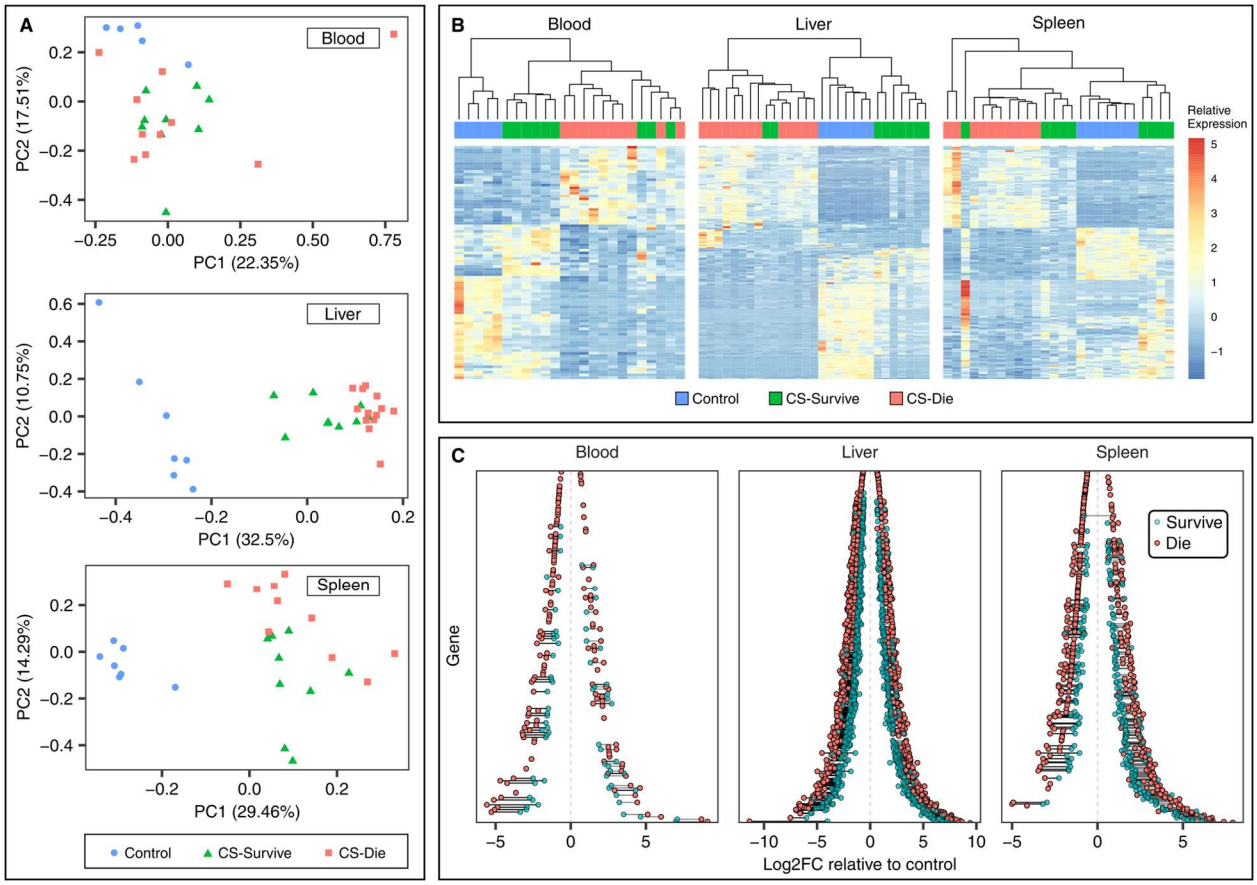

Figure 3-1. Likely survivors and likely non-survivors of cecal slurry challenged pups have different gene expression profiles and survivors appear more like healthy controls. (A) Principal component analyses showed clear clustering of likely survivors (CS-Survive) and likely non-survivors (CS-Die) is in both liver and spleen, with less obvious but still observable clustering in the blood. (B) Heatmaps showing relative gene expression of differentially expressed genes in blood, liver, and spleen between control pups (healthy, non-challenged). CS-challenged likely survivors (CS-Survive), and CS-challenged likely non-survivors (CS-Die). Predicted survivors cluster more similarly to healthy controls than predicted non-survivors. (C) The visualization of relative change in gene expression against healthy controls between likely survivors and likely non-survivors showed a greater magnitude of difference in likely non-survivors than likely survivors, both in terms of upregulation and downregulation.

3.3.2 Gene set enrichment analysis on hallmark and curated pathways

I performed a gene set enrichment analysis (GSEA) wherein I imported the list of differentially expressed genes between likely survivors and likely non-survivors in blood, spleen, and liver and tested for overrepresentation in a curated list of 50 hallmark gene sets (Table 3-2). These gene sets have been previously identified by the GSEA and MSigDB teams and are publicly available online. While limited in scope, this analysis did identify the hallmark set associated with "TNFα signaling via NF-κB" as the most enriched (by Benjamani-Hochberg, BH, adjusted p-value) term in RNA-Seq data from the blood and liver, as well as the third most enriched term in data from the spleen. Gene sets associated with IFNα and IFNβ responses were enriched in both the spleen and liver. The liver exhibited the most enrichment overall by far, with enrichment in a variety of gene sets beyond those classically associated with a basic inflammatory response.

Table 3-2. Gene set enrichment analysis in DE genes between likely survivors and likely non-survivors across all biological compartments. The "setSize" column refers to the number of enriched genes identified within the set, and "enrichmentScore" reflects the degree that a gene set is enriched in the data and "p.adjust" refers to significance, measured by Benjamani-Hochberg adjusted p-value.

Compartment	ID	setSize	enrichmentScore	p.adjust
Blood	TNFA_SIGNALING_VIA_NFKB	15	0.715	4.24E-06
Blood	IL2_STAT5_SIGNALING	10	0.537	0.017463
Spleen	INTERFERON_GAMMA_RESPONSE	34	0.684	1.60E-09
Spleen	INTERFERON_ALPHA_RESPONSE	19	0.708	1.80E-07
Spleen	TNFA_SIGNALING_VIA_NFKB	23	0.482	0.006149
Spleen	ESTROGEN_RESPONSE_LATE	15	-0.491	0.047802
Liver	TNFA_SIGNALING_VIA_NFKB	57	0.544	3.10E-09
Liver	INFLAMMATORY_RESPONSE	40	0.535	1.16E-07
Liver	HYPOXIA	43	0.525	1.16E-07
Liver	INTERFERON_GAMMA_RESPONSE	41	0.535	2.19E-07
Liver	INTERFERON_ALPHA_RESPONSE	18	0.623	4.53E-05

Liver	APOPTOSIS	23	0.523	0.000256
Liver	IL6_JAK_STAT3_SIGNALING	15	0.591	0.00051
Liver	ALLOGRAFT_REJECTION	25	0.478	0.000937
Liver	IL2_STAT5_SIGNALING	30	0.393	0.004624
Liver	BILE_ACID_METABOLISM	23	-0.798	0.007495
Liver	EPITHELIAL_MESENCHYMAL_TRANSITION	25	0.417	0.007624
Liver	P53_PATHWAY	26	0.392	0.012208
Liver	MTORC1_SIGNALING	25	0.378	0.018905
Liver	UNFOLDED_PROTEIN_RESPONSE	13	0.480	0.039057

I performed an additional GSEA on a larger curated dataset of "canonical pathways", which was another publicly available set which included 2868 canonical pathways. After p-value adjustment, this analytical method yielded no enriched pathways for either the blood or the spleen. Curiously, the only enriched pathways which emerged from the analysis were in the liver and were almost all associated in some way with the extracellular matrix (Table 3-3). The most enriched term by BH adjusted p-value was the gene set titled "MATRISOME" which mSigDB defines as an "Ensemble of genes encoding extracellular matrix and extracellular matrix-associated proteins".[185] Also included within the five enriched gene sets were "MATRISOME_ASSOCIATED" (genes associated with the matrisome), "ECM_GLYCOPROTEINS" and "ECM_REGULATORS". Thus 80% (4/5) of the gene sets in the liver identified as enriched were associated in some fashion with the extracellular matrix.

Table 3-3. GSEA on 2868 "canonical pathway" gene sets comparing DE genes between likely survivors and likely non-survivors. The "setSize" column refers to the number of enriched genes identified within the set, and "enrichmentScore" reflects the degree that a gene set is enriched in the data and "p.adjust" refers to significance, measured by BH adjusted p-value.

Compartment	ID	setSize	enrichmentScore	p.adjust

Liver	MATRISOME	78	0.359	1.28E-05
Liver	SECRETED_FACTORS	30	0.513	3.24E-05
Liver	MATRISOME_ASSOCIATED	65	0.343	0.000318
Liver	ECM_GLYCOPROTEINS	10	0.591	0.005881
Liver	ECM_REGULATORS	21	0.361	0.040487

3.3.3 Reactome pathway analysis

I next decided to run the DE genes between likely survivors and likely non-survivors against the pathways curated by Reactome (Table 3-4), a more specific set than the broad "hallmark" and "curated pathway" GSEA performed using MSigDB. The most enriched pathway in the blood was "Biosynthesis of specialized proresolving mediators (SPMs)", driven by DE in five genes: Alox15, Ltc4s, Alox5, Ptgs2, and Alox5ap. Only one of these genes, Ptgs2 (also known as COX-2) was increasing in likely non-survivors relative to likely survivors – the rest were all higher in likely survivors (Appendix B.2). Other significantly enriched pathways in the blood included "Arachidonic acid metabolism" and "Fatty acid metabolism", along with pathways classically associated with inflammatory / immune responses (i.e. "neutrophil degranulation, antimicrobial peptides, etc.). This was the only pathway / gene set enrichment analysis wherein the there were the fewest pathways enriched in the liver. The two most significantly enriched liver pathways were biologically right next to one another: "biological oxidations", referring to the breakdown and oxidation of xenobiotics or endogenous compounds by the CYP gene family, and "Phase I - Functionalization of compounds", which refers to the next direct step in that process. Both pathways were driven by an enrichment cluster of Cyp450 genes which will be discussed in greater detail in the next section.

Finally, Reactome pathways enriched between likely survivors and likely non-survivors in the spleen were diverse and relatively broad in their function. The common theme I observed was enrichment in pathways loosely affiliated with the ECM or other structural components, such as: "ECM organization", "Keratinization", "Formation of the cornified envelope", and "Collagen degradation", just to name a few.

Table 3-4. Reactome pathway enrichment analysis of DE genes between likely survivors and likely non-survivors following CS challenge. Lists of DE genes between likely survivors and likely non-survivors were compared against publicly available Reactome datasets. P-value was adjusted using the Benjamani-Hochberg procedure. "Count" refers to the number of genes identified within the set contributing to the enrichment.

Compartment	Description	Count	p.adjust
Blood	Biosynthesis of specialized proresolving mediators (SPMs)	5	0.000188
Blood	Neutrophil degranulation	24	0.000188
Blood	Antimicrobial peptides	6	0.000427
Blood	Chemokine receptors bind chemokines	6	0.000528
Blood	Peptide ligand-binding receptors	8	0.000528
Blood	Arachidonic acid metabolism	6	0.000615
Blood	G alpha (i) signalling events	12	0.001313
Blood	Class A/1 (Rhodopsin-like receptors)	9	0.002115
Blood	GPCR ligand binding	9	0.007648
Blood	Signaling by GPCR	15	0.007648
Blood	GPCR downstream signalling	14	0.01465
Blood	Fatty acid metabolism	8	0.037789
Liver	Biological oxidations	33	2.70E-05
Liver	Phase I - Functionalization of compounds	21	5.72E-05
Liver	Bile acid and bile salt metabolism	12	0.007227
Liver	Signaling by GPCR	45	0.008934
Liver	GPCR downstream signalling	43	0.013856
Liver	G alpha (s) signalling events	13	0.016238
Spleen	Peptide ligand-binding receptors	17	7.32E-06
Spleen	Extracellular matrix organization	27	5.71E-05

Spleen	Keratinization	9	5.71E-05
Spleen	Formation of the cornified envelope	9	5.71E-05
Spleen	Degradation of the extracellular matrix	15	0.000199
Spleen	Class A/1 (Rhodopsin-like receptors)	19	0.000199
Spleen	GPCR ligand binding	20	0.000602
Spleen	Collagen degradation	9	0.001905
Spleen	GPCR downstream signalling	32	0.002375
Spleen	Signaling by GPCR	32	0.00411
Spleen	Muscle contraction	14	0.006425
Spleen	O-glycosylation of TSR domain-containing proteins	7	0.007096
Spleen	Collagen chain trimerization	7	0.007096
Spleen	Chemokine receptors bind chemokines	7	0.008433
Spleen	Nicotinamide salvaging	5	0.010235
Spleen	Adherens junctions interactions	6	0.024657
Spleen	Antimicrobial peptides	6	0.024657
Spleen	Potassium Channels	7	0.028013
Spleen	O-linked glycosylation	10	0.03439
Spleen	Molecules associated with elastic fibres	6	0.039551

3.3.4 MeSH term enrichment

The next analytical approaches I used both generated far too many significantly expressed terms to attempt to use them in their entirety. In a Medical Subject Headings (MeSH) "Chemicals and Drugs" term enrichment analysis, again on the DE genes between likely survivors and likely non-survivors, there were 343 significant terms in the blood, 2673 in the liver and 2401 in the spleen. I therefore narrowed my focus to only the 30 most enriched terms (by BH-adjusted p-value) in each biological compartment (Fig 3-2). This form of MeSH term enrichment compared the DE gene list between likely survivors and likely non-survivors against a curated database of gene sets associated with a specific chemical or drug. In this approach the most enriched terms in the blood were more classical inflammatory pathways ("Inflammation Mediators", "IL-10", "Immunologic Adjuvants") and this largely held true across most of the top 30 terms. Among these there was also a through-line of arachidonic acid-related terms such as "Eicosanoids",

"Arachidonate 5-Lipoxygenase", and "Lipoxygenase Inhibitors", similar to those observed in the Reactome pathway enrichment analysis previously described.

The top enriched MeSH "Chemicals and Drugs" terms in the spleen were dominated by elements related to the ECM and the vascular endothelium. The top five terms were: "Matrix Metalloproteinase 2", "Hypoxia-Inducible Factor 1, alpha Subunit", "Vascular endothelial Growth Factors", and "Endothelial Growth Factors". Also among the top 30 were "Angiotensin I", "Collagen Type 1", "Collagenases", "Vascular Cell Adhesion Molecule-1", and "Matrix Metalloproteinases". These signals mirrored those observed in the GSEA and Reactome enrichment results – structural components, perhaps the vascular endothelium, were critical points of differentiation between likely survivors and likely non-survivors. Enrichment associated with HIF1α was the most significant signal identified in the liver, making the HIF1α term one of the strongest in both the spleen and liver. Collagans also emerged as significantly enriched among the top 30 terms in the liver as well, as did "Angiotensin II" and "Cyclooxygenase 2". Notably I identified the emergence of reactive oxygen species (ROS) and reactive nitrogen species (RNS) terms, marked by enrichment of Nitric Oxide Donors", "Superoxides", and "NG-Nitroarginine Methyl Ester" (also known as L-NAME).

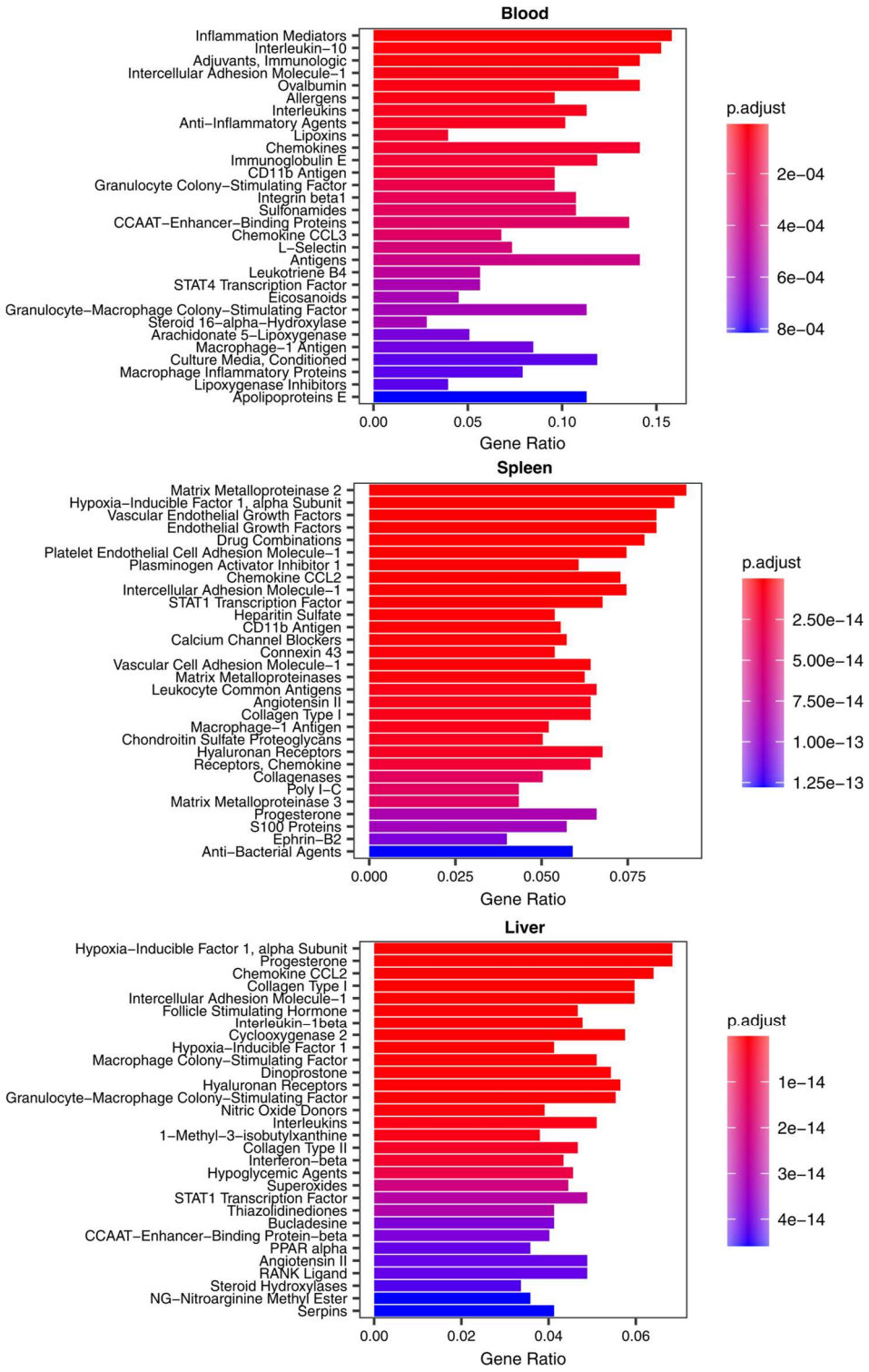
Blood
Inflammation Mediators
Interleukin−10
Adjuvants, Immunologic
Intercellular Adhesion Molecule−1
Ovalbumin
Allergens
Interleukins
Anti−Inflammatory Agents
Lipoxins
Chemokines
Immunoglobulin E
CD11b Antigen
Granulocyte Colony−Stimulating Factor
Integrin beta1
Sulfonamides
CCAAT−Enhancer−Binding Proteins
Chemokine CCL3
L−Selectin
Antigens
Leukotriene B4
STAT4 Transcription Factor
Eicosanoids
Granulocyte−Macrophage Colony−Stimulating Factor
Steroid 16−alpha−Hydroxylase
Arachidonate 5−Lipoxygenase
Macrophage−1 Antigen
Culture Media, Conditioned
Macrophage Inflammatory Proteins
Lipoxygenase Inhibitors
Apolipoproteins E
0.00
0.05
0.10
0.15
Gene Ratio
p.adjust
2e−04
4e−04
6e−04
8e−04
Spleen
Matrix Metalloproteinase 2
Hypoxia−Inducible Factor 1, alpha Subunit
Vascular Endothelial Growth Factors
Endothelial Growth Factors
Drug Combinations
Platelet Endothelial Cell Adhesion Molecule−1
Plasminogen Activator Inhibitor 1
Chemokine CCL2
Intercellular Adhesion Molecule−1
STAT1 Transcription Factor
Heparitin Sulfate
CD11b Antigen
Calcium Channel Blockers
Connexin 43
Vascular Cell Adhesion Molecule−1
Matrix Metalloproteinases
Leukocyte Common Antigens
Angiotensin II
Collagen Type I
Macrophage−1 Antigen
Chondroitin Sulfate Proteoglycans
Hyaluronan Receptors
Receptors, Chemokine
Collagenases
Poly I−C
Matrix Metalloproteinase 3
Progesterone
S100 Proteins
Ephrin−B2
Anti−Bacterial Agents
0.000
0.025
0.050
0.075
Gene Ratio
p.adjust
2.50e−14
5.00e−14
7.50e−14
1.00e−13
1.25e−13
Liver
Hypoxia−Inducible Factor 1, alpha Subunit
Progesterone
Chemokine CCL2
Collagen Type I
Intercellular Adhesion Molecule−1
Follicle Stimulating Hormone
Interleukin−1beta
Cyclooxygenase 2
Hypoxia−Inducible Factor 1
Macrophage Colony−Stimulating Factor
Dinoprostone
Hyaluronan Receptors
Granulocyte−Macrophage Colony−Stimulating Factor
Nitric Oxide Donors
Interleukins
1−Methyl−3−isobutylxanthine
Collagen Type II
Interferon−beta
Hypoglycemic Agents
Superoxides
STAT1 Transcription Factor
Thiazolidinediones
Bucladesine
CCAAT−Enhancer−Binding Protein−beta
PPAR alpha
Angiotensin II
RANK Ligand
Steroid Hydroxylases
NG−Nitroarginine Methyl Ester
Serpins
0.00
0.02
0.04
0.06
Gene Ratio
p.adjust
1e−14
2e−14
3e−14
4e−14

Figure 3-2. MeSH "Chemicals and Drugs" enrichment analysis from DE genes between likely survivors and likely non-survivors after CS challenge. Only the 30 most enriched terms (by BH adjusted p-value) are shown. "Gene ratio" refers to the proportion of genes identified in the dataset which comprise the entirety of the MeSH term, i.e. a gene ratio of 1.0 means all genes which were associated with a term were differentially expressed between likely survivors and likely non-survivors.

3.3.5 GO term enrichment

The final pathway / term enrichment analytical approach I took was to run a gene ontology (GO) term enrichment, specifically focused on the "biological processes" subcategory. This again yielded too many results to explore the entire set so I narrowed the focus on to the 20 most enriched terms in each biological compartment (Fig 3-3). Many of the most enriched terms in the blood were broadly associated with sepsis and were unsurprising, such as: innate immune response, response to bacterium, inflammatory response, etc. A term cluster affiliated with leukocyte migration and cell chemotaxis emerged as a strongly enriched subsection of this inflammatory response. The other prominent cluster that was strongly enriched were terms related to fatty acid derivatives, specifically metabolites of arachidonic acid known as eicosanoids. The top five most enriched terms in the blood, again by BH-adjusted p-value, were "inflammatory response", "response to bacterium", "eicsosanoid biosynthetic process", "eicosanoid metabolic process", and "fatty acid derivative biosynthetic process"; clearly enrichment of these eicosanoids were extremely strong in the blood.

This pattern was mirrored and amplified in the liver: the strongest cluster of enriched terms by far were those related to fatty acid metabolism broadly and arachidonic acid metabolism

specifically. Specifically, the top five most enriched terms were “unsaturated fatty acid metabolic process”, “arachidonic acid metabolic process”, “long-chain fatty acid metabolic process”, “eicosanoid metabolic process”, and “monocarboxylic acid metabolic process”. The magnitude of enrichment of the arachidonic acid terms in the liver was large enough that very few GO terms not affiliated with this pathway made it into the top 20 most enriched genes. Among those that did were “cellular response to interferon-beta” and “anion transport”, as well as the standard cluster comprised of “inflammatory response” and “response to bacterium”.

Among all biological compartments the cellular response to interferon beta was strongest in the spleen, with two IFN-β terms placing in the top 5 most enriched. The most enriched term overall was “angiogenesis”, again implicating the vascular endothelium as a potential critical point in differentiating likely survivors and likely non-survivors in this model. In addition to a chemotaxis cluster similar to the one identified in the blood, a strong group of terms affiliated with the circulatory system emerged in the top 20 spleen terms. As eicosanoids are major regulators of both angiogenesis and the circulatory system, in essence linking the signals across compartments, these maps of GO term enrichment indicated that arachidonic acid metabolism was a strong target to further analyze moving forward.

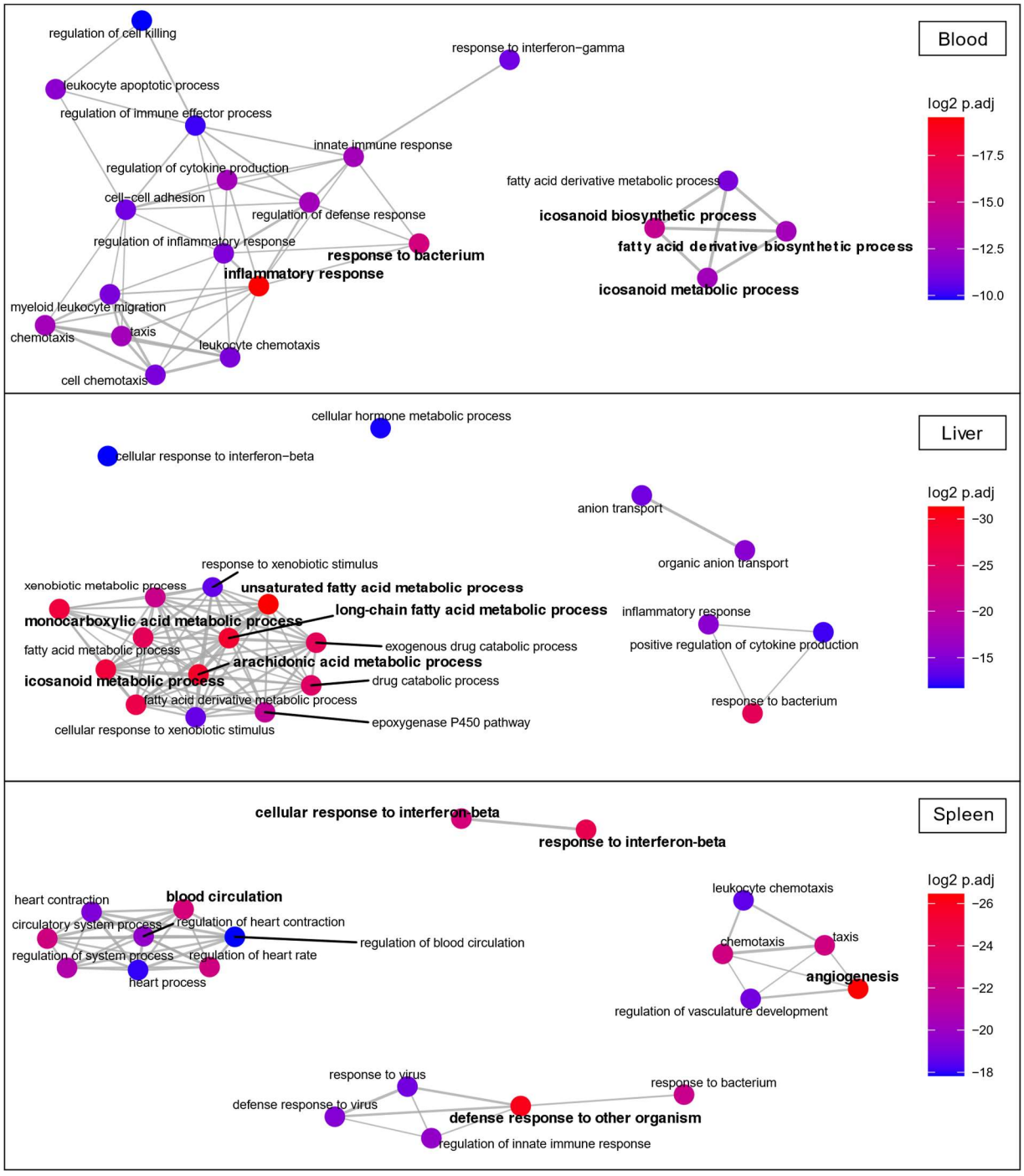

Figure 3-3. GO term enrichment from differentially expressed genes between likely survivors and likely non-survivors after CS challenge. The five most enriched terms (by adjusted p-value) for each biological compartment

are in bold. Only the top 20 most enriched terms by adjusted p-value shown here. Color of each node corresponds to log2 adjusted p-value.

Enrichment of the "eicosanoid metabolic process" term in blood was driven by a handful of eicosanoid-related genes spread across the arachidonic acid metabolic pathways (Fig 3-4A). Genes associated with the synthesis of prostaglandins, leukotrienes, and EETs / HETEs were identified as differentially expressed between likely survivors and likely non-survivors. In the liver, the enrichment was driven largely by downregulation of the Cyp2c family of genes, with the downregulation occurring to a larger magnitude in likely non-survivors compared to likely survivors (Fig 3-4B). The largest exception to this was the observed increase in expression of Cyp1b1, where both likely survivors and non-survivors exhibited enhanced expression relative to non-challenged controls.

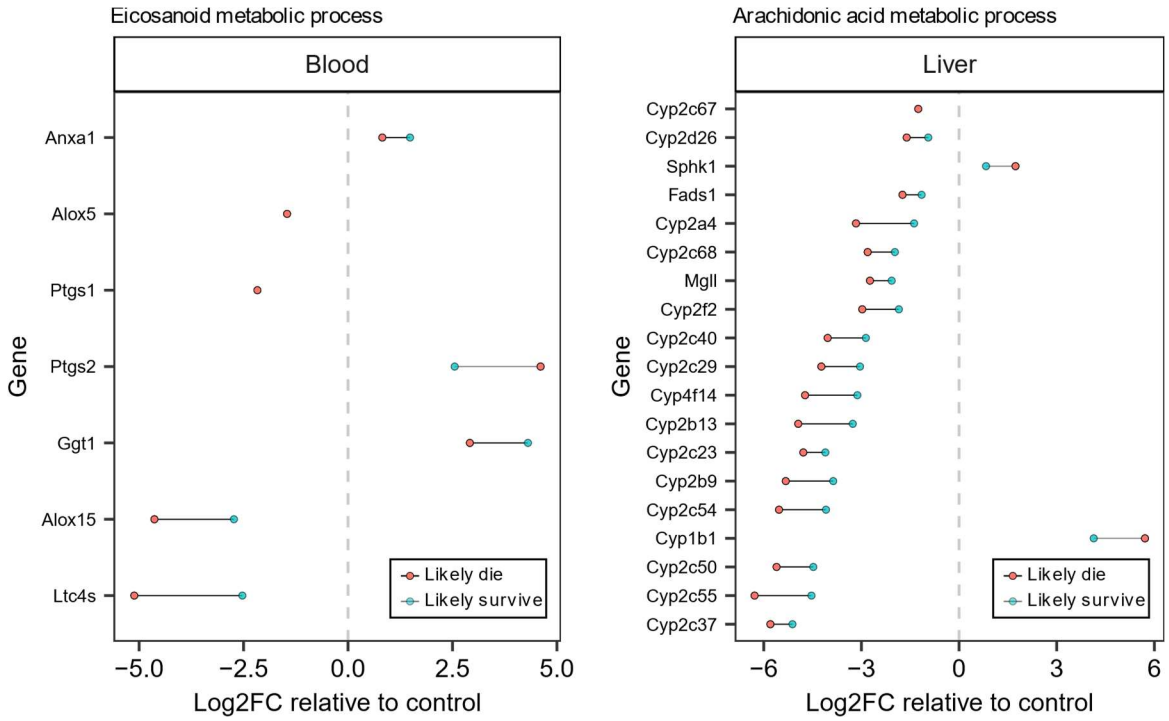

Figure 3-4. Relative changes in expression among genes related to eicosanoids and arachidonic acid metabolism in blood and liver. Average log2 fold change (Log2FC) of specific genes relative to healthy, unchallenged controls between pups predicted to die and pups predicted to survive. Genes included were the differentially expressed genes identified between likely survivors and non-survivors which drove the GO enrichment of the GO term "eicosanoid metabolic process" (blood) and "arachidonic acid metabolic process" (liver).

3.4 Discussion

The relative gene expression pattern between healthy controls and the likely survivors/non-survivors was revealing in its starkness and consistency. Pups that were likely to die exhibited a greater level of deviance from healthy controls in both directions – genes that were upregulated during sepsis were farther upregulated, and genes that were downregulated were farther downregulated. This captures the essence of sepsis: dysregulation on a global scale. Sepsis is

now understood to not simply be an overexuberant inflammatory response or even a failure in controlling the immune response – sepsis is a systemic breakdown of the myriad, delicately balanced feedback loops across the entire body. Any host-focused interventions must either be far enough upstream to help the body return to homeostasis or be acting on the pathways are directly responsible for death.

Different approaches to pathway analysis often yield slightly different results. The most difficult and critical part of interpreting the data was deciding which signals and which approaches to focus on in the validation step. Across most of the approaches there was a persistent signal surrounding eicosanoid processes in the blood, arachidonic acid processes in the liver, and cardiovascular / vascular endothelial processes in the spleen. I decided to focus on this pathway simply because there was an easy way to connect all three – arachidonic acid metabolism. Arachidonic acid is metabolized into eicosanoids, so the presence of those two pathways across blood and liver already strongly indicated that this would perhaps warrant further consideration. When I considered that one of the downstream effects of non-classical eicosanoids is to regulate the cardiovascular system and vascular endothelial integrity, a picture began to emerge that enabled me to connect the data from all three biological compartments. Notably, the arachidonic acid enrichment in the liver was very much driven by the Cyp450 family, responsible for the breakdown of arachidonic acid into the non-classical EETs and HETEs. EETs and HETEs which have been linked to angiogenesis through, among other regulatory points, Hypoxia Inducible Factor 1-α (HIF-1α).[186] HIF-1α was another of the persistent signals across all compartments, particularly in the MeSH enrichment wherein it was the most significantly enriched term in the

liver and the second most enriched term in the spleen. This still fit directly into the arachidonic acid → non-classical eicosanoids → vascular endothelium pathway that was the apparent differentiating (likely to die vs. likely to survive) factor.

It would of course be an oversimplification to pretend this was the only signal identified in this analysis. Terms associated with interferons were a consistent presence in all biological compartments across multiple analytical approaches, particularly in the GSEA and GO term enrichment. Many other signals associated with changes in innate immunity were clearly present in all biological compartments, such as neutrophil degranulation and cell chemotaxis. Moving forward, I decided not to focus on these other innate immune differences and inflammatory differences for two reasons: 1) I could trace a coherent pathway connecting signals identified across all organs which started with arachidonic acid, and 2) there was less chance to discover something novel in pursuing inflammatory differences between likely survivors and likely non-survivors. While certainly not unimportant, sole focus on inflammation was unlikely to generate any new treatments as anti-inflammatories in sepsis (and especially within animal models of sepsis) are unlikely to result in groundbreaking new therapeutics. Similarly, though COX-2 aka prostaglandin-2 did emerge as a major individual point of differentiation between likely survivors and likely non-survivors, COX2 inhibitors have been tried repeatedly in sepsis with little to no translational success.[187] Further, it is the non-classical eicosanoids which have previously been identified as major regulators of vascular endothelial integrity. For this reason, I decided to focus on the hypothesis that targeting this arachidonic acid pathway would be able to alter outcomes in this model of neonatal sepsis.

Chapter 4: Hypothesis testing: Arachidonic acid, nitric oxide, and the Angiopoietin-TIE2 axis

4.1 Introduction

An unbiased comparison of gene expression profiles of likely survivors and likely non-survivors in our mouse model of neonatal sepsis performed in the previous chapter revealed one signal that stood above the rest as a potential target for intervention: non-classical, arachidonic acid (ARA) metabolism acting downstream on the vascular endothelium. If this pathway truly was the discriminatory factor between survival and death in neonatal sepsis then it should have been possible to target the pathway at different points and improve or worsen outcomes, depending on the type of intervention. This chapter aimed to test this hypothesis by looking at the upstream component of the pathway, ARA, and the downstream component – changes to the vascular endothelium.

One of the only ARA metabolism-related genes which was strongly upregulated in CS challenged mice across blood, liver, and spleen was Cyp1b1. Mutations in Cyp1b1 lead to a rare, juvenile-onset open-angle glaucoma, which is only relevant here as mutations to Angiopoetin-1 (Angpt1) happen to result in the same disease.[140,141,188] This was the initial link that led me to begin examining the Angpt-TIE2 axis as a potential target in this model. The ratio of serum Angpt1:Angpt2 has been shown in adult mice to be directly correlated to the integrity of the vascular endothelium – a Angpt1-skewed ratio exists in a healthy, steady-state and is associated with normal levels of endothelial integrity.[142] An increase in Angpt2 and corresponding decrease

in Angpt1 is associated with neovascularization, oxidative stress, and a breakdown of the vascular endothelium. A higher ratio of Angpt2:Angpt1 has been directly associated with worse outcomes in human neonates and has been proposed as a potential marker of sepsis severity.[144,145] Administration of Angpt1 prior to endotoxin challenge in adult mice was protective and this protective effect was the result of maintaining the stability of the vascular endothelium.[147] I therefore used the Angpt1:Angpt2 ratio as a marker of vascular endothelial stability through the rest of this chapter.

4.2 Materials and methods

4.2.1 Mice and Monitoring

All animal work conducted was approved by the Animal Ethics Committee at Telethon Kid's Institute. Specific pathogen-free mouse breading pairs for C57BL/6J mice were bought in from The Jackson Laboratory and bred in-house. To generate neonatal mice, paired matings were established weekly, females were isolated from males after being visually identified as pregnant and then monitored daily thereafter to ensure an accurate date of birth was determined for all experimental mice. All experimental mice were monitored as describe in Chapter 2. Briefly, mice were monitored every 8 to 12 hours for the first 2 days post challenge and then daily thereafter. Humane endpoint was determined using a righting reflex and mobility score as previously described.

4.2.2 Survival Experiments

For prophylactic ARA experiments, 2µL of ARA (Fisher Scientific: #ICN19462510) was diluted in 30µL of corn oil which was administered 4-6 prior to CS challenge via oral gavage as described and demonstrated in our 2019 JoVE publication[189]. Monitoring and assigning humane endpoints was always performed by a researcher blinded to the treatment group. For exogenous Angpt1 and Angpt2 experiments, 500ng of Angpt1 or Angpt2 (R&D Systems: #9936-AN and #7186-AN respectively) was administered via intraperitoneal injection 1-hour prior to CS challenge as described in chapter 2. For anti-Angiopoietin-2 antibody treatment 50µg of the neutralizing antibody (Adipogen: #AG-27B-0016PF) was administered concurrently with CS challenge via intraperitoneal injection. For L-NAME experiments, 10µg of L-NAME (Abcam: #120136) reconstituted in water was injected via intraperitoneal injection concurrently with CS challenge. For L-Arginine experiments, 5mg of L-Arginine (Cayman Chemical: #23703) reconstituted in distilled water was administered via intraperitoneal injection 4-6 hours prior to CS challenge. The schedules described above were maintained for all combinational interventions described. Finally, for LPS challenge pups were injected IP with 8g/kg LPS from E. coli strain O55:B5 (Sigma: #L2880) in 50µL water. Monitoring schedule and humane endpoint criteria were identical to CS challenge.

4.2.3 ROS Quantification

For ROS detection, mice were treated according to the schedule described above. 8 hours post CS challenge we administered dihydroethidium (DHE) (Cayman Chemical: #12013) reconstituted in DMSO at a concentration of 10µg/g body weight via intraperitoneal injection.

After a 20-minute incubation period, mice were euthanized, and livers were excised and immediately drop fixed in 4% paraformaldehyde (Thermo Scientific: #AAJ19943K2) for 48 hours. Livers were then snap frozen, sectioned and DAPI counterstained. DHE was visualized using fluorescence microscopy utilizing a standard ethidium bromide filter. Relative ROS quantification was performed using ImageJ. Briefly, a DAPI counter-mask was created to isolate nuclei, background fluorescence was subtracted, and MFI of DHE was calculated. All results are normalized relative to batch-matched unchallenged controls that were imaged under identical parameters.

4.2.4 Murine Angiopoietin 1 and 2 ELISA

Mice were treated according to the above-described schedule. Blood was drawn 2- or 4-hours post-CS challenge via cardiac puncture. Blood was allowed to clot at room temperature for 15 minutes at room tempura prior to centrifugation to isolate serum. Mouse Angpt1 (Sapphire Biosciences: #LS-F2956) and Angpt-2 (R&D Systems: #MANG20) ELISAs were performed according to manufacturer instructions.

4.2.5 Statistical analyses

All statistical analysis and figure generation was performed in R v4.0.2. Sample size calculations for survival experiments used an expected effect size of 0.5 and always included at least 15 pups per group.

4.3 Results

4.3.1 Prophylactic arachidonic acid and angiopoietin-1 / angiopoietin-2

Pups were given arachidonic acid or vehicle control (corn oil) 4-6 hours prior to CS challenge via oral gavage, which alone significantly improved survival ($p = 0.003$, log-rank test) (Fig 4-1A). In a separate cohort of pups also treated with ARA and CS challenged but sacrificed 12 HPC, we found a significant reduction of liver superoxide in ARA treated pups ($p < 0.0001$, Wilcoxon-rank sum test) relative to challenged, untreated controls (Fig 4-1B). The ARA prophylactic treatment was so effective at reducing liver ROS that there was no statistical difference between the ARA-CS challenged pups and healthy, unchallenged controls. ARA treatment also simultaneously prevented a CS-challenge associated decrease in Angpt1 and a corresponding increase in Angpt2 – in both experimental set-ups, pups which received the ARA treatment looked more like healthy, unchallenged pups than their challenged counterparts despite receiving an identical dose of CS (Fig 4-1C).

Angpt1 treatment prior to CS challenge significantly improved survival, yet exogenous Angpt2 had no effect on survival in either direction (Fig 4-2A). Despite this, anti-Angpt2 antibody did have a protective effect (Fig 4-2B). As the body naturally releases a massive amount of Angpt2 during inflammatory or oxidative stress and it acts by directly outcompeting Angpt1 for binding of the TIE2 receptor, I suspect the lack of effect of exogenous Angpt2 may reflect receptor saturation – if the natural release of Angpt2 functionally binds all available TIE2 receptors then we would not anticipate additional Angpt2 to have any additional affect. We did find anti-

Angpt2 antibody to significantly improve survival after CS challenge which conforms to the hypothesis that a cap on TIE2 availability limits the impact of additional Angpt2 to the system.

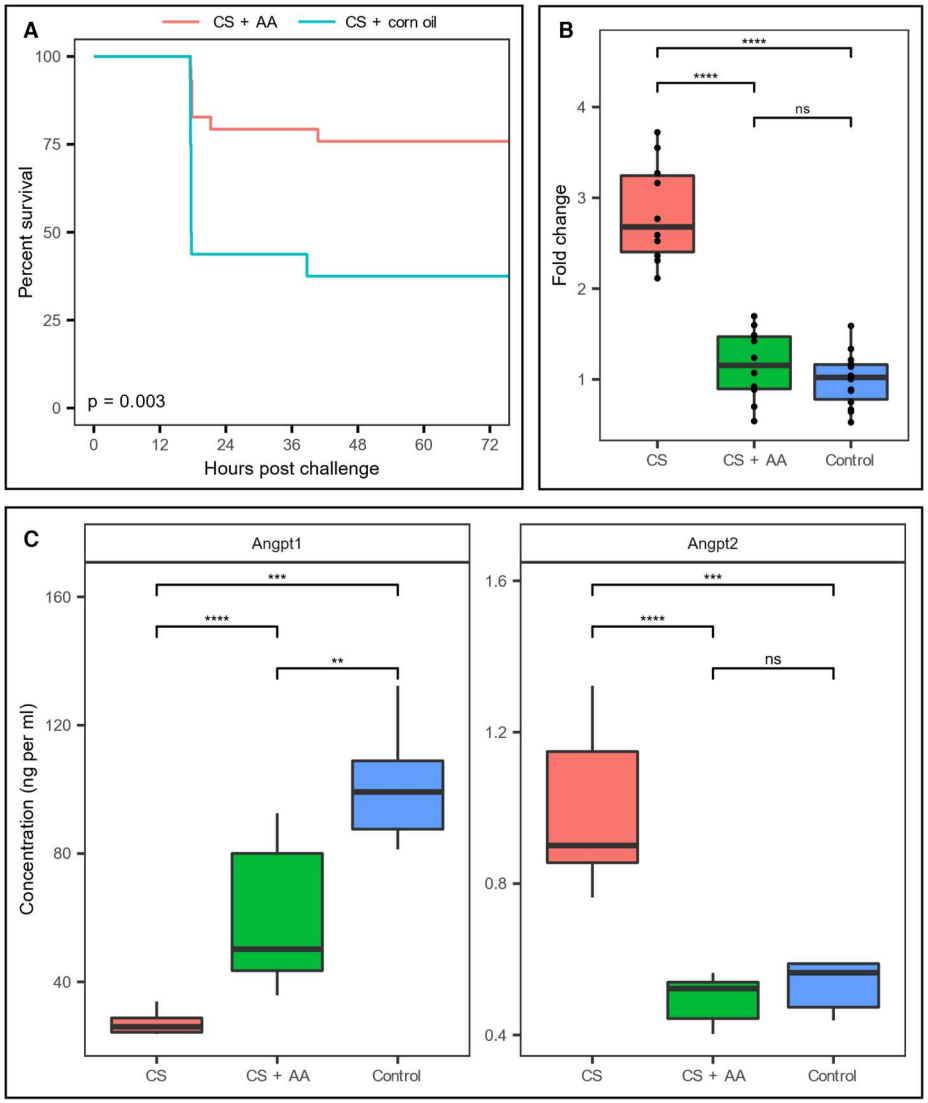

Figure 4-1. Arachidonic acid protects against CS challenge, reduces oxidative stress and impacts Angpt1/Angpt2 levels. A) Overall survival of CS challenged mice with or without prophylactic AA treatment 4-6 hours prior to challenge (n = 29 ARA, n = 16 corn oil, p=0.003, log rank test). B) ROS (O2-) accumulation in the liver of CS challenged mice (n = 5) 8 hours after CS challenge as determine by relative fluorescence integrity of

dihydroethidium normalized to untreated controls ($p<0.001$, Wilcoxon rank-sum test). C) Serum angiopoietin-1 and angiopoietin-2 levels in control (n = 5), challenged (CS, n = 5) and CS + AA (n = 5) challenged mice as determined by ELISA.

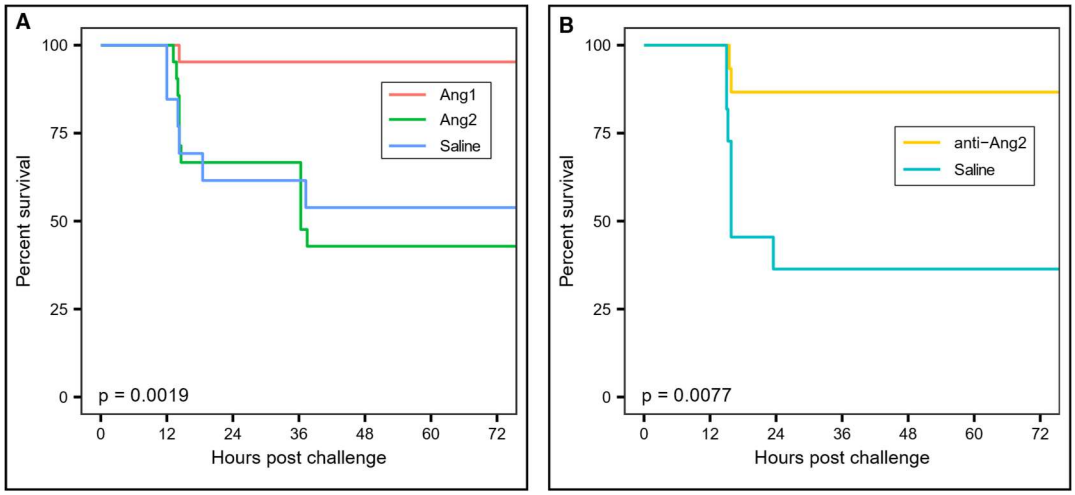

Figure 4-2. Exogenous angiopoietin-1 and anti-angiopoietin-2 antibody both improve survival in mouse pups prior to CS challenge. A) Overall survival of CS challenged pups after prophylactic administration of exogenous Angiopoietin-1 (Ang1, n=21), Angiopoietin-2 (Ang2, n = 21), or saline (n = 13) (p = 0.0019, log-rank). B) Overall survival of CS challenged mice after prophylactic administration of anti-Ang-2 blocking antibody (n = 15) or saline control (n=11) (p = 0.0077, log-rank).

To begin to parse if the protective effect of Angpt1 and ARA was a result of an antimicrobial or anti-inflammatory response, I titrated an LPS challenge to approximate an LD50 and repeated the experiment by giving either Angpt1 or ARA prior to challenge. Both treatments significantly improved survival (Fig 4-3) – Angpt1 led to an improvement similar to when pups were challenged with cecal slurry (increase from about 40% to ~80% survival, n = 15) while not a

single pup which received ARA died from LPS challenge (n = 32). Angpt1 and ARA are either just as or even more effective against preventing death against LPS challenge.

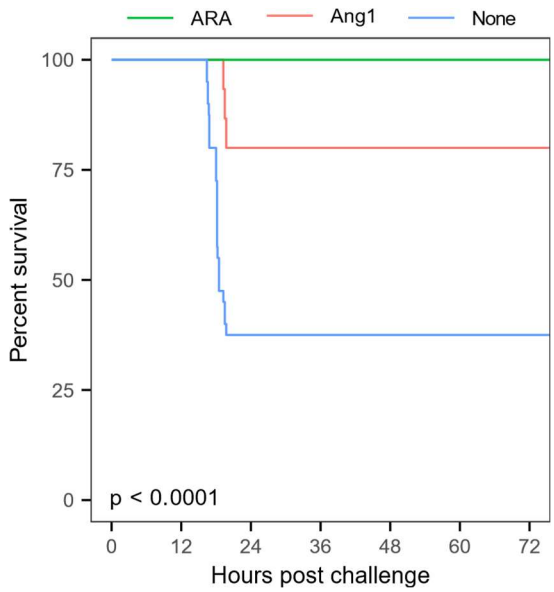

Figure 4-3. Arachidonic acid and angiopoietin-1 are effective against LPS challenge. Pups received either arachidonic acid (ARA, n = 32), antiopoietin-1 (Ang1, n = 15) or saline control (n = 40) prior to LPS challenge (8 mg/kg).

4.3.2 Role of nitric oxide in the arachidonic acid / angiopoietin pathway

I originally hypothesized that arachidonic acid's protective effect would be dependent on nitric oxide (NO) as it is one of the major regulators of the angiopoietin-TIE2 axis and vascular endothelial integrity in general. To test this, I first wanted to confirm the role of NO and its relationship to the angiopoietins in our neonatal sepsis model. Prior to challenge I administered N(gamma)-nitro-L-arginine methyl ester (L-NAME), a powerful inhibitor of eNOS, and found that it resulted in significantly greater mortality in mouse pups than vehicle control (p = 0.0013)

(Fig 4-4A). I then gave L-Arginine (L-Arg), the substrate for eNOS which has the opposite effect on bioavailable NO and found that it indeed had the opposite effect on survival as well; L-Arg prior to challenge significantly improved survival ($p = 0.0013$) (Fig 4-4B). To test if the protection provided by ARA administration prior to challenge was eNOS dependent, I pre-treated pups with either L-NAME alone or L-NAME and ARA simultaneously. When co-administered with L-NAME, ARA had no protective effect ($p = 0.38$) (Fig 4-4C). Simultaneous pre-treatment of L-NAME with Angpt1, on the other hand, did not impact the protection afforded by Angpt1 ($p < 0.0001$) (Fig 4-4D).

Curiously, I found L-NAME administration prior to CS challenge had no impact on serum Angpt1 levels compared to challenge, untreated controls (Angpt1 dropped precipitously in both instances) but it did prevent an increase in serum Angpt2 (Fig 4-4E). Perhaps even more surprising was how L-NAME pre-treatment prior to CS challenge affected liver ROS – not only did L-NAME not lead to higher levels of oxidative stress but it significantly decreased ROS relative to healthy, unchallenged controls (Fig 4-4F). This was likely a reflection of the magnitude of ROS which are generated from the uncoupling of eNOS during sepsis. Oxidative stress or inflammation begins a positive feedback loop in eNOS where it ceases to produce NO and instead produces superoxide directly.[138] Through the inhibition eNOS with L-NAME, this uncoupling could not occur and there was no surge of superoxide.

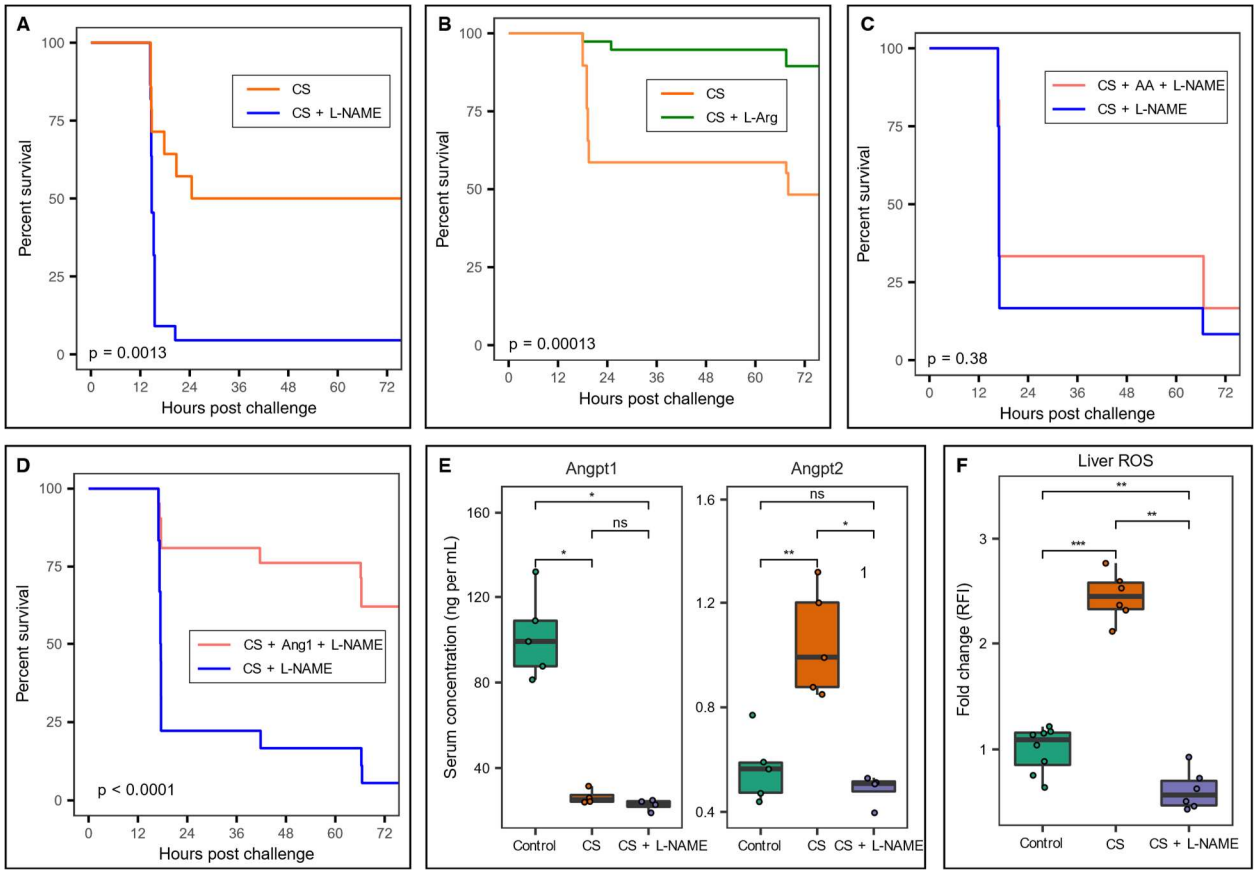

Figure 4-4. Nitric oxide synthase and reactive oxygen species play an important role in the arachidonic acid / angiopoietin pathway in murine neonatal sepsis. (A) N(gamma)-nitro-L-arginine methyl ester (CS + L-NAME, n = 22) given prior to challenge significantly worsened percent survival vs CS challenge alone (n = 14) (p = 0.0013, log-rank test). (B) L-Arginine (CS + L-Arg, n = 38) pre-treatment significantly improved survival relative to CS challenge control (n = 29) (p = 0.0013, log-rank test). (C) The protective effect of arachidonic acid (CS + AA + L-NAME, n = 12) was not maintained when co-administered with L-NAME compared to L-NAME alone (CS + L-NAME, n = 12) (p = 0.38, log-rank test), though (D) the protective effect of angiopoitin-1 (CS + Ang1 + L-NAME, n = 21) was still present when co-administered with L-NAME, versus CS + L-NAME alone (n = 18) (p < 0.0001, log-rank test). (E) Serum levels of Angpt1 and Angpt2 were measured 4 hours post challenge in unchallenged, healthy controls ("Control", n = 5), untreated, cs-challenged pups ("CS", n = 4) and pups which received L-NAME prior to cs challenge ("CS + L-NAME", n = 6). L-NAME had no impact on Angpt1 but prevented an increase in

Angpt2. (F) Relative fluorescence intensity (RFI) of superoxide ($O2^-$) in the liver was measured in the same three groups – L-NAME administration prevented expansion of ROS relative to cs untreated pups.

4.3.3 Therapeutic interventions after CS challenge

Up to this point all treatments had been given prior to CS challenge, in a sense prophylactically. While certainly useful in a basic science sense, the clinical relevance of a treatment that must be given directly prior to the onset of sepsis is nil. I decided to give two of the most successful prophylactics alone or together directly prior to challenge, Angpt1 and L-Arg, as well as 1 hour after CS challenge, as this was the approximate onset of 'clinical' symptoms. To adapt the model for assessing therapeutic efficacy, I challenged pups with a higher dose (1.4 mg CS / g mouse). I observed significant improvements in survival among both Angpt1 and L-Arg treated pups (Figure 4-5). Since NO has many stabilizing functions beyond those acting through the Angpt-TIE2 axis, I decided to give a combinatorial therapy of Angpt1 and L-Arg simultaneously. This combinatorial group saw significantly less mortality than either individual treatment alone, and a massive improvement over the control group - where 95% (19/20) of untreated, CS challenged pups reached humane endpoint, only a single pup (~5%, 1/21) which received the combinatorial therapeutic intervention was sacrificed.

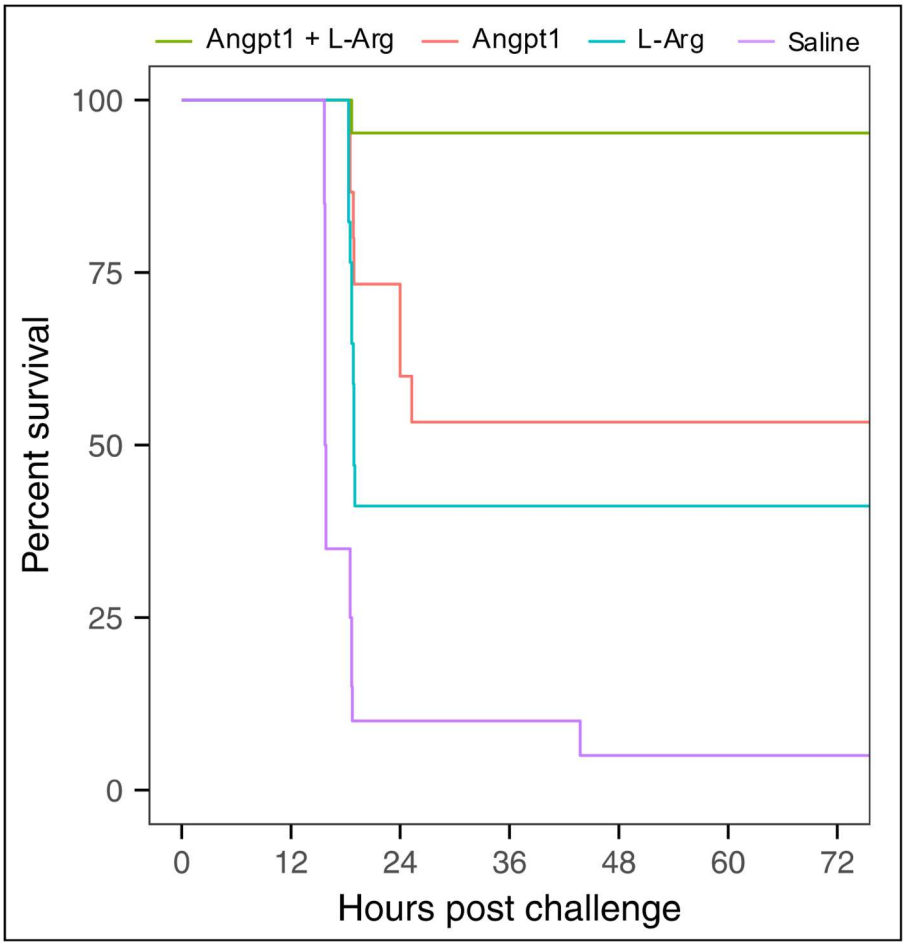

Figure 4-5. Combinatorial treatments of Angiopoietin-1 and L-Arginine at the onset of clinical symptoms significantly improved survival. Survival following cecal slurry challenge increased from 5% in saline control (1/20, n = 20) to 54% (8/15) following Angiopoietin-1 (Angpt1, n = 15) treatment ($p < 0.0001$, log-rank test). L-Arginine (L-Arg, n = 17) treatment also improved survival from 5% to 42% ($p = 0.00022$, log-rank test). The combinatorial treatment of Angpt1 and L-Arg (n = 21) led to only a single death after cecal slurry challenge and had a survival rate of 95% (20/21), significantly improving outcomes from both L-Arg alone ($p = 0.00022$) and Angpt1 alone ($p = 0.0032$).

4.4 Discussion

The breadth and significance of arachidonic acid (ARA) metabolic pathways in neonatal sepsis had not been recognized yet clearly represented a strong candidate for interventions moving forward. I showed that ARA itself significantly improved survival against CS challenge (and even more so against LPS challenge) and this protective effect was lost when co-administered with eNOS inhibitor L-NAME. This represents the first instance where a single dose of arachidonic acid has been shown to improve survival in any model of infection. It also clearly is dependent on NOS to some capacity. ARA also minimized liver oxidative stress associated with sepsis and prevented a skewing of the serum Angpt1/2 ratio, which functionally means it helped to maintain a healthy state of vascular endothelial stability. The data presented here represent the first direct connection between arachidonic acid and the angiopoietin-TIE2 axis – this is significant not only in the context of neonatal sepsis but also as part of the pathway building that so much of basic science relies upon.

There is much more to be learned about the protective effect of arachidonic acid – this work assumed that giving ARA directly would in some manner alter the ARA metabolic pathways identified as significant in Chapter 3. Given the number of different eicosanoids that could be produced from ARA administration an important follow-up experiment would be to run an eicosanoid panel on pups which received / did not receive ARA treatment in the context of this infection model. My hypothesis would be that the non-classical eicosanoids EETs / HETEs would be the driving factor as they have been previously identified to be major regulators of the vascular endothelium, but this certainly needs to be tested moving forward. ARA is also present

in low concentrations in human breastmilk[126] and has shown to be a critical fatty acid for neonatal brain development.[126] Despite this, very little research has been done looking at ARA alone as a therapeutic or supplement for neonates. This data indicates that this may be a worthwhile avenue to pursue moving forward.

The importance of the angiopoietin-TIE2 axis (and its direct function in regulating stability of the vascular endothelium) has also clearly been overlooked in neonatal sepsis. Multiple papers have been published identifying the balance of Angpt1 to Angpt2 as a potential sepsis biomarker[144,145], but there has been no mechanistic follow-up on this aimed at explaining why the switch occurs or, more importantly, testing if there is potential for novel interventions. The upregulation of Angpt2 appears to be directly tied to oxidative stress – when L-NAME was given prior to CS challenge I did not observe any increase in liver ROS, nor did I observe and increase in Angpt2. The combinatorial treatment of L-Arginine and Angpt1 was extremely successful in minimizing death in this murine model of neonatal sepsis – survival improved from 5% to about 95% when receiving the treatment after the onset of clinical symptoms. This speaks to just how significant the maintenance of the vascular endothelium is in surviving neonatal sepsis. There is little reason to think that this intervention would be detrimental in humans – at the very least, this now must be tested in clinical trials. This clearly represents a strong jumping-off point for future clinical work.

The effectiveness of the combinatorial treatment of L-Arginine and Angpt1 raises questions that remain unanswered in the scope of this work. The broad biological impact of NO is well known,

and it is unlikely that L-Arginine was acting solely through the Angpt-TIE2 axis. It is possible that the improved protection of L-Arg with Angpt1 was simply a result of increased Angpt1 production pushed through by the activation of eNOS, but I would hypothesize that the increased bioavailability of NO impacts many pathways related to vascular endothelial stability beyond that controlled by Angpt-TIE2. There is an abundance of future work tied to this aspect of the results: staining to look at the vascular endothelium directly, measurements of serum Angpt1 after combinatorial Angpt1 / L-Arg treatment, testing L-Arg protection when co-administered with an anti-Angpt1 antibody, etc. In the context of this dissertation this result shows that the vascular endothelium is a key player in neonatal sepsis and can be targeted to substantially improve outcomes. The findings from this section are graphically summarized below in Fig 4-6.

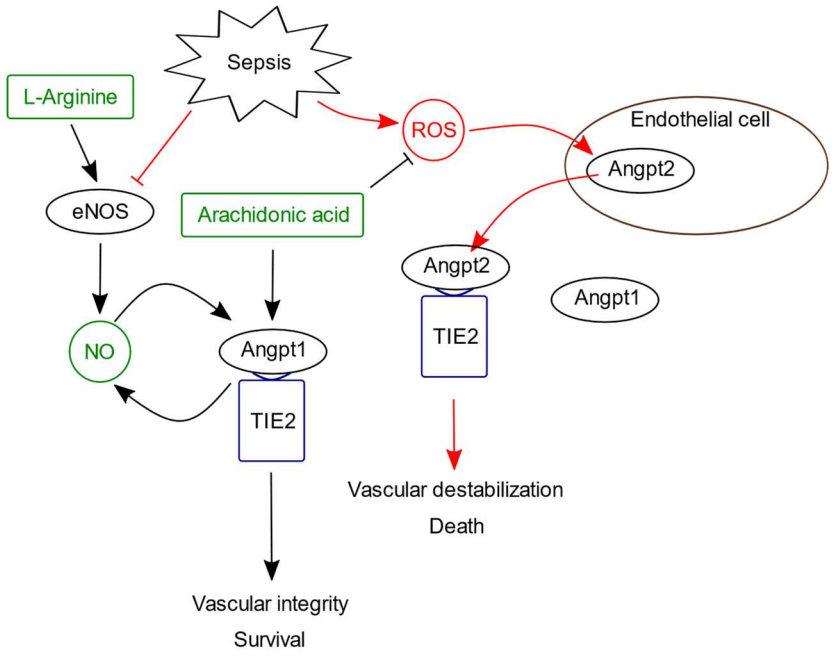

Figure 4-6. Graphical summary of the relationship between ARA and the Angpt/TIE2 axis.

There are other gaps and weakness to this study, the first that should always be addressed is that mice are simply not humans and it is never clear to what degree a mechanism identified in mice can be extrapolated to humans. I also did not touch on one of the most important regulators of the vascular endothelium, vascular endothelial growth factor (VEGF), which we know must be a critical component of this puzzle. The omission of VEGF is not an attempt to claim otherwise, rather I found the possible connection between ARA and the Angpt-TIE2 axis more novel and compelling – but it is unlikely sepsis can be reduced to a single biological node. The study would have benefited from a direct examination of vascular endothelial integrity as well, instead of relying solely on the Angpt1/Angpt2 ratio as a marker of such. There is, however, plenty of evidence directly linking Angpt1 and Angpt2 to endothelial integrity[190,191], but it is always better to be observed directly. The organ of focus within this chapter was the liver, but it very well could have been the lungs or heart – organs perhaps more associated with failure during severe septic shock. Further work may be warranted examining ROS in these organs in addition to what was described here.

Any intervention which improves survival from 5% to 95%, even in an imperfect mouse model of neonatal sepsis, warrants further research into its translation potential. These data also indicate that exogenous arachidonic acid could help to control the dysregulated immune response that defines sepsis – whether this is through a direct dampening of ROS or through increased eicosanoid production which enables better regulatory control remains to be seen. Regardless, there were multiple treatments and prophylactics identified in this chapter that have never been studied in the context of neonatal sepsis. These worked not only in the cecal slurry model but

also in an LPS-challenge model – not a single mouse pup died from an LD50, LPS challenge (n = 40) when pre-treated with ARA. Perhaps this means the protection conferred by ARA pre-treatment is more related to control of inflammation rather than a heightened antimicrobial response. Regardless of the details, this chapter demonstrated that the pathways identified in Chapter 2 which reportedly discriminated likely survivors and likely non-survivors were at least accurate in the context of this model. Arachidonic acid metabolism and vascular endothelial integrity must be major points of interest for researchers moving forward in neonatal sepsis.

Chapter 5: Conclusion

5.1 Major contributions

5.1.1 Scoring system and predicting outcomes in murine neonatal sepsis

Despite growing acceptance in the field that it is inappropriate to extrapolate data generated in adult mouse models to neonates, little work has been done to rigorously construct neonatal mouse models, especially not in a manner that can be assessed for consistency across laboratories. One of the most critical components of this is establishing health scoring systems and endpoints. This was accomplished by publishing our simple health scores and showing they directly correlate with a quantitative measure of disease state (bacterial burden). Prior to this there were virtually no published resources outlining how to determine if a mouse pup should be sacrificed or how to quantify disease progression without sacrificing the animal. In its simplest and most direct form, publishing this scoring system makes it easier for researchers to begin running their own neonatal mouse models which is important to accelerate our understanding of neonatal disease. An existing, validated, and robust health scoring system for neonates also can enable groups to operate in a sublethal range; for example, the observation that a drug significantly improves average scores over some period can be a helpful and straightforward experiment for assessing a new intervention's efficacy. An established humane endpoint is also important for standardizing results across multiple laboratories – it is correctly considered unethical to allow mice to proceed to the point of natural death. Most research institutions have

established protocols for this in adult mice but very little exists in neonatal mice: this work fills a clear gap in the literature.

The other component of this publication that can independently act as a contribution to the field was the gradient boosting machine classifier which is freely available in the appendices of the paper. One of the biggest difficulties of working with neonatal mice is that they are extremely difficult to serially bleed or even bleed a single time without having to sacrifice them. This may result in an overreliance on biomarkers as a substitute for mortality. There is nothing fundamentally wrong with using a biomarker such as inflammatory cytokine levels or bacterial load as an indicator of disease progression, but when operating in the space of neonatal infectious disease where so much is unknown, the accessibility of a classifier to confirm the suspected relationship between said biomarker and a relevant clinical outcome (survival) is invaluable. The utility of such a classifier was also demonstrated immediately through its use in subsequent chapters where it enabled me to begin to identify mechanistic differences between likely survivors and likely non-survivors.

5.1.2 Arachidonic acid and angiopoietins in neonatal sepsis

Another major contribution of this work was from the comparison of transcriptomes between likely survivors and like non-survivors of this model. As mouse pups are too small to collect samples from without sacrifice, there is very little published work that exists which compares survivors and non-survivors in neonatal sepsis. This is extremely important in sepsis as we have historically been unsure about what exactly leads to organ failure and have failed to consistently

produce new, effective interventions or treatments. The comparison of gene expression profiles between likely survivors and likely non-survivors in liver and spleen tissue was entirely novel and provided immediate insights into the pathology underlying neonatal sepsis. Much has been published on comparisons between healthy and septic neonates, and the signals picked up in those studies are critical to biomarker discovery and future diagnostics. Much less has been published with the explicit goal of discriminating survivors and non-survivors – this approach quickly identified arachidonic acid metabolism and integrity of the vascular endothelium as major targets for future interventions.

The importance of the vascular endothelium and its relevance to sepsis has become more apparent in the scientific community over the last few years. A recent, comprehensive review on the vascular endothelium in neonatal sepsis published in 2019 concluded that the vascular endothelium "has emerged as a fundamental sepsis mediator interposed between the effect of the invading pathogens, the systemic immune response of the host, and the multi-organ damage that ultimately leads to death."[137] The data presented in this dissertation strongly supports this emergent hypothesis that the vascular endothelium represents a critical component of the final common pathway in neonatal sepsis. Moreover, I demonstrated here that a major regulatory point of vascular endothelial integrity, the angiopoietin-TIE2 axis, is a central target in sepsis and was extremely effective at preventing murine neonatal death from sepsis. Angiopoietin-1 had been shown to be improve survival in an adult endotoxin model of sepsis when given prior to challenge – here we showed that it not only works in neonates, but it works therapeutically when given as an intervention after/around the onset of symptoms. Angpt1 was even more effective

when given in concert with NO precursor L-Arginine, increasing survival from near zero to near 100%. The effect size of this combinatorial treatment is large enough alone that in a vacuum of additional evidence would warrant further research, but this finding does not exist in a vacuum. This therapeutic intervention was the direct result of identifying discriminatory pathways between likely survivors and likely non-survivors and pushes forward an already growing movement in the field of neonatal sepsis.

Finally, the use of a single bolus of arachidonic acid (ARA) as a sepsis intervention is novel emphasizes this importance of eicosanoids and fatty acid metabolites in treating and understanding neonatal sepsis. For too long, ARA has been viewed as simply the precursor for prostaglandins and the substrate for COX2 – the research on other eicosanoids and especially non-classical eicosanoids (EETs and HETEs) is limited, and these data indicate they are probably important players in neonatal sepsis. The complex regulatory role that eicosanoids fill in the body as simultaneous regulators of inflammation and anti-inflammation, vasoconstriction and vasodilation, immune cell activation and immune cell suppression, and myriad other tasks should itself implicate them as critical pieces in sepsis. Showing that ARA itself is sufficient to improve survival should encourage more research into eicosanoids and neonatal sepsis. The connection of ARA to the Angpt-TIE2 axis is also novel and demonstrates the utility of attempting to treat sepsis from high up in a cascade, in addition to attempting to treat sepsis at the endpoint.

5.2 Limitations and future work

5.2.1 Mouse models and preterm sepsis

There are inherent limitations to mouse models that must always be addressed when one implies animal findings have direct human relevance. In this instance we attempted to build upon signals which already existed in human literature and to validate the animal work in an independent cohort, but this certainly does not mean that the results described here will translate directly to humans. Perhaps just as significant as the biological differences between mice and humans are the differences in medical care between a sick newborn in a NICU and a sick mouse pup in an experiment. A septic human neonate will certainly receive aggressive antibiotic treatment and fluid resuscitation, as well as an array of other therapies such as steroids, vasoactive and/or inotropic agents, vasopressin, granulocyte transfusions, etc. None of this is to say the data generated in mouse models is meaningless. Rather, one must always attempt to parse the findings in the context of humans and ensure the data is reflected in human cohorts wherever possible. None of these experiments were constructed to address questions of long-term effects or side-effects of treatment; this is an extremely important consideration when discussing new interventions to be given in the critical neonatal period. Certainly, further research is warranted before administering any treatments described here directly to a human newborn.

Age also becomes an issue when working with neonatal mouse models; it is generally accepted that mouse pups around day of life 7 approximately cross over to term human neonates in respect to immune cell development[28] but this is a dart throw at best. Working with mouse pups on the first day of life inevitably results in high cannibalization rates and a host of other practical issues.

This makes data presented here even more difficult to try to extrapolate onto preterm neonates, the population at greatest risk of sepsis. It would be valuable to look through existing, preterm neonatal sepsis datasets to confirm the existence of the same signals (sPLA2, Angpt1/2) which were found in term infants.

5.2.2 Exogenous arachidonic acid

This novel connection between ARA, ROS, NO, and the Angpt-TIE2 axis forms a mechanistic skeleton to build upon but certainly demands further research to fill out the details. The protective effect of ARA in sepsis is almost certainly mediated by some of its eicosanoid metabolites and there is still question of which family is most relevant. The signal in the liver was very much driven by Cyp450s which implicate HETEs and EETs as the critical mediators, but this proved difficult to test *in vivo*: as this is a fatty acid metabolic pathway, all signaling molecules and potential inhibitors are hydrophobic lipids which we were insoluble in any solution suitable for *in vivo* injection. Previous work with these ARA pathway inhibitors has been performed *in vitro.* We were therefore unable to identify the role of each eicosanoid family downstream of ARA administration. A valuable future study would be to perform full lipidomics or metabolomics on samples collected from likely survivors and likely non-survivors, as well as pups which received ARA or vehicle control. Most of the non-classical eicosanoids are short-lasting and unstable, but it may still be possible to give some directly to pups to test if they are sufficient for maintaining the protective effect observed in ARA alone. One curious observation that was not explored in depth here was the enhanced protective effect of ARA in the LPS challenge model. We found ARA to be 100% protective (n = 40) against LPS challenge that

resulted in about 60% mortality in vehicle controls. This enhanced protection raises questions about the differences between LPS and CS challenge that was beyond the scope of this investigation but could be potentially relevant moving forward.

5.2.3 Metabolism and disease tolerance

As discussed in the introduction, there is some intriguing evidence supporting the hypothesis that the energy demands of early life dictate a disease tolerance (DT)-like phenotype in the neonatal immune response. This hypothesis was not explicitly tested within this dissertation, primarily due to the time it took to acquire a direct animal calorimeter sensitive enough to collect meaningful data from a three-gram mouse pup. Access to this sort of data would enable direct comparisons of energy expenditure between challenged vs non-challenged pups, survivors vs non-survivors, and pups vs adults. This would also allow us to examine what effect, if any, the interventions studied here (i.e. arachidonic acid, Angpt1) as well as other interventions of interest (i.e. probiotics, BCG, etc) had on energy expenditure and could represent an extremely interesting and important line of work.

5.3 Concluding remarks

Sepsis most likely represents the breakdown of the tight, regulatory feedback loops that exist in the immune system and all over the body. The failure of these regulatory systems leads to simultaneous, uncontrolled production of inflammatory as well as anti-inflammatory cytokines, eventually leading to organ failure and death. The intricacies of sepsis are still poorly understood

in adults and even more so in neonates, where we are faced with an extra layer of the unknown in the form of a developing immune system constrained by demands unlike any other time in life.

To conclude this dissertation, I wanted to revisit the objectives as outlined in the introduction:

1) Develop a way to reliably classify likely survivors and likely non-survivors at time of experimental sacrifice.

My co-authors and I were able generate a large enough dataset of neonatal mouse data that we were able to train and construct a gradient-boosting machine model which confidently classified pups as likely survivors or likely non-survivors prior to the onset of mortality. While I initially set out with to achieve this objective solely to enable a comparison of the transcriptomes of pups which were likely to survive a CS challenge and those likely to die, I ended up with the creation of a scoring system which had utility beyond this scope.

2) Use gene expression data in organs and whole blood to identify pathways which differentiate likely survivors and likely non-survivors and generate leads to target in further investigations.

In the pursuit of this objective, I generated a large amount of useful data which not only helped to directly inform my subsequent experiments, but also will exist as a valuable repository for future experiments. The strongest and most obvious signal which emerged out of these data was

the arachidonic acid cluster of enriched GO terms in the liver, which is why I pursued that pathway into the next section. There were of course many other signals that likely warrant future research and the entire dataset will be available upon publication.

3) Directly administer drugs or metabolites to modify the critical pathways identified in the dry-lab portion to see if the improve survival.

The pursuit of this objective was the culmination of the entire body of work and yielded tremendously successful results. Multiple new interventions for neonatal sepsis which massively improved survival both prior to and after cecal slurry challenge – these mouse data have already begun discussions around clinical trials in human neonates, which is the goal for any mouse experiment. Prior to commencement of these trials, it would be critical to repeat the work done here and monitor the animals for changes in longer term outcomes, such as brain development and normal growth. Safety trials would be required before moving straight into real clinical trials. I not only identified a novel pathway connecting arachidonic acid to Angpt1 but showed also that the pathway could be modified at both the beginning (exogenous arachidonic acid) and the end (exogenous angiopoietin-1) in order to significantly and substantially improve survival. Combinatorial treatment of angiopoietin-1 and L-Arginine reduced the mortality rate to near 0. This represents a substantial contribution to the field of neonatal sepsis and a promising new intervention with concrete potential for translation and clinical utility. These novel connections are summarized in Figure 5-1.

The approach taken in this dissertation was to try to work backwards from death in neonatal sepsis to a) learn about the mechanism underlying mortality and b) generate new treatments for neonatal sepsis. I believe this work represents a substantial and important contribution to the field and has genuine potential for direct clinical translation and impact. Future work must focus on arachidonic acid metabolites and vascular endothelial integrity – these pathways are clearly critical components of the neonatal immune response and are major players in dictating outcomes in sepsis.

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Appendices

Appendix A Chapter 2 supplementary material

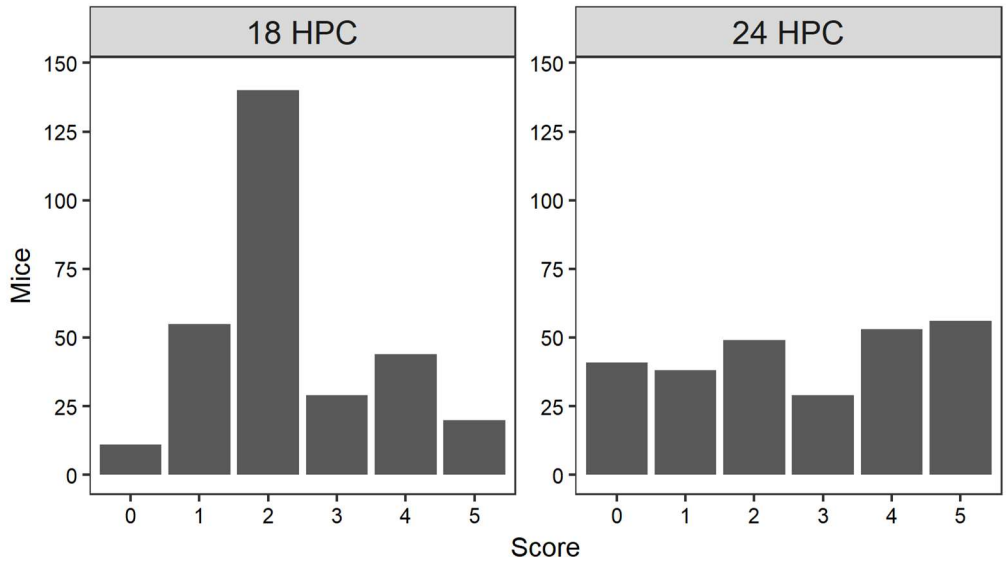
A.1 Data repository and source code URL

https://github.com/radaniba/Sepsis_Project.

A.2 Table of AUCs for various methods with or without feature selection

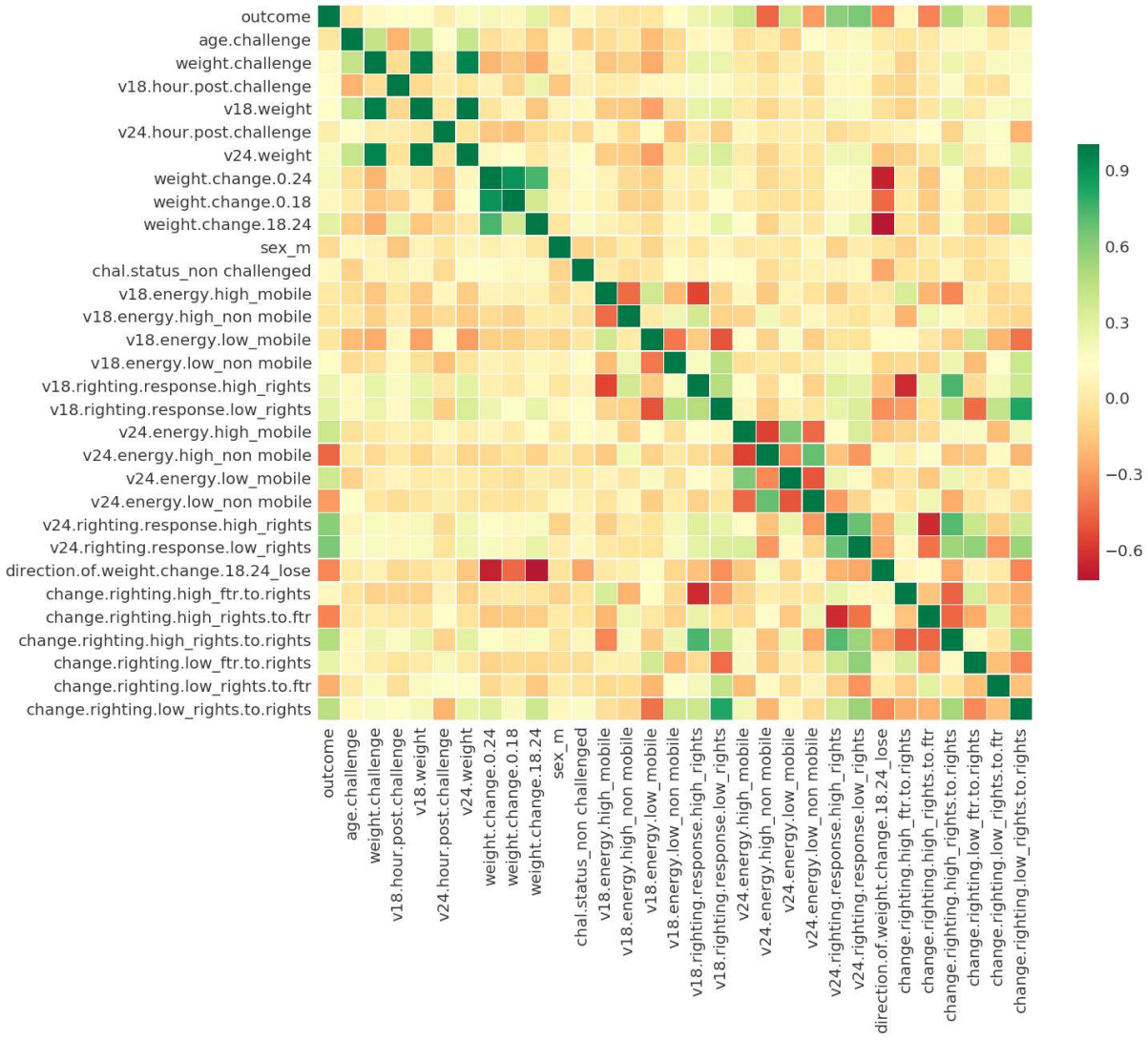
Method	GridSearch (No feature selection)	Correlation + GridSeach	Hypothesis + GridSearch	Model based + GridSearch
Logistic Regression	0.86	0.874	0.874	0.874
K Nearest Neighbor	0.827	0.833	0.851	0.856
Decision Tree	0.82	0.842	0.842	0.869
Random Forest	0.878	0.869	0.874	0.878
Gradient Boosting	0.883	**0.887**	0.887	0.883
XGBoost	0.883	**0.892**	0.892	0.887
Average	**0.859**	**0.866**	**0.87**	**0.875**

A.3 **Distributions of scores assigned to neonatal mice at 18 and 24 hours post challenge.**



Scores assigned to neonatal mice 18 and 24 hours post IP challenge with cecal slurry (HPC). At 18 HPC the scores are poorly distributed, with the vast majority of mice assigned a score of 2 (failure to right, mobile) indicating that most mice have not progressed towards survival or non-survival. By 24 HPC the scores are evenly distributed as mice have begun to succumb to or recover from sepsis.

A.4 Feature selection visualization using Pearson correlation



Heatmap of feature correlations of with Pearson correlation. Scores were split into components, starting with looking at righting reflex and mobility independent from one another and then further separated by the lower and higher measurements of each score taken in duplicate. Change in righting reflex reflects the difference between the monitoring timepoints at 18 HPC and 24 HPC.

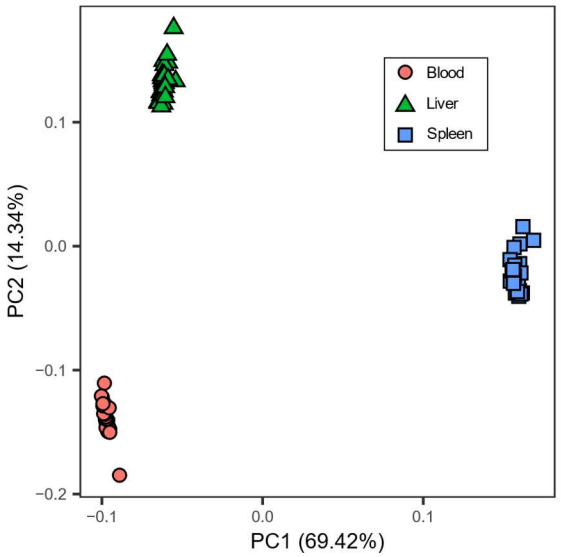
A.5 Confusion matrix showing classifier applied to external dataset

True class		
Survivor	**9**	2
Non-survivor	1	**9**
	Survivor	Non-Survivor
	Predicted class	

Appendix B Chapter 3 supplementary material

This is appendix contains supplementary material referenced in Chapter 3

B.1 PCA of combined gene expression data across blood, liver, and spleen.



Only genes retained after data cleaning and normalization in all compartments (blood, liver, and spleen) included in this PCA.

B.2 Fold change of five DE genes in blood comprising the most significantly enriched reactome pathway

SYMBOL	log2FoldChange	p.adjust
Alox15	-1.903	0.0019
Ltc4s	-2.587	0.0010
Alox5	-0.809	0.0351
Ptgs2	2.056	0.0035
Alox5ap	-0.738	0.0146

Relative changes between likely survivors and likely non-survivors of the five genes which drove the enrichment of the "Biosynthesis of specialized proresolving mediators (SPMs)" reactome pathway. Negative log2FC means expression of the gene was higher in likely survivors than in likely non-survivors. P-value adjusted via the BH procedure.