**Identifying Altered Metabolic Signatures in Septic Shock: Steps Toward Precision Pharmacotherapy**

by

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## Dedication

*This work is dedicated to my parents, Ted and Lisa Jennaro, who have shown me the value of hard work, the power of kindness, and the importance of cherishing loved ones.*

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## Table of Contents

## List of Tables

## List of Figures

## List of Appendices

## Abstract

Sepsis is a life-threatening clinical syndrome characterized by a dysregulated host-response to infection and organ dysfunction. There are variable clinical course trajectories in patients with sepsis, and the precise reasons why the illness resolves in some individuals, causes long-term sequela in others, and is deadly to many remains poorly understood. This heterogeneity has contributed to the litany of failed clinical trials for novel therapeutics such that sepsis therapy remains largely supportive and non-specific.

This dissertation leverages patient data and biospecimen from a recently completed clinical trial to investigate the metabolic pathways that drive sepsis mortality and patient response to a metabolic therapeutic, levocarnitine (L-carnitine). Using data from the placebo arm of the trial, I determined that baseline clusters of metabolomics data can identify patient subgroups characterized by differential organ function and mortality. I also modeled the early trajectory of metabolite changes over the first 48-hours of septic shock and found several acylcarnitines and IL-8 were persistently elevated in patients who died. I then found higher concentrations of acylcarnitines and amino acids in serum were related to non-mortality endpoints, including the continued need for vasopressors and mechanical ventilation.

With logistic regression models and a grid search methodology of the entire metabolomics dataset from the trial, I discovered a signal that L-carnitine treatment response varies depending on a patient's baseline metabolic status. Namely, that those

patients with elevated acetylcarnitine and/or valine derived a mortality benefit upon having been randomized to high dose L-carnitine over placebo. I also built a population pharmacokinetic model that describes L-carnitine concentrations over time in patients with sepsis. I then validated that renal function is a key patient covariate on the elimination rate of the drug, particularly when renal function estimates were built from equations using serum cystatin C without a patient's self-identified race. These results add to growing calls to reconsider renal function estimates based on serum creatinine and/or race.

In aggregate, this thesis explores sepsis-induced perturbations in metabolism and how they relate to patient outcomes and the pharmacokinetic and clinical response to L-carnitine. This work implicates energetic and mitochondrial metabolic dysfunction, particularly related to the impairment of fatty acid beta-oxidation, in explaining some of the observed heterogeneity in sepsis outcomes and response to treatment. Future pre-clinical and clinical follow-up studies are necessary to understand: 1) the mechanisms driving perturbed host metabolism and the development of sepsis-induced organ dysfunction and 2) if these metabolic measures hold further promise as prognostic and/or predictive biomarkers in patients with sepsis.

# Chapter 1: Introduction

Aspects of this work have been published as an original review article in *Pharmacotherapy*.[1]

## 1.1 Sepsis is a heterogeneous clinical syndrome

Sepsis is a life threatening, dysregulated host response to infection, which is characterized by systemic organ dysfunction.[2] One in three Americans who die in the hospital have sepsis, and in 2017 there were an estimated 48.9 million cases worldwide.[3] Moreover, sepsis prevalence is rising, and in 2013 cost an estimated $24 billion, making it the most expensive inpatient diagnosis.[4]

Sepsis is best described as a highly heterogenous clinical syndrome, with patients presenting along a continuum of clinical signs, symptoms, and severity of illness.[5] Multi organ failure, as measured by the Sequential Organ Failure Assessment (SOFA) score, is a hallmark of sepsis and can impact all major systems of the body including the heart, kidney, liver, lungs, central nervous system, and circulatory systems.[6,7] The mechanism and pathophysiology underlying highly variable clinical trajectories and the development of specific organ failures are complex, and the precise reasons some patients exhibit severe dysregulated responses while others recover from their initial infection in an uncomplicated fashion remains poorly understood. Such host-response heterogeneity muddies the interpretation of treatment response and is a major reason why novel pharmacotherapy often fails. Absence of adequate stratification of

patients based on their underlying pathophysiology may contribute to this.[8] The need to advance mechanistic understanding of sepsis heterogeneity has led to funding calls from the National Institutes of Health (NIH), Institute of General Medical Sciences (NIGMS) for studies that seek to determine the effect of patient characteristics on differential treatment response (NIH Notice of Information, GM-19-054).[9] Teasing out this variability is necessary to bring about a precision medicine approach to sepsis.

### 1.2 Perturbed metabolism is a pillar of sepsis pathophysiology

Ample evidence suggests a hypermetabolic component and derangement of host metabolism is central to sepsis pathophysiology.[10] Recently revised consensus guidelines define the most severe manifestation, septic shock, as infection with sustained hypotension despite recommended evidence-based treatment interventions (e.g., fluid resuscitation), and pertinent to this discussion, metabolic dysfunction and/or tissue hypoperfusion as evidenced by an elevated blood lactate concentration.[2] Altered energy utilization results in hyperglycemia, protein catabolism, and lipolysis and contributes to poor patient outcomes.[11] While several studies have targeted lactate level as a resuscitation goal[12-14], these trials have typically utilized fluids, vasopressors, or other agents designed to improve organ perfusion under the assumption that lactate elevations are predominantly explained by ongoing tissue ischemia, which may not necessarily be true.[15]

Current pharmacotherapy neither targets nor corrects these metabolic perturbations, although restoration of host bioenergetics offers a promising therapeutic target. Moreover, given the prevalence, persistent mortality, and lack of specific treatment paradigms, there is a critical need to advance understanding of the metabolic

consequences of sepsis. This includes capturing dynamic alterations in host metabolism, measuring a greater diversity of potential metabolite biomarkers, and moving beyond observational studies.

### 1.3 Metabolomics as a tool to understand sepsis heterogeneity

Metabolomics seeks to identify and quantify small molecule metabolites, the full collection of which define the metabolome, in a given biofluid.[16] The metabolome encompasses both endogenous and exogenous compounds (i.e., not produced by the body) that play critical roles in cellular homeostasis and human health/disease. With well over 200,000 metabolite entries in the Human Metabolome Database (HMDB)[17], the diversity of the metabolome presents both an exciting opportunity for describing molecular phenotypes as well as an analytical challenge.[18] The two most common methods for measuring metabolites include nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS), typically coupled with a chromatography column to separate compounds based on chemical characteristics.[19] While NMR is highly reproducible, it suffers from a lack of sensitivity and provides a more limited read of the metabolome compared to most MS-based methods.[20] Both techniques can provide quantitative information about the abundance of individual metabolites in a given sample are used extensively throughout the remainder of this dissertation.

Importantly, metabolites provide a molecular phenotype of the host, since they are the product of the body's biochemistry. An advantage of metabolomics is that phenotypes are downstream of genetic, transcriptomic, and proteomic influence and sensitive to microbiotic and environmental variability (**Figure 1-1**).[21] As such, metabolomics reflects the culmination of these regulators on the host. In addition,

metabolism is dynamic on a practical and physiological time-scale, and this sensitivity informs heterogeneity in disease trajectory and treatment response.[20,22,23] Finally, metabolomics findings are translatable. They can direct the development of point of care measurements that are feasible in existing care models, and most diagnostic assays are measurements of small molecules.[24]

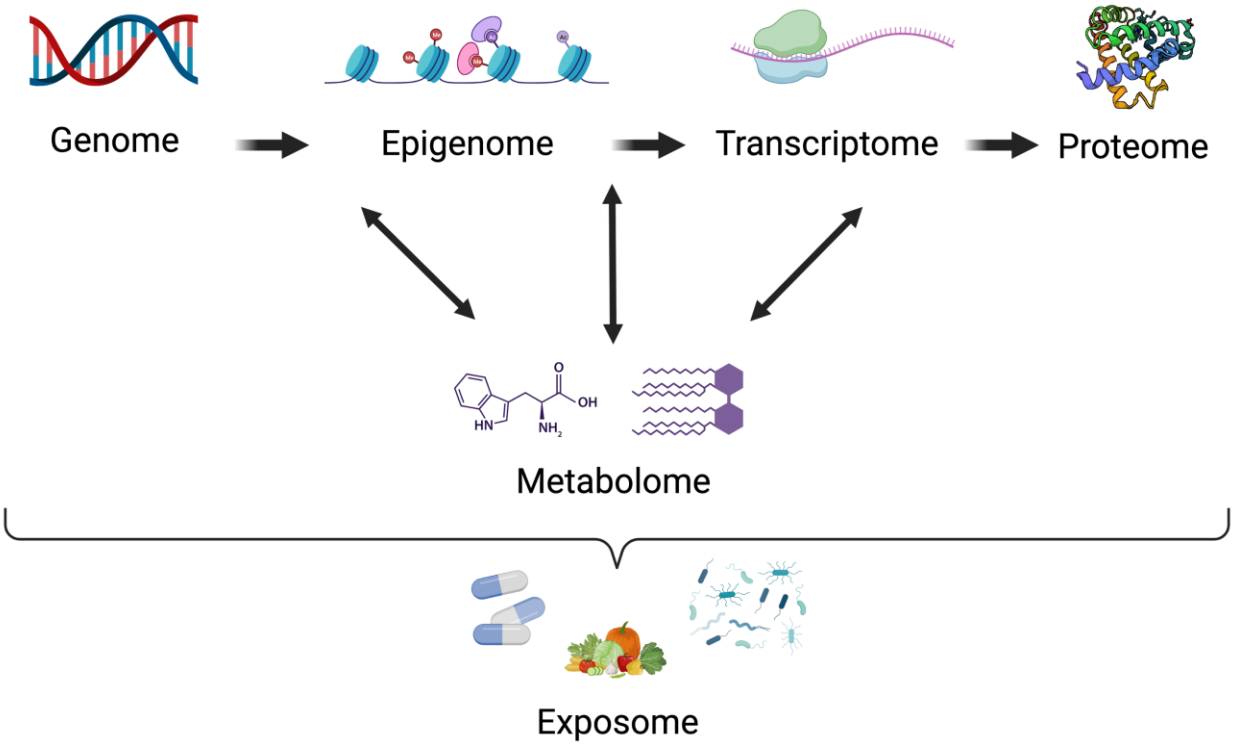


**Figure 1-1. Overview of biology's central dogma and molecular phenotypes.**

Conventional wisdom displays the central dogma of molecular biology as a linear process by which DNA is transcribed to RNA that is then translated to protein. In reality, the processes of systems biology are nonlinear with important feedback loops and self-sustaining mechanisms. Metabolites include a diverse class of chemical compounds including but not limited to sugars, organic acids, ketones, aldehydes, amines, amino acids, lipids, steroids, alkaloids and drugs. These compounds play vital roles in maintaining homeostasis, cellular signaling, and the balance between health and disease. The metabolome is thought of as 'down-stream' and thus a closer description of molecular phenotype compared to other multi-'omic' discovery sciences, as small molecules are the product of cellular biochemistry. The exposome, including the bacteria, nutrients, and exogenous compounds in our environment, interact with systems biology at all levels, but their impact can be most directly measured through the metabolome. Figure created with BioRender.com.

Known perturbations of host metabolism has pushed metabolomics to the forefront for studying sepsis. Sepsis induces derangements in amino acid metabolism, mitochondrial function and oxidation of fatty acids (FAs), and the tricarboxylic acid cycle.[20,22] Previous work has shown differences in the metabolome are associated with mortality, organ function, and differentiation of sepsis severity.[25-31] While these have provided insight into the metabolic component to sepsis pathology, there are important drawbacks that deserve consideration. Past work has largely relied on single, point-in-time samples of convenience. These 'snapshots' cannot capture the dynamic and time-sensitive nature of metabolism. Consequently, little is known about sepsis metabolism over time. Moreover, the metabolome encompasses a diverse set of chemical compounds, which cannot be detected by a single analytical approach. Innovations in technology and instrumentation have increased the ability to detect and quantify low abundant compounds.[32] These, 'untargeted' approaches hold promise in defining molecular phenotypes of sepsis, yet can suffer from correlated, redundant features.[33] Finally, prior work in metabolomics of sepsis has been largely observational and has not delved into understanding heterogeneity of treatment effect, where a treatment may only be efficacious when used in a more homogenous, metabolic subgroup. In this dissertation, I leveraged a completed clinical trial of levocarnitine (L-carnitine) in patients with septic shock and the ancillary metabolomics study to understand the metabolic alterations of sepsis and variable response to treatment.

## 1.4 Leveraging a completed clinical trial to study sepsis heterogeneity

### *1.4.1 Physiological role of L-carnitine*

L-carnitine is an endogenous, polar small-molecule derived from lysine and methionine, which plays a well-established, crucial role in transport of long-chain fatty acids into the mitochondria for β-oxidation. Other key roles during times of metabolic stress include maintenance of coenzyme A homeostasis, metabolic flexibility and promotion of normal TCA cycle function, and further oxidation of fatty acids by peroxisomes.[34] A full, in-depth review of carnitine and acylcarnitine homeostasis and biochemistry has been extensively reviewed elsewhere.[34,35] Briefly, the carnitine shuttle allows for fatty acid entrance to the mitochondria for oxidation and subsequent energy production through transfer of acyl groups and conversion into acylcarnitines (**Figure 1-2**). Acylcarnitines are esters formed in peroxisomes and the mitochondria from the conjugation of fatty acids with L-carnitine and are numbered based on the chain length and saturation of the parent fatty acid.[36] Together, this class of compounds are important markers of metabolic mitochondrial dysfunction, particularly related to β-oxidation, and perturbed systemic concentrations have been found in cardiovascular disease, diabetes, and cancer.[37]

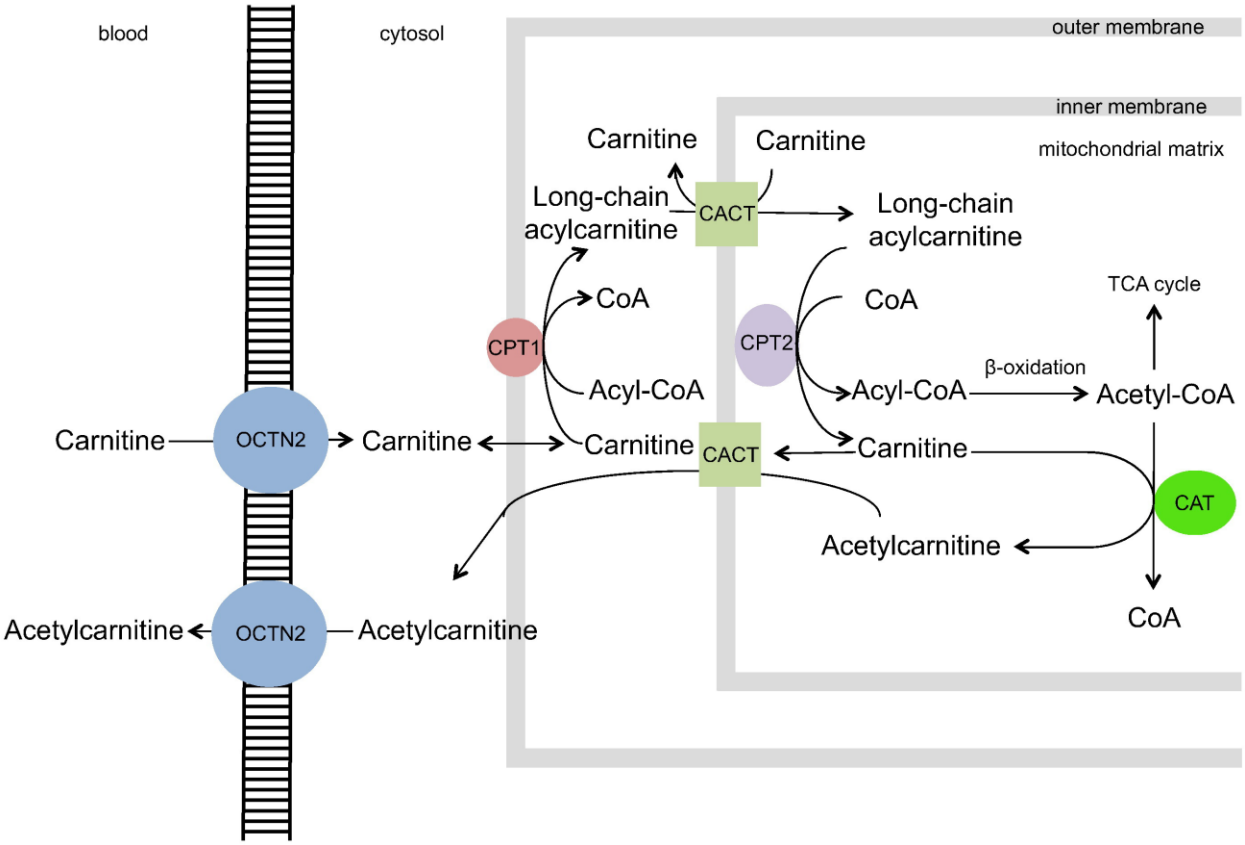


**Figure 1-2. Overview of carnitine transport into the cell and its enzymatic conversions in the mitochondria**

Carnitine enters the cell from the blood through an organic cation transporter (OCTN2), after which carnitine palmitoyl transferase I (CPT1) facilitates the conversion of carnitine and long chain fatty acid-CoAs to acylcarnitines and coenzyme A (CoA). The transporter carnitine-acylcarnitine translocase (CACT) moves the newly formed long-chain acylcarnitines into the mitochondrial matrix in exchange for free carnitine. Here, long chain acyl groups are transferred back to CoA by carnitine palmitoyl transferase II (CPT2). The newly regenerated acyl-CoA undergoes β-oxidation into Acetyl-CoA, which feeds into the TCA cycle. Alternatively, carnitine acetyl-transferase (CAT) converts free carnitine and Acetyl-CoA to acetylcarnitine, which can freely diffuse through CACT and OCTN2 back into the bloodstream. This latter process may be enhanced during sepsis and times of metabolic stress, serving as a crucial sink for excess acetyl groups that may be toxic to the cell. The ladder cartoon represents the plasma membrane separating the blood and the cytosol of the cell, while grey boxes represent the outer and inner membranes of a mitochondrion. (Open-source through the Creative Commons Attribution, obtained with permission from https://doi.org/10.1016/j.ebiom.2017.01.026).[38]

In sepsis, mitochondrial dysfunction has been increasingly reported as a critical factor in persistent organ failure and altered peripheral cell mitochondrial function is known to be associated with sepsis mortality.[39,40] Further evidence of mitochondrial dysfunction includes elevations of systemic acylcarnitines, indicating incomplete β-

oxidation of fatty acids, and the presence of mitochondrial DNA in plasma.[25,26] Sepsis alterations in mitochondrial metabolic function and lipid metabolism are associated with kidney and liver function that are driven in part through inhibition of the pyruvate dehydrogenase complex and decreased activity of carnitine palmitoyltransferase I.[41,42] Prior clinical studies of intravenous (IV) L-carnitine and acetylcarnitine given to patients in cardiogenic and circulatory shock found an overall benefit on hemodynamic parameters and patient survival.[43-45]

### *1.4.2 L-carnitine as a targeted metabolic therapeutic in sepsis*

These principles served as the basis for two recent clinical trials of L-carnitine in septic shock. The first was a phase I, randomized, double-blind clinical trial of L-carnitine (12 g IV) vs. saline placebo conducted in 31 patients with septic shock enrolled within 16 hours of diagnosis.[46] Study drug was given as an IV bolus (33% of total dose), followed by a 12-hour infusion that delivered the remaining drug. This study found no difference in the reduction of Sequential Organ Failure Assessment (SOFA) score at 24 hours, but there was an improvement in mortality at 28 days (4/16 vs. 9/15, p=0.048) and 1-year (8/16 vs. 12/15, p = 0.081) in L-carnitine treated patients. Adverse events sometimes attributable to L-carnitine, including gastrointestinal distress, body odor, and a decreased seizure threshold were not observed in the study. In addition, serious adverse events were not significantly different between the L-carnitine and placebo treatment arms. A follow-up phase II multicenter, double-blind, adaptive dose-finding trial randomized 250 patients within 24 hours of identified septic shock to IV L-carnitine (6 g, 12 g, or 18 g) vs. placebo.[47] In the primary analysis, the highest dose (18 g) of L-carnitine was not found to be superior to placebo in reducing the total SOFA score at 48

hours, and the predicted probability of success of a subsequent phase III trial in reducing mortality at 28 days did not exceed the *a priori* threshold of 90%. The 6 g and 12 g L-carnitine doses underperformed in the trial and were adaptively dropped from the randomization scheme as the trial progressed.

However, the primary endpoints of both clinical studies do not describe a critical component of drug response to supplemental L-carnitine in patients with septic shock. The pharmacometabolomics data from the Phase I trial reveal substantial interpatient variability in serum carnitine and acetylcarnitine concentrations post-infusion.[29,30] Patients receiving L-carnitine in the phase I study had 24-hour post infusion (T24) serum carnitine levels ranging from 30 µM to over 1600 µM (median = 368 µM). The temporal changes in carnitine and acetylcarnitine for the treatment and placebo arms are shown in **Figure 1-3**. Critically, L-carnitine treated non-survivors (based on 1-year mortality) had elevated carnitine and acetylcarnitine (C2), short chain acylcarnitines (C3, C4, and C5), and long chain acylcarnitines (C14 and C16) compared to L-carnitine treated survivors. This suggests the observed variability in measured peak concentrations and metabolic response profiles are associated with clinical outcomes. As such, identification of the patient-level factors associated with peak carnitine/acylcarnitine concentrations may help identify patient most likely to derive a mortality benefit from L-carnitine and inform the design of future clinical studies.

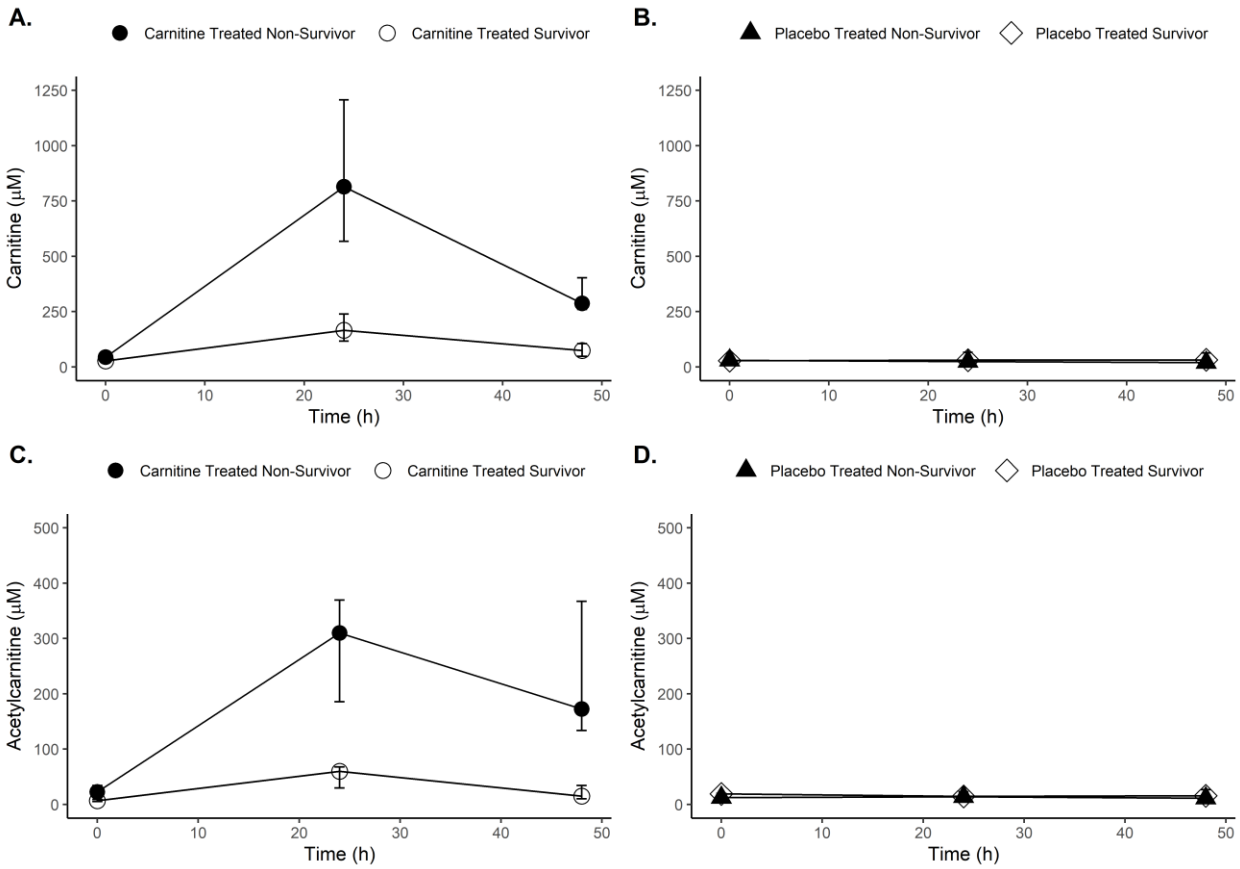


**Figure 1-3. Levocarnitine induced metabolic phenotype.**

Serum carnitine and acetylcarnitine concentrations are elevated in sepsis non-survivors. Concentrations of carnitine and acetylcarnitine are plotted over time for patients treated with either L-carnitine (panels A and C) or saline placebo (panels B and D). Data plotted are the median, 25th, and 75th percentile of observed serum concentrations, and the Mann-Whitney U test was used to determine significant differences between non-survivors and survivors at each timepoint. All p-values are corrected for multiple comparison using a false discovery rate method according to Storey et. al and are reported as q-values.[48] L-carnitine treated non-survivors (N=7-8) at 1-year had significantly higher concentrations of carnitine relative to survivors (N=8) at baseline (BL, q=0.02); 24-hours (T24, q=0.004); and 48-hours (T48, q=0.02) post-treatment. Similar trends were observed for acetylcarnitine (BL, q=0.01; T24, q=0.003; and T48, q=0.02). No significant differences in carnitine or acetylcarntine concentrations were observed between placebo treated non-survivors (N=8-12) and survivors (n=3).

### *1.4.3 Candidate mechanisms of interpatient variability of drug response*

*Pharmacogenomics*

Pharmacogenomics seeks to explain variability in drug exposure and response based on genetic differences between individuals. Genetic variation in drug metabolizing enzymes, transporters, and targets impacts an individual's exposure

and/or response to a given pharmacologic therapy, which can manifest as distinct drug-response phenotypes. Genetic variability is known to alter patient response to across disease states and in medications commonly used in the intensive care unit (ICU).[49] Treatment and dosing paradigms which incorporate patient-specific pharmacogenomic data hold promise in decreasing adverse drug events (ADRs) and improving efficacy.[50] Moreover, rationale clinical trial enrollment based on pharmacogenomic phenotypes can foster a more homogenous patient cohort and target patient populations most likely to benefit from therapy (**Table 1-1**).

**Table 1-1. Patient variables that could influence clinical trials of sepsis therapeutics**

| ***Candidate mechanisms of interpatient variability*** | ***Impact on levocarnitine trial design and interpretation*** | ***Influence on improving precision medicine in sepsis*** |
|---|---|---|
| Pharmacogenomics | Genetic variance in the transport receptor of L-carnitine (OCTN2) may influence drug concentration at site of action | Stratify patients by genotype at the time clinical trial enrollment |
| Pharmacometabolomics | Baseline and dynamic metabolic signatures are associated with elevated drug concentrations and patient mortality | Target metabolic subgroups for trial enrollment and measure metabolic response signatures post treatment |
| Morphomics | Patient muscle mass and body composition may influence metabolic adaptability, energetic stores, and drug distribution | Consider variation in body size and composition when testing targeted metabolic therapeutics |
| Renal function and Pharmacokinetics (PK) | Altered renal clearance and reabsorption of drug and acyl- | Embedded clinical pharmacology studies to quantify sepsis- |

| | | |
|---|---|---|
| | metabolites may influence drug concentrations and patient outcomes | pathophysiology induced alterations in drug PK |

Genetic variability in a number of enzymes and transporters could contribute to L-carnitine drug response including those highlighted in the carnitine shuttle (**Figure 1-2**). Carnitine acts intracellularly and is highly sequestered in skeletal muscle and other tissues of the body.[34] Given the polar structure of carnitine, active sodium-dependent transport by organic cation/carnitine transporters (OCTNs) is required for entry from the blood into the cell and subsequent facilitation of fatty acid β-oxidation. The primary carnitine transporter, OCTN2, thus represents the focus of this section.

The OCTN2 transporter is encoded by the SLC22A5 gene located on chromosome 5q31.1. Spanning 25 kb, the 10 exons of this gene encode the full length 557 amino acid protein. Numerous autosomal recessive mutations in the SLC22A5 gene are responsible for primary carnitine deficiency and results in low serum carnitine levels due to the kidney's impaired ability to reabsorb the molecule.[51] Missense mutations are exceedingly rare, result in severe metabolic and mitochondrial dysfunction, and manifest clinically as a primary carnitine deficiency at a young age. As such, loss of function mutations are unlikely to play a role in explaining variability in L-carnitine concentrations or response in clinical studies of adults with septic shock. Nonetheless, given the vital role of OCTN2 in carnitine uptake into the cell, and considering the large doses administered in these trials, more common genetic polymorphisms in OCTN2 resulting in reduced function and / or expression may improve understanding of the mechanisms that explain the broad dynamic range of carnitine concentrations following supplementation.

Common polymorphisms (i.e., minor allele frequency greater than 1%) in the OCTN2 gene and their impact on carnitine transport outside the context of primary carnitine deficiency are rare.[52-54] Three SNPs (Phe17Leu, Tyr449Asp, Val481Asp) were associated with reduced OCTN2 function compared to wild-type (WT), and a SNP in the promoter region of the gene (-207C>G) was associated with increased carnitine transport capacity and trended toward increase mRNA expression in cell lines.[52] Out of these, only the promoter region variant (-207C>G, rs2631367) could be considered common according to the National Center for Biotechnology Information database of genetic variation (dbSNP).[55] Further studies have observed a tissue-specificity to the -207C>G variant's effect on mRNA expression levels.[53,54]

To supplement the limited literature regarding common polymorphisms effecting OCTN2, we conducted a systematic bioinformatics search for potentially relevant SNPs. We queried the Genotype-Tissue Expression (GTEx) Project (available at https://gtexportal.org/home/), which seeks to explain variability in mRNA expression levels from previously healthy human cadavers with whole genome sequencing.[56] The goal of this query was to determine common genetic variants (i.e., SNPs) that significantly alter gene expression of the OCTN2 transporter. Using expression quantitative trait loci (eQTL) analysis, approximately 1500 variants were found to be associated with altered gene expression at the tissue level. Summing across more than 6,000 SNP/tissue pairs, the variant with the largest effect on net OCTN2 gene expression was the promoter region variant (-207C>G, rs2631367).

In previously unpublished data from our group, patients treated with L-carnitine in the phase I trial[46] were genotyped for the OCTN2 (-207C>G) SNP. In this preliminary

study, fourteen patients had both genomic and serum carnitine concentrations measured at 24 hours (T24). Among these, four patients were wild-type (CC), while ten carried one or two copies of the G allele. Patients with the C/G or G/G genotype trended toward lower T24 plasma levels of L-carnitine (p=0.11), suggesting that genetic variation in the OCTN2 transporter may contribute to variability and persistent elevations in L-carnitine following supplementation during septic shock. More pharmacogenetic studies are needed and are underway in the phase II trial[47] to determine if variation in OCTN2 and other carnitine-specific enzymes and / or transporters explain interpatient variability in L-carnitine drug response.

*Pharmacometabolomics*

Metabolomics seeks to identify and quantify small molecules, the full collection of which define the metabolome, in a given biofluid.[16] The metabolome constitutes a read-out of underlying cellular and biochemical events that reflect the genetic makeup of the host, transcriptomic and proteomic influence, as well as variability in the microbiome and environmental exposure (**Figure 1-1**). As such, metabolomics represents the culmination of these important regulators on the host. In addition, given that metabolism is dynamic on a practical and physiological time-scale, this sensitivity can inform heterogeneity in disease trajectory and treatment response. Pharmacometabolomics exploits this paradigm and is aimed at understanding and predicting response to drug treatment. In short, clinical application of metabolomics holds great promise in improving the diagnosis and risk stratification of critically ill patients, furthering drug

discovery through metabolic signatures of drug response and/or ADRs, and elucidating biochemical pathways involved in the pathophysiology of critical illness (**Table 1-1**).

A pharmacometabolomic approach was utilized to understand baseline metabolic differences in patients treated in the Phase I study of L-carnitine.[30] Patients treated with L-carnitine who had low baseline levels of the ketone body, 3-hydroxybutyrate, also had lower post-treatment carnitine levels at 24 hours. The L-carnitine treated, low-ketone patients also had better clinical outcomes as evidenced by a timelier reduction in vasopressor requirement and decreased 1-year mortality. A follow-up, an untargeted metabolomics approach was employed in male patients from the Phase I study.[33] L-carnitine treated non-survivors were found to have post-treatment elevations in metabolites related to vascular inflammation including histamine, allysine, and fibrinopeptide A. Along with the differential metabolic response of survivors and non-survivors highlighted in **Figure 1-2**, these data suggest both baseline metabolic signatures and metabolic profiles over time may be predictive of L-carnitine treatment responsiveness.

*Morphomics*

Analytic morphomics is a new and rapidly growing scientific discipline within precision pharmacotherapy that studies how variation in body size, composition and structure are associated with drug and disease response.[57] In sepsis, two recent meta-analyses have observed a paradox between body composition and survival, whereby particularly overweight (BMI between 25 kg/m$^2$ and 29.9 kg/m$^2$), and to a lesser extent obese (BMI between 30 kg/m$^2$ and 40 kg/m$^2$), patients tend to have better mortality outcomes compared to normal weight individuals (BMI between 18.5 kg/m$^2$ and 24.9

kg/m$^2$).[58,59] Notably, underweight (BMI less than 18.5 kg/m$^2$) and morbidly obese (BMI greater than 40 kg/m$^2$) patients were found to have similar risk of mortality relative to normal weight individuals. Neither measured peak concentrations of L-carnitine nor mortality were significantly associated with BMI in patients who received study drug in the phase I study. However, the observed "obesity paradox" reinforces the concept of a metabolic and energy-driven component to sepsis pathophysiology and has a number of possible pathophysiological explanations including increased energy stores, anti-inflammatory mediator release from adipose tissue, and lipoprotein binding of bacterial cellular components.[60]

Another possible explanation is that increased muscle mass offers energetic and metabolic adaptability to patients within a window of the BMI spectrum. Protein catabolism and subsequent myopathy is observed in critically ill patients, and skeletal muscle, an important energetic source to the host, sustains mitochondrial injury over the course of sepsis.[61] Indeed, recent studies have found an association between low muscle mass and increased risk of mortality for patients with sepsis. In 74 patients with liver cirrhosis and sepsis, patients with low muscle mass (defined as mid-arm muscle circumference lower than the 5$^{th}$ percentile of the population) had increased mortality compared to patients with normal muscle mass (47% compared to 26%, p=0.06).[62] In a separate retrospective review of 627 patients with a diagnosis of sepsis and an available abdominal computed tomography scan of the psoas muscle, muscle mass depletion was associated with 28-day mortality in both univariate and multivariate logistic regression (OR 2.79, p=0.01).[63] Given the extent of protein catabolism, the sepsis-obesity paradox, and the known sequestering of carnitine into muscle tissue,

morphomics and variability in body composition offers a currently untapped field that could aid in explaining the observed variability in response to supplemental L-carnitine and patient mortality in sepsis broadly (**Table 1-1**).

*Pharmacokinetics and Renal Function*

Pharmacokinetics (PK) as a science seeks to understand what the body does with and to drugs. More specifically, it is the study of how drugs are absorbed, distributed, metabolized, and eliminated from the body. Previous studies have highlighted that there is profound sepsis-induced variation in drug PK. The reasons for this are likely multifaceted but include altered protein binding, perturbed vascular and tissue permeability, decreased hepatic and renal blood flow, and lower activity of drug metabolizing enzymes.[64] High interpatient variability in drug PK in sepsis clinical trials contributes to overall heterogeneity of the patient cohort and may confound trial results unless careful analysis of drug exposure is considered (**Table 1-1**).

The PK of L-carnitine has been described, however no studies have determined the precise PK of L-carnitine in sepsis or at such high intravenous doses. As discussed above, OCTN2 is a critical carnitine transporter that is responsible for carnitine uptake into cells/tissues, however it is also responsible for reabsorption of carnitine in the kidney proximal tubule. As such, kidney function may play a vital role in the interpatient variability in serum carnitine concentrations that result following supplementation. Previous reviews, report an average renal clearance of endogenous carnitine of 1-3 mL/min, indicating that, at physiologically relevant concentrations, up to 99% of carnitine is reabsorbed by the kidney.[65] Exogenous carnitine administered to healthy volunteers, increased renal clearance of carnitine and acetylcarnitine, indicating saturation of the

OCTN2 transporter and the reabsorption process, which may be relevant for supraphysiologic doses of intravenous carnitine like those given in septic shock trials.[65] Unfortunately, urine samples were not collected in these studies, which prevents us from estimating renal clearance of relevant carnitine species in these patients. Both studies reported similar serum creatinine levels among survivors and non-survivors indicating renal function alone does not explain heterogeneity in L-carnitine and acylcarnitine concentrations among patients. However, the reliability of creatinine as a biomarker in the setting of acute kidney injury (AKI), sepsis and other critical illness, and in drug development broadly been called into question.[66,67] New investigations of biomarkers of kidney injury and function are underway, but have yet to be widely adapted or clinically validated. Further investigations of the variability in L-carnitine drug response stratified by the presence of AKI and acute liver injury, and among other measures of organ dysfunction are warranted before precise clinical recommendation can be made in these patient groups. Moreover, modeling the impact of patient-level biological variables such as sex, age, and race is critical to understand the observed heterogeneity in L-carnitine drug response.

### 1.5 Thesis aims and objectives

Sepsis is a clinical syndrome characterized by substantial clinical and biological heterogeneity such that the individual host response to infection and treatment strategies are highly variable. This profound variability has led to an incomplete understanding of sepsis pathophysiology at the patient level and resulted in a litany of failed clinical trials. Part of the variability in patient outcomes and response to therapy in sepsis may be explained by metabolomics, particularly by a more complete and

dynamic assessment of an individual's metabolic status. The *central hypothesis* of this dissertation is that there are distinct metabolic signatures related to carnitine homeostasis and mitochondrial metabolic function that are associated with sepsis mortality and other clinical outcomes. To advance precision pharmacotherapy and address knowledge gaps in the mechanisms that underlie sepsis heterogeneity I leveraged time-series blood samples and metabolomics data from the phase II, placebo-controlled, clinical trial – RACE (Rapid Administration of Carnitine in sEpsis).[47,68]

My first aim was *to determine baseline and dynamic metabolic signatures associated with mortality in the 'natural' sepsis phenotype (i.e., placebo treated patients).* This aim is addressed in chapters 2 and 3 using baseline and longitudinal metabolomics data from patients who were randomized to receive saline placebo. Chapter 2 focuses on the association between dynamic metabolic changes and mortality, outcomes among metabolic subgroups derived through unsupervised machine learning, and the correlation between metabolites and protein biomarkers of the host-response. In contrast, chapter 3 emphasizes the relationship between metabolites and non-mortality endpoints, namely the need for persistent organ support and life sustaining interventions.

My second aim was *to identify metabolic, genomic, and patient-level factors associated with L-carnitine pharmacokinetics and patient outcomes.* Chapter 4 describes the search for heterogeneity of treatment effect to L-carnitine based on an individual's baseline metabolic status, while chapter 5 explains a population PK model

for L-carnitine in septic shock and the important patient factors that impact response to the drug.

Finally, in chapter 6 I summarize the implications and the outlook of this work. This includes an assessment of its strengths and limitations, as well as a glimpse at future projects that could leverage data from the RACE cohort.

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## Chapter 2: Sustained Perturbation of Metabolism and Metabolic Subphenotypes are Associated with Mortality and Protein Markers of the Host Response

This work has been submitted as an original research manuscript and is under revision. It was presented in abstract form at the 2022 American Thoracic Society International Meeting.[1]

### 2.1 Chapter Abstract

Perturbed host metabolism is increasingly recognized as a pillar of sepsis pathogenesis, yet the dynamic alterations in metabolism and its relationship to other components of the host response remain incompletely understood.

We sought to characterize the early host-metabolic response in patients with septic shock and to explore bio-physiological phenotyping and differences in clinical outcomes among metabolic subgroups.

We measured serum metabolites and proteins reflective of the host immune and endothelial response in patients with septic shock. We considered patients from the placebo arm of a completed phase II, randomized controlled trial. Patients had moderate organ dysfunction and were identified within 24-hours of septic shock. Serum was collected at baseline (within 24-hours of the identification of septic shock), 24-, and 48-hours post enrollment. Linear mixed models were built to assess the early trajectory of protein analytes and metabolites stratified by 28-day mortality status. Unsupervised

clustering of baseline metabolomics data was conducted to identify subgroups of patients.

Fifty-one metabolites and 10 protein analytes were measured longitudinally in 72 patients with septic shock. In the 30 (41.7%) patients who died prior to 28-days, systemic concentrations of short-chain acylcarnitines and IL-8 were elevated at baseline and persisted at T24 and T48 throughout early resuscitation. Concentrations of pyruvate, IL-6, TNF-α, and angiopoietin-2 decreased at a slower rate in patients who died. Two groups emerged from clustering of baseline metabolites. Group 1 was characterized by higher levels of acylcarnitines, greater organ dysfunction at baseline and post-resuscitation ($p<0.05$), and greater mortality over one-year ($p<0.001$).

Among patients with septic shock, non-survivors exhibited a more profound and persistent dysregulation in protein analytes and metabolism related to mitochondrial function and neutrophil activation.

## 2.2 Introduction

Sepsis is a syndrome characterized by a dysregulated host response to an infection, which manifests as life-threatening multiorgan dysfunction [2]. The clinical trajectory and outcomes for patients with sepsis is highly heterogeneous, with mortality in septic shock exceeding 40% [3]. The mechanisms underlying this variability in patient outcomes remain poorly understood, threatening the development of novel pharmacotherapy and improvement of care for patients with sepsis [4]. Identification of the biological mechanisms underlying sepsis heterogeneity holds promise in personalizing care for patients with sepsis through predictive and prognostic enrichment, disease subphenotyping, and rational drug discovery [5].

Altered metabolism and host bioenergetics are increasingly recognized as cornerstones of sepsis pathophysiology and sepsis heterogeneity [6]. The Surviving Sepsis Campaign recently listed an improved understanding of sepsis-induced metabolic disruptions as one of five key translational research questions in the field, and elevated lactate concentrations are associated with mortality and are formally codified in the definition of septic shock [2,7]. Clearance of lactate has also been proposed as a metabolic biomarker and physiologic endpoint of randomized controlled clinical trials [8-10], yet the kinetics and bioenergetics of lactate are complex and remediation of elevated concentrations have not consistently translated to improved survival [11-14]. This stresses the need for additional prognostic and predictive metabolic biomarkers beyond lactate in patients with sepsis.

Metabolomics is a discovery science that seeks to identify and quantify small molecules in a given biospecimen [15]. In sepsis and other critical illnesses, metabolomics studies have consistently identified differences among patients stratified by mortality and organ function [16-18], and shown promise in understanding variable response to treatment [19,20]. These studies have implicated mitochondrial dysfunction, protein catabolism, and perturbed lipid metabolism as potential drivers of poor patient outcomes. However, most studies in critical care metabolomics have been cross-sectional, leveraging single blood samples of convenience, and included patients with highly variable illness severity and/or diagnosis at presentation.

In this study, we analyzed longitudinal serum samples from patients in the placebo arm of the Rapid Administration of Carnitine in sEpsis (RACE) clinical trial [21]. Our primary goal was to characterize the early host-metabolic response in patients with

vasopressor-dependent shock. We also sought to determine if metabolomics data alone could identify clusters or subphenotypes of septic shock and assess the relationship between metabolites and protein biomarkers of the host response.

## 2.3 Methods

### *2.3.1 Study population and design*

We performed a secondary analysis of patients who were randomized to the saline placebo arm of the RACE clinical trial [21]. Abbreviated inclusion criteria for the parent trial included: 1) enrollment within 24-hours of the identification of septic shock; 2) a blood lactate level exceeding 18 mg/dL; 3) a Sequential Organ Failure Assessment (SOFA) score of at least six; and 4) receipt of high-dose vasopressors within 4 hours of enrollment [22]. Patients in the placebo arm received a 20 mL bolus of 0.9% normal saline (NS) followed by a 1 liter 12-hour infusion, rather than an equivalent volume of L-carnitine. Patients in this analysis were also required to have at least one serum sample available for metabolomics. We excluded patients who were allocated to receive intravenous L-carnitine to focus on the metabolic trajectory of the disease course without any interference that may be introduced by the study drug. Furthermore, we have previously identified phenotypes of L-carnitine response [20]. The trial protocol was approved by the institutional review board of all 16 centers and registered with clinicaltrials.gov (NCT01665092) prior to patient enrollment. All patients or their legally authorized representative provided informed consent prior to randomization.

### *2.3.2 Serum sampling and assays*

Blood samples were collected at enrollment (baseline or Day 0), 24-hours (Day 1), and 48-hours (Day 2) after saline initiation (**Figure 2-1**). Serum was obtained from whole blood, frozen at -80°C, and shipped on dry ice to the University of Michigan College of Pharmacy NMR Metabolomics Laboratory to await metabolomics assays. Further details regarding sample processing from this trial have been reported elsewhere [20,23].


**Figure 2-1. Abbreviated inclusion criteria and study flow diagram**

Patients with vasopressor dependent septic shock from the Rapid Administration of Carnitine in Sepsis (RACE) trial were considered. Patients randomized to receive saline placebo and who had at least one

serum sample available for analysis were included in this secondary analysis. Longitudinal serum samples were analyzed for acylcarnitines by liquid chromatography – mass spectrometry (LC-MS), small polar metabolites by nuclear magnetic resonance (NMR), and protein markers of the host response by immunoassays.

For our analysis we used existing metabolomics data that was generated as part of an ancillary metabolomics study to the RACE trial [20]. We employed the same metabolomics strategy as our prior work which included the measurement of low-molecular weight, polar metabolites as measured by NMR spectroscopy using previously described methods [19]. Briefly, spectra were acquired on a Varian (now Agilent, Inc., Santa Clara, CA) 11.74 Tesla (500 MHz) NMR spectrometer [24]. Spectra were acquired at room temperature using a 1 H,1 H-NOESY (METNOSEY) pulse sequence and analyzed using the Processor and Profiler modules of the Chenomx NMR Suite 8.2 (Edmonton, AB, Canada) software. Acylcarnitines were measured by reverse-phase liquid chromatography with tandem mass spectrometry (LC-MS/MS) [25]. Internal standards (NSK-B Cambridge Isotope Laboratories) were used to allow for absolute quantification of L-carnitine and C2, C3, C4, C5, C8, C14, and C16, while other acylcarnitine species were measured using relative quantification by peak area.

Residual serum was used to measure inflammatory and immune-related cytokines of the host-response on a Milliplex® magnetic bead immunoassay panel run on a Luminex™ 200™ Instrument. Angiopoietin-2 (ANG-2) was measured using a commercially available enzyme-linked immunoassay (Invitrogen™, Thermo Fisher) in accordance with the manufacturer's instructions.

### *2.3.3 Statistical analyses and outcomes*

Missing data for metabolites quantified by NMR and protein markers were assumed to be left-censored and below the limit-of-detection [26]. For each analyte, we

imputed missing observations as the minimum concentration observed divided by two. Following imputation, concentrations were log-transformed and z-scaled to have a mean of zero and standard deviation of one. There were no missing data for acylcarnitines quantified by LC-MS.

For the primary analysis, we fit a series of general linear mixed models to assess metabolite concentrations over time in patients stratified by their mortality status at 28-days. Similar approaches have been successfully used in the analysis of multi-omics biomarker data in other cohorts of patients with critical illness [27,28]. For each metabolite we built: 1) a null model consisting of a patient-level (random) intercept; 2) a fixed-effect model which added *Time* (Baseline, Day 1, Day 2) and *Mortality Status* (Survivor vs. Non-Survivor at 28-days); and 3) an interaction model which further added a *Time*Mortality* interaction. All models contained patient age, sex, and baseline Sequential Organ Failure Assessment (SOFA) Score as covariates.

Models were compared based on the approximate F-test according to the Kenward-Roger approach with the 'pbkrtest' R package [29,30]. We selected between the fixed-effect and interaction models, opting for the latter when the interaction p-value was less than 0.05. The overall p-value value was then determined by comparing the selected model to the null model. The resulting p-values were rank-ordered to determine the most perturbed signals and corrected for multiple comparison according to the false discovery procedure of Benjamini and Hochberg [31]. Analyte concentrations of sepsis survivors and non-survivors were visualized on the log10-scale, with differences at each timepoint assessed by the Mann-Whitney U test. We assessed the

correlation between metabolites and protein biomarkers over time using repeated measure correlation analysis using the 'Rmcorr' R package [32].

We also sought to identify if there were clusters or metabolic subtypes of patients with septic shock at trial enrollment. We employed an unsupervised clustering approach (i.e., naïve to patient demographics, outcomes, and clinical status) using baseline metabolomics data and the K-means algorithm [33]. To prevent overfitting, clustering was done on the first five principal components of the metabolomics data, representing ~70% of the variance in the dataset. The optimal number of clusters was selected using the Silhouette method and the 'NbClust' R package [34], and the individual metabolites driving cluster separation were visualized using the 'ComplexHeatMap' R package [35].

We assessed for patient differences in demographics, comorbidities, and organ dysfunction between metabolic clusters using linear mixed models, the Mann-Whitney U-test, or Chi-Square test, as appropriate. We also tested the impact of metabolic cluster assignment on mortality status at 28-days using logistic regression and on mortality out to one-year using Kaplan-Meir analysis and the log-rank test. All data analysis and figure generation were completed using the statistical programming language R (version 4.1.0) [36]. All raw data and the code used to conduct our analysis are available on Github (https://github.com/UMichNMR-Metabolomics, accessed 06/22/2022).

## 2.4 Results

### *2.4.1 Patient characteristics, metabolites and protein analytes of the host response*

Seventy-two patients from the RACE trial were randomized to receive saline placebo and had at least one serum sample available for metabolomics (**Figure 2-1**). Of these, 30 patients (41.7%) died within 28-days of trial enrollment. Patients who died had greater organ dysfunction but were similar with respect to age, sex, and baseline co-morbidities as measured by a modified version of the Charlson Comorbidity Index [1,37] (**Table 2-1**).

**Table 2-1. Patient demographics, laboratory values, and physiologic parameters stratified by 28-day mortality.**

| **Patient Characteristic** | **28-Day Survivor, N = 42[1]** | **28-Day Non-Survivor, N = 30[1]** | **p-value[2]** |
|---|---|---|---|
| Age (years) | 62 (54, 70) | 62 (47, 72) | 0.8 |
| Sex | | | 0.4 |
| *Female* | 22 (52%) | 13 (43%) | |
| *Male* | 20 (48%) | 17 (57%) | |
| Self-Reported Race | | | >0.9 |
| *African American* | 13 (31%) | 9 (30%) | |
| *Caucasian* | 29 (69%) | 21 (70%) | |
| Total SOFA Score | 11 (7, 13) | 13 (9, 15) | 0.016 |
| Charlson Comorbidity Index | 4.00 (2.25, 4.75) | 5.00 (3.00, 5.75) | 0.4 |
| Clinical Lactate (mmol/L) | 3.15 (2.58, 6.20) | 4.20 (2.90, 6.50) | 0.9 |
| *Unknown (N)* | 6 | 5 | |
| Creatinine (mg/dL) | 1.90 (1.47, 2.70) | 1.81 (1.12, 2.88) | 0.6 |
| Platelet Count (cells/mm$^3$) | 165 (101, 227) | 146 (75, 215) | 0.4 |
| *Unknown (N)* | 0 | 1 | |
| Total Bilirubin (mg/dL) | 0.95 (0.40, 1.99) | 2.40 (0.70, 4.20) | 0.005 |
| *Unknown (N)* | 0 | 1 | |
| White Blood Cell Count (cells/mm$^3$) | 25 (14, 31) | 18 (12, 25) | 0.7 |
| *Unknown (N)* | 9 | 9 | |
| Body Mass Index | 29 (23, 37) | 24 (21, 35) | 0.14 |

| | | | |
|---|---|---|---|
| *Unknown (N)* | 0 | 1 | |
| Respiratory Rate (breaths/min) | 20 (17, 24) | 20 (16, 26) | 0.7 |
| Heart Rate (beats/min) | 101 (94, 113) | 104 (92, 114) | >0.9 |
| Cumulative Vasopressor Index | 4 (3, 8) | 6 (4, 8) | 0.092 |
| *Unknown (N)* | 2 | 1 | |

[1]Described as median (IQR) for continuous variables and n (%) for categorical variables
[2]Calculatd by one-way ANOVA for continuous variables or Pearson's Chi-squared test for categorical variables.

A total of 51 metabolites and 10 protein biomarkers of the host response were measured at baseline, 24-hours, and 48-hours. Metabolites included 24 acylcarnitines measured by LC-MS and 27 small, predominately polar, molecules by NMR spectroscopy. Protein analytes included markers of the host-immune and inflammatory response and endothelial cell activation. A full list of the measured analytes and the analytical platform used are provided in the first two columns of **Supplementary Table S-1**.

### *2.4.2 Early host response over time stratified by 28-day mortality status*

We fit linear mixed models for each protein analyte and metabolite and found the inclusion of fixed effects for time and mortality status improved model fit across 39 (76%) metabolites and 10 (100%) protein markers of the host response (overall FDR < 0.05). This indicates the concentration of analytes and metabolites indicative of the host response significantly changed throughout the early course of shock and/or by a patient's mortality status. In addition, the linear trajectory of 15 of these analytes and metabolites varied based on mortality status, as indicated by a significant *Time*Mortality* interaction, $p<0.05$.

The most perturbed signals across analytical platforms are highlighted in **Figure 2-2** and included the acylcarnitines, C2, C6, and C8 (LC-MS, **Figure 2-2A**); lactate, pyruvate, and isoleucine (NMR, **Figure 2-2B**); and IL-6, IL-8, and TNFα (protein immunoassays, **Figure 2-2C**). Patients who died experienced greater inflammation and mitochondrial dysfunction that were sustained over the two days post-enrollment, as evidenced by persistent elevations in IL-8 and short-chain acylcarnitines. IL-6, TNFα, and pyruvate concentrations were similar at individual timepoints (Mann Whitney U-test, $p>0.05$), but decreased, or were cleared, at a much slower rate in patients who died, as indicated by a significant and positive *Time*Mortality* interaction ($\beta_{Time*Mortality} > 0$, $p<0.05$). Lactate levels tended to steadily decline in both groups ($\beta_{Time} = -0.456$, 95% CI: -0.583, -0.33), while isoleucine levels increased ($\beta_{Time} = 0.346$, 95% CI: 0.223, 0.469).

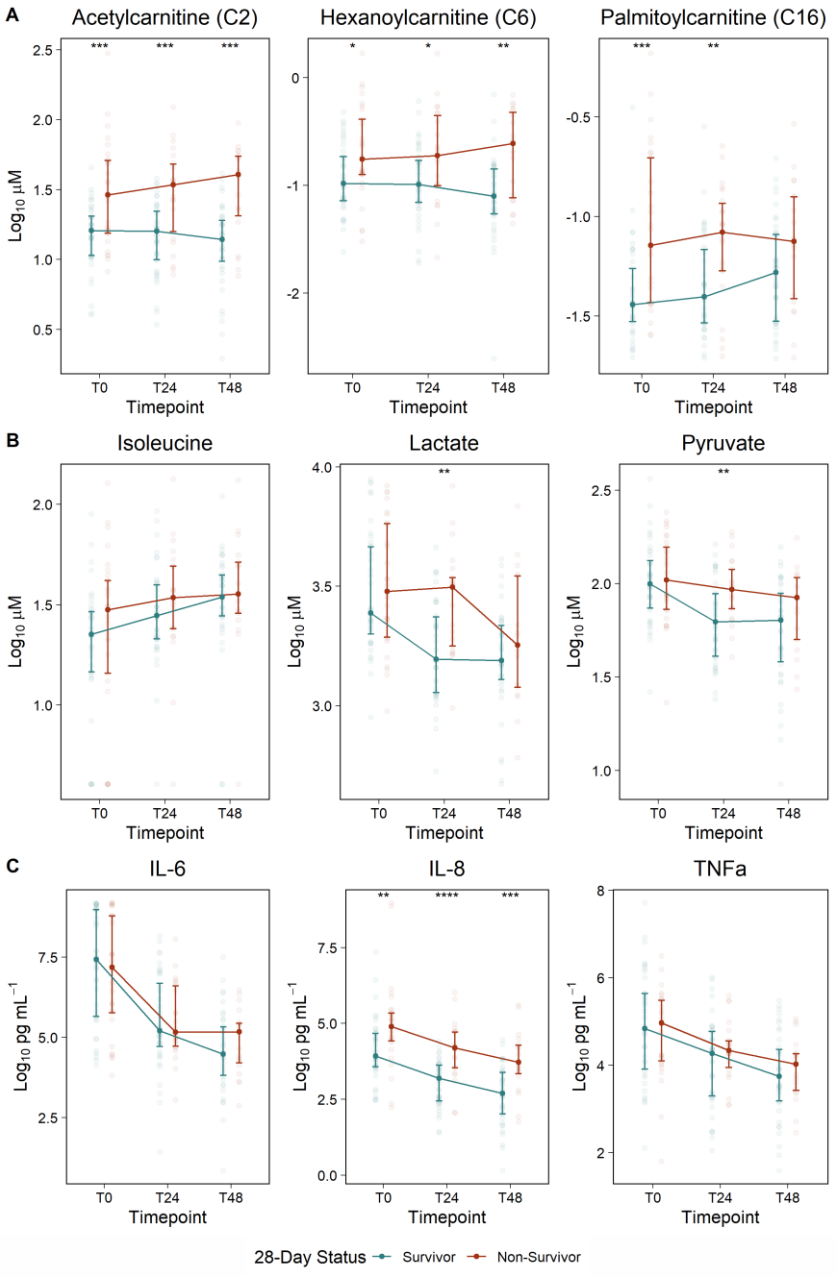


**Figure 2-2. Early host response in patients with septic shock stratified by 28-day mortality status.**

The most perturbed analytes across time and 28-day mortality status measured using three analytical platforms: (A) acylcarnitines measured by liquid chromatography – mass spectrometry (LC-MS); (B) Metabolites measured by nuclear magnetic resonance (NMR); and (C) Proteins measured by immunoassays. Analyte concentrations at each timepoint are visualized with the median ± 25$^{th}$ and 75$^{th}$ percentiles, with differences between mortality groups assessed by the Mann-Whitney U test. Analytes presented here were chosen according to the rank-ordered overall p-value from linear mixed modeling as described in the Methods section.

The full linear mixed modeling results for all protein analytes and metabolites are shown in Supplementary **Table S-1**. Numerous metabolites were elevated in patients

who did not survive past 28-days, indicated by a $\beta_{Mortality}$ coefficient significantly greater than zero. This included many acylcarnitines of varying chain length, 2-hydroxybutyrate and 3-hydroxybutyrate (a ketone body), propylene glycol, and amino acids (methionine, glycine, alanine, tyrosine, and glutamine). Among patients who died, concentrations of angiopoietin (ANG)-2 decreased more slowly ($\beta_{Time*Mortality} > 0$), while concentrations of nine long-chain acylcarnitines decreased more rapidly ($\beta_{Time*Mortality} < 0$) than in survivors.

Repeated measure correlations between protein analytes and metabolites are shown in **Figure 2-3**. Notably, strong positive correlations included those between numerous inflammatory cytokines and markers of endothelial activation (ANG-2 and fractalkine) with lactate and pyruvate. In contrast, branched-chain and numerous other amino acids tended to be negatively correlated with inflammatory cytokines. Similarly, long-chain acylcarnitines were negatively correlated with endothelial markers.

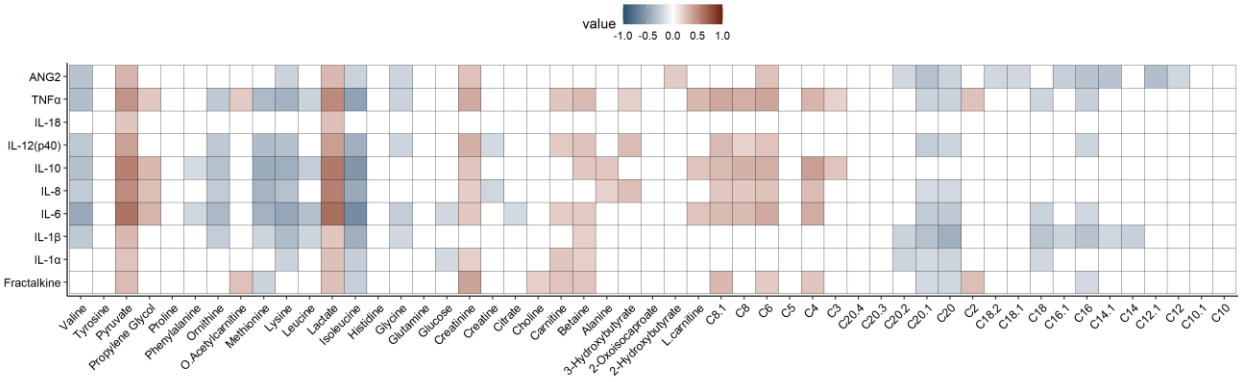


**Figure 2-3. Repeated-measure correlations between protein and metabolite analytes.**

The Rmcorr R package was used to determine the repeated-measure correlation coefficient between proteins and metabolites measured across three timepoints. The ggcorrplot R package was used to visualize the results. Positive correlations are indicated in red, while negative correlations are shown in blue, and only significant ($p<0.05$) correlation pairs are included.

### *2.4.3 Baseline Metabolic Clustering*

Unsupervised clustering of baseline metabolomics data revealed two distinct groups of patients. The heatmap of metabolites driving separation demonstrates significant metabolic heterogeneity among patients with septic shock at trial enrollment (**Figure 2-4A**). Clusters differed most dramatically across medium and long chain acylcarnitines. Across clusters, patient age, sex, self-identified race, and baseline comorbidities were similar (**Figure 2-4B**).

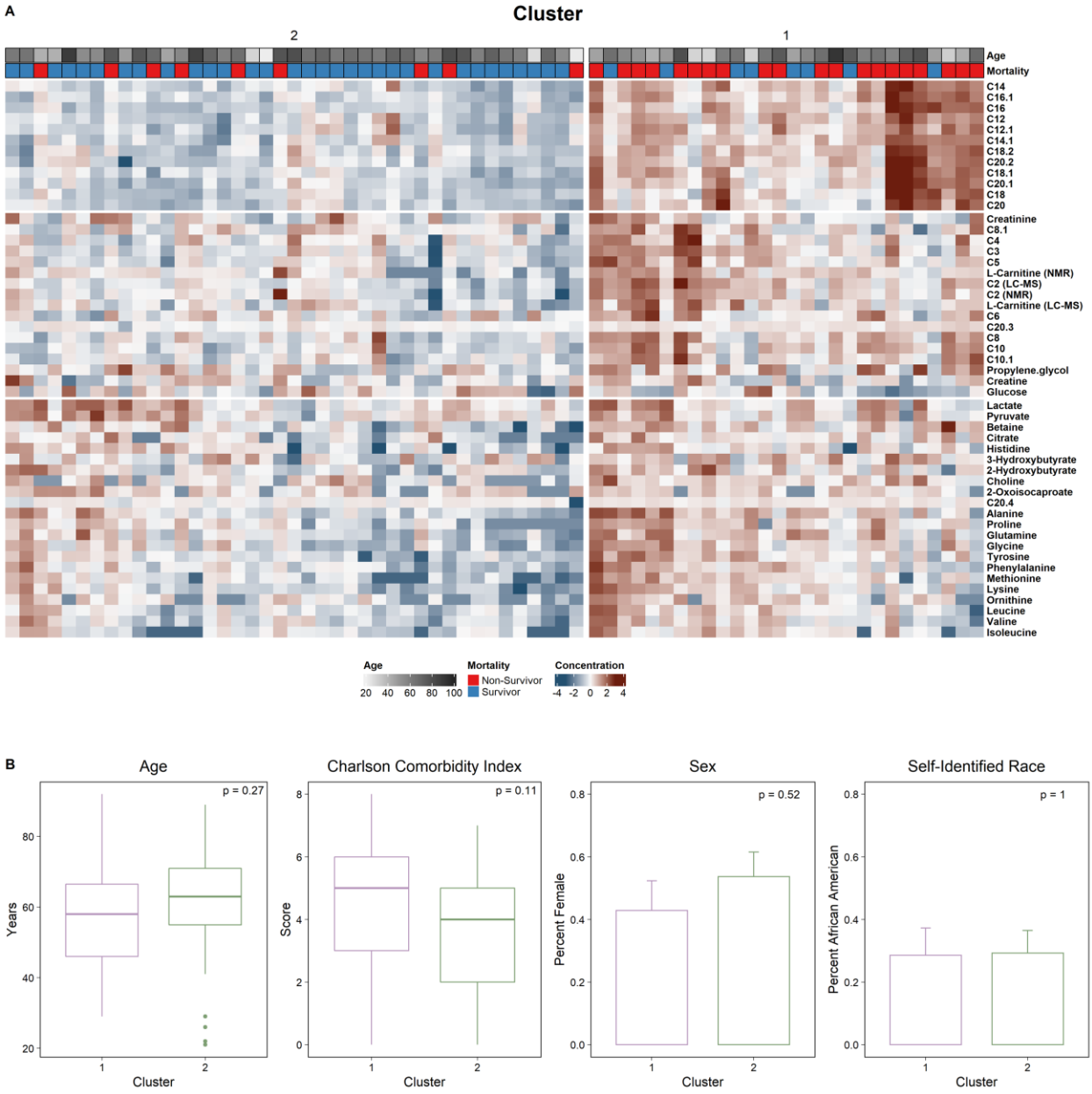


**Figure 2-4. Metabolite concentration and patient characteristics stratified by cluster assignment.**

(A) Heatmap comparison of metabolites stratified by cluster assignment. Concentrations of metabolites were log-transformed and z-scaled as described in the methods. Patients were clustered after principal component analysis of the baseline metabolomics data, with two groups best separating the data (Cluster 1: N=28 and Cluster 2: N=41). Patient age and 28-day mortality are shown as annotation above the heatmap. (B) Patient demographics, comorbidities, sex, and self-identified race stratified by metabolic cluster assignment. P-values reported are from the Mann-Whitney U-test or Chi-Square test, as appropriate.

We assessed the trajectory of organ dysfunction between groups using linear mixed models with a patient-level intercept and time, cluster, and their interaction as

fixed effects (**Figure 2-5A**). The linear SOFA score trajectory was not different between metabolic clusters ($\beta_{Time*Cluster1}$ = 0.536, 95% CI: -0.15, 1.22, p=0.12); however, patients in cluster one had worse organ function at baseline (Mann-Whitney U-test, p <0.05), which was sustained over the early course of their illness (Mann Whitney U-test at T24 and T48, p<0.01).

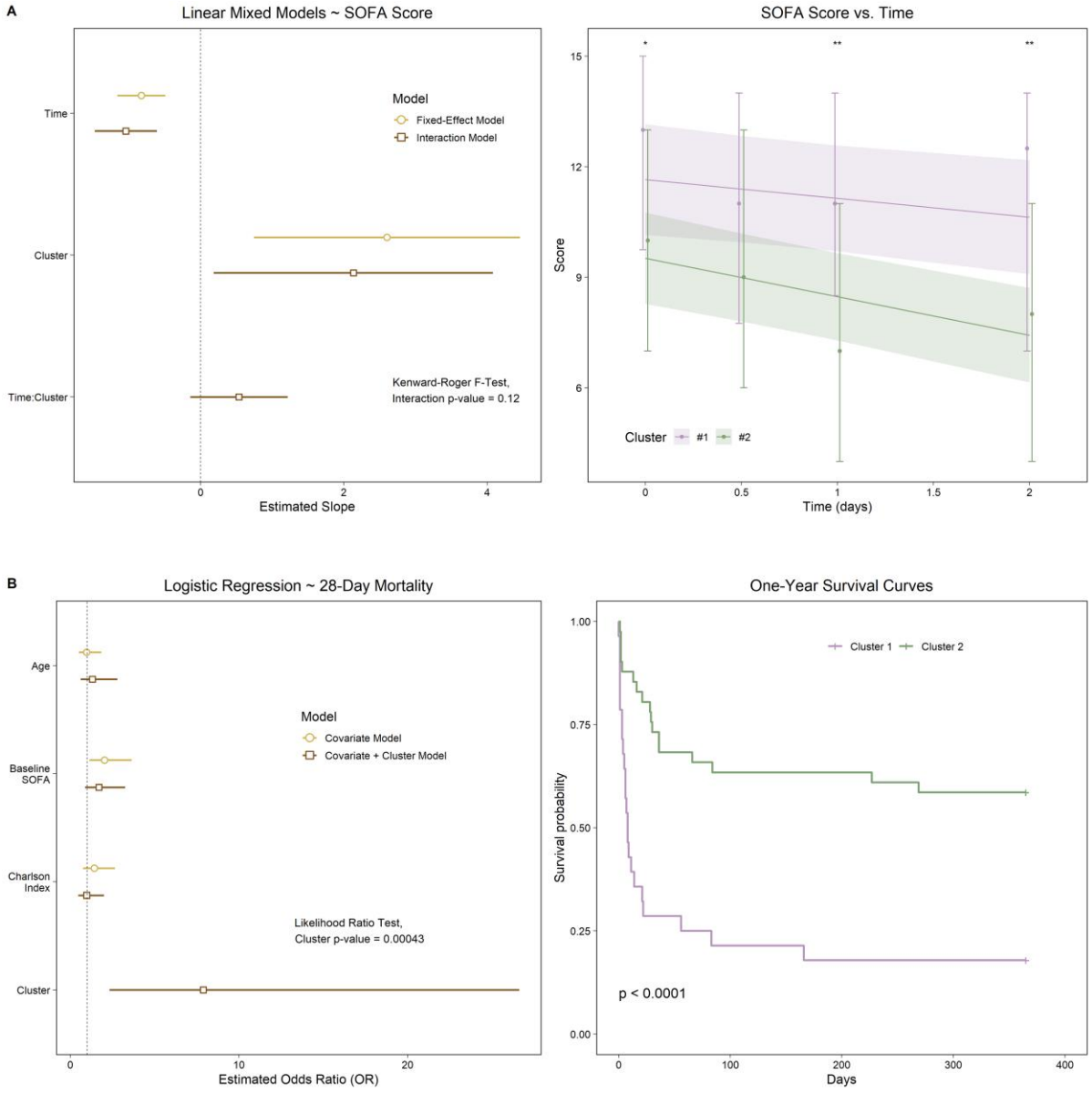


**Figure 2-5. Patient outcomes stratified by metabolic cluster assignment.**

(A) Model coefficients (left) and predictions (right) from linear mixed models, with SOFA score as the outcome variable. The fixed-effect model included the *Time* when SOFA was measured and *Cluster*

assignment. The interaction model also included the *Time*Cluster* interaction, and its inclusion was assessed with the Kenward-Roger F-test. The SOFA score predictions for each cluster (cluster 1 in purple, cluster 2 in green) from the interaction model are shown with their 95% confidence interval. The median and interquartile range are also plotted for each timepoint, with between-cluster differences assed by the Mann-Whitney U-test. (B) Model coeffiicents from logistic regression, with 28-day mortality (left) and survival curves out to one-year (right) between cluster. The probability of 28-day mortality was modeled with a covariate model that included age, baseline SOFA score, and the Charlson Comorbidity Index. A second model added cluster assignment as a predictor variable, and the likelihood ratio test was used to determine the impact of its inclusion. Survival curves for each metabolic cluster were plotted and assessed with the log-rank test.

We also assessed differences in mortality between the metabolic clusters. In a logistic regression model adjusted for age, baseline SOFA score, and comorbidities, assignment to cluster 1 was associated with a greater probability of 28-day mortality (**Figure 2-5B**, OR = 7.8, 95%CI: 2.45, 28.62, p< 0.001). One year survival curves between the two metabolic clusters were also significantly different (log-rank test, p<0.001).

## 2.5 Discussion

In this study, we found a distinct metabolic and inflammatory signature in the early host response in 28-day non-survivor patients with septic shock. This signature was derived from measurements of serum acylcarnitines, small polar metabolites, and protein biomarkers of inflammation and endothelial activation. Specifically, patients who died had sustained elevations in IL-8 and acylcarnitines throughout the first 48 hours after the identification of septic shock. In addition, levels of pyruvate, IL-6, TNF$\alpha$, and ANG-2 declined at a slower rate in patients who died. Importantly, we also found a metabolic pattern of inflammation, whereby inflammatory cytokines were correlated with glycolysis products and branched-chain amino acid catabolism. Taken together, our work reinforces that mitochondrial dysfunction and host inflammation are related to

clinical outcomes, continue early during septic shock, and persist in patients who die by 28 days.

In addition to our main findings, unsupervised clustering revealed two distinct metabolic groups. These subgroups of patients with septic shock had similar demographics and comorbidities, however patients in the first cluster were characterized by greater organ dysfunction and had a greater likelihood of one-year mortality. Our findings demonstrate the substantial metabolic heterogeneity of patients within a well-defined cohort of septic shock, highlighting the syndromic nature of the disease and the potential for multi-omic data to inform biologically-driven endotyping of sepsis.

Metabolic perturbations are a well-established aspect of sepsis pathophysiology and have reliably been linked to acute illness severity and patient mortality [4]. Described disruptions include a hypermetabolic state that results in catabolism of protein and fat, a glycolytic shift with a subsequent upregulation of the TCA cycle, and mitochondrial dysfunction [38-40]. Our work contributes toward the Surviving Sepsis Campaign's goal to better understand sepsis-induced metabolic disruptions [7] by providing a more comprehensive mapping of the dynamic metabolic changes in sepsis and its relationship to the host-immune response. This information is needed to direct the rationale design of targeted, metabolic pharmacotherapy and to inform biologic mechanisms underlying sepsis phenotypes and heterogeneity.

The enhancement of analytical platforms and the growth of metabolomics as a field have shown promise in addressing these questions of variable patient outcomes in sepsis and other critical illness [41,42]. An integrated metabolomic and proteomic analysis

by Langley et al. found alterations in fatty acid oxidation in sepsis non-survivors [16]. Namely, patients who died had consistent elevations in acylcarnitines of various chain lengths and down regulation of fatty-acid transport proteins, suggesting a decreased capacity for β-oxidation of fats by the mitochondria. Subsequent studies have been largely consistent with these findings and demonstrated elevations in acylcarnitines are related to not only differential mortality and organ dysfunction [17,43,44], but also systemic levels of mitochondrial DNA [45] and concentrations of inflammatory cytokines [18]. Our work here further corroborates that sepsis non-survivors are characterized by persistent elevation in acylcarnitines, mitochondrial dysfunction, and perturbed fatty acid metabolism and adds a longitudinal component. By leveraging the serially collected blood samples of the RACE trial, we show that these elevations persist early in the course of illness despite shock resuscitation treatment.

Endothelial dysfunction and hyperinflammation are also well characterized components of sepsis pathophysiology, associated with patient outcomes, and represent potential avenues for targeted therapeutic development. Elevated concentrations of IL-8 have been associated with patient mortality in multiple cohorts of patients with sepsis and have been proposed as a prognostic biomarker for clinical trial enrichment [46-48]. Similar findings have been reported in sepsis or sepsis-induced ARDS for IL-6 [49-51] and ANG-2 [52-54]. Consistent with these studies, we found that concentrations of IL-8 were elevated in sepsis non-survivors over the first two days. While we did not observe the same differences for IL-6 or ANG2, the rate of decline for these protein analytes was slower in patients who died. These differences may be

attributed to the fact that this cohort was comprised exclusively of patients with septic shock, presenting at a more advanced stage of infection.

Our study also further informs details of the interaction between the host metabolic and immune response, finding a positive correlation between numerous hyper-inflammatory cytokines and the glycolysis end-products, pyruvate and lactate. In contrast, the branched chain amino acids (BCAA), leucine, isoleucine, and valine, tended to be lower in patients with a more hyper-inflamed state. Alanine, known to be released from skeletal muscle following oxidation of BCAA [55], was positively correlated to both IL-8 and IL-10. Our findings here are consistent with a signature of BCAA catabolism that may result from a shifting preference for different energy substrates and a variable metabolic response that tracks with the host-immune response to infection [44]. Conditions of chronic, low-grade inflammation such as obesity and type 2 diabetes are associated with increased systemic levels of BCAA (e.g., less catabolism) [56], while the inverse or normal levels have been reported in sepsis [57,58]. While the precise mechanism for this has not been elucidated, we hypothesize that accelerated oxidation of BCAA results from a heighted acute inflammatory response.

The failure of numerous clinical trials and the lack of any targeted therapy beyond antibiotics has led to calls to reevaluate the approach to defining and treating sepsis and other critical care syndromes [5,59]. This shift toward disease subphenotyping (or endotyping) and defining treatable traits leverages individual patient characteristics and laboratory values, -omic based descriptions of an individual's biological response, and unsupervised statistical or machine learning methods to cluster similar patients. These efforts have recently been reviewed [60], but notably include the identification of

distinct patient clusters based on electronic health record data [61-63] and gene expression data [64,65]. In ARDS, similar approaches have defined hyper-inflammatory and hypo-inflammatory subphenotypes, with dramatic differences in clinical outcomes and response to therapy [66-70]. Rogers et al. recently used metabolomics data from patients with sepsis and found distinct groups separated by concentrations of plasma lipids and with variable organ dysfunction and mortality [71]. Here, we employed a similar approach on a smaller, but well-defined, cohort of patients exclusively with septic shock. Using metabolomics and a widely-used clustering algorithm, we found two distinct groups of patients driven primarily by increased systemic concentrations of long-chain acylcarnitines, suggesting excess fatty acid supply and/or incomplete β-oxidation [72]. Patients assigned to cluster one tended to have significantly more organ dysfunction at baseline and post-resuscitation and a greater risk of death. These findings demonstrate that metabolomic data is beneficial for defining subclasses of sepsis and directing future work that seeks to re-define critical illness based on biological underpinnings of disease pathobiology.

Our work has several limitations that are worthy of consideration. Sepsis heterogeneity is multifaceted. The sample size of the cohort limited our ability to assess the impact of variability in the site of infection and treatment with specific pharmacologic agents (antibiotics, corticosteroids, and specific sedative agents or vasopressors). We acknowledge too that this only permits the introduction of this concept and that a larger cohort of patients will be needed for reproducibility and validation. As part of future validation, while we assayed samples using the same analytical platform, assessment of plasma analyte measurements may be warranted since we used residual serum

volume for our analyte assays [73]. For our analysis we used an existing metabolomics data set that was generated as part of an ancillary study to the parent clinical trial [20]. Directed by our prior work, these assays were targeted and relatively limited in scope, measuring only acylcarnitines and a modest number of polar molecules [19]. Use of larger cohorts of patients using untargeted metabolic profiling will likely find additional signals related to patient outcomes and may result in additional clusters of metabolically distinct patients. These studies should ideally integrate other patient characteristics and measures of the host-biological response to provide a more comprehensive understanding of the biological changes during sepsis. Finally, metabolomic and other -omic data are not currently readily available within clinical practice. Point of care testing and thoughtful assay development and implementation will be required for the translation of metabolomics and sub-phenotyping findings.

### 2.6 Conclusions

In summary, we found that early concentrations of acylcarnitines and IL-8 are persistently elevated in patients with septic shock who do not survive. Unsupervised clustering of baseline metabolomics data also revealed two groups of patients with differentiating organ function and mortality. These findings reinforce that metabolic derangements of the host, particularly related to fatty acid metabolism and mitochondrial dysfunction, continue post-resuscitation and may be useful for prognostic and/or predictive enrichment to combat sepsis heterogeneity.

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## Chapter 3: Serum Levels of Acylcarnitines and Amino Acids Are Associated with Liberation from Organ Support in Patients with Septic Shock

This work has been published as an original research manuscript in the *Journal of Clinical Medicine*.[1]

### 3.1 Chapter Abstract

Sepsis-induced metabolic dysfunction is associated with mortality, but the signatures that differentiate variable clinical outcomes among survivors are unknown. Our aim was to determine the relationship between host metabolism and chronic critical illness (CCI) in patients with septic shock. We analyzed metabolomics data from mechanically ventilated patients with vasopressor-dependent septic shock from the placebo arm of a recently completed clinical trial. Baseline serum metabolites were measured by liquid chromatography-mass spectrometry and $^{1}$H-nuclear magnetic resonance. We conducted a time-to-event analysis censored at 28 days. Specifically, we determined the relationship between metabolites and time to extubation and freedom from vasopressors using a competing risk survival model, with death as a competing risk. We also compared metabolite concentrations between CCI patients, defined as intensive care unit level of care ≥ 14 days, and those with rapid recovery. Elevations in two acylcarnitines and four amino acids were related to the freedom from organ support (Subdistributional Hazard Ratio <1 and False Discovery Rate <0.05). Proline, glycine, glutamine, and methionine were also elevated in patients who

developed CCI. Our work highlights the need for further testing of metabolomics to identify patients at risk of CCI and to elucidate potential mechanisms that contribute to its etiology.

### 3.2 Introduction

Sepsis is a prevalent, costly, and life-threatening syndrome, formally defined as organ dysfunction occurring secondary to an infection resulting from a dysregulated host response.[2-4] Mortality from the most severe form of sepsis, septic shock, approaches 40.[5] Furthermore, survivors of sepsis experience highly variable clinical trajectories[6], with some patients rapidly recovering (within days) and others developing chronic critical illness (CCI) and suffering profound morbidity, long-term sequela, and an increased risk of late mortality.[7-9] The poor outcomes of this latter phenotype are driven in part by the initial sepsis-induced organ injury and dependence on mechanical ventilation or other organ support measures.[10] Furthermore, these patients also have prolonged stays in the intensive care unit (ICU), characterized by cascading, late-onset organ failures.[11] Improved understanding of the patient risk-factors and biologic mechanisms driving CCI are key for the development of novel pharmacotherapy and improvement of long-term sepsis outcomes.

Previous attempts to discriminate who is at risk for developing CCI using electronic health record data at ICU admission have proven unsuccessful.[11-13] These findings imply a need for deeper phenotyping of patients, derived from biologic signals of the host-response to infection, for full risk stratification and identification of modifiable drug targets. Recent work provides evidence of biologic differences among patients who develop CCI at the transcription, protein, and metabolism levels[14-17], though further

studies in different patient cohorts that leverage and integrate other ‘omic’-technologies are still needed.

Sepsis induces a deranged energy metabolism that manifests as elevated blood lactate and glucose levels, muscle catabolism and perturbed amino acid concentrations, and mitochondrial dysfunction.[18-20] Metabolomics, as an applied science, identifies small molecules in a biological sample.[21] In doing so, metabolomics provides a physiological “snapshot” and molecular phenotype of the host and has proven useful in differentiating patient outcomes and response to drug therapy in patients with sepsis.[22-28] As such, we sought to determine among mechanically ventilated patients with septic shock, if the baseline patient metabolic status could help distinguish CCI from patients with rapid recovery.

## 3.3 Materials and Methods

### *3.3.1 Patient population*

The Effect of Levocarnitine vs Placebo as an Adjunctive Treatment for Septic Shock -the Rapid Administration of Carnitine in Sepsis (RACE) clinical trial was a multicenter, placebo-controlled, phase II study that adaptively randomized patients with vasopressor-dependent septic shock and moderate organ dysfunction to saline placebo or low (6 g), medium (12 g), or high dose (18 g) levocarnitine.[29] Enrolled patients included adults with a confirmed or suspected bloodstream infection who were identified within 24 hours of recognized septic shock and initiation of a standardized sepsis treatment guidelines. In addition, inclusion required a total sequential organ failure assessment (SOFA) score[30] greater than 6, a clinical lactate level greater than 2 mmol/L, and treatment with high-dose vasopressors within 4 hours of enrollment.

Additional inclusion criteria for this secondary analysis were: (a) allocation to the saline placebo treatment arm; (b) receipt of invasive mechanical ventilation; and (c) a serum blood sample available for metabolomics collected within 36 hours of onset of mechanical ventilation (**Figure 3-1**). These criteria were chosen to provide a more homogeneous cohort that required multiple types of organ support (both endotracheal intubation and exogeneous vasopressors) and were at high risk of developing CCI. The inclusion of only placebo-treated patients removed any potential modifying effect from a putative metabolic treatment (L-carnitine).

All patients or their legally authorized representative provided informed consent and the trial protocol was registered with clinicaltrials.gov (NCT01665092) and approved by the Institutional Review Board of all participating study sites. The trial was conducted ethically according to Good Clinical Practice guidelines and in accordance with local and federal guidelines and statutes.

### *3.3.2 Blood sampling and metabolomics*

Detailed descriptions of the blood sampling, handling, and processing from this cohort have been previously reported.[27,31] Briefly, baseline whole blood samples were collected at the time of clinical trial enrollment. Samples were allowed to clot at room temperature for at least 30 minutes, aliquoted, and centrifuged to obtain serum. Technical replicates were frozen (-80°C), de-identified, and shipped on dry ice to the NMR Metabolomics Laboratory at the University of Michigan.

Acylcarnitines were measured by reverse-phase liquid chromatography mass spectrometry (LC-MS/MS) at the Michigan Regional Comprehensive Metabolomics Research Core as previously described.[26] Samples were analyzed using an Agilent

1200 LC coupled to an Agilent 6410 tandem quadrupole (Santa Clara, CA). Absolute quantification using stable isotope internal standards was completed for the following carnitine species: Levocarnitine, C2, C3, C4, C5, C8, C14, C16. Relative quantification by peak area was utilized for 16 additional acylcarnitine compounds.

Abundant polar compounds were measured by quantitative proton nuclear magnetic resonance ($^{1}$H-NMR) on a Varian (now Agilent, Inc., Santa Clara, CA) 11.74 Tesla (500 MHz) spectrometer consistent with our prior methods.[32,33] Spectra were processed using Chenomx NMR Suite 8.2 (Edmonton, AB, Canada) software as previously described.[33] Briefly, compounds were identified in the profile module using the Chenomx spectral library and quantified relative to the area of a formate internal standard (50µL of 9.64 mM). The complete list of acylcarnitines and NMR metabolites is available in the online supplement (Supplementary **Table S-2**).

In preparation for downstream statistical analysis, missing concentration data in the NMR dataset were assumed to be left-censored and missing not at random due to falling below the limit of detection.[34] As such, missing data was imputed for each metabolite as the minimum concentration observed divided by 2. There were no missing data present in the acylcarnitine dataset. After imputation, metabolite concentrations were log-transformed and standardized to have a mean of zero and standard deviation of one.[35,36]

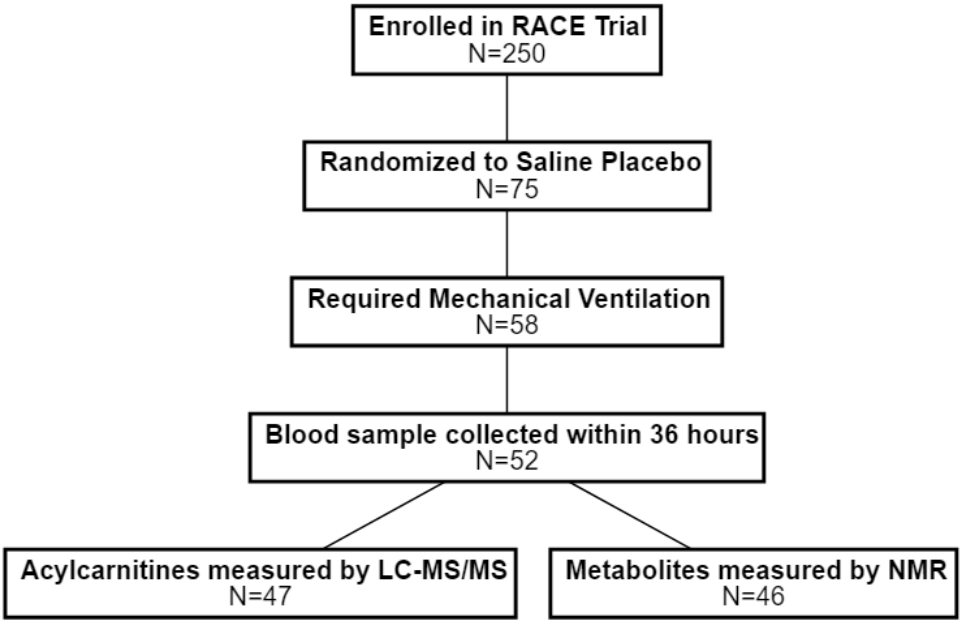


**Figure 3-1. Study Flow Diagram**

Patients were considered for this secondary analysis of the RACE clinical trial if they were randomized to (a) receive saline placebo; (b) required mechanical ventilation; and (c) had a blood sample collected within 36 hours of the initiation of mechanical ventilation. Metabolomics data were generated and available for a subset of patients. RACE = Rapid Administration of Carnitine in Sepsis; LC-MS/MS= liquid chromatography mass spectrometry; NMR= nuclear magnetic resonance.

### *3.3.3 Clinical outcomes and statistical analysis*

For the primary outcome, we considered each patient's ventilator free days and vasopressor free days as a time-to-event analysis censored at 28 days, with death as a competing risk.[37,38] Specifically, an endpoint of successful extubation without continued need of vasopressors was modeled as a function of time, with death over 28 days considered the competing event. We then fit a series of competing risk survival models, first for patient characteristics at enrollment and then one for each metabolite measured and determined the subdistributional hazard ratio (SHR). Patient characteristics included demographics, clinical laboratory values, and other physiologic parameters (**Figure 3-2**). Covariates were chosen *a priori*, and we adjusted metabolite models for sex, baseline SOFA score, and a modified Charlson Comorbidity Index (see methods in

the online supplement), which also accounts for patient age.[30,39] Given the use of sedation in this patient population and its impact on the Glasgow Coma Score, we excluded the neurological component of the SOFA score.[40,41] The p-value corresponding to each metabolite's adjusted SHR was corrected for multiple comparisons according to the false discovery rate (FDR) procedure of Benjamini–Hochberg.[42] Metabolites with an FDR <0.05 were then rank ordered by the adjusted SHR and plotted with the 95% confidence interval.

To help further visualize the results of the competing risk models, we dichotomized the cohort based on the median value of the top metabolic predictor. We then plotted time to event curves for successful extubation and freedom from vasopressors over 28 days in patients at or above and those below the median metabolite concentration. Patients who died during the 28-days were considered to have zero ventilator free days and vasopressor free days to account for the competing risk of death.[43]

As an exploratory analysis, we sought to determine the relationship between host metabolism and CCI. Importantly, enrollment and blood sampling in the RACE clinical trial were anchored to onset of septic shock rather than ICU admission. While total patient days in the ICU were recorded, it was not possible to determine how long a patient had been in the ICU prior to enrollment in the study. This complicated our ability to classify patients into common phenotypes of CCI that are derived from ICU length of stay.[6,44-46] As such, we classified patients based on a competing risk of death and the continued need for vasopressors and/or mechanical ventilation. Patients were classified as follows: (a) 'Death': mortality within 28 days of enrollment; (b) 'CCI': survival at 28-

days with a continued need for mechanical ventilation and/or vasopressors for at least 14 days; and (c) 'Rapid Recovery': survival at 28-days and free from mechanical ventilation and vasopressors before 14 days. We then used Metaboanalyst to perform principal component analysis (PCA) and conducted a one-way ANOVA for each metabolite to determine if there were metabolic differences stratified across outcomes.[47] The ANOVA p-values were corrected for multiple comparison as described above, and post-hoc testing for between-group differences was performed for metabolites with an FDR <0.05 according to Fisher's Least Square Difference. All data analysis and figure generation were completed in Metaboanalyst (v 5.0; https://www.metaboanalyst.ca/) or RStudio with R (version 3.6.2; Boston, MA).[47,48]

## 3.4 Results

### *3.4.1 Patient characteristics and time to freedom from organ support*

A total of 52 patients from the RACE trial were randomized to the placebo arm and had a baseline blood sample taken within 36 hours of the onset of mechanical ventilation. Acylcarnitines data generated by LC-MS/MS were available for 47 patients and corresponding data generated by NMR were available for all but one patient (**Figure 3-1**). Of these, 21 patients (N = 21/47; 44.7%) were successfully extubated and free of vasopressors over 28-days, 6 (N= 6/47; 12.8%) required persistent mechanical ventilation and/or vasopressors, and 20 (N=20/47; 42.6%) died prior to 28-days. Patient characteristics at baseline are provided in the supplement (**Supplementary Table S-3**).

First, we compared the impact of patient characteristics on time to successful extubation and freedom from vasopressors, with any death over 28-days as a competing risk (**Figure 3-2**). Female sex was associated with a higher rate of intact

extubation and freedom from vasopressors (SHR: 2.49, 95% CI: 1.05 – 5.90), while respiratory rate (SHR: 0.92, 95% CI: 0.86 – 0.99) and baseline total bilirubin (SHR: 0.69, 95% CI: 0.53 – 0.9) were associated with a lower likelihood. In addition, baseline SOFA score (SHR: 0.87, 95% CI: 0.74 – 1.01) and clinical lactate levels (SHR: 0.79, 95% CI: 0.59 – 1.05) were moderately related to time to successful extubation. Patient characteristics were otherwise similar based on the primary outcome.

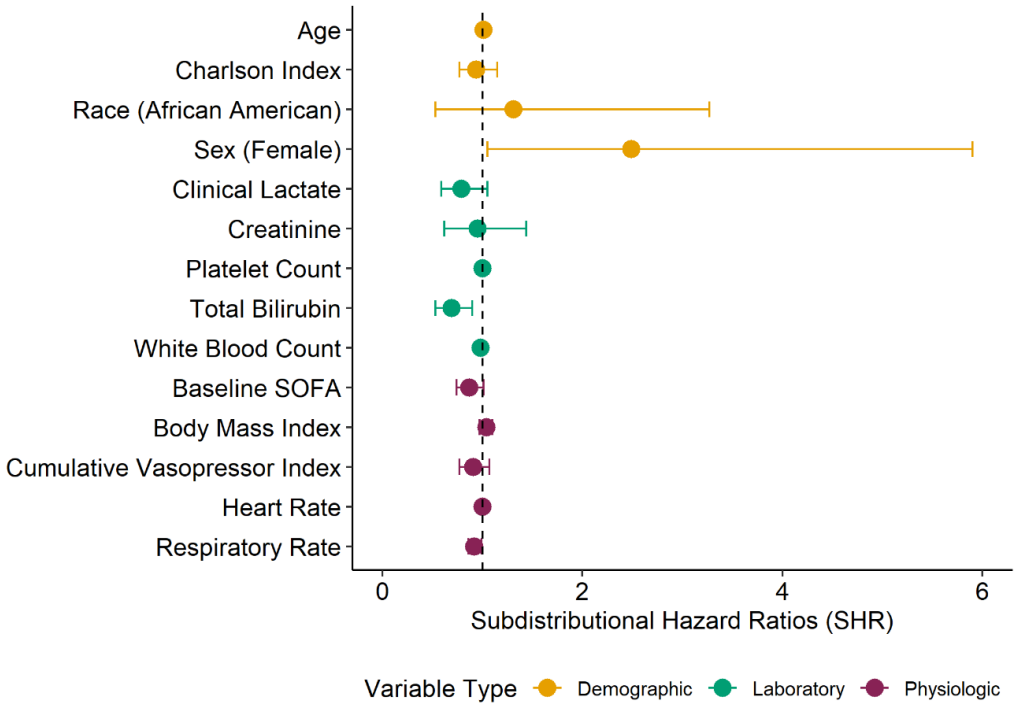


**Figure 3-2. Time to successful extubation and freedom from vasopressors based on baseline patient characteristics.**

Comparison of time to successful extubation and freedom from vasopressors based on patient characteristics at baseline. The unadjusted subdistributional hazard ratio (SHR) was determined for demographic, clinical laboratory, and physiologic characteristics of patients at time of enrollment. The SHR was determined using a competing risk survival model for time to extubation and freedom from vasopressors, with death in the first 28 days as a competing risk. Here, an SHR < 1 indicates that, with increases in the predictor variable, there is a lower incidence of intact extubation and freedom from vasopressors. Female sex and African American self-reported race were coded as 1, while male sex and Caucasian race were coded as 0. Complete data (N=47) were available for all variables except Race (N=46); Clinical Lactate (N=37); Platelet Count and Cumulative Vasopressor Index (N=46); and White Blood Count (N=34). Patient characteristics can be found in Supplementary Table S-3.

### *3.4.2 Metabolite concentrations and time to freedom from organ support*

We identified and measured twenty-four acylcarnitine species by LC-MS/MS (N=47) and 27 small, polar molecules by NMR (N=46). Levocarnitine (LC) and acetylcarnitine (C2) are measured by both methods. A comprehensive list of these metabolites is available in the supplement (**Supplementary Table S-2**). All metabolomics data are publicly available through the National Institutes of Health Metabolomics Workbench (https://www.metabolomicsworkbench.org/; accession number ST001319).

In adjusted competing risk survival models, a significant difference was detected in the incidence of successful extubation and freedom from vasopressors based on the baseline concentration of six metabolites (**Figure 3-3A**, FDR<0.05). These metabolic features included acetylcarnitine (C2), valerylcarnitine (C5-carnitine), and four amino acids (glutamine, glycine, proline, and methionine). All significant features had an adjusted SHR less than 1, indicating elevations in the baseline metabolite concentration were associated with a reduction in the incidence of the event, in this case no longer requiring ICU-level of care.[49] To help further visualize the results of the competing risk models, we stratified patients based on the top metabolic signature, acetylcarnitine (adjusted SHR: 0.23, 95% CI: 0.13 – 0.40), as measured by LC-MS/MS (**Figure 3-3A**). Patients with acetylcarnitine concentrations at or above the median were designated as `High-C2` and those below the median as `Low-C2` (**Figure 3-3B**). As a dichotomous variable, the adjusted SHR for acetylcarnitine was 0.28 (95% CI: 0.11 – 0.75).

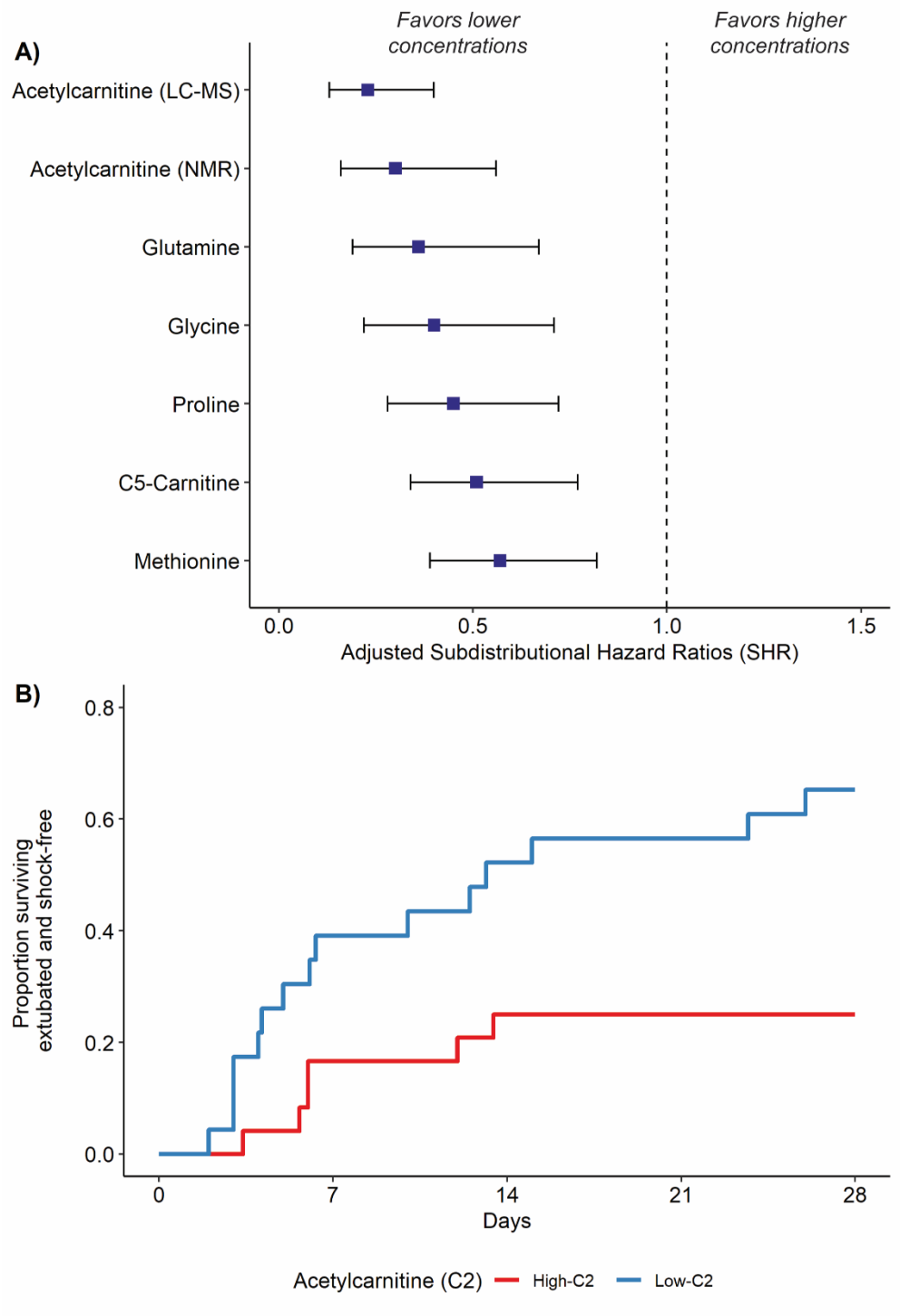


**Figure 3-3.Time to successful extubation and freedom from vasopressors based on baseline serum metabolite levels.**

A) The adjusted sub-distributional hazard ratio (SHR) for top metabolic features (FDR <0.05) related to time to extubation and freedom from vasopressors. The SHR was determined using a competing risk survival model for time to extubation, with death in the first 28 days as a competing risk. Each model was adjusted for baseline SOFA score, sex, and the Charlson comorbidity index. For all metabolites displayed

above, lower concentrations were associated with a greater incidence of successful extubation and freedom from vasopressors. B) Visualization of time to breathing unassisted upon dichotomizing the top metabolic feature, acetylcarnitine (C2), above and below the median value. There was a higher proportion of patients with low C2 that survived, were extubated and shock-free over time versus patients with high C2.

### *3.4.3 Metabolic differences between CCI and rapid recovery with death as a competing risk*

In the exploratory analysis, among patients with both acylcarnitine and NMR data, 9 patients developed CCI (N= 9/46; 19.6%), 17 (N=17/46; 37.0%) experienced a rapid recovery, and 20 (N= 20/46; 43.5%) died prior to 28-days. Patients who experienced a rapid recovery were more likely to be alive at one-year compared to patients who developed CCI (82.4% vs. 66.7%, p = 0.03). In multivariable PCA analysis, metabolic differences were most pronounced in the mortality outcome group. There was substantial overlap between patients who developed CCI and those who experienced rapid recovery (**Supplementary Figure S-1**). In our univariate one-way ANOVA analysis, 24 metabolites were significantly different among the three groups (ANOVA FDR<0.05). Post-hoc testing for between-group differences by Fisher's Least Square Differences revealed this was largely driven by metabolic differences in the mortality outcome group (**Supplementary Table S-4**). Nonetheless, after post-hoc testing, the same four amino acids identified in our organ failure support analysis were also elevated in patients who went on to develop CCI relative to those who had a rapid recovery (FDR <0.05, **Figure 3-4**, **Supplementary Table S-4**).

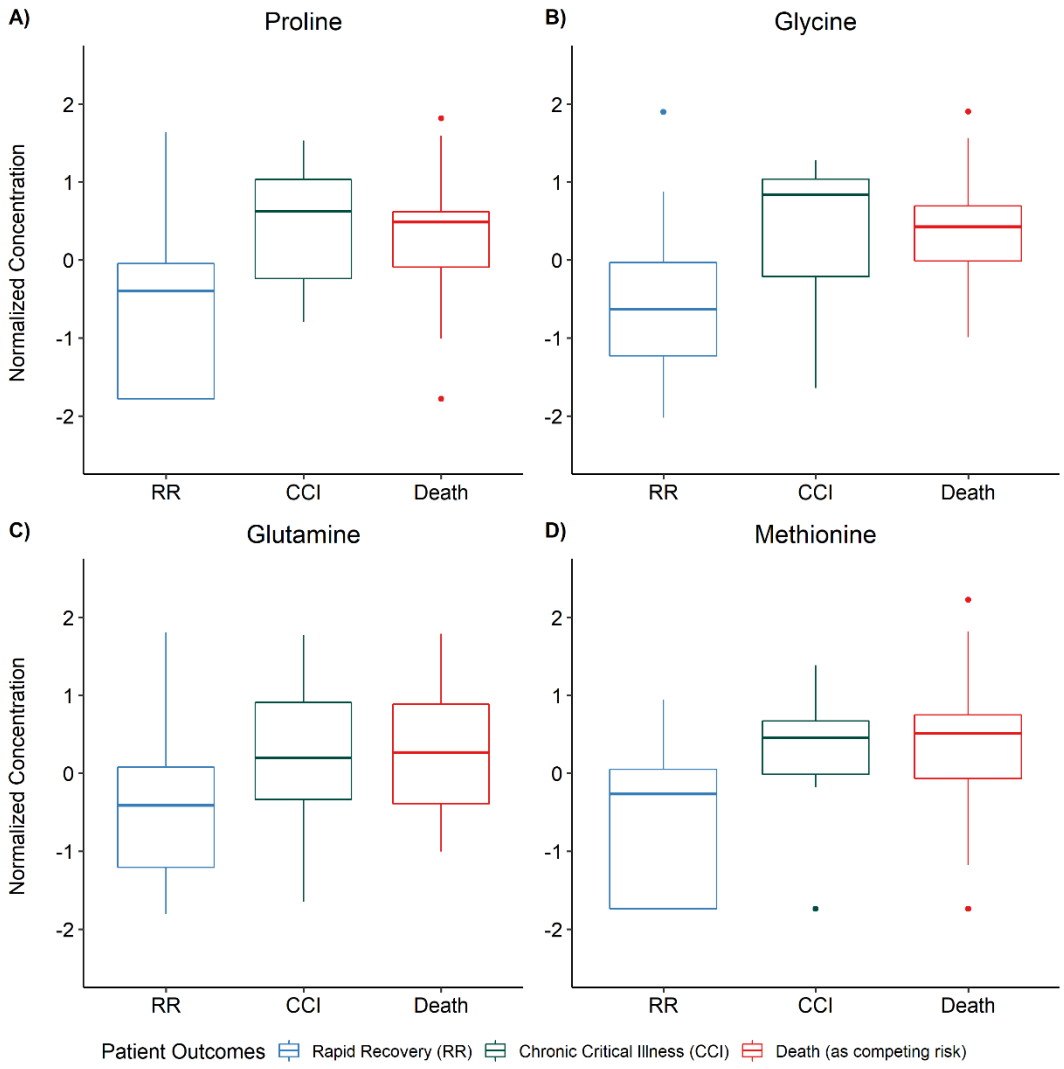


**Figure 3-4. Serum amino acid concentration differences between chronic critical illness (CCI) and rapid recovery (RR) patients**

One-way analysis of variance (ANOVA) was used to determine differences in metabolite concentrations stratified by patient outcomes. The ANOVA p-values were corrected for multiple comparisons according to the false discovery rate (FDR) procedure of Benjamini–Hochberg and post-hoc testing for between-group differences was done according to Fisher's Least Square Difference when the FDR was less than 0.05. Four metabolites (proline, glycine, glutamine, and methionine) were different (FDR < 0.05) between patients who developed CCI and those who experienced a RR.

## 3.5 Discussion

Our study sought to determine if metabolic differences among mechanically ventilated patients with septic shock were associated with the liberation from organ support and duration of ICU-level of care. In a competing risk, time-to-event analysis we

demonstrated that serum concentrations of short chain acylcarnitines (C2 and C5) and four amino acids (proline, glycine, glutamine, and methionine) are related to liberation from mechanical ventilation and vasopressors over 28-days. Additionally, we found that these same amino acids were elevated in CCI patients who required at least 14 days of mechanical ventilation and/or vasopressors relative to those who rapidly recovered. Our findings provide new insights into candidate biochemical pathways that are perturbed in sepsis-survivors and suggest metabolomics may provide prognostic detail beyond mortality outcomes.

A dysregulated host metabolic response is formally defined in the Sepsis-3 definition and increasingly understood as a hallmark of sepsis pathophysiology.[2,50] This perturbation of metabolism has been consistently linked with alterations in energy utilization, mitochondrial dysfunction, organ failure, and mortality.[20,51-55] Our work supports this growing body of evidence, finding that the mortality group in our study was the most metabolically disrupted (**Supplementary Figure S-1** and **Supplementary Table S-4**). Perhaps more importantly however, our study introduces the utility of metabolomics to differentiate sepsis survivor phenotypes, CCI, and rapid recovery. The role of deranged metabolism in CCI following sepsis survival is best understood in work surrounding the Persistent Inflammation, Immunosuppression and Catabolism Syndrome.[56-58] This syndrome is characterized in part by persistent inflammation leading to profound muscle catabolism and a cachexia-like response.[6,59] Elevations in two of key metabolites from our study, acetylcarnitine and valerylcarnitine (**Figure 3-3**), are broadly indicative of altered energy demand, β-oxidation of fatty acids, mitochondrial dysfunction, and metabolic inflexibility[60]; and acetylcarnitine was recently further linked

with the systemic inflammatory response in patients with sepsis.[53] Here, we implicate short-chain acylcarnitines as markers of not only mortality, but also the differential need for life-supporting measures in the ICU.

Other proposed metabolic biomarkers of CCI have included low serum albumin and increased frailty (as a surrogate for poor nutritional status)[61], and the urea to creatinine ratio, a biochemical signature related to muscle catabolism.[16,62] We were not able to assess the impact of serum albumin or measures of frailty in our cohort, and although creatine, a key metabolite of skeletal muscle energy homeostasis, was detected by our NMR assay, it was not found to be a strongly related to the time to extubation and freedom from vasopressors. However, serum concentrations of four amino acids (all of which are non-essential except for methionine) were strongly related in both our competing risk models and when CCI was defined with a 14-day cut point.

Differentiating blood levels of amino acids are metrics of the overall energy economy of the host and are related to patient outcomes in critical illnesses.[63,64] We have previously shown that serum levels of methionine are increased in patients with persistent septic shock compared with those whose shock resolved.[65] Methionine is important for immune function and its dietary restriction has been shown to decrease inflammation and improve skeletal muscle health in animal models.[66,67] Glutamine is the most abundant amino acid in humans and sources numerous metabolic pathways many of which are important in maintaining energy homeostasis.[68] It and glycine are precursors of the antioxidant, glutathione[69], and glutamine, glycine and proline are all precursors of bacterial (microbiome) production of short-chain fatty acids (e.g., butyrate) which participate in maintaining immune function.[70] In critically ill patients, both low[71,72]

and high levels[73,74] of glutamine have been previously shown to be related to mortality, and supplementation has failed to consistently demonstrate clinical benefit.[75,76] This has led many to question the indiscriminate supplementation of glutamine[77,78] and suggests a precision approach may be warranted. In this cohort of patients with septic shock, high levels of glutamine were related to a prolonged need for organ support and poor clinical outcomes; further studies in this specific patient population are warranted. In aggregate, our findings suggest that metabolic differences among patients with septic shock may lend insight into mechanisms that contribute to sepsis outcome phenotypes and could be used as predictive biomarkers of CCI. Future metabolomics studies in patients at risk of CCI will permit further assessment of the prognostic value of candidate metabolite biomarkers and inform targeted metabolic pharmacotherapy and/or adjunctive nutritional support.

Our study has important limitations that warrant further consideration. First, our study was cross-sectional in nature, leveraging only a single metabolic timepoint. While the collection of serum samples was carefully anchored to a clinical event (onset of mechanical ventilation), future work that follows the trajectory of metabolic changes in the ICU may provide additional prognostic value and mechanistic insight. In addition, we considered only a limited read of the serum metabolome using normalized concentrations and acknowledge that our metabolomics data are not comprehensive. Absolute quantification of potential biomarkers will be essential for ultimate clinical translation, while a broader read of the metabolome combined with data acquired at the transcription and protein level offers an exciting and potentially more fruitful assessment of the pathophysiology of CCI. Moreover, we used a definition of CCI based on the

continuous need for mechanical ventilation and vasopressors, while much of the CCI literature relies on ICU length of stay. Finally, our study was observational and thus our findings are hypothesis-generating and require rigorous validation in prospective cohorts.

### 3.6 Conclusion

Among mechanically ventilated patients with septic shock, serum concentrations of two acylcarnitines and four amino acids were related to the time to extubation and freedom from vasopressors. Our work supports the feasibility of metabolomics to interrogate the mechanisms of CCI and the hypothesis that altered host metabolism is a sign of and/or contributes to CCI.

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# Chapter 4: Pharmacometabolomics Identifies Candidate Predictor Metabolites of a L-Carnitine Treatment Mortality Benefit in Septic Shock

This work has been published as an original research manuscript in *Clinical Translational Science*.[1]

## 4.1 Chapter Abstract

Sepsis-induced metabolic dysfunction contributes to organ failure and death. Levocarnitine (L-carnitine) has shown promise for septic shock, but a recent phase II study of patients with vasopressor-dependent septic shock demonstrated a non-significant reduction in mortality. We undertook a pharmacometabolomics study of these patients ($n$ = 250) to identify metabolic profiles predictive of a 90-day mortality benefit from L-carnitine. The independent predictive value of each pretreatment metabolite concentration, adjusted for L-carnitine dose, on 90-day mortality was determined by logistic regression. A grid-search analysis maximizing the Z-statistic from a binomial proportion test identified specific metabolite threshold levels that discriminated L-carnitine responsive patients. Threshold concentrations were further assessed by hazard ratio and Kaplan-Meier estimate. Accounting for L-carnitine treatment and dose, 11 $^{1}$H-NMR metabolites and 12 acylcarnitines were independent predictors of 90-day mortality. Based on the grid-search analysis numerous acylcarnitines and valine were identified as candidate metabolites of drug response. Acetylcarnitine emerged as highly viable for the prediction of an L-carnitine mortality benefit due to its abundance and

biological relevance. Using its most statistically significant threshold concentration, patients with pretreatment acetylcarnitine greater than or equal to 35 μM were less likely to die at 90 days if treated with L-carnitine (18 g) versus placebo ($p$ = 0.01 by log rank test). Metabolomics also identified independent predictors of 90-day sepsis mortality. Our proof-of-concept approach shows how pharmacometabolomics could be useful for tackling the heterogeneity of sepsis and informing clinical trial design. In addition, metabolomics can help understand mechanisms of sepsis heterogeneity and variable drug response, because sepsis induces alterations in numerous metabolite concentrations.

### 4.2 Introduction

Sepsis represents the leading cause of death in the intensive care unit and the single most expensive inpatient diagnosis, representing more than $17 billion in healthcare costs annually in the United States.[2-4] Septic shock carries a particularly poor prognosis, with short-term mortality rates of approximately 40%. Among the many physiologic disturbances associated with sepsis is a profound shift in metabolism.[5] Hyperlactatemia represents one of the hallmarks of sepsis and is now considered a criterion for the diagnosis of septic shock.[6] However, hyperglycemia, lipolysis, and protein catabolism are also common and similarly associated with increased mortality.[5,7] Manipulation of these processes represents an underdeveloped but promising target for novel pharmacotherapies.

Despite the concerning sepsis mortality statistics and an increasingly focused research effort on the condition, clinical trials of novel sepsis pharmacotherapies have traditionally yielded disappointing results. While the causes of the failure of clinical trials

to further novel treatments are multifactorial, the highly heterogeneous nature of sepsis certainly contributes to these results.[8,9] This highlights the need to forge a better understanding of the heterogeneity and complexity of the clinical illness by identifying sepsis endotypes.[10] In doing so, strategies for enriched patient selection could be used to improve the precision of clinical trials. Importantly, predictive and prognostic enrichment strategies for clinical trials have been advocated by many and have been issued as guidance by regulatory agencies like the U.S. Food and Drug Administration.[11-13]

We recently completed a phase II, Bayesian adaptive dose-finding randomized control trial comparing L-carnitine (6, 12, or 18 g) treatment to saline (placebo) for the early treatment of septic shock. None of the tested doses of L-carnitine resulted in a significant reduction in sequential organ failure assessment (SOFA) score at 48 hours, though the highest and best performing dose (18 g) demonstrated a non-significant 3% and 6% absolute mortality reduction at 28 days in the intention to treat and per protocol analyses compared to saline placebo, respectively.

In parallel with the planning of the original trial, we designed an ancillary metabolomics study, the L-carnitine Pharmacometabolomics in Sepsis (CaPS) study, to identify candidate metabolites of drug response that could serve to endotype a heterogeneous septic shock cohort and direct the design of a clinical enrichment strategy for a phase III trial. A number of studies have demonstrated the importance of energy-related metabolites for the differentiation of sepsis survivors and the identification of sepsis endotypes,[5,7,14-17] most of which are readily detected by nuclear magnetic resonance (NMR) spectroscopy[7,15,16] and targeted liquid chromatography –

mass spectroscopy (LC-MS) assays.[17] Furthermore, we have previously demonstrated the utility of metabolomics in predicting drug response (pharmacometabolomics) in sepsis[16] employing relatively quantified NMR metabolites and acylcarnitines generated by an LC-MS assay. With this background in mind, we hypothesized that serum concentrations of acylcarnitines and/or other metabolites could differentiate patients that disproportionately benefit from L-carnitine treatment as measured by mortality.

## 4.3 Methods

### *4.3.1 Study design*

This study utilized pre-treatment serum samples collected from 236 of the 250 patients enrolled in the Rapid Administration of Carnitine (RACE) in Sepsis clinical trial.[18] The parent trial was approved by each site's institutional review board, all patients or their surrogate gave written informed consent, and it was registered at clinicaltrials.gov prior to initiation (NCT 01665092). Details of the blood samples included in the study are provided in the supplementary material, **Figure S-2**. Serum samples were assayed for acylcarnitines by LC-MS[17] and by quantitative proton ($^1$H) nuclear magnetic resonance (NMR) as previously described.[19,20] More details about the methods for these measurements can be found in the supplementary material.

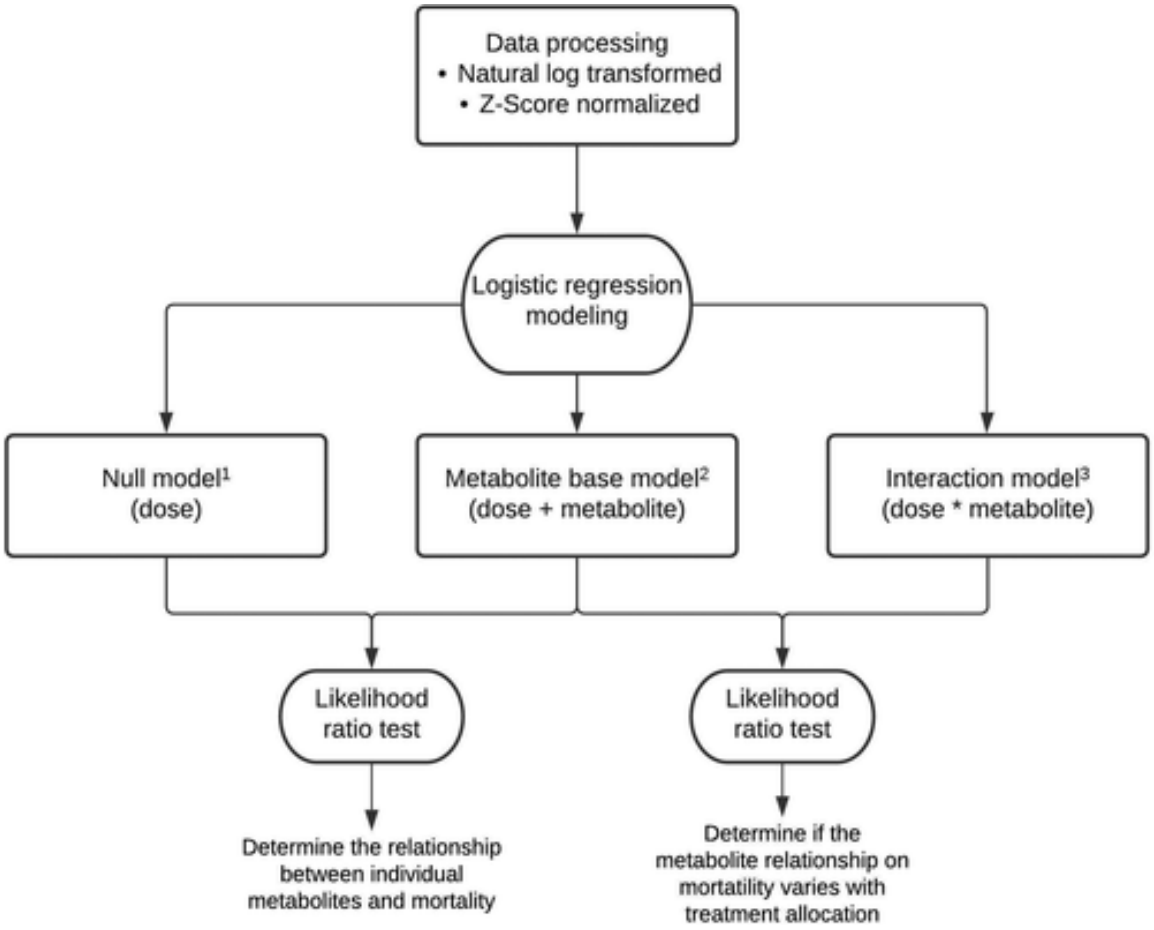



**Figure 4-1. Statistical and logistic regression modeling workflow.**

We first natural log transformed and normalized each metabolite to have a mean of 0 and SD of 1. For each metabolite, we then considered a series of logistic regression models with an outcome of 90-day mortality (*p*). The full model descriptions are provided below. In the metabolite base model, the *p* value corresponds to the likelihood ratio test for inclusion of the metabolite coefficient, $B_M$, compared to the nested null model with only L-carnitine dose ($B_D$) as a predictor. For the interaction model, the *p* value corresponds to the likelihood ratio test for inclusion of the interaction coefficient, $B_{MD}$, compared to a nested model with dose ($B_D$) and metabolite concentration ($B_M$) as predictors. [1]Null model: $\text{logit}(p) = B_0 + B_D * \text{Dose}$. [2]Metabolite base model: $\text{logit}(p) = B_0 + B_D * \text{Dose} + B_M * \text{Metabolite}_i$. [3]Interaction model: $\text{logit}(p) = B_0 + B_D * \text{Dose} + B_M * \text{Metabolite}_i + B_{MD} * \text{Metabolite}_i * \text{Dose}$

### *4.3.2 Outcomes*

We elected to use mortality as the outcome of our analysis because the primary end point of the RACE trial (reduction in SOFA score at 48 h) was not met, but the 18 g dose of L-carnitine resulted in a trend towards a reduction in mortality. Based on data suggesting a substantial continued decline in mortality among sepsis patients beyond

28 days and preliminary data from our phase I data suggesting continued benefit from L-carnitine treatment on longer term mortality rates,[21] we elected to assess the cumulative distribution mortality function to find the optimal time frame for assessment of mortality (28, 90, 180, or 365 days). By 90-days, ~90% of the deaths had occurred, (see **Figure S-3** in the supplementary material), based on this analysis, we chose 90-day mortality as the primary clinical outcome.

### *4.3.3 Statistical Analyses*

Descriptive data are reported as means and standard deviations, medians with interquartile ranges, or proportions as appropriate. Differences in categorical outcomes were compared using chi-square tests, while Student t-tests and Wilcoxon rank sum tests were used to compare continuous variables. The aims of our primary analyses were to: 1) determine the relationship between individual metabolites and 90-day mortality; 2) determine if the relationship between a predictive metabolite and mortality depends on treatment allocation; and 3) using metabolites most associated with mortality, determine the optimal (threshold) metabolite level that could be used to identify patients with septic shock most likely to respond favorably to L-carnitine treatment. Collectively, and similar to other secondary analyses or ancillary studies of clinical and observational trials,[22-26] achievement of these goals would provide clinical proof of concept of a metabolically informed strategy to tackle the heterogeneity of sepsis that also could be used for a predictive enrichment design of a phase III study.[11,12]

Since metabolomics data are on different scales due to varying abundance, in preparation for statistical analyses, data were natural-log transformed and Z-score

normalized to have a mean of 0 and a standard deviation of 1.[27,28] We began our analysis using partial least squares-discriminant analysis (PLS-DA)[29] to visualize the overall metabolic heterogeneity of the study participants and determine whether there were metabolic differences between sexes and the treatment groups.

We followed PLS-DA by an assessment of the predictive value of individual metabolites on 90-day mortality. To accomplish this, we constructed a series of logistic regression models and adjusted for treatment assignment (**Figure 4-1**).[30] We then tested if the relationship between metabolite predictors and mortality varied across treatment groups using a logistic regression interaction model. The likelihood ratio test was used to determine the impact of baseline concentration and the interaction between concentration and dose for each metabolite (**Figure 4-1**). Age[31] and SOFA score[32] were considered as covariates in further multivariable modelling since they are clinically available at the time of therapeutic decision making.

To test the potential clinical application of our pharmacometabolomics approach, after identifying metabolites strongly related to 90-day mortality that also had a significant interaction with treatment allocation, we aimed to identify the specific concentration or levels of these candidate metabolites that could be used to predict which patients would be most likely to derive a mortality benefit from L-carnitine (**Figure 4-2**). To achieve this, we used a grid-search methodology to compute the Z-statistic from the binomial proportion test at every possible threshold metabolite concentration or level.[33] For this example, since the 18g dose of L-carnitine was the most efficacious in the RACE trial and would be the one most likely to be tested in a phase III trial, we used the Z-statistic to quantify the standardized difference in the proportion of deaths

between those patients who received L-carnitine (18 g) and those who received placebo. For this analysis, the metabolite level at each threshold was used as the criterion for inclusion into the proportion test. We then computed a two-sample (binomial) proportion test[34] which compared the proportion of patients treated with L-carnitine who died by 90 days to those that were treated with placebo. This permitted the identification of metabolite levels associated with a range of Z-statistics including the maximum Z-statistic and their corresponding p values. The Z-statistic simultaneously accounts for the difference in the proportion of patients who died in the treatment versus placebo groups and the sizes of each group, thereby suggesting the most optimal metabolite threshold level. Metabolites were then ranked by descending maximum Z-statistic. Similar approaches have been used by other studies that have sought to identify the responder population in clinical and observational trials.[22-26] To further illustrate the implications of the use of different metabolite concentrations as predictors of mortality, hazard ratios were calculated using the Mantel-Haenszel method and Kaplan-Meier curves were constructed (log-rank [Mantel-Cox] test). Metabolite concentration cut points were selected according to different trial scenarios and the grid-search analysis described above. All statistical tests except for hazard ratios (Mantel-Haenszel) and log-rank (Mantel-Cox) tests which were done using PRISM, were performed in R studio (R version 3.6.2 (2019-12-12) Copyright 2019 The R Foundation for Statistical Computing) and figures were constructed in R and PRISM (version 8.4.3, June 10, 2020).

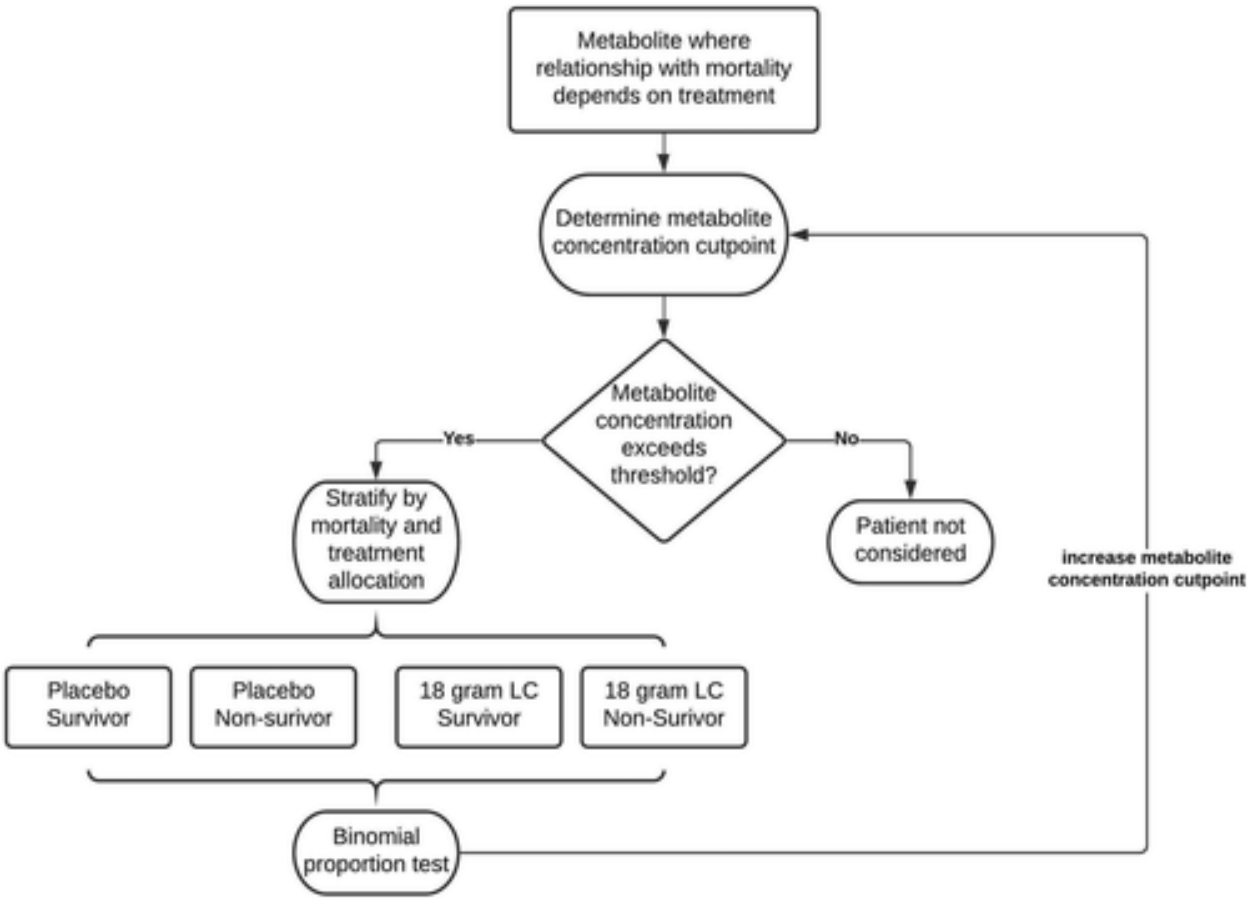



**Figure 4-2. Grid-search methodology workflow.**

After identifying metabolites with the strongest interaction in the logistic regression modeling, the metabolite concentration threshold or cut point that maximized the interaction was determined. For every possible threshold concentration, patients randomized to receive either placebo or 18 g L-carnitine were considered. For patients whose values exceeded the concentration threshold, we stratified patients by treatment allocation and 90-day mortality status and calculated the Z-statistic from the two-sample binomial proportion test. This was done iteratively for each metabolite, and the maximum Z-statistic was identified from the grid-search (see Table S-8). LC, L-carnitine

## 4.4 Results

Of the 250 participants randomized in the parent trial, $^{1}$H-NMR metabolomics and acylcarnitine data were available from 228 and 236 patient serum samples, respectively (see **Figure S-2** in the supplementary material). We identified and quantified 27 serum metabolites by $^{1}$H-NMR and 24 acylcarnitines by LC-MS (see **Table S-5** in the supplementary material). Representative $^{1}$H-NMR and LC spectra are shown in **Figures S-4 and S-5** in the supplementary material. All-cause 90-day mortality was 124/236

(52.5%), while 28-day and 1-year mortality were 104/236 (44.1%) and 136/236 (57.6%), respectively. Clinical and demographic variables of the cohort stratified by the primary outcome are summarized in **Table 4-1**. As expected, patients who died were older and had a higher SOFA score. The PLS-DA plots of the acylcarnitine data and the NMR metabolites by treatment category (supplementary **Figure S-6A and B**) and sex (supplementary **Figure S-7A and B**) illustrate the metabolic heterogeneity of the study cohort and do not demonstrate any significant metabolic distinction between these groups.

**Table 4-1. Demographics and clinical characteristics of the cohort, stratified by 90-day mortality**

| | Survived (n = 111) | Died (n = 125) | p value |
|---|---|---|---|
| Age, years (IQR) | 61 (49, 69) | 66 (57, 76) | 0.002 |
| Male, n (%)<br>Female, n (%) | 60 (54)<br>51 (46) | 74 (41)<br>51 (59) | 0.43 |
| Race<br>Black, n (%)<br>Asian, n (%)<br>White, n (%)<br>Other, n (%) | <br>33 (30)<br>3 (3)<br>68 (61)<br>7 (6) | <br>39 (31)<br>2 (2)<br>74 (59)<br>10 (8) | 0.88 |
| Ethnicity<br>Hispanic, n (%) | <br>5 (4) | <br>7 (6) | 0.70 |
| Diabetes, n (%) | 34 (31) | 46 (37) | 0.32 |
| Liver disease, n (%) | 11 (10) | 25 (20) | 0.03 |

| | | | |
|---|---|---|---|
| Renal disease, n (%) | 10 (10) | 24 (20) | 0.03 |
| Heart rate, beats per minute (IQR) | 100 (84, 113) | 100 (87, 114) | 0.70 |
| Respiratory rate, breaths per minute (IQR) | 20 (16, 24) | 21 (18, 26) | 0.09 |
| Cumulative vasopressor index (IQR) | 4 (3, 8) | 6 (4, 8) | <0.001 |
| Body mass index (IQR) | 28 (25, 36) | 27 (22, 35) | 0.10 |
| White blood count, cells/$mm^3$ (IQR) | 22.0 (12.3, 28.7) | 16.1 (11.4, 23.7) | 0.24 |
| Platelet count, cells/$mm^3$ (IQR) | 161 (99, 232) | 129 (65, 210) | 0.02 |
| Creatinine, mg/dL (IQR) | 1.6 (1.1, 2.4) | 2.1 (1.4, 3.0) | 0.003 |
| Total Bilirubin, mg/dL (IQR) | 0.9 (0.5, 1.7) | 1.6 (0.7, 3.7) | <0.001 |
| Clinical lactate, mmol/L (IQR) | 3.1 (2.3, 4.8) | 4.9 (2.7, 8.4) | <0.001 |
| SOFA score | 10 (8, 12) | 12 (9, 15) | <0.001 |

We then conducted multivariate logistic regression using L-carnitine dose and metabolites as covariates (base model) and applied a conservative Bonferroni correction for multiple comparisons. The base model identified in 11/27 $^1$H-NMR metabolites and 12/24 acylcarnitines that significantly discriminated 90-day mortality (**Table 4-2**; the complete list can be found in **Table S-6** of the supplementary material). We then tested whether the relationship between predictive metabolites and mortality depends on treatment allocation. This was done with the addition of an interaction term between L-carnitine dose and metabolite level (interaction model) which reduced the number of significant metabolites from 23 to 14, of which all but three metabolites were

acylcarnitines (**Table 4-3**; a comprehensive list can be found in **Table S-7** in the supplementary material); these were not in range to withstand a conservative adjustment (e.g., Bonferroni) for multiple comparisons. In this analysis, a statistically significant and negative interaction term indicates that the predicted probability of 90-day mortality for a given metabolic feature is lower at higher doses of L-carnitine. To determine whether the signals found in the base and interaction models was merely due to factors associated with the risk of death, we controlled for both age[31] and SOFA score[32]. Several acylcarnitines and choline tolerated this adjustment (see **Table S-8** in the supplementary material for the full list of metabolites); notably, lactate was not significant in either model (p=0.96 and p=0.22, respectively).

**Table 4-2. Logistic regression model for the prediction of 90-day mortality adjusted for treatment.**

| | **Base model*** | | |
|---|---|---|---|
| **Metabolite predictor‡** | **Metabolite Coefficient ($\beta_M$)** | **$\beta_M$ Standard Error** | **$\beta_M$ P value (Bonferonni)** |
| Acetylcarnitine (C2)§ | 0.85 | 0.16 | <0.0001 |
| C18:1§ | 0.84 | 0.17 | <0.0001 |
| Acetylcarnitine (C2)‖ | 0.76 | 0.16 | <0.0001 |
| C20:1§ | 0.74 | 0.16 | <0.0001 |
| Tyrosine‖ | 0.68 | 0.16 | 0.0002 |
| Betaine‖ | 0.68 | 0.16 | 0.0002 |
| Propionylcarnitine (C3)§ | 0.64 | 0.15 | 0.0002 |
| Propylene glycol‖ | 0.66 | 0.16 | 0.0003 |
| C16:1§ | 0.60 | 0.15 | 0.001 |
| Lysine‖ | 0.58 | 0.15 | 0.002 |
| Glycine‖ | 0.56 | 0.15 | 0.003 |

| | | | |
|---|---|---|---|
| C20-carnitine§ | 0.56 | 0.15 | 0.004 |
| Glutamineǁ | 0.55 | 0.15 | 0.01 |
| C14-carnitine§ | 0.53 | 0.15 | 0.01 |
| C16-carnitine§ | 0.52 | 0.15 | 0.01 |
| Methionineǁ | 0.51 | 0.15 | 0.01 |
| Lactateǁ | 0.51 | 0.15 | 0.02 |
| C12:1-carnitine§ | 0.51 | 0.15 | 0.02 |
| C4-carnitine§ | 0.48 | 0.14 | 0.02 |
| C20:2-carnitine§ | 0.49 | 0.15 | 0.03 |
| Prolineǁ | 0.47 | 0.14 | 0.03 |
| C8-carnitine§ | 0.46 | 0.14 | 0.04 |
| Alanineǁ | 0.46 | 0.14 | 0.05 |

*The base model is described as logit(p) = $B_0$ + $B_D$ * Dose + $B_M$ * $Metabolite_i$, where p is the probability of mortality in 90 days
‡compounds with Bonferonni adjusted p values ≤0.05 ranked in ascending order; for the complete list see supplementary Table S-6 in additional file 1
§as measured by LC-MS
ǁas measured by $^1$H-NMR

**Table 4-3. Logistic regression interaction model.**

Tests the relationship between metabolite predictors and mortality by treatment (L-carnitine dose or placebo) for the prediction of 90-day mortality ranked by descending p value.

| | Interaction model† | | |
|---|---|---|---|
| **Metabolite predictor‡** | **Interaction Coefficient ($\beta_{M*D}$)** | **$\beta_{M*D}$ Standard Error** | **$\beta_{M*D}$ P value# (Raw)** |
| C10:1-carnitine§ | -1.22 | 0.37 | <0.0001 |
| C8:1-carnitine§ | -1.07 | 0.35 | 0.001 |
| C8-carnitine§ | -0.97 | 0.36 | 0.01 |
| C10-carnitine§ | -0.97 | 0.36 | 0.01 |
| C18:2-carnitine§ | -0.96 | 0.35 | 0.01 |

| | | | |
|---|---|---|---|
| C14:1-carnitine[§] | -0.90 | 0.34 | 0.01 |
| C12-carnitine[§] | -0.77 | 0.33 | 0.02 |
| C16:1-carnitine[§] | -0.84 | 0.38 | 0.02 |
| Choline[ǁ] | -0.74 | 0.33 | 0.02 |
| C16-carnitine[§] | -0.82 | 0.38 | 0.02 |
| Oxoisocaproate[ǁ] | -0.74 | 0.34 | 0.03 |
| C5-carnitine[§] | -0.70 | 0.36 | 0.04 |
| Valine[ǁ] | -0.69 | 0.35 | 0.05 |
| Acetylcarnitine (C2)[§] | -0.81 | 0.42 | 0.05 |

†The interaction model is described as $\text{logit}(p) = B_0 + B_D * \text{Dose} + B_M * \text{Metabolite}_i + B_{MD} * \text{Metabolite}_i * \text{Dose}$
[§]as measured by LC-MS
[ǁ]as measured by $^1$H-NMR
[#]Raw p values are not adjusted for multiple comparisons.

As these findings were not evident in the parent clinical trial and they suggest that there may be a sepsis endotype that may derive a therapeutic benefit from supplement L-carnitine, we hypothesized that a pharmacometabolomics approach may aid in defining this sub-group of patients. To identify the candidate metabolites, we took a hypothesis-generating approach and considered all metabolites with significant ($\leq$ 0.05) unadjusted p values (n=14 in the logistic regression interaction model; **Table 4-3**) and assessed the Z-statistic of each. Based on this analysis, the metabolites with significant (FDR-corrected p values $\leq$ 0.05) maximum Z-statistics included a number of acylcarnitines as well as the branched chain amino acid, valine (**Table 4-4**; also see supplementary **Table S-9**). In addition to the Z-statistic values, to identify candidate metabolites, we also considered the prevalence of the acylcarnitine signal, the known potential of acetylcarnitine (C2) to predict drug responsiveness[16] and its close metabolic relationship with L-carnitine. Furthermore, the maximum Z-statistic of C12 and C8:1

represented a lower percentage of the clinical cohort than either C5 or acetylcarnitine (C2). As such, we selected acetylcarnitine (C2) as the most viable metabolite candidate to demonstrate the utility of our pharmacometabolomics approach. As examples, we assessed several concentrations of both acetylcarnitine (C2) and valine, including the ones at the maximum Z-statistic, 35 µM (p=0.002; as measured by LC-MS) (**Figure 4-3**) and 88µM (p=0.009), respectively **(**also see **Figure S-8** and **Table S-9** in the supplementary material). These analyses illustration how a pharmacometabolomics may aid in the design of a precision trial of L-carnitine for the treatment of septic shock using the scheme as illustrated in **Figure 4-4**.

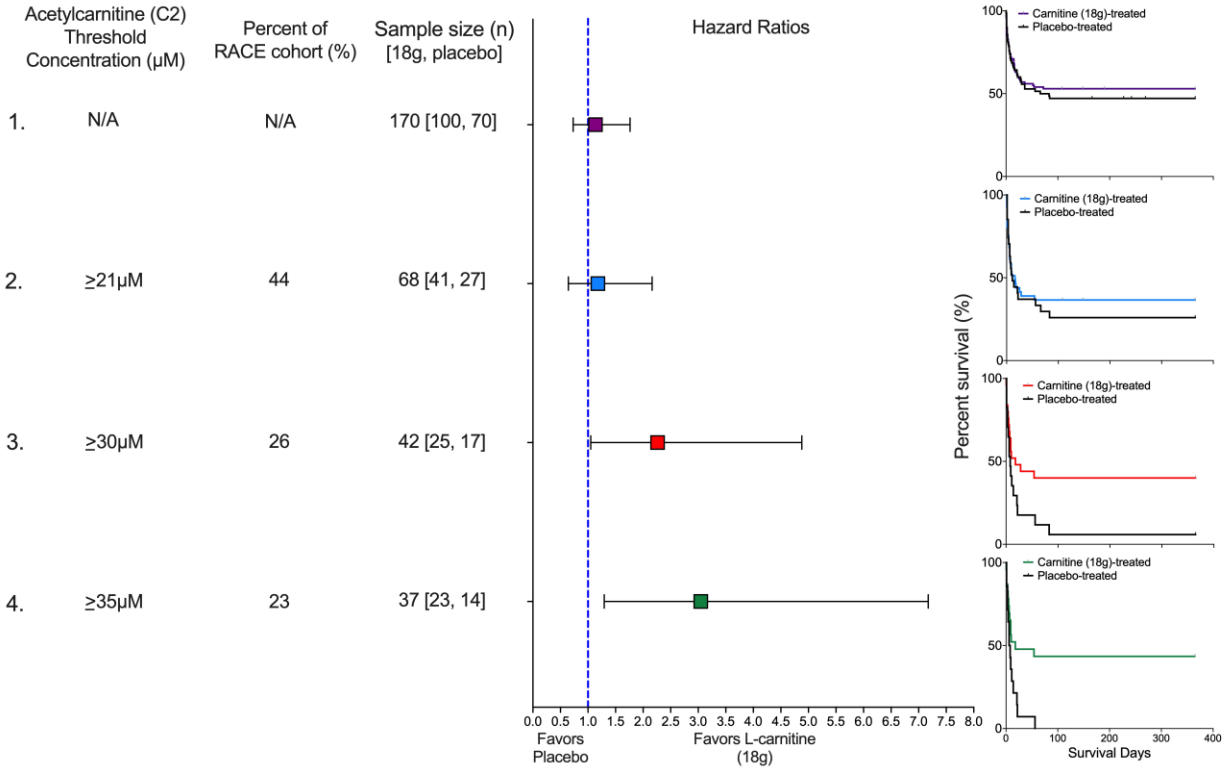
**Table 4-4. Significant metabolites from logistic regression interaction model ranked by descending maximum Z-statistic**

| Metabolite predictor | Maximum Z-statistic | P value | FDR (%)* |
|---|---|---|---|
| C10:1-carnitine§ | 3.71 | 0.0002 | 0.30 |
| C8:1-carnitine§ | 3.50 | 0.0005 | 0.33 |
| C10-carnitine§ | 3.10 | 0.002 | 0.73 |
| Acetylcarnitine (C2)§ | 3.06 | 0.002 | 0.73 |
| C8-carnitine§ | 3.01 | 0.003 | 0.73 |
| C5-carnitine§ | 2.77 | 0.01 | 1.29 |
| Valineǁ | 2.63 | 0.01 | 1.73 |
| C12-carnitine§ | 2.58 | 0.01 | 1.73 |
| C14:1-carnitine§ | 2.54 | 0.01 | 1.75 |
| C18:2-carnitine§ | 2.50 | 0.01 | 1.75 |
| C16-carnitine§ | 2.40 | 0.02 | 1.96 |
| C16:1-carnitine§ | 2.39 | 0.02 | 1.96 |
| Cholineǁ | 1.72 | 0.08 | 9.19 |
| Oxoisocaproateǁ | 1.68 | 0.09 | 9.25 |

§as measured by LC-MS

[ll]as measured by [1]H-NMR
*calculated in MS Excel using the method of Storey, et al[35]

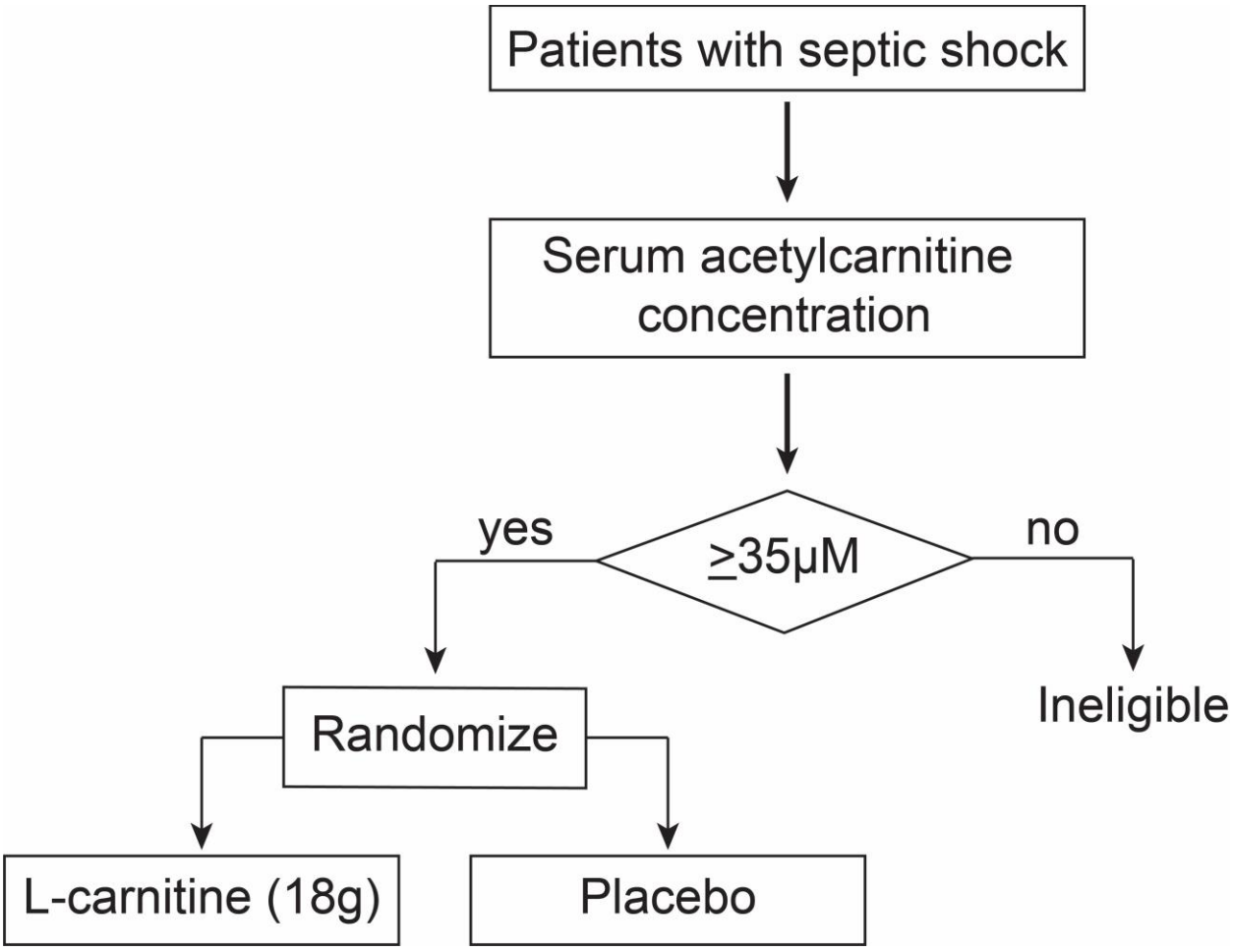
**Figure 4-3. Pretreatment acetylcarnitine (C2) concentration as a predictive clinical trial enrichment strategy.**




Four scenarios illustrate how different threshold concentrations of acetylcarnitine (C2), a high abundant acylcarnitine would have impacted the outcome of the Rapid Administration of Carnitine (RACE) in Sepsis clinical trial in patients treated with either L-carnitine (18 g) or placebo. In scenario one, no threshold concentration is used so the entire RACE cohort (*n* = 236) is eligible. The sample size of 170 patients represents those that received either L-carnitine (18 g; *n* = 100) or placebo (*n* = 70). The hazard ratio is not significant, and consistent with the parent trial, the Kaplan-Meier curve shows no mortality benefit of L-carnitine (*p* = 0.57). In scenario two, an acetylcarnitine (C2) threshold concentration of greater than 21 μM is used. Forty-four percent (*n* = 104) of the RACE cohort met this criterion and of these, 68 patients received either L-carnitine (18 g) or placebo. The hazard ratio is not improved, and the Kaplan-Meier curve shows no mortality benefit of L-carnitine (*p* = 0.59). In scenario three, an acetylcarnitine (C2) threshold concentration of greater than 30 μM is used. Twenty-seven percent (*n* = 64) of the RACE cohort met this criterion and of these, 42 patients received either L-carnitine (18 g) or placebo. The hazard ratio is significant and favors L-carnitine (18 g); the Kaplan-Meier curve shows a mortality benefit of L-carnitine (*p* = 0.04). Finally, scenario four uses the acetylcarnitine (C2) concentration associated with the maximum Z-statistic (Table S-9), greater than 35 μM. Twenty-three percent (*n* = 54) of the RACE cohort met this criterion and of these, 37 patients received either L-carnitine (18 g) or placebo. The hazard ratio is significant, and the Kaplan-Meier curve shows a mortality benefit of L-carnitine (*p* = 0.01). The number of patients at risk at each time point and the number of censored subjects, which was due to the end of the study (1 year), can be found here: https://doi.org/10.7302/vvqp-ma61. N/A, not applicable

**Figure 4-4. A clinical trial enrichment strategy for heterogeneous critical illnesses like sepsis.**

An example of a scheme for a hypothetical phase III clinical trial of supplement L-carnitine for the treatment of septic shock that uses an a priori determined acetylcarnitine (C2) threshold concentration to determine whether a patient is enrolled and randomized to receive either L-carnitine (18 g) or placebo.




## 4.5 Discussion

Our pharmacometabolomics study, CaPS, aimed to identify pre-treatment, sepsis-induced metabolic derangements in survivors and non-survivors treated with L-carnitine. We found that there are likely metabolically distinct groups (endotypes) of patients that do proportionally better when they receive an 18 g dose of supplemental L-carnitine. These findings imply that a precision, clinical trial enrichment strategy using

pharmacometabolomics could help combat the heterogeneity of sepsis and drug response, which is known to have contributed to numerous negative clinical studies.[8]

Here we show that a pharmacometabolomics approach identified clinically indistinguishable sepsis endotypes that are more likely to derive a mortality benefit from treatment with L-carnitine (18 g), a finding not evident in the metabolically naive parent trial. To accomplish this, we used a metabolomics analysis to capture high abundant polar compounds (quantitative $^{1}$H-NMR) and acylcarnitines (LC-MS) in serum samples collected from patients enrolled in a phase II clinical trial of L-carnitine therapy.[18] Using this approach, similar to our prior study[17], we found a prevalent acylcarnitine signal. From this profile, we selected acetylcarnitine (C2) and valine to illustrate how different threshold concentrations could influence mortality outcome in patients randomized to either placebo of L-carnitine (18 g). Specifically, patients with higher (e.g., $\geq$ 30μM) acetylcarnitine (C2) levels at enrolment may be more likely to derive a treatment benefit as defined by decreased intermediate term (90-day) mortality; this benefit is maximized at acetylcarnitine (C2) concentrations $\geq$ 35μM. While severity of illness could contribute to this finding, clinical variables alone do not seem to account for the identification of the drug-responsive endotype since the finding is retained when accounting for factors associated with the risk of death (age and SOFA score, see supplementary **Table S-8**). Notably, we also found that serum concentrations of the branched chain amino acid, valine, could also be used to identify a mortality benefit of L-carnitine but not to as great an extent as acetylcarnitine (C2). Collectively, these data suggest that there are patients that are in clinically occult subgroups. Should these data be validated, metabolically informed clinical trial design[36] and ultimately, precision treatment strategies, could

represent a new paradigm of sepsis care. These data provide the groundwork and rationale for a pharmacometabolomics directed clinical trial to test L-carnitine therapy efficacy for septic shock using a specific concentration of a key metabolite (e.g., acetylcarnitine (C2) to guide inclusion criteria (**Figure 4-2**).

Importantly, the current study also shows that numerous metabolites may have predictive value for sepsis mortality, even after controlling for factors associated with the risk of death (see **Table S-8** in the supplementary material). These data provide further evidence that sepsis induces broad metabolic disruption that is linked to patient outcomes, corroborating prior studies.[37,38] Of note, numerous acylcarnitines, including unsaturated acylcarnitines, predicted mortality, suggesting significant disruption in fatty acid metabolic pathways.[38] Overall, the broad range in disruption of acylcarnitines may reflect differential and variable mobilization of fatty acids,[39] rather than disruption of a specific enzyme or pathway. We have previously demonstrated this in a smaller cohort of septic shock patients.[17] Despite this variance, acetylcarnitine (C2) was the most robust predictor of overall sepsis mortality. This corroborates a previous study that identified acetylcarnitine (C2) as being associated with the severity of sepsis-induced organ dysfunction, inflammation and infection.[37] Acetylcarnitine (C2) also happens to be one of only two compounds (with L-carnitine) detected by both the LC-MS and NMR analytical platforms; regardless of the detection method, it performed similarly in the regression models.

Interestingly, acetylcarnitine (C2) outperformed the more clinically ubiquitous lactate level in predicting sepsis mortality. After correcting for age and SOFA score, lactate was not a significant independent predictor (supplementary **Table S-8**) whereas

acetylcarnitine (C2) retained its predictive value following this correction, which suggests the potential for its use as an adjunctive clinical test for risk prognosis. However, as our cohort was highly selected and involved only participants receiving vasopressors (which affect glycolysis and lactate production)[40,41] who were already resuscitated, it would be inappropriate to interpret these data to imply that lactate does not serve an important role in the early identification and prognosis patients with suspected infection. In particular, serial lactate levels and its clearance rate have been used to assess the adequacy of resuscitation and lactate is included in the sepsis definition.[6,42-44] Nevertheless, limitations of lactate have been recognized[42] and, notably, others have demonstrated that acylcarnitines outperform lactate in predicting sepsis mortality.[38] Our data suggest that acetylcarnitine (C2) may represent a superior risk stratification tool in a selected cohort of fully resuscitated patients undergoing treatment with vasopressor infusions.

We also learned from the CaPS study that pre-treatment serum L-carnitine concentrations did not predict a L-carnitine treatment mortality benefit, suggesting against the hypothesis that serum L-carnitine deficiency drives the response to supplemental L-carnitine in sepsis patients. Rather, in aggregate, these data provide evidence to support the hypothesis that sepsis induces an impairment in the mobilization of acetyl groups. While there may be a number of biologically plausible hypotheses, our findings could be due to sepsis-induced increased intracellular accumulation of acetyl-CoA secondary to its decreased metabolism via the tricarboxylic acid cycle (TCA) or enhanced acetyl-CoA production via fatty acid (beta-oxidation) metabolism (**Figure S-9** in the supplementary material). Consequently, increases in

acetyl-CoA are managed by a number of mechanisms one of which is via the mitochondrial enzyme, carnitine acetyltransferase (EC 2.3.1.7). Carnitine acetyltransferase transfers acetyl groups to carnitine, displacing the hydrogen atom in its hydroxyl group[45] converting it to the membrane-permeable, acetylcarnitine (C2) (**Figure S-9** in the supplementary material). Acetylcarnitine (C2), the shortest of the acylcarnitines, is important because it plays a controlling role over acetyl-CoA on metabolic substrate switching and therefore, enables metabolic flexibility.[45] As the need for ATP increases, acetyl-CoA is diverted to the TCA cycle. However, in sepsis, the TCA cycle may fail to metabolize these groups resulting in excess acetyl-CoA and subsequent elevation in measured serum acetylcarnitine (C2) concentrations. The elevation in acetylcarnitine (C2) may reflect the ability of L-carnitine to serve as route for the disposal of excess acetyl groups which has been demonstrated in the myocardium[46] and during exercise.[47] However, unlike acetylcarnitine (C2), the metabolic link between L-carnitine therapeutic response and BCAA concentrations is less clear. We and others have shown that levels of branched chain amino acids (BCAA) influence sepsis outcome[16,38] and shock resolution.[48] It is possible that patients with elevated BCAA blood concentrations represent those with a metabolic reserve that enables them to more efficiently utilize supplemental L-carnitine[49] but in general, the mechanisms of BCAA signaling and metabolic mechanisms of action are poorly understood.[50] In aggregate, our findings suggest that the magnitude of sepsis-induced disruptions in energy metabolism may be associated with a therapeutic benefit of L-carnitine. This relationship and the mechanisms that underlie it warrant further interrogation.

Despite the encouraging results of our study, we acknowledge that there are several important weaknesses. We recognize that “real-time” metabolomics is not feasible in clinical practice and that routine measurement of these compounds, including acetylcarnitine (C2), for routine clinical use is not currently available. We also employed a limited, focused metabolomic approach, measuring high abundant polar compounds ($^{1}$H-NMR) and acylcarnitines. We acknowledge that a broad, untargeted approach may have yielded additional compounds predictive of outcomes or treatment response. With our targeted approach, we still made multiple comparisons testing involving over 50 metabolites in this study, which opens the door to false positive findings. Our findings persisted after application of a conservative Bonferroni correction, but we acknowledge that the predictive capacity of acetylcarnitine (C2) and valine, when accounting for interactions between baseline metabolite and treatment assignment (interaction model), was not amenable to correction for multiple comparisons. As such, and given that this was an ancillary study, we acknowledge that any conclusions regarding the accurate prediction of clinical drug responsiveness are only hypothesis generating and will require rigorous prospective testing. We did, however, highlight how the use of a number of different acetylcarnitine (C2) and valine concentrations would influence the mortality outcome of the RACE trial (**Figure 4-**3 and supplementary **Figure S-8**). These were merely used as examples to illustrate the utility of a pharmacometabolomics approach and despite including almost 250 patients, we acknowledge that our results may overestimate the true effect size and will require validation in an external cohort. Nevertheless, even though these subgroups represent $\leq$ 50% of the total RACE trial cohort, they highlight the value of a predictive enrichment strategy that could be used to

design a phase III clinical trial of L-carnitine supplementation for septic shock. Importantly, the pharmacometabolomics approach was developed concurrent with the design of the parent trial, and the conceptual model was based on and is consistent with our preliminary work in a unique, though smaller cohort,[16] strengthening the validity of the findings.

In summary, an ancillary pharmacometabolomics study, CaPS, of the parent clinical trial, RACE, found numerous predictors, independent of intervention, age and SOFA score, for 90-day mortality in septic shock including many acylcarnitines and other metabolites such as tyrosine, betaine, lysine and glycine. We also demonstrate the translational value of the work by showing how the application of a pharmacometabolomics-based clinical trial enrichment strategy, using pre-treatment acetylcarnitine (C2) concentrations as an example, could be used to identify the responder population, a sepsis endotype, that may derive a mortality benefit from L-carnitine supplementation. This represents a unique clinical trial enrichment strategy that could be employed to improve the efficiency of a phase III L-carnitine efficacy study in patients with septic shock[10] and other emerging therapeutics in heterogeneous critical illnesses. These findings also support the notion that distinct metabolic endotypes contribute to sepsis heterogeneity.

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# Chapter 5: Population Pharmacokinetics to Understand the Disposition of High-Dose L-Carnitine in Septic Shock

This work is in preparation and will be submitted as an original research manuscript in Spring of 2023. It was presented in abstract form at the 2022 American College of Clinical Pharmacology Annual Meeting.[1]

## 5.1 Chapter Abstract

Levocarnitine (L-carnitine), an endogenous regulator of fatty acid oxidation and mitochondrial function, has shown promise as a metabolic-therapeutic for septic shock, where mortality approaches 40%. In these patients, high-dose (≥ 6 grams) intravenous supplementation results in a broad range of concentrations, the highest of which are associated with mortality. This is not attributable to L-carnitine toxicity but may reflect inherent differences in host-related factors. We sought to describe the population pharmacokinetics (PK) of high-dose L-carnitine in patients with septic shock and test whether metabolic status, genetic variability in a transporter, or clinical outcomes were related to estimated individual parameters.

We leveraged serial serum samples collected from the Rapid Administration of Carnitine in Sepsis (RACE) phase II clinical trial. Patients with vasopressor-dependent septic shock were adaptively randomized to receive intravenous L-carnitine (6 grams, 12 grams, or 18 grams) vs. placebo. Serum was collected at baseline (T0); end-of-infusion (T12); and 24, 48, and 72 hours after treatment. L-carnitine and acylcarnitine

derivatives were measured by liquid-chromatography – mass spectrometry and low-molecular weight, polar metabolites were measured using nuclear magnetic resonance spectrometry. Patients were genotyped by TaqMan at a single nucleotide polymorphism (rs2631367) in the predominant organic carnitine transporter (OCTN2). Population PK analysis was done with baseline normalized L-carnitine concentrations using nonlinear mixed effect models in Monolix (version 2021R1). Age, sex, body mass index, weight, the Sequential Organ Failure Assessment score, transporter genotype, and estimated kidney function were tested as covariates. Final model selection was based on the Bayesian Information Criterion (BIC), visual predictive checks (VPC), and other diagnostics.

We measured LC concentrations in 542 serum samples from 130 patients who received treatment with study drug. A two-compartment model with linear elimination and a fixed volume of distribution (17.1 liters) best described the data and was chosen as a base model. Estimated glomerular filtration rate (eGFR), measured by the 2021 CKD-EPI Creatinine and Cystatin C equation, as a covariate on the elimination rate constant (k) significantly improved model fit and outperformed creatinine clearance (Cockcroft-Gault) and CKD-EPI equations, some of which use an adjustment for self-identified race. Other clinical and demographic covariates on transfer rate constants did not improve model fit. Patients who died prior to 28-days had significantly lower elimination rates (Wilcoxon Signed-Rank, $p<0.001$). The correlation between individual parameter estimates and baseline metabolites were determined and indicated that short chain acylcarnitines were related to both k and $k_{21}$. Genotype at rs2631367 in the gene

encoding OCTN2 was not related to individual patient parameters (Kruskal–Wallis ANOVA, $p>0.05$).

L-carnitine population PK is well-described by a two-compartment model following high-dose supplementation in patients with septic shock. Kidney function is an important driver of L-carnitine elimination in this patient group, and baseline metabolic status and clinical outcomes are further associated with interindividual variability. Future investigations are warranted to differentiate which patient factors drive drug response and define subgroups of patients most likely to benefit from therapy.

## 5.2 Introduction

Sepsis is a clinical syndrome defined by life-threatening organ dysfunction and a dysregulated host response to infection.[2] In 2017, nearly 50 million cases were identified worldwide, with one in five deaths recorded attributable to sepsis.[3] Beyond antimicrobials, treatment for sepsis remains largely non-specific and supportive with a litany of failed clinical trials for more targeted interventions.[4]

L-carnitine is an endogenous molecule that plays a vital bioenergetic role in the mobilization of fatty acids into the mitochondria for subsequent beta oxidation. Given the hypermetabolic state and mitochondrial dysfunction that ensues in many patients with sepsis[5,6], L-carnitine was recently tested as a targeted, metabolic agent in patients with sepsis. In a phase I, randomized, double-blind trial, high-dose L-carnitine was found to be safe in 31 patients with septic shock and demonstrated a modest, but significant improvement in patient mortality.[7] A follow-up phase II-b trial did not find evidence that L-carnitine significantly improved patient mortality or organ dysfunction[8], as measured by the Sequential Organ Failure Assessment (SOFA) score.[9] However,

pharmacometabolomic analyses of the phase I trial have demonstrated significant interpatient variability in L-carnitine and acylcarnitine concentrations post-treatment[10] and that genetic variability in the organic cation transporter (OCTN2), body size, and renal function may be important drivers of the observed variability.[11] Furthermore, a significant mortality benefit was observed in the phase IIb trial in patients who were metabolically more perturbed at baseline.[12] Taken together, these findings suggest heterogeneity in the pharmacokinetics (PK) and effectiveness (pharmacodynamics, PD) of high-dose L-carnitine in septic shock.

The overall goal of our study was to describe the population PK of high-dose, IV L-carnitine in an acutely ill cohort of patients with septic shock. We also sought to determine the patient covariates that improved the model's fit and description of drug concentrations over time. Given that L-carnitine is extensively cleared by renal elimination[13], we recognized an additional opportunity to leverage trial data and contribute to the ongoing debate regarding the ideal approach to estimate kidney function. A secondary goal of this study was to test different equations that estimate kidney function based on serum creatinine ($S_{cr}$), serum cystatin C ($S_{cys}$), and self-identified race in critically ill patients using L-carnitine as a probe molecule.

## 5.3 Methods

### *5.3.1 Study design and participants*

This was a secondary analysis of the Rapid Administration of Carnitine in Sepsis (RACE) clinical trial (NCT01665092).[8] The RACE study was a multicenter, placebo-controlled, phase IIb clinical trial that adaptively randomized patients with septic shock to saline placebo or three dosing arms of IV L-carnitine: 6 grams, 12 grams, or 18

grams. The Bayesian adaptive randomization scheme selected the highest dose as the most efficacious.[14] Study drug or an equivalent volume of saline placebo was given as an IV bolus (33% of dose) immediately followed by a 12-hour infusion. The trial was conducted in accordance with the Declaration of Helsinki, where all patients or their legal representatives provided informed consent and all sites were approved by their local Institutional Review Board.

Adult patients were eligible for the trial if they were: 1) identified within 24-hours of the identification of septic shock; 2) required high-dose vasopressors; 3) presented with moderate organ dysfunction (SOFA $\geq$ 6); and 4) had a blood lactate of at least 18 mg/dL (2 mmoles/L). Patients who were pregnant, breastfeeding, immunocompromised, or had a history of seizures were excluded. Serum samples for drug and other metabolomics analysis were collected at baseline (T0), end-of-infusion (T12), and 24 hours (T24), 48 hours (T48), and 72 hours (T72) after treatment initiation. Full inclusion and exclusion criteria, as well as detailed sample collection and processing have been previously described.[8,12]

### *5.3.2 Drug and Metabolite Quantification*

*Carnitine and acylcarnitines*

L-carnitine and acylcarnitines, which are esters formed from the conjugation of L-carnitine and fatty acids of various chain length, were measured by reverse phase, liquid-chromatography mass-spectrometry at the Michigan Regional Comprehensive Metabolomics Resource Core at the University of Michigan as previously described.[10,12] Absolute quantification for L-carnitine and several acylcarnitines (C2, C3, C4, C5, C8, C14, and C16) was achieved through stable isotope internal standards at a known

concentration (NSK-B Cambridge Isotope Laboratories). An additional eight acylcarnitines were relatively quantified by peak area.

*Small polar molecules*

Proton nuclear magnetic resonance spectroscopy ($^{1}$H-NMR) was used to measure 27 low-molecular weight metabolites at the University of Michigan College of Pharmacy NMR Laboratory using standard methods.[15] Metabolites identified included several amino acids, intermediates of the tricarboxylic acid (TCA) cycle, and other bioenergetic compounds.

### *5.3.3 Renal Function Estimates*

*Quantification of serum creatinine and cystatin C*

Serum creatinine was measured clinically as part of the RACE study, and baseline measures were abstracted from the trial's research electronic data capture (REDCap) database.[16] Cystatin C was measured using biobanked residual serum samples using a standard, commercially available enzyme-linked immunoassay (ELISA) assay according to the manufacturer's instructions (R & D Systems).

*Equations to estimate renal function*

The Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) has established equations to determine a patient's estimated glomerular filtration rate (eGFR) based on $S_{cr}$ and/or $S_{cys}$, patient age, sex, and race. Given the increasing controversy about inclusion of patient race as a variable[17] and the drawbacks of serum creatinine as a renal biomarker[18], we estimated eGFR using four iterations of the CKD-EPI equation: the 2009 CKD-EPI[19] (includes patient race and $S_{cr}$); the 2021 CKD-EPI

equation[20] (uses $S_{cr}$ but drops patient race); the 2012 CKD-EPI[21] (uses only $S_{cys}$); and the 2021 CKD-EPI[20] (includes both $S_{cr}$ and $S_{cys}$ without an adjustment for race). All eGFR calculations were calculated according to standard body surface area and are in the units of mL/min per 1.73 $m^2$.

### *5.3.4 Transporter Genotyping*

L-carnitine is transported into the cell through the OCTN2 transporter, which is also responsible for its renal tubular reabsorption.[13] Given L-carnitine's critical role in metabolic homeostasis, loss of function variants in the gene encoding OCTN2 (SLC22A5) are rare and result in-born errors of metabolism. Nonetheless, a single nuclear polymorphism (rs2631367, −207C>G) has been associated with lower mRNA levels in previous studies and in the Genotype-Tissue Expression (GTEx) Project.[22-24] We isolated DNA from buffy coat collected in the RACE trial and genotyped patients at the rs2631367 loci using a commercially available TaqMan genotyping assay (ThermoFisher®, assay ID C__26479161_30).

### *5.3.5 Pharmacokinetic modeling*

We restricted our secondary analysis to patients who were randomized to receive study drug and who had a baseline and at least one post-treatment serum sample available. For population PK analysis, post-treatment L-carnitine concentrations were baseline normalized in accordance with FDA guidance for modeling endogenous molecules.[25]

All data were cleaned in RStudio, and population PK analysis was performed in Monolix (Version 2021R1, Lixoft SAS, Antony, France). Given the sparse sampling

scheme of the RACE trial in relationship to the drug infusion time, we opted for a fixed population parameter for the volume of distribution (Vd) based on the median weight of the cohort and previous PK reports that Vd for IV L-carnitine is 0.2 to 0.3 L/kg.[13]

To determine the optimal number of compartments and linear versus nonlinear elimination, we first built a base model with one compartment and with a linear elimination rate constant (k). We then built two and three compartment models with linear and Michaelis Menten elimination and selected the model based on the Akaike information criterion (AIC) and model diagnostic plots. In all model comparisons, a two-point reduction or more in the AIC value was considered meaningful.

For the best performing model, we assessed the impact of different renal function parameters as a covariate on the elimination rate constant. We tested the performance of eGFR as estimated by the CKD-EPI equations described above; the CrCL according to Cockgroft-Gault; and $S_{cr}$ and $S_{cys}$ as standalone biomarkers. In addition, we considered the sarcopenia index, calculated as $100*( S_{cr} / S_{cys} )$, which is biomarker of muscle mass rather than true renal function.[26]

We considered additional clinical and demographic patient variables as covariates using the available automated stepwise covariate model (SCM) building algorithm in Monolix. Once the final model was selected, we assessed the relationship between individual patient PK parameters to baseline metabolites, OCTN2 genotype, and patient mortality using standard nonparametric statistical methods.

## 5.4 Results

### *5.4.1 Patients and pharmacokinetic data*

Of the 175 patients randomized to receive L-carnitine in the RACE trial, 130 patients had a baseline and follow-up serum sample available for population PK analysis. In these patients, we measured drug concentrations in 542 serums samples. Observations at end-of-infusion (T12) and T72 were underrepresented (**Table 5-1**), as these samples were only collected during the an initial 'burn-in' phase of the trial, where the first 40 patients were randomized equally to all trial arms.[14] As such, 60% of the cohort in this secondary analysis was randomized to the 18g treatment arm, which was selected as the most efficacious by the Bayesian adaptive design.

**Table 5-1. Characteristics for patients considered in population pharmacokinetic modeling.**

| **Patient characteristics** | **Total Patients, N = 130[1]** |
|---|---|
| L-carnitine dose received | |
| *Low dose (6 grams)* | 27 (21%) |
| *Medium dose (12 grams)* | 25 (19%) |
| *High dose (18 grams)* | 78 (60%) |
| Sex | |
| *Female* | 50 (38%) |
| *Male* | 80 (62%) |
| Age | 62 (53, 70) |
| Weight | 85 (70, 102) |
| Self-Identified Race | |
| *Black* | 41 (32%) |
| *White, Asian, or Other* | 89 (68%) |
| Body Mass Index | 28 (23, 35) |
| Serum Creatinine | 1.93 (1.29, 2.79) |
| Serum Cystatin C | 2.44 (1.57, 3.66) |
| Baseline SOFA Score | 10.0 (8.0, 13.0) |
| OCTN2 genotype at rs2631367 | |
| *CC* | 23 (21%) |
| *CG* | 50 (45%) |
| *GG* | 37 (34%) |
| *Unknown* | 20 |
| Serum samples analyzed for pharmacokinetics | |
| *Baseline, T0* | 130 (100%) |
| *End-of-infusion, T12* | 23 (18%) |
| *24-hours after treatment initiation, T24* | 127 (98%) |
| *48-hours after treatment initiation, T48* | 114 (88%) |
| *72-hours after treatment initiation, T72* | 18 (14%) |

[1]n (%); Median (IQR)

Baseline $S_{cr}$ was available for all patients. Four patients did not have a sufficient volume of residual baseline serum to measure $S_{cys}$, and values were imputed from a simple linear model using $S_{cr}$ and patient age, sex, and weight as predictors (**Supplementary Figure S-10**). Genotype data for OCTN2 at rs2631367 was available for 110 patients (**Table 5-1**).

### *5.4.2 Population pharmacokinetic modeling*

A two-compartment model with a linear elimination and a fixed volume of distribution (17.1 L) provided an optimal model fit (**Table 5-2**). Renal function as a covariate on the elimination rate constant reliably improved model fit regardless of the equation or biomarker used. **Table 5-2** shows the impact on the AIC after including various renal parameters as a covariate on the elimination rate constant. The $eGFR_{cr,cys}$, estimated according to the the 2021 CKD-EPI equation and using both $S_{cr}$ and $S_{cys}$, provided the largest reduction in AIC (*-52.93 points).* The $eGFR_{cr}$ (2021 CKD-EPI using only $S_{cr}$) and $eGFR_{Cys}$ (2012 CKD-EPI using only $S_{cys}$) provided a similar improvement over the base model, while the $eGFR_{cr,\ race}$ (2009 CKD-EPI using both self-identified race and $S_{cr}$) resulted in marginally 'worse' model. All estimates of eGFR outperformed CrCL, according to Cockgroft-Gault. Inclusion of Scys concentration (Δ AIC = -38.62 points) outperformed Scr (Δ AIC = -34.68 points) as a single covariate. The sarcopenia index, a measure of muscle loss rather than true renal function, provided no improvement over the base model (Δ AIC = -1.56 points).

**Table 5-2. Comparison of pharmacokinetic models with various renal function parameters as a covariate on the elimination rate.**

| Model performance with covariate on k | Δ AIC |
|---|---|

| | |
|---|---|
| **Systemic Biomarkers** | |
| Serum Creatinine ($S_{cr}$) | -34.68 |
| Cystatin C ($S_{cys}$) | -38.62 |
| Sarcopenia Index | -1.56 |
| **Renal Function Estimates** | |
| Creatinine Clearance | -39.48 |
| eGFR$^{+}$ 2009 (Race, SCr) | -42.19 |
| eGFR 2012 (CysC) | -45.14 |
| eGRR 2021 (SCr) | -44.78 |
| eGFR 2021 (SCr, CysC) | -52.93 |

+eGFR = estimated glomerular filtration rate

The population parameters for the 'best' performing model with $eGFR_{cr,Cys}$ as a covariate on the elimination rate is shown in **Table 5-3**. The visual predictive check (VPC, **Figure 5-1A**), the probability of individual weighted residual (IWRES) plot (**Figure S-11**), and plot of observed versus predicted concentrations (**Figure S-12**) show model diagnostics and performance. Automated SCM did not identify any additional patient demographics or clinical covariates that substantially improved model performance.

**Table 5-3. Top performing population pharmacokinetic model for high-dose intravenous L-carnitine.**

| Population Parameters | Value | S.E. | R.S.E.(%) |
|---|---|---|---|
| **Fixed Effects** | | | |
| V_pop (L) | 17.1 | - | - |
| k_pop ($hr^{-1}$) | 0.21 | 0.012 | 5.51 |

| | | | |
|---|---|---|---|
| $\beta_{eGFR}$ on k ($hr^{-1}$) | 0.49 | 0.068 | 13.8 |
| k12_pop ($hr^{-1}$) | 0.38 | 0.05 | 13.2 |
| k21_pop ($hr^{-1}$) | 0.16 | 0.017 | 10.8 |
| **Standard Deviation of the Random Effect** | | | |
| ωV | 0.32 | 0.066 | 20.7 |
| ωk | 0.44 | 0.045 | 10.2 |
| ωk12 | 0.39 | 0.08 | 20.8 |
| ωk21 | 0.53 | 0.087 | 16.3 |
| **Error Model Parameters** | | | |
| b | 0.37 | 0.025 | 6.69 |

The population pharmacokinetic model shown below is a two-compartment model with linear elimination and a fixed volume distribution (17.1 L). The $eGFR_{cr,cys}$ as a covariate on the elimination rate constant is estimated according to the the 2021 CKD-EPI equation with both serum cystatin C and serum creatinine. The V_pop parameter was fixed based on the median weight of the cohort and literature estimates for L-carnitine's volume of distribution. S.E. = standard error. R.S.E. = residual standard error as a percentage.

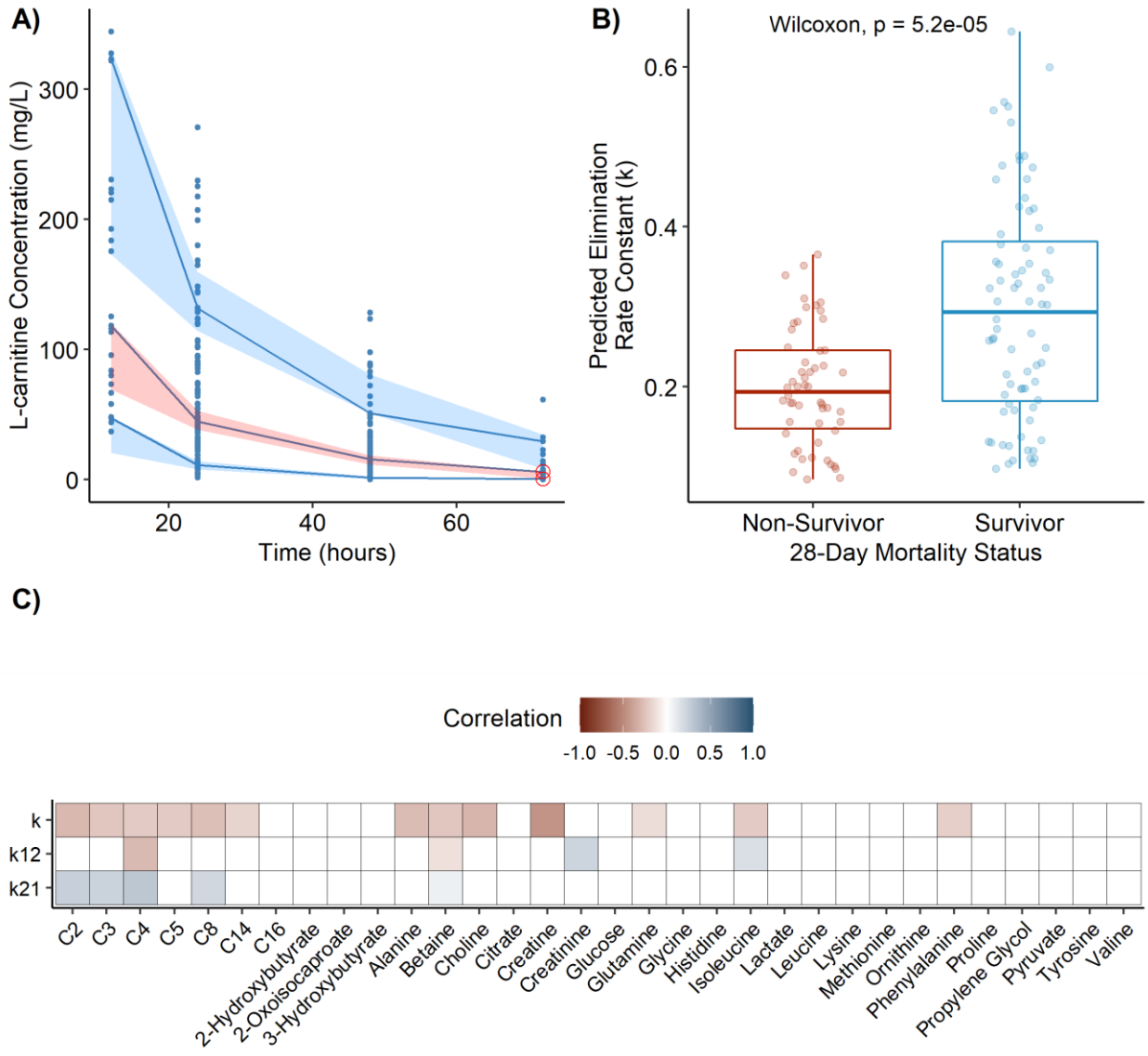


**Figure 5-1. L-carnitine population pharmacokinetics and individual parameter associations with sepsis survival and metabolic status.**

A) Visual predictive check (VPC) for the predicted concentration of L-carnitine over time. B) Boxplots of estimated elimination rate constant (k) stratified by 28-day mortality status. C) Correlation heatmap of correlations between conditional mode estimated individual parameters and baseline metabolites. Individual parameters considered were k and rate in to ($k_1$) and out of ($k_2$) tissue.

### *5.4.3 Other patient factors and individual variation in pharmacokinetics*

From the final model, we determined the individual population PK parameters for the elimination rate (k), the rate out of compartment one ($k_{12}$), and the rate out of compartment two ($k_{21}$). These individual parameters were compared to OCTN2

genotypes (rs2631367), baseline metabolite concentrations, and patient mortality at 28-days.

Twenty-three patients were wildtype (CC) at rs2631367, while 87 patients contained either one (CG, 50 patients) or two (GG, 37 patients) copies of the G allele, which has been associated with greater transporter expression. There was no evidence of a relationship between OCTN2 genotype and any individual PK parameters (by the Kruskal–Wallis rank test, $p>0.05$). Patients who died before 28-days had a lower predicted value for k (Wilcoxon signed rank test, p=5.2e-05, **Figure 5-1B**), but similar values for $k_{12}$ and $k_{21}$. **Figure 5-1C** shows the correlation between individual PK parameters and baseline metabolites measured by LC-MS or NMR. Baseline acylcarnitines tended to be negatively correlated to k and positively correlated to $k_{21}$. Lactate and creatinine were also negatively correlated to k, while lactate and glucose were positively correlated to $k_{12}$.

## 5.5 Discussion

The host-response to infection and pharmacotherapy in sepsis is often highly heterogeneous.[27] A phase I trial of intravenous L-carnitine in patients with septic shock demonstrated a high degree of interindividual variability in the response to the candidate metabolic therapeutic.[7,10] In this secondary population PK analysis of the subsequent phase IIb trial, a two-compartment model with a fixed population parameter for the volume of distribution and eGFR as covariate on the elimination rate constant best fit the observed data. Patient mortality and baseline metabolic status, but not transporter genomics, were related to individual drug response.

L-carnitine is taken orally as a supplement and prescribed intravenously to treat primary carnitine deficiency in patients with end-stage renal disease or with in-born errors of metabolism. As such, the PK of L-carnitine has been previously reported[13,28], albeit at lower routinely clinically used doses and in patients who are not acutely ill. The lack of PK data for L-carnitine given at high-doses in patients with septic shock served as the primary justification for our analysis.

Administration of radiolabeled L-carnitine has demonstrated a renally eliminated drug that can be represented as a 3 compartment model with a central pool (approximating extracellular fluid), a faster equilibrating compartment (likely generalizing to kidney and liver), and a slowly equilibrating compartment (i.e., skeletal muscle).[29] Moreover, endogenous L-carnitine is extensively (>98%) reabsorbed in the renal tubules, and single intravenous doses demonstrate saturation of this process and increased clearance of the compound.[30,31] Although we tested more complex population PK models with nonlinear elimination and multiple compartments, these models were characterized by a higher AIC and poor predictions compared to the 2-compartment model with linear elimination. However, our work does strongly support the importance of renal function in the elimination of high-dose exogenous L-carnitine, as each renal function estimate we considered as a covariate on k dramatically lowered the AIC compared to the base model (**Table 5-1**). This strengthens our justification in using L-carnitine as a probe drug to test alternative approaches when estimating renal function in critically ill patients.

Current clinical and drug development standards rely on the measurement of $S_{cr}$ as a biomarker of renal function. While wide use and the international standardization of

the analytical method to quantify $S_{cr}$ are strengths of this paradigm, there are increasing calls to adopt alternative renal biomarkers. Another endogenous biomarker, $S_{cys}$, has demonstrated modest improvement in estimating kidney function for renally eliminated drugs.[32] Recent work using vancomycin as a renally eliminated probe molecule has shown the value of $S_{cys}$ over $S_{cr}$ in population PK models.[33] In cases when only $S_{cr}$ is available, inclusion of patient race to estimate eGFR has resulted in worse performing models for vancomycin and aminoglycosides.[33,34] In this analysis, we find that $S_{cys}$ outperformed $S_{cr}$ as an individual renal biomarker and covariate on the elimination rate of a renally cleared compound. We also found eGFR equations that leverage $S_{cys}$ provided superior model performance and that inclusion of race to estimate eGFR hurt model fit. Our work adds to growing calls to reconsider the approach to estimating renal function in clinical practice and drug development.

In the phase I trial of L-carnitine in septic shock there was considerable interpatient variability in carnitine and acylcarnitine concentrations post-treatment, with elevated levels associated with mortality.[7,10] Here, we see a similar broad-dynamic range in concentrations following treatment, with non-survivors characterized by lower individual values for the elimination rate and consequently higher concentrations (**Figure 5-1**). Similarly, all acylcarnitines measured were negatively correlated with individual parameters for the elimination rate (**Figure 5-2**). Adverse drug reactions due to L-carnitine were assessed in the phase I and II trials of L-carnitine but known toxicity to the compound including an increased potential for seizures, gastrointestinal side effects, and body odor were not widely reported. This suggests the higher mortality in patients with elevated concentrations is not directly attributable to L-carnitine toxicity,

however this cannot be completely ruled out. Rather, we speculate that the patients with elevated concentrations had worse renal function and/or greater metabolic dysfunction over the course of the study and that decreased clearance of L-carnitine and its acyl derivatives account for our observations.

Genetic variability at rs2631367 in the OCTN2 transporter were associated with peak concentrations of L-carnitine in the smaller phase I study.[11] The G allele has been associated with increased mRNA expression of the transporter in eQTL analysis, potentially granting systemic tissue a greater ability to sequester exogenous L-carnitine.[11] Our results here found that the elimination rate and the rates into and out of tissue were not meaningfully related to OCTN2 genotype. Given that OCTN2 is a highly conservative transporter, owing to its critical in biochemistry, it is possible the impact of altered gene transcription was insufficient to impact drug response in a heterogeneous, acutely ill clinical cohort. Moreover, we were unable to account for the potential of drug-transporter interactions that could impact tissue sequestration of L-carnitine given the lack of detailed concomitant medications in the RACE trial data.

Our study has several strengths and limitations that warrant further consideration. We employed rigorous metabolomics and PK methods to build a well-performing population model of high-dose, intravenous L-carnitine in the setting of septic shock. In building the population model, we chose to test the impact of only patient covariates commonly available in the clinical setting. However, we also assessed the relationship between less commonly available patient information including OCTN2 transporter genotype and baseline metabolic status. We were also able to assess different approaches to estimating renal function in critical illness using a

probe drug candidate in the setting of a multicenter clinical trial. Nonetheless, the blood sampling scheme for the trial was rather sparce, particularly early during the drug's infusion, which superseded our ability to fit a population parameter for the volume of distribution. In addition, we were forced to use baseline normalization when considering drug concentrations post-treatment, as L-carnitine is an endogenous molecule and the investigative product administered was not radio-labeled. Finally, our measurement of $S_{cys}$ was done using residual, biobanked serum and a commercially available ELISA kit rather than a clinical measurement from a fresh patient sample. As such our results regarding the optimal method for estimating eGFR must be interpreted as exploratory and requires rigorous further validation using additional cohorts of critically ill patients and probe drug molecules.

In conclusion, we found that high-dose intravenous L-carnitine in patients with septic shock can be reliably modeled at the population level using a two-compartment model with linear elimination. Renal function as a covariate on the elimination dramatically improved model performance, with methods that incorporate $S_{cys}$ but not patient race providing the greatest improvement. We also found that patient mortality and baseline metabolites were strongly related to individual patient PK parameters.

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# Chapter 6: Concluding Remarks and Future Directions

## 6.1 Summary of project goals

Sepsis is a severe and dysregulated response to infection that is also characterized by organ dysfunction.[1] Despite significant research investment, the mortality in septic shock approaches 40%, and new paradigms in the pharmacotherapy of sepsis have remained elusive in large part due to the clinical heterogeneity of the disease.[2] In addition, sepsis is mechanistically complex and the underpinnings for why some patients have an uncomplicated trajectory, while others suffer from chronic critical illness and/or death is poorly understood.

Sepsis induces profound changes in host metabolism that are associated with organ dysfunction, the inflammatory and immune responses, and patient's clinical outcomes.[3,4] Levocarnitine (L-carnitine), a key regulator of fatty acid oxidation and mitochondrial function, has shown promise as a targeted metabolic-therapeutic agent for the treatment of septic shock.[5] However, L-carnitine's administration is associated with substantial interpatient variability in pharmacokinetics (PK), and patients with elevated peak concentrations of carnitine and acyl-carnitines (ACs) have increased mortality.[6]

The overall objective of this dissertation was to determine metabolic pathways that drive sepsis mortality and patient response to L-carnitine. In these pursuits, I have leveraged time-series metabolomic and patient data from a randomized, double-blinded,

placebo-controlled clinical trial of intravenous l-carnitine vs. saline placebo in patients with septic shock (RACE).[7]

### 6.2 Dysregulated metabolism is associated with variable patient outcomes in septic shock

In Chapters 2 and 3, I explore the relationship between baseline and dynamic metabolic signatures associated with patient outcomes in the natural' sepsis phenotype (i.e., patients randomized to the placebo arm of the trial). I found differentiating trajectories for several acylcarnitines and IL-8 over the first 48-hours among septic shock survivors and non-survivors at 28-days.[8] We also described the correlation between metabolic analytes and protein markers of the host response showing that hyper-inflammatory cytokines were strongly related to lactate and pyruvate, the products of glycolysis. In addition, unsupervised clustering of baseline metabolomics data identified two clinically occult groups that were characterized by distinct organ function over the first 48-hours and mortality outcomes out to one year. The concentration of two acylcarnitines and four amino acids were also found to be associated with prolonged need for organ support (vasopressors and mechanical ventilation) in competing risk regression.[9] Continued need for these life-supporting measures for more than 14 days (defined as chronic critical illness) was similarly related to elevated concentrations of amino acids in the systemic circulation. Together, our findings add to the existing evidence that dysregulated host metabolism, particularly related to impaired β-oxidation of fatty acids and mitochondrial dysfunction, are associated with not only death, but also sepsis morbidity.

### 6.3 Patient factors drive variable drug response to L-carnitine in septic shock

In Chapters 4 and 5, I identified metabolic, genomic, and other patient covariates associated with drug response to L-carnitine. Using logistic regression interaction models and a grid-search methodology across the metabolomics dataset, I tested for candidate metabolic predictors of L-carnitine drug response.[10] I discovered that subgroups of patients who were more metabolically perturbed, namely those with elevated acetylcarnitine and valine, appeared to derive a mortality benefit from L-carnitine that was not present in the trial population at large.[10] This shows the power of metabolomics in limiting the heterogeneity of drug response in sepsis, as predictive metabolic biomarkers could enrich clinical trial design and establish subgroups of patients most likely to respond to specific therapy. I then established a population PK model for high-dose, intravenous L-carnitine in septic shock and explored the impact of different renal function estimates and patient covariates on model performance.[11] Renal function estimates as a covariate on the elimination rate reliably improved model fit. Specifically, renal function calculated with equations that leverage serum cystatin C proved superior to equations based on serum creatinine and self-identified race. Patient mortality and concentrations of other baseline metabolites were related to individual pharmacokinetic parameters; however, I was unable to validate our previous work that implicated a single nucleotide polymorphism in the carnitine organic cation transporter as an important driver of drug response. In summary, this work has established patient variables that are related to the pharmacokinetic and pharmacodynamic response to supplemental L-carnitine in patients with sepsis.

## 6.4 Future directions

In future work, metabolomics and other patient data from the clinical trials of L-carnitine can be leveraged to further the understanding of the metabolic nature of sepsis and identify patients who may derive a benefit from supplemental L-carnitine.

Recent work combining data science with biological and clinical patient characteristics has consistently identified two subphenotypes of acute respiratory distress syndrome.[12,13] Importantly, the hyper- and hypo-inflammatory subgroups are associated with differential mortality (up to 20% higher in inflamed patients), differential response to treatment[14,15], and are generalizable to sepsis.[16,17] Acetylcarnitine, which our work has identified with as a metabolic signal of drug response to L-carnitine, is strongly associated with inflammatory cytokines in patients with sepsis[4], and recent work has shown a glycolytic shift and impaired lipid metabolism among hyper-inflammatory patients with sepsis and ARDS.[18] Taken together, these findings imply a greater metabolic dysfunction within hyper- compared to hypo-inflammatory patients that may have been mitigated by a targeted metabolic agent, L-carnitine. The hyper- and hypo-inflammatory subphenotypes of patients in the RACE trial could be predicted with either machine learning models of patient covariates in the electronic health record[19], or from parsimonious models of a few key biomarkers measured in residual blood samples.[20] This work would show if L-carnitine drug response varies among inflammatory phenotypes with point-of-care potential that have been identified across several cohorts and thousands of patients.

Acetylcarnitine has previously been consistently associated with mortality, organ dysfunction, and inflammation in patients with sepsis.[4,21,22] The work in this dissertation

has largely corroborated these findings in septic shock.[9,23] Our group is collecting available metabolomics data in patients with sepsis to conduct an individual participant data meta-analysis. We will compare acetylcarnitine and lactate, a clinical defining metabolic biomarker of sepsis, and their relationship to mortality and organ dysfunction across hundreds of patients. Our hypothesis is that acetylcarnitine has additional prognostic value for patient outcomes in sepsis.

Metabolomics remains primarily a discovery science, yet its potential to impact patient care and treatment decisions in critically patients is growing. Outside of sepsis, metabolomics has been associated with patient outcomes and employed to understanding the pathophysiology of other critical illness syndromes including ARDS and COVID-19.[24,25] Our group has collected baseline and follow-up blood samples in patients admitted to Michigan Medicine during the first wave of the COVID-19 pandemic. We will employ an untargeted metabolomics assay to determine the relationship between individual metabolites and patient outcomes including mortality, the need for prolonged respiratory support (e.g., mechanical ventilation), and development of additional non-pulmonary organ failures.

### 6.5 Concluding remarks

In determining metabolic pathways that drive sepsis mortality and patient response to L-carnitine, I hypothesized that there are distinct metabolic signatures related to L-carnitine homeostasis and mitochondrial metabolic function that are associated with mortality and drug response in patients with sepsis. Using data from the RACE trial, I have completed several analyses that implicate metabolic dysfunction as a factor that contributes to heterogenous outcomes and response to L-carnitine in patients

with sepsis. However, this line of research is largely embedded in a single clinical trial, and as such the results and conclusions here should be interpreted as hypothesis-generating. Nonetheless, our work demonstrates the potential for integrating metabolomics with other patient and multi-'omics' data to advance the molecular basis for patient outcomes and differential response to therapy in patients with sepsis. Further pursuit of this research in combination with the principles of clinical pharmacology can help transform the care of sepsis and dramatically improve patient lives.

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# Appendices

# Appendix A – Supporting Information for Chapter 2

## A.1 Tables

**Table S-1. Linear mixed model results for measured metabolite and protein analytes.**

For each analyte a fixed vs. interaction model was selected as described in the methods. The overall p-value was determined by comparing the selected model to a null model, which only included a patient-level intercept and covariates. Resulting p-values were corrected for multiple comparisons according to Benjamini and Hochberg and are reported as q values. Model coefficients for Time, Mortality, and their Interaction are reported with their 95% confidence interval. Blue coefficients indicate a negative slope significantly different from zero, while red coefficients indicate a positive slope significantly different from zero.

| Metabolite/Protein | Model | Q-Value | Time | Mortality | Interaction |
|---|---|---|---|---|---|
| C6[1] | FE | 8.10e-05 | **-0.196 (-0.284, -0.107)** | **0.433 (0.027, 0.839)** | |
| C2 (Acetylcarnitine)[1] | FE | 2.76e-04 | **-0.093 (-0.173, -0.014)** | **0.779 (0.382, 1.176)** | |
| C8[1] | FE | 2.76e-04 | **-0.115 (-0.193, -0.037)** | **0.667 (0.28, 1.055)** | |
| C16[1] | IM | 3.02e-04 | **0.167 (0.068, 0.267)** | **0.958 (0.498, 1.418)** | **-0.275 (-0.448, -0.103)** |
| C4[1] | FE | 3.05e-04 | **-0.154 (-0.234, -0.074)** | **0.497 (0.07, 0.924)** | |
| C20.1[1] | IM | 4.36e-04 | **0.163 (0.059, 0.267)** | **0.972 (0.508, 1.437)** | **-0.194 (-0.375, -0.014)** |
| C14.1[1] | IM | 4.66e-04 | 0.074 (-0.02, 0.169) | **1.026 (0.57, 1.482)** | **-0.225 (-0.39, -0.061)** |
| C12[1] | FE | 6.19e-04 | -0.056 (-0.138, 0.027) | **0.869 (0.439, 1.299)** | |
| C16.1[1] | IM | 6.97e-04 | 0.072 (-0.022, 0.166) | **0.98 (0.533, 1.428)** | **-0.216 (-0.38, -0.053)** |
| C12.1[1] | IM | 1.08e-03 | 0.057 (-0.03, 0.144) | **0.98 (0.522, 1.438)** | **-0.18 (-0.332, -0.028)** |
| C18.1[1] | FE | 1.28e-03 | -0.023 (-0.094, 0.048) | **0.848 (0.422, 1.273)** | |
| C14[1] | IM | 1.42e-03 | 0.023 (-0.083, 0.128) | **0.919 (0.475, 1.363)** | **-0.19 (-0.372, -0.008)** |
| C18.2[1] | IM | 1.85e-03 | 0.02 (-0.069, 0.109) | **0.848 (0.374, 1.323)** | **-0.207 (-0.362, -0.052)** |
| C10.1[1] | FE | 2.64e-03 | -0.002 (-0.073, 0.07) | **0.776 (0.368, 1.183)** | |
| C3[1] | FE | 2.77e-03 | -0.067 (-0.148, 0.015) | **0.705 (0.282, 1.128)** | |
| C8.1[1] | FE | 3.00e-03 | **-0.178 (-0.276, -0.081)** | 0.146 (-0.294, 0.586) | |
| C20.2[1] | IM | 3.93e-03 | 0.056 (-0.046, 0.157) | **0.913 (0.437, 1.389)** | **-0.189 (-0.365, -0.013)** |
| C20[1] | FE | 4.07e-03 | **0.103 (0.022, 0.184)** | **0.607 (0.156, 1.059)** | |

| | | | | | |
|---|---|---|---|---|---|
| C10[1] | FE | 5.15e-03 | -0.028 (-0.113, 0.058) | **0.689 (0.289, 1.09)** | |
| C18[1] | IM | 6.18e-03 | **0.142 (0.025, 0.259)** | **0.817 (0.339, 1.295)** | **-0.207 (-0.41, -0.004)** |
| L-carnitine[1] | FE | 1.18e-02 | **-0.097 (-0.17, -0.025)** | 0.38 (-0.073, 0.833) | |
| C5[1] | FE | 2.37e-02 | -0.025 (-0.098, 0.048) | **0.591 (0.175, 1.008)** | |
| C20.4[1] | FE | 2.26e-01 | 0.062 (-0.052, 0.176) | -0.272 (-0.657, 0.112) | |
| C20.3[1] | FE | 2.67e-01 | -0.028 (-0.188, 0.132) | 0.291 (-0.057, 0.639) | |
| | | | | | |
| Lactate[2] | FE | 1.72e-09 | **-0.456 (-0.583, -0.33)** | 0.309 (-0.045, 0.664) | |
| Pyruvate[2] | IM | 2.39e-09 | **-0.53 (-0.683, -0.377)** | 0.089 (-0.336, 0.515) | **0.287 (0.023, 0.551)** |
| Isoleucine[2] | FE | 7.82e-06 | **0.346 (0.223, 0.469)** | 0.144 (-0.28, 0.567) | |
| Lysine[2] | FE | 7.82e-06 | **0.293 (0.187, 0.399)** | 0.336 (-0.073, 0.745) | |
| Propylene glycol[2] | FE | 5.66e-05 | **-0.274 (-0.398, -0.15)** | **0.401 (0.033, 0.769)** | |
| Methionine[2] | FE | 3.53e-04 | **0.263 (0.138, 0.389)** | **0.402 (0.026, 0.779)** | |
| Valine[2] | FE | 3.96e-04 | **0.28 (0.156, 0.405)** | 0.132 (-0.279, 0.543) | |
| C2 (Acetylcarnitine)[2] | FE | 4.66e-04 | **-0.113 (-0.221, -0.005)** | **0.679 (0.312, 1.046)** | |
| L-carnitine[2] | FE | 1.19e-03 | **-0.179 (-0.31, -0.047)** | **0.533 (0.153, 0.914)** | |
| 3-Hydroxybutyrate[2] | FE | 2.80e-03 | **-0.182 (-0.325, -0.039)** | **0.45 (0.094, 0.807)** | |
| Leucine[2] | FE | 4.07e-03 | **0.21 (0.093, 0.327)** | 0.18 (-0.243, 0.602) | |
| Ornithine[2] | FE | 5.37e-03 | **0.223 (0.095, 0.351)** | -0.033 (-0.461, 0.395) | |
| Creatinine[2] | FE | 6.16e-03 | **-0.172 (-0.275, -0.069)** | 0.133 (-0.289, 0.555) | |
| Glycine[2] | FE | 1.33e-02 | **0.115 (0.019, 0.21)** | **0.443 (0.05, 0.835)** | |
| 2-Hydroxybutyrate[2] | FE | 3.20e-02 | -0.092 (-0.195, 0.011) | **0.425 (0.012, 0.837)** | |
| Alanine[2] | FE | 3.32e-02 | -0.083 (-0.186, 0.021) | **0.44 (0.035, 0.846)** | |
| Tyrosine[2] | FE | 3.35e-02 | 0.077 (-0.024, 0.179) | **0.521 (0.097, 0.945)** | |
| Citrate[2] | FE | 5.51e-02 | **0.128 (0.013, 0.243)** | 0.317 (-0.105, 0.74) | |
| Betaine[2] | FE | 1.12e-01 | -0.085 (-0.176, 0.006) | 0.229 (-0.176, 0.634) | |
| Glutamine[2] | FE | 1.43e-01 | -0.008 (-0.114, 0.098) | **0.422 (0.019, 0.826)** | |
| Phenylalanine[2] | FE | 2.06e-01 | 0.093 (-0.018, 0.204) | 0.234 (-0.204, 0.672) | |
| 2-Oxoisocaproate[2] | FE | 4.04e-01 | -0.07 (-0.219, 0.078) | 0.196 (-0.197, 0.59) | |
| Creatine[2] | FE | 4.10e-01 | 0.026 (-0.048, 0.099) | 0.29 (-0.164, 0.743) | |
| Proline[2] | FE | 4.10e-01 | 0.02 (-0.095, 0.135) | 0.278 (-0.121, 0.677) | |
| Glucose[2] | FE | 5.74e-01 | 0.091 (-0.075, 0.258) | -0.015 (-0.366, 0.335) | |

| | | | | | |
|---|---|---|---|---|---|
| Histidine[2] | FE | 6.16e-01 | 0.052 (-0.056, 0.16) | 0.088 (-0.32, 0.495) | |
| Choline[2] | FE | 9.47e-01 | -0.024 (-0.167, 0.119) | -0.007 (-0.408, 0.395) | |
| | | | | | |
| IL-6[3] | IM | 2.10e-26 | **-0.759 (-0.87, -0.647)** | -0.224 (-0.625, 0.177) | **0.228 (0.038, 0.419)** |
| IL-8[3] | IM | 9.56e-24 | **-0.577 (-0.674, -0.48)** | **0.654 (0.231, 1.076)** | **0.185 (0.018, 0.352)** |
| TNFα[3] | IM | 1.80e-22 | **-0.531 (-0.619, -0.444)** | -0.164 (-0.628, 0.301) | **0.17 (0.019, 0.322)** |
| IL-10[3] | FE | 6.40e-19 | **-0.456 (-0.535, -0.377)** | **0.496 (0.084, 0.909)** | |
| IL-12(p40)[3] | FE | 1.57e-11 | **-0.342 (-0.422, -0.263)** | 0.001 (-0.455, 0.457) | |
| ANG2[3] | IM | 2.39e-09 | **-0.327 (-0.412, -0.242)** | -0.194 (-0.636, 0.249) | **0.314 (0.165, 0.463)** |
| IL-1β[3] | FE | 2.21e-08 | **-0.252 (-0.326, -0.177)** | 0.332 (-0.131, 0.796) | |
| Fractalkine[3] | FE | 3.44e-06 | **-0.221 (-0.299, -0.143)** | 0.296 (-0.154, 0.746) | |
| IL-1α[3] | FE | 1.08e-03 | **-0.156 (-0.242, -0.07)** | 0.403 (-0.045, 0.851) | |
| IL-18[3] | FE | 1.63e-03 | **-0.104 (-0.157, -0.052)** | 0.025 (-0.491, 0.542) | |

[1]Measured by liquid-chromatography mass-spectrometry
[2]Measured by nuclear magnetic resonance spectroscopy
[3]Measured by protein immunoassay
FE = fixed effects model
IM = interaction model

# Appendix B – Supporting Information for Chapter 3

## B.1 Supporting Methods

*Definition of modified Charlson Comorbidity Index:* Patient demographics, comorbidities, and clinical outcomes were recorded and are maintained in a secure research electronic data capture (REDCap) database.[1] However, it was not possible to obtain all comorbidities necessary to calculate a full comorbidity score according to Charlson.[2] Specifically, we lacked information on peptic ulcer disease and connective tissues diseases and could not distinguish between uncomplicated diabetes vs. diabetes with residual organ damage or HIV positive vs. Acquired immunodeficiency syndrome. Thus, our modified scale assigns a score of +1 to any patient with documented diabetes and does not account for the remaining disease states.

## B.2 Figures

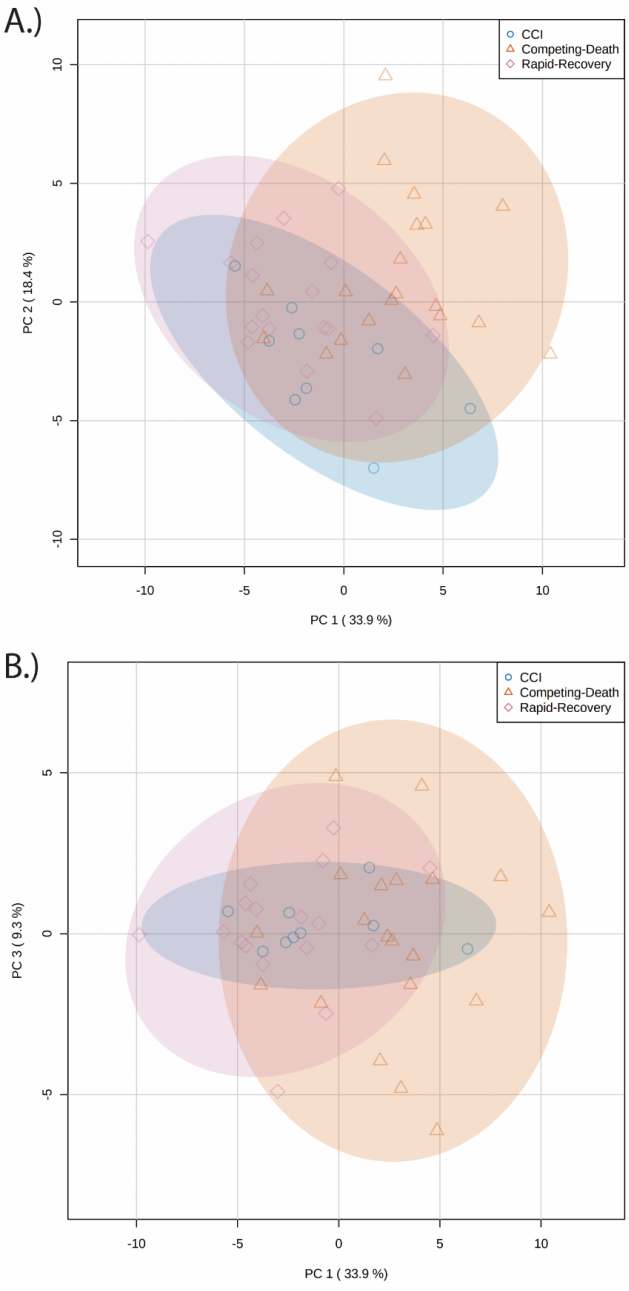


**Figure S-1. Principal component analysis (PCA) stratified by patient outcomes.**

The first three principal components explained ~62% variation; 33.9% (PC1), 18.4% (PC2), and 9.3% (PC3). Patients who required at least 14 days of mechanical ventilation or vasopressors were classified as chronic critical illness ('CCI', blue circles) and were more similar to patients who experienced rapid recovery ('Rapid-Recovery', purple diamonds) than patients who died at or prior to 28 days ('Competing-Death', orange triangles).

## B.3 Tables

**Table S-2. Metabolites detected and quantified by LC-MS/MS and 1H-NMR.**

The Kyoto Encyclopedia of Genes and Genomes (KEGG) identification (ID) number is provided when available (+). When a corresponding KEGG ID does not exist, the human metabolomics database (HMDB) ID number is provided (*). When neither a KEGG nor HMDB ID is available, a metabolite is assigned N/A. The missingness for NMR metabolites represents the percentage of samples for which that respective compound did not have a reported value.

| **LC-MS/MS Metabolites** | | **$^{1}$H-NMR Metabolites** | | |
|---|---|---|---|---|
| Compound Name | KEGG ID$^{+}$ | Compound Name | KEGG ID$^{+}$ | Missingness |
| Levocarnitine | C00318 | 2-Hydroxybutyrate | C05984 | 0% |
| Acetylcarnitine (C2) | C02571 | 2-Oxoisocaproate | C00233 | 17.4% |
| Propanoylcarnitine (C3) | C03017 | 3-Hydroxybutyrate | C01089 | 2.2% |
| Butanoylcarnitine (C4) | C02862 | Acetylcarnitine (C2) | C02571 | 2.2% |
| Valerylcarnitine (C5) | HMDB0013128* | Alanine | C00041 | 0% |
| Hexanoylcarnitine (C6) | HMDB0000756* | Betaine | C00719 | 2.2% |
| C8:1-carnitine | HMDB0013324* | Levocarnitine | C00318 | 10.9% |
| Octanoylcarnitine (C8) | C02838 | Choline | C00114 | 19.6% |
| C10:1-carnitine | HMDB0240585* | Citrate | C00158 | 15.2% |
| Decanoylcarnitine (C10) | C03299 | Creatine | C00300 | 0% |
| C12:1-carnitine | HMDB0013326* | Creatinine | C00791 | 0% |
| Dodecanoylcarnitine (C12) | HMDB0002250* | Glucose | C00221 | 0% |
| C14:1-carnitine | HMDB0002014* | Glutamine | C00064 | 0% |
| Tetradecanoylcarnitine (C14) | HMDB0005066* | Glycine | C00037 | 0% |
| C16:1-carnitine | HMDB0006317* | Histidine | C00135 | 8.7% |
| C20:4-carnitine | HMDB0006455* | Isoleucine | C00407 | 15.2% |
| C18:2-carnitine | HMDB0006469* | Lactate | C00186 | 0% |
| C20:3-carnitine | N/A | Leucine | C00123 | 0% |
| Palmitoylcarnitine (C16) | C02990 | Lysine | C00047 | 2.2% |
| C18:1-carnitine | HMDB0006464* | Methionine | C00073 | 15.2% |
| C20:2-carnitine | N/A | Ornithine | C00077 | 26.1% |
| Stearoylcarnitine (C18) | HMDB0000848* | Phenylalanine | C00079 | 0% |
| C20:1-carnitine | N/A | Proline | C00148 | 15.2% |
| Arachidonoylcarnitine (C20) | HMDB0006455* | Propylene glycol | C00583 | 0% |
| | | Pyruvate | C00022 | 0% |
| | | Tyrosine | C00082 | 0% |
| | | Valine | C00183 | 0% |

**Table S-3. Patient demographics and clinical characteristics.**

| Patient characteristic | N = 47 |
|---|---|
| Age (years)[1] | 61 (46, 70) |
| Sex[2] | |
| Female | 21 (45%) |
| Male | 26 (55%) |
| Race[2] | |
| African American | 14 (30%) |
| Caucasian | 32 (70%) |
| Unknown | 1 |
| Charlson Comorbidity Index[1] | 4.00 (2.00, 5.50) |
| Heart Rate (beats/minute)[1] | 103 (94, 113) |
| Respiratory Rate (breaths/minute)[1] | 20.0 (16.5, 25.0) |
| Cumulative vasopressor index[1] | 4.00 (4.00, 8.00) |
| Unknown | 1 |
| Body mass index[1] | 27 (22, 35) |
| SOFA score[1,3] | 9.00 (8.00, 11.00) |
| White blood count (cells/mm$^3$)[1] | 24 (12, 31) |
| Unknown | 13 |
| Platelet (cells/mm$^3$)[1] | 147 (126, 214) |
| Unknown | 1 |
| Creatinine (mg/dL)[1] | 1.60 (1.16, 2.33) |
| Total bilirubin (mg/dL)[1] | 1.30 (0.60, 3.30) |
| Clinical lactate (mmol/L)[1] | 3.60 (2.30, 6.50) |
| Unknown | 10 |

[1]Median (IQR)
[2]N (%)
[3]Neurological component removed

**Table S-4. One-way analysis of variance (ANOVA) for differences in metabolite concentrations stratified by patient outcomes.**

The ANOVA p-values were corrected for multiple comparisons according to the false discovery rate (FDR) procedure of Benjamini–Hochberg. For metabolites with an FDR <0.05, post-hoc testing for between group differences was done according to Fisher's Least Square Difference and reported when significant (FDR <0.05). CCI = chronic critical illness; RR = rapid recovery.

| Metabolite | F-Value | P-Value | FDR | Post-hoc Testing |
|---|---|---|---|---|
| C16:1-carnitine | 14.38 | 1.65E-05 | 8.43E-04 | Death vs. CCI; Death vs. RR |
| C14:1-carnitine | 12.36 | 5.75E-05 | 1.47E-03 | Death vs. CCI; Death vs. RR |
| Dodecanoylcarnitine (C12) | 11.56 | 9.61E-05 | 1.63E-03 | Death vs. CCI; Death vs. RR |
| Tetradecanoylcarnitine (C14) | 10.95 | 1.43E-04 | 1.83E-03 | Death vs. CCI; Death vs. RR |
| Palmitoylcarnitine (C16) | 10.20 | 2.38E-04 | 2.42E-03 | Death vs. CCI; Death vs. RR |
| Acetylcarnitine (C2, LC-MS) | 9.69 | 3.35E-04 | 2.82E-03 | Death vs. CCI; Death vs. RR |
| Decanoylcarnitine (C10) | 9.22 | 4.66E-04 | 2.82E-03 | Death vs. CCI; Death vs. RR |
| C12:1-carnitine | 9.10 | 5.05E-04 | 2.82E-03 | Death vs. CCI; Death vs. RR |
| C20:2-carnitine | 9.04 | 5.29E-04 | 2.82E-03 | Death vs. CCI; Death vs. RR |
| C18:1-carnitine | 8.97 | 5.53E-04 | 2.82E-03 | Death vs. CCI; Death vs. RR |
| C18:2-carnitine | 8.32 | 8.79E-04 | 4.08E-03 | Death vs. CCI; Death vs. RR |
| C20:1-carnitine | 7.93 | 1.17E-03 | 4.97E-03 | Death vs. CCI; Death vs. RR |
| Acetylcarnitine (C2, NMR) | 7.81 | 1.28E-03 | 5.02E-03 | Death vs. RR |
| Stearoylcarnitine (C18) | 7.06 | 2.23E-03 | 8.13E-03 | Death vs. CCI; Death vs. RR |
| C20:3-carnitine | 6.62 | 3.11E-03 | 1.06E-02 | Death vs. CCI; Death vs. RR |
| Proline | 6.41 | 3.66E-03 | 1.17E-02 | CCI vs. RR; Death vs. RR |
| C10:1-carnitine | 5.68 | 6.48E-03 | 1.94E-02 | Death vs. CCI; Death vs. RR |
| Octanoylcarnitine (C8) | 5.45 | 7.80E-03 | 2.03E-02 | Death vs. CCI; Death vs. RR |
| Glycine | 5.43 | 7.90E-03 | 2.03E-02 | CCI vs. RR; Death vs. RR |
| C20-carnitine | 5.42 | 7.95E-03 | 2.03E-02 | Death vs. CCI; Death vs. RR |
| Propanoylcarnitine (C3) | 5.05 | 1.07E-02 | 2.60E-02 | Death vs. RR |
| Glutamine | 4.61 | 1.53E-02 | 3.55E-02 | CCI vs. RR; Death vs. RR |
| Methionine | 4.32 | 1.95E-02 | 4.32E-02 | CCI vs. RR; Death vs. RR |
| C20:4-carnitine | 4.11 | 2.32E-02 | 4.93E-02 | Death vs. CCI |
| L-Carnitine (LC-MS) | 4.03 | 2.48E-02 | 5.06E-02 | NA |
| Valerylcarnitine (C5) | 3.76 | 3.13E-02 | 6.14E-02 | NA |
| L-carnitine (NMR) | 3.46 | 4.05E-02 | 7.65E-02 | NA |
| Betaine | 3.25 | 4.83E-02 | 8.81E-02 | NA |
| Propylene Glycol | 3.09 | 5.56E-02 | 9.77E-02 | NA |
| Alanine | 2.94 | 6.36E-02 | 1.08E-01 | NA |
| Citrate | 2.78 | 7.31E-02 | 1.20E-01 | NA |
| 3-Hydoxybutyrate | 2.55 | 8.98E-02 | 1.43E-01 | NA |
| Tyrosine | 2.45 | 9.80E-02 | 1.52E-01 | NA |
| Phenylalanine | 2.34 | 1.08E-01 | 1.63E-01 | NA |

| | | | | |
|---|---|---|---|---|
| Choline | 2.05 | 1.42E-01 | 2.06E-01 | NA |
| Leucine | 1.87 | 1.67E-01 | 2.37E-01 | NA |
| Histidine | 1.82 | 1.75E-01 | 2.41E-01 | NA |
| 2-Hydroxybutyrate | 1.69 | 1.97E-01 | 2.58E-01 | NA |
| Butanoylcarnitine (C4) | 1.65 | 2.03E-01 | 2.58E-01 | NA |
| Hexanoylcarnitine (C6) | 1.65 | 2.04E-01 | 2.58E-01 | NA |
| Lysine | 1.63 | 2.07E-01 | 2.58E-01 | NA |
| Valine | 1.50 | 2.34E-01 | 2.84E-01 | NA |
| Lactate | 1.38 | 2.62E-01 | 3.10E-01 | NA |
| C8:1-carnitine | 1.05 | 3.60E-01 | 4.17E-01 | NA |
| Glucose | 0.81 | 4.52E-01 | 5.12E-01 | NA |
| Isoleucine | 0.77 | 4.70E-01 | 5.21E-01 | NA |
| Creatine | 0.70 | 5.02E-01 | 5.45E-01 | NA |
| Pyruvate | 0.60 | 5.53E-01 | 5.88E-01 | NA |
| Ornithine | 0.25 | 7.78E-01 | 8.09E-01 | NA |
| 2-Oxoisocaproate | 0.12 | 8.89E-01 | 9.07E-01 | NA |
| Creatinine | 0.07 | 9.32E-01 | 9.32E-01 | NA |

## B.4 References


1. Harris PA, Taylor R, Thielke R, Payne J, Gonzalez N, Conde JG. Research electronic data capture (REDCap)--a metadata-driven methodology and workflow process for providing translational research informatics support. *J Biomed Inform*. Apr 2009;42(2):377-81.

2. Charlson ME, Pompei P, Ales KL, MacKenzie CR. A new method of classifying prognostic comorbidity in longitudinal studies: development and validation. *J Chronic Dis*. 1987;40(5):373-83.

## Appendix C – Supporting Information for Chapter 4

### C.1 Methods

*Patient selection:* Detailed inclusion and exclusion criteria have been previously reported.[1] Briefly, patients presenting with suspected or confirmed infection and meeting consensus criteria for septic shock including administration of continuous vasopressors despite adequate fluid resuscitation, an elevated lactate >2 mmol/L, and a SOFA score of at least 5 were eligible for inclusion. All patients enrolled in the clinical trial were included in the present analysis with the exception of 2 patients who withdrew consent following randomization and 12 that lacked a T0 sample (**Figure S1**).

*Blood sampling:* Following consent but prior to initiation of therapy, whole blood was drawn into serum separator tubes (SST®; Vacutainer, Becton Dickinson, Franklin Lakes, NJ) and was allowed to clot for at least 30 min at room temperature. A portion of whole blood was aliquoted, while the remainder was centrifuged to yield serum. All samples were then frozen at -80°C within 60 minutes of the initial sample acquisition. Since blood samples were collected from study participants enrolled in a clinical trial, the collection time (T0) of the pre-treatment sample was at the time of enrollment. This was based on when the patient met the study's inclusion criteria and was not anchored to the timing of ICU or hospital admission. This approach has been previously utilized by our group to yield samples appropriate for metabolomics analyses.[2] At the

completion of the clinical trial, de-identified serum samples were shipped on dry ice to the University of Michigan (NMR Metabolomics Laboratory) for assay. The CaPS study was deemed “not regulated” by the University of Michigan’s institutional review board since only de-identified samples and data would be used (HUM00104311).

*Sample handling:* Upon receipt, technical replicate frozen serum samples were transported on dry ice from the NMR Metabolomics Laboratory to the Michigan Regional Comprehensive Metabolomics Research Core. Samples were inventoried, assigned a unique identifier and stored (-80°C) until the time of assay. In preparation for assay, samples were randomized and batched. Samples from a total of 236 of the 250 patients enrolled in the parent clinical trial were assayed; 40 from the dose-response phase of RACE, and 196 from the block randomization phase.[1] Dose response samples were received, processed, and were assayed in advance and separately from the block randomization samples but the randomization schemes were the same for both and the resulting data were combined. For both metabolomics assays, each sample was assigned a random number and samples were ordered by this number for assay. A small volume (50µL) from each sample was reserved for assay of free hemoglobin.[3] The dose response samples were analyzed with Cayman Chemicals hemoglobin colorimetric assay (Ann Arbor, MI; catalog #: 700540), and block randomization samples were analyzed with Arbor Assays hemoglobin high sensitivity detection kit (Ann Arbor, MI; catalog #: K013-HX1). Sample handling was done in accordance with BRISQ guidelines.[4]

*Measurement of Acylcarnitines:* Analysis was performed consistent with our prior methods.[5] Briefly, at the time of analysis, serum samples were thawed on ice and extraction solvent was prepared consisting of a 1:1:1 mixture of methanol:acetonitrile:acetone plus a 1:200 dilution of a stock mixture of stable-isotope labeled acylcarnitine standards dissolved according to manufacturer instructions (NSK-B, Cambridge Isotope Laboratories). Acylcarnitine species were then analyzed without derivatization by RPLC-MS/MS using an Agilent 1200 LC coupled to an Agilent 6410 tandem quadrupole (Santa Clara, CA). A pooled sample generated by combining a small volume of all study samples was injected every $10^{th}$ run and was used for intra- and inter-batch drift correction as described below. Absolute quantitation by isotope dilution mass spectrometry was performed for acylcarnitine species with exact-matching stable isotope internal standards (L-carnitine, C2, C3, C4, C5, C8, C14, C16) by multiplying the unlabeled / labeled peak area ratio by the known concentration of acylcarnitine in the extraction solvent, then adjusting for dilution of the original serum sample. Other acylcarnitine species were limited to relative quantitation by peak area.

*Quantitative Nuclear Magnetic Resonance (NMR) metabolomics:* Serum samples were prepared by methanol precipitation followed by ultrafiltration as previously described.[6] $^{1}$H-NMR spectra were acquired at the University of Michigan's Biochemical NMR Core Laboratory on a Varian (now Agilent, Inc., Santa Clara, CA) 11.74 Tesla (500 MHz) NMR spectrometer with a VNMRS console operated by host software VNMRJ 4.0 and equipped with a 5-mm Agilent "One-probe." NMR spectra were recorded using 32 scans of the first increment of a 1 H,1 H-NOESY (commonly referred to as a 1D-NOESY or

METNOESY) pulse sequence. Spectra were acquired at a room temperature of 295.45 ± 0.3 K. The NMR pulse sequence was as follows: a 1 s recovery delay, a 990 ms saturation pulse of 80 Hz (gB1) induced field strength empirically centered on the water resonance, 2 calibrated 90° pulses, a mixing time (tmix) of 100 ms, a final 90° pulse, and an acquisition period of 4 s. Optimal excitation pulse widths were obtained by utilizing an array of pulse lengths as previously described.[7] Individuals, blinded to the treatment designation of each sample, analyzed NMR spectra (e.g., carnitine treatment) using Chenomx NMR Suite 8.2 (Edmonton, AB, Canada) software. The Processor module was used to phase shift, baseline correct and excise water from each spectrum. Compounds were identified and relatively quantified using the Profiler module of the software, which allows metabolites to be quantified relative to the area of the internal standard (formate, 50µL of 9.64 mM) added to each sample. Before statistical analysis, data were scaled to correct for differences in initial sample volumes. Only metabolites detected in at least 70% of all samples were considered in the analysis and some samples were excluded because of technical issues (**Figure S1**).

*Preparation of metabolomics data for statistical analyses*: Patients with any metabolomic data were included in the analyses. Assayed acylcarnitines were detected in all samples. Because the samples for the acylcarnitine assay were batched and assayed by LC-MS, we assessed and corrected for batch related differences and within-batch intensity using custom-written scripts operating in R. These scripts use local estimate of scatterplot smoothing (LOESS) to correct for within- and between-batch intensity drift in each acylcarnitine species individually, based on peak intensity

measured in a pooled QC sample run every 10th sample through all batches.[8,9] For the NMR data set, in preparation for statistical analysis and since concentration values for metabolites that are typically detected can be being missing in the final data set, missing data were replaced with half of the minimum concentration of the respective metabolite across all samples. All detected metabolites and their Kyoto Encyclopedia of Genes and Genomes (KEGG) identification numbers are shown in **Table S1**. The metabolomics data sets, subject demographics and R code used in the manuscript's data analyses can be found at: https://github.com/UMichNMR-Metabolomics. All analytical protocols will be made available upon request.

### C.2 Results

The acquisition of the T0 blood sample occurred at a mean (+SD) of 11(+7) hours from the time of meeting inclusion criteria.

*Serum free hemoglobin*: We assayed all samples for free hemoglobin since we have previously shown that, in serum samples, it may contribute to a distortion in the concentration of certain metabolites particularly if the hemoglobin concentration is greater than 0.1 g/dL.[3] All but one (0.2g/dL) of the assayed samples had a free hemoglobin concentration below 0.1 g/dL (data not shown).

## C.3 Figures

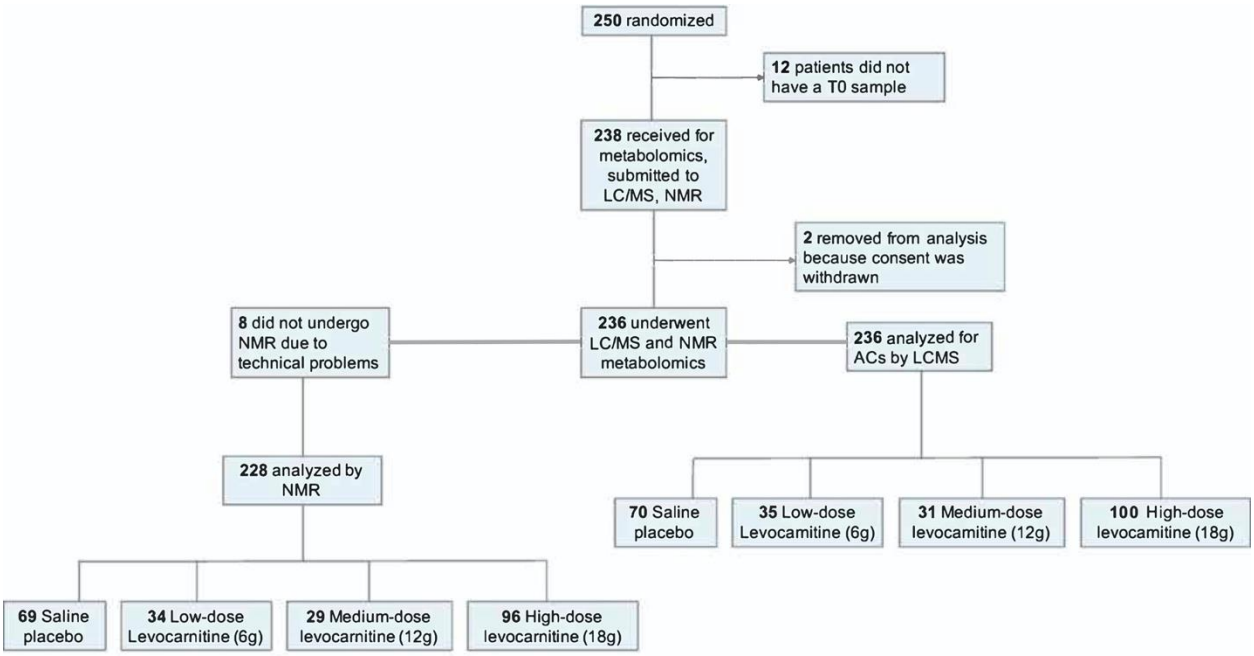


**Figure S-2. Consort diagram of patient samples for metabolomics analysis.**

Consort diagram of patient samples for metabolomics analysis. A total of 250 patients were enrolled and randomized in the Rapid Administration of Carnitine in Sepsis (RACE) trial [1]. Twelve participants did not have a T0 blood sample because the sample was not collected. Of the total of 238 samples that were received for metabolomics analysis, two were excluded from analysis because of withdrawal of consent. Of the 236 samples that were submitted for NMR analysis, there were 8 that had insufficient volume or did not extract properly. The final data set are represented by 228 samples with NMR metabolomics data and 236 with acylcarnitine data.

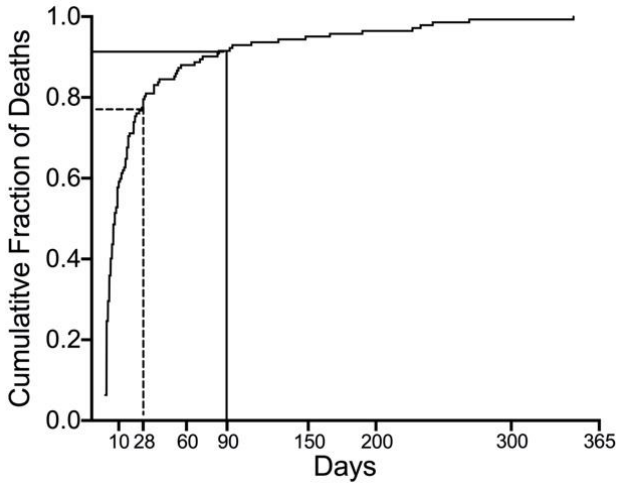


**Figure S-3. Cumulative fraction of deaths of the patients who died in the Rapid Administration of Carnitine in Sepsis (RACE) clinical trial.**

The dotted line corresponds to the cumulative fraction of deaths at 28 days (0.78) and the solid line corresponds to the cumulative fraction of deaths at 90 days (0.91).

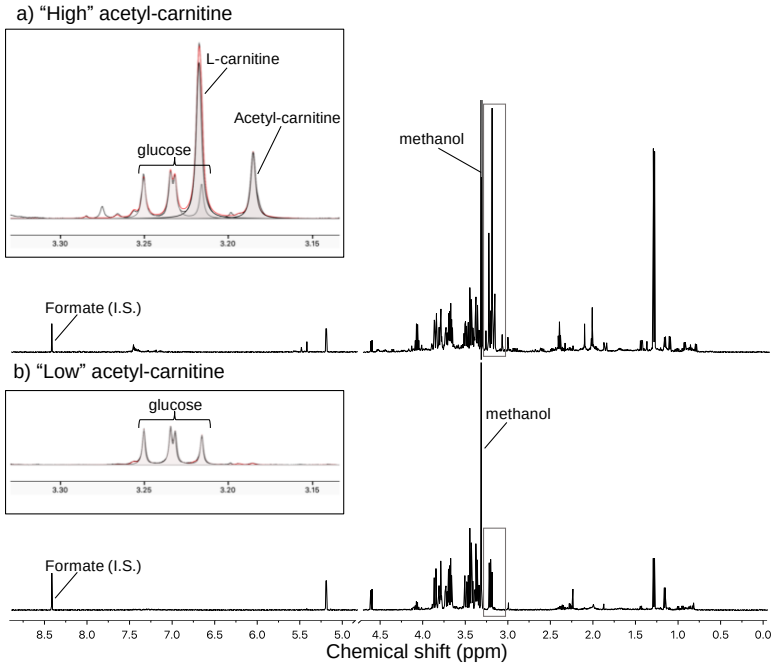


**Figure S-4. Representative proton ($^{1}$H) nuclear magnetic resonance (NMR) spectra.**

A) a serum sample from a patient with “high” acetylcarnitine (C2) concentration (≥ 35 µM) and (B) a “low” acetylcarnitine (C2) concentration (< 35µM). Formate was added to each sample as the internal standard (I.S.). The compounds (glucose, L-carnitine and acetylcarnitine) are the most abundant in the spectral region shown in the insets. Methanol is introduced into samples via the precipitation process.

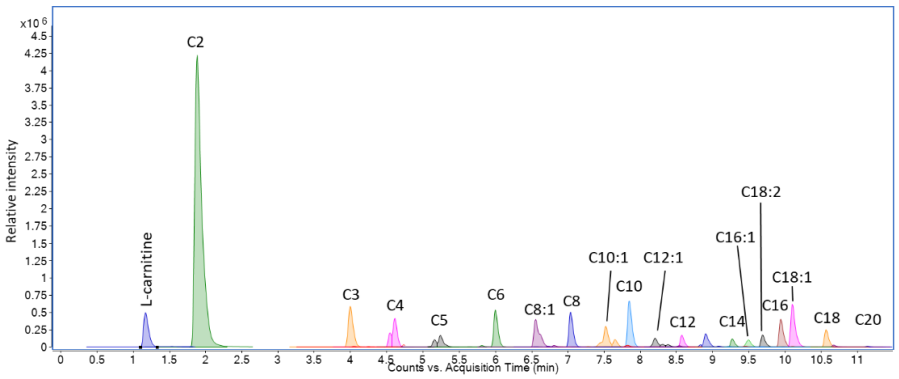


**Figure S-5. Representative liquid chromatography-mass spectroscopy chromatogram of acylcarnitines in a pooled plasma sample.**

The chromatogram was generated by overlaying multiple reaction monitoring transitions for all detected acylcarnitine species; major species were identified by peak labels in the chromatogram. Isotopically-labeled internal standard peaks are omitted from this chromatogram for clarity, but essentially co-eluted with the matching unlabeled acylcarnitine species.

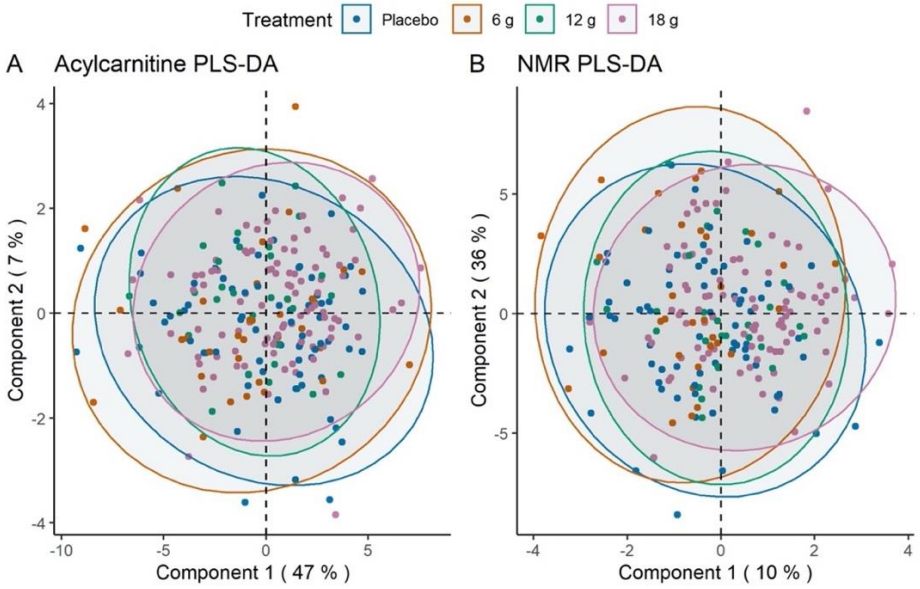


**Figure S-6. Partial Least Squares-Discriminant Analysis (PLS-DA) does not discriminate detected metabolites stratified by treatment.**

PLS-DA (and associated multivariate t distribution) of serum acylcarnitines (**A**) and NMR-detected metabolites (**B**) from the 236 and 228 patients, respectively, of the 250 patients enrolled in the Rapid Administration of Carnitine (RACE) in Sepsis clinical trial.[1] The results highlight the metabolic heterogeneity of the cohort and demonstrate that there were no detectable metabolic differences across the treatment groups.

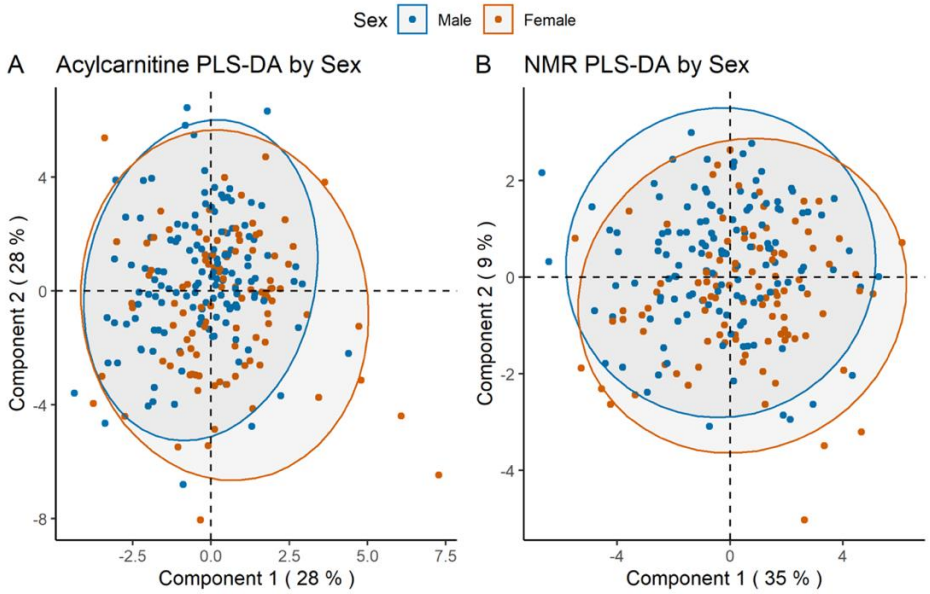


**Figure S-7. Partial least squares-discriminant analysis (PLS-DA) does not differentiate metabolites stratified by sex.**

PLS-DA (and associated multivariate t distribution) of serum acylcarnitines (**A**) and the NMR-detected metabolites (**B**) from the 236 (102, female) and 228 (100, female) patients, respectively, of the 250 patients enrolled in the Rapid Administration of Carnitine (RACE) in Sepsis clinical trial.[1]

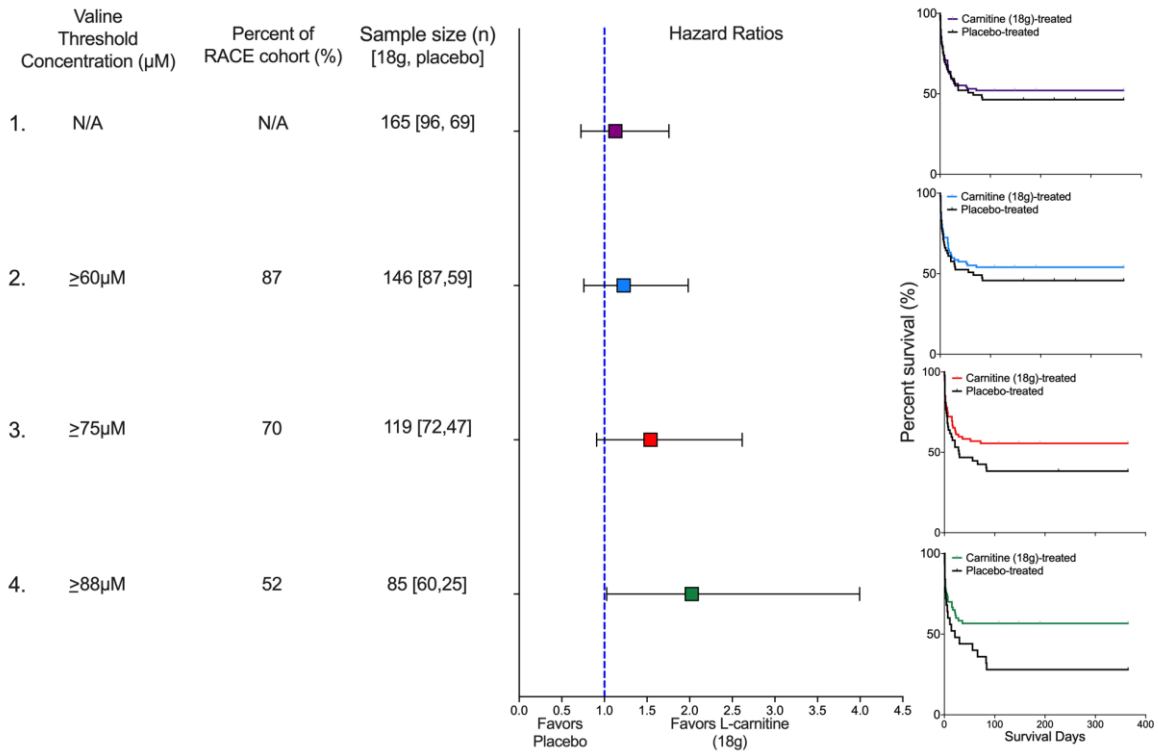


**Figure S-8. Pre-treatment valine concentration may be predictive of an L-carnitine treatment mortality benefit in patients with septic shock.**

We present four scenarios to illustrate how different threshold concentrations of valine, a branched chain amino acid, would have impacted the outcome of the Rapid Administration of Carnitine (RACE) in Sepsis clinical trial in patients treated with either L-carnitine (18 g) or placebo. In scenario 1, no threshold concentration is used so the entire RACE cohort (n=228) is eligible. The sample size of 165 patients represents those that received either L-carnitine (18 g; n=96) or placebo (n=69). The hazard ratio is not significant, and consistent with the parent trial, the Kaplan-Meier curve shows no mortality benefit of L-carnitine (p=0.59). In scenario 2, a valine threshold concentration of ≥60μM is used. Eighty-seven percent (n=198) of the RACE cohort met this criterion and of these, 146 patients received either L-carnitine (18 g) or placebo. The hazard ratio is not improved, and the Kaplan-Meier curve shows no mortality benefit of L-carnitine (p=0.41). In scenario 3, valine threshold concentration of ≥75μM is used. Seventy percent (n=160) of the RACE cohort met this criterion and of these, 119 patients received either L-carnitine (18 g) or placebo. The hazard ratio is modestly improved but is not significant; the Kaplan-Meier curve shows no mortality benefit of L-carnitine (p=0.11). Finally, scenario 4 uses the valine concentration associated with the maximum Z-statistic (see Table S4), ≥88μM. Fifty-two percent (n=119) of the RACE cohort met this criterion and of these, 85 patients received either L-carnitine (18 g) or placebo. The hazard ratio is significant, and the Kaplan-Meier curve shows a mortality benefit of L-carnitine (p=0.04). Kaplan-Meier curves were interpreted using log-rank (Mantel-Cox) tests; hazard ratios were calculated using Mantel-Haenszel. The number of patients at risk at each time point and the number of censored subjects, which was due to the end of the study (1 year), can be found here: https://doi.org/10.7302/vvqp-ma61.

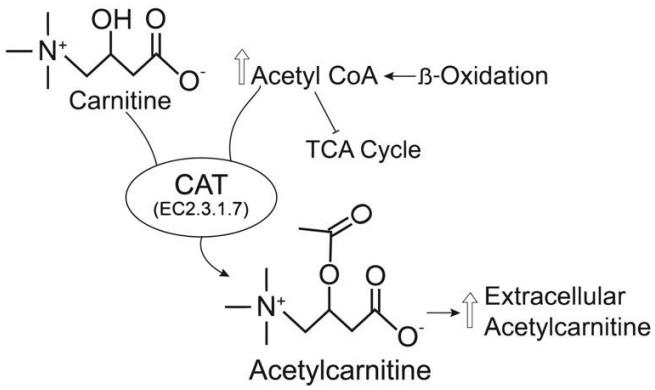


**Figure S-9. Illustration of sepsis energy alterations and proposed mechanism of L-carnitine treatment benefit.**

In the mitochondria, sepsis induced altered flux through β-oxidation and TCA cycle energy pathways, including a possible TCA cycle "stall", may lead to increased levels of acetyl-CoA. These excess acetyl-groups are removed via carnitine acetyl-transferase (CAT) mediated metabolism of carnitine converting it to acetylcarnitine (C2). In the setting of sepsis, this could be a plausible mechanism that explains the broad range of extracellular (serum) acetylcarnitine (C2) concentrations in sepsis patients. In patients with higher acetylcarnitine (C2), the mortality benefit of supplemental L-carnitine may be driven by it serving as a "sink" for excess acetyl-CoA/acetyl-groups.

## C.4 Tables

**Table S-5. Acylcarnitines and $^{1}$H-NMR Detected and Quantified Metabolites**

| **Acylcarnitines** | | **$^{1}$H-NMR Metabolites$^{\#}$** | |
|---|---|---|---|
| Compound Name | KEGG ID$^{+}$ | Compound Name | KEGG ID$^{+}$ |
| **L-Carnitine** | C00318 | 2-Hydroxybutyrate | C05984 |
| **C2-carnitine** | C02571 | 2-Oxoisocaproate | C00233 |
| **C3-carnitine** | C03017 | 3-Hydroxybutyrate | C01089 |
| **C4-carnitine** | C02862 | Acetylcarnitine (C2) | C02571 |
| **C5-carnitine** | HMDB0013128* | Alanine | C00041 |
| C6-carnitine | HMDB0000756* | Betaine | C00719 |
| C8:1-carnitine | HMDB0013324* | Carnitine | C00318 |
| **C8-carnitine** | C02838 | Choline | C00114 |
| C10:1-carnitine | HMDB0240585* | Citrate | C00158 |
| C10-carnitine | C03299 | Creatine | C00300 |
| C12:1-carnitine | HMDB0013326* | Creatinine | C00791 |
| C12-carnitine | HMDB0002250* | Glucose | C00221 |
| C14:1-carnitine | HMDB0002014* | Glutamine | C00064 |
| **C14-carnitine** | HMDB0005066* | Glycine | C00037 |
| C16:1-carnitine | HMDB0006317* | Histidine | C00135 |
| C20:4-carnitine | HMDB0006455* | Isoleucine | C00407 |
| C18:2-carnitine | HMDB0006469* | Lactate | C00186 |
| C20:3-carnitine | N/A | Leucine | C00123 |
| **C16-carnitine** | C02990 | Lysine | C00047 |
| C18:1-carnitine | HMDB0006464* | Methionine | C00073 |
| C20:2-carnitine | N/A | Ornithine | C00077 |
| C18-carnitine | HMDB0000848* | Phenylalanine | C00079 |
| C20:1-carnitine | N/A | Proline | C00148 |
| C20-carnitine | HMDB0006455* | Propylene glycol | C00583 |
| | | Pyruvate | C00022 |
| | | Tyrosine | C00082 |
| | | Valine | C00183 |

+Kyoto Encyclopedia of Genes and Genomes (KEGG) identification number
*Human metabolomics database (HMDB) identification number; KEGG ID not available
N/A = neither a KEGG nor HMDB ID is available
**Bolded** acylcarnitines were quantified using an exact-matching stable isotope internal standard; all others were relatively quantified based on peak-area. $^{\#}$NMR metabolites were quantified from the addition of a known concentration of an internal standard (formate) that was added to each sample.

**Table S-6. Logistic regression base model for the prediction of 90-day mortality (all metabolites) ranked by ascending Bonferroni-corrected p value.**

| | Base model* | | |
|---|---|---|---|
| **Metabolite predictor** | **Metabolite Coefficient ($\beta_M$)** | **$\beta_M$ Standard Error** | **$\beta_M$ P value (Bonferroni)** |
| Acetylcarnitine (C2)§ | 0.85 | 0.16 | <0.0001 |
| C18:1-carnitine§ | 0.84 | 0.17 | <0.0001 |
| Acetylcarnitine (C2)‖ | 0.76 | 0.16 | <0.0001 |
| C20:1-carnitine§ | 0.74 | 0.16 | <0.0001 |
| Tyrosine ‖ | 0.68 | 0.16 | 0.0002 |
| Betaine ‖ | 0.68 | 0.16 | 0.0002 |
| Propionylcarnitine (C3)§ | 0.64 | 0.15 | 0.0002 |
| Propylene glycol‖ | 0.66 | 0.16 | 0.0003 |
| C16:1-carnitine§ | 0.60 | 0.15 | 0.001 |
| Lysine‖ | 0.58 | 0.15 | 0.002 |
| Glycine‖ | 0.56 | 0.15 | 0.003 |
| C20-carnitine§ | 0.56 | 0.15 | 0.004 |
| Glutamine‖ | 0.55 | 0.15 | 0.006 |
| C14-carnitine§ | 0.53 | 0.15 | 0.007 |
| C16-carnitine§ | 0.52 | 0.15 | 0.01 |
| Methionine‖ | 0.51 | 0.15 | 0.01 |
| Lactate‖ | 0.51 | 0.15 | 0.02 |
| C12:1-carnitine§ | 0.51 | 0.15 | 0.02 |
| C4-carnitine§ | 0.48 | 0.14 | 0.02 |
| C20:2-carnitine§ | 0.49 | 0.15 | 0.03 |
| Proline‖ | 0.47 | 0.14 | 0.03 |
| C8-carnitine§ | 0.46 | 0.14 | 0.04 |
| Alanine‖ | 0.46 | 0.14 | 0.05 |
| C18-carnitine§ | 0.44 | 0.15 | 0.09 |
| Creatinine‖ | 0.43 | 0.14 | 0.09 |
| C10-carnitine§ | 0.41 | 0.14 | 0.13 |
| C5-carnitine§ | 0.41 | 0.14 | 0.14 |
| Phenylalanine‖ | 0.39 | 0.15 | 0.29 |
| Citrate‖ | 0.36 | 0.14 | 0.45 |
| Carnitine‖ | 0.35 | 0.14 | 0.52 |
| Histidine‖ | 0.35 | 0.14 | 0.52 |
| C10:1-carnitine§ | 0.34 | 0.14 | 0.58 |
| C6-carnitine§ | 0.34 | 0.14 | 0.59 |
| C12-carnitine§ | 0.31 | 0.14 | 1.00 |

| | | | |
|---|---|---|---|
| C18:2-carnitine[§] | 0.30 | 0.14 | 1.00 |
| Ornithine[ll] | 0.29 | 0.14 | 1.00 |
| C14:1-carnitine[§] | 0.29 | 0.14 | 1.00 |
| Creatine[ll] | 0.25 | 0.14 | 1.00 |
| Carnitine[§] | 0.25 | 0.14 | 1.00 |
| Pyruvate[ll] | 0.23 | 0.14 | 1.00 |
| Leucine[ll] | 0.21 | 0.14 | 1.00 |
| Choline[ll] | 0.20 | 0.14 | 1.00 |
| Valine[ll] | 0.18 | 0.14 | 1.00 |
| 2-hydroxybutyrate[ll] | 0.18 | 0.14 | 1.00 |
| C20:3-carnitine[§] | 0.17 | 0.14 | 1.00 |
| C8:1-carnitine[§] | 0.16 | 0.13 | 1.00 |
| C20:4-carnitine[§] | 0.11 | 0.14 | 1.00 |
| 3-hydroxybutyrate[ll] | 0.10 | 0.14 | 1.00 |
| Isoleucine[ll] | 0.08 | 0.13 | 1.00 |
| Glucose[ll] | 0.07 | 0.14 | 1.00 |
| Oxoisocaproate[ll] | -0.06 | 0.14 | 1.00 |

*The base model is described as $\text{logit}(p) = B_0 + B_D * \text{Dose} + B_M * \text{Metabolite}_i$, where p is the probability of mortality in 90 days
[§]as measured by LC-MS
[ll]as measured by $^1$H-NMR

**Table S-7. Logistic regression interaction model for the prediction of 90-day mortality (all metabolites) ranked by ascending p value.**

| | Interaction model† | | |
|---|---|---|---|
| **Metabolite predictor** | **Interaction Coefficient ($\beta_{M*D}$)** | **$\beta_{M*D}$ Standard Error** | **$\beta_{M*D}$ P value‡ (Raw)** |
| C10:1-carnitine§ | -1.22 | 0.37 | <0.0001 |
| C8:1-carnitine§ | -1.07 | 0.35 | 0.001 |
| C8-carnitine§ | -0.97 | 0.36 | 0.01 |
| C10-carnitine§ | -0.97 | 0.36 | 0.01 |
| C18:2-carnitine§ | -0.96 | 0.35 | 0.01 |
| C14:1-carnitine§ | -0.90 | 0.34 | 0.01 |
| C12-carnitine§ | -0.77 | 0.33 | 0.02 |
| C16:1-carnitine§ | -0.84 | 0.38 | 0.02 |
| Choline‖ | -0.74 | 0.33 | 0.02 |
| C16-carnitine§ | -0.82 | 0.38 | 0.02 |
| Oxoisocaproate‖ | -0.74 | 0.34 | 0.03 |
| C5-carnitine§ | -0.70 | 0.36 | 0.04 |
| Valine‖ | -0.69 | 0.35 | 0.05 |
| Acetylcarnitine (C2)§ | -0.81 | 0.42 | 0.05 |
| C12:1-carnitine§ | -0.70 | 0.37 | 0.06 |
| Isoleucine‖ | -0.57 | 0.31 | 0.06 |
| Carnitine‖ | -0.64 | 0.36 | 0.07 |
| Glycine‖ | -0.62 | 0.35 | 0.08 |
| C14-carnitine§ | -0.56 | 0.35 | 0.10 |
| C18-carnitine§ | -0.56 | 0.36 | 0.11 |
| Proline‖ | -0.52 | 0.34 | 0.12 |
| Acetylcarnitine (C2)‖ | -0.57 | 0.40 | 0.14 |
| C6-carnitine§ | -0.47 | 0.33 | 0.14 |
| Lactate‖ | 0.52 | 0.37 | 0.15 |
| Propylene glycol‖ | -0.54 | 0.39 | 0.16 |
| Citrate‖ | -0.47 | 0.34 | 0.16 |
| Leucine‖ | -0.45 | 0.34 | 0.17 |
| Carnitine§ | -0.44 | 0.33 | 0.18 |
| C20:4-carnitine§ | 0.44 | 0.44 | 0.21 |
| C20:3-carnitine§ | -0.39 | 0.32 | 0.22 |
| Methionine‖ | -0.40 | 0.34 | 0.23 |
| Propionylcarnitine (C3)§ | -0.42 | 0.36 | 0.24 |
| Pyruvate‖ | 0.36 | 0.33 | 0.28 |
| C20:2-carnitine§ | -0.37 | 0.36 | 0.30 |
| 2-hydroxybutyrate‖ | -0.30 | 0.31 | 0.34 |
| 3-hydroxybutyrate‖ | -0.30 | 0.32 | 0.34 |

| | | | |
|---|---|---|---|
| C20-carnitine[§] | -0.27 | 0.38 | 0.47 |
| Phenylalanine[ll] | -0.23 | 0.35 | 0.51 |
| Creatine[ll] | -0.18 | 0.33 | 0.58 |
| Tyrosine [ll] | 0.16 | 0.39 | 0.68 |
| Ornithine[ll] | 0.13 | 0.32 | 0.69 |
| Alanine[ll] | -0.13 | 0.35 | 0.71 |
| Creatinine[ll] | -0.10 | 0.33 | 0.75 |
| C20:1-carnitine[§] | -0.10 | 0.39 | 0.80 |
| C18:1-carnitine[§] | -0.10 | 0.39 | 0.80 |
| Glucose[ll] | 0.06 | 0.33 | 0.86 |
| C4-carnitine[§] | -0.03 | 0.34 | 0.92 |
| Histidine[ll] | 0.03 | 0.34 | 0.93 |
| Glutamine[ll] | -0.03 | 0.37 | 0.94 |
| Betaine [ll] | 0.02 | 0.38 | 0.96 |
| Lysine[ll] | 0.01 | 0.35 | 0.97 |

†The interaction model is described as $\text{logit}(p) = B_0 + B_D * \text{Dose} + B_M * \text{Metabolite}_i + B_{MD} * \text{Metabolite}_i * \text{Dose}$
[§]as measured by LC-MS
[ll]as measured by $^1$H-NMR
[#]Raw p values are not adjusted for multiple comparisons.

**Table S-8. Logistic regression models for the prediction of 90-day mortality adjusted for age and Sequential Organ Failure Assessment (SOFA) Score**

| | Base model* | | | Interaction model† | | |
|---|---|---|---|---|---|---|
| **Metabolite predictor** | **Metabolite Coefficient ($\beta_M$)** | **$\beta_M$ Standard Error** | **$\beta_M$ P value‡ (Bonferonni)** | **Interaction Coefficient ($\beta_{M*D}$)** | **$\beta_{M*D}$ Standard Error** | **$\beta_{M*D}$ P value‡ (Raw)** |
| C18:1-carnitine§ | 0.73 | 0.18 | 0.0004 | -0.02 | 0.41 | 0.96 |
| Acetylcarnitine (C2) § | 0.70 | 0.17 | 0.001 | -0.82 | 0.43 | 0.05 |
| C20:1-carnitine § | 0.67 | 0.18 | 0.002 | -0.05 | 0.41 | 0.90 |
| Betaine‖ | 0.62 | 0.18 | 0.009 | -0.12 | 0.41 | 0.76 |
| Acetylcarnitine (C2)§ | 0.57 | 0.17 | 0.02 | -0.46 | 0.41 | 0.25 |
| Tyrosine ‖ | 0.55 | 0.17 | 0.02 | 0.10 | 0.40 | 0.79 |
| C16-carnitine§ | 0.53 | 0.16 | 0.03 | -0.85 | 0.41 | 0.03 |
| C16:1-carnitine§ | 0.53 | 0.16 | 0.03 | -0.90 | 0.41 | 0.02 |
| C20-carnitine§ | 0.53 | 0.16 | 0.03 | -0.17 | 0.40 | 0.67 |
| Propionylcarnitine (C3)§ | 0.49 | 0.16 | 0.06 | -0.35 | 0.38 | 0.35 |
| C18-carnitine§ | 0.46 | 0.16 | 0.11 | -0.49 | 0.39 | 0.20 |
| Lysine‖ | 0.47 | 0.16 | 0.12 | 0.00 | 0.37 | 0.99 |
| C20:2-carnitine§ | 0.45 | 0.16 | 0.16 | -0.39 | 0.38 | 0.29 |
| Glycine‖ | 0.47 | 0.16 | 0.16 | -0.69 | 0.38 | 0.07 |
| Glutamine‖ | 0.47 | 0.17 | 0.17 | -0.12 | 0.40 | 0.76 |
| C14-carnitine§ | 0.44 | 0.15 | 0.18 | -0.57 | 0.37 | 0.12 |
| C12:1-carnitine§ | 0.44 | 0.16 | 0.25 | -0.83 | 0.40 | 0.03 |
| Proline‖ | 0.41 | 0.16 | 0.36 | -0.57 | 0.37 | 0.11 |
| Propylene glycol‖ | 0.44 | 0.17 | 0.36 | -0.39 | 0.40 | 0.33 |
| Methionine‖ | 0.40 | 0.16 | 0.46 | -0.39 | 0.36 | 0.26 |
| Creatinine‖ | 0.35 | 0.15 | 0.92 | -0.03 | 0.35 | 0.94 |
| Lactate‖ | 0.37 | 0.16 | 0.96 | 0.47 | 0.39 | 0.22 |
| C10--carnitine§ | 0.34 | 0.15 | 0.98 | -0.94 | 0.37 | 0.01 |
| Carnitine§ | 0.08 | 0.15 | 1.00 | -0.35 | 0.36 | 0.32 |
| C4-carnitine§ | 0.31 | 0.15 | 1.00 | 0.16 | 0.36 | 0.66 |
| C5-carnitine§ | 0.24 | 0.15 | 1.00 | -0.57 | 0.38 | 0.12 |
| C6-carnitine § | 0.18 | 0.15 | 1.00 | -0.37 | 0.34 | 0.28 |
| C8:1-carnitine§ | 0.09 | 0.14 | 1.00 | -1.06 | 0.37 | 0.003 |
| C8--carnitine§ | 0.31 | 0.15 | 1.00 | -0.87 | 0.37 | 0.02 |
| C10:1-carnitine§ | 0.26 | 0.15 | 1.00 | -1.23 | 0.39 | 0.001 |
| C12-carnitine§ | 0.30 | 0.15 | 1.00 | -0.88 | 0.37 | 0.01 |
| C14:1-carnitine§ | 0.27 | 0.15 | 1.00 | -0.98 | 0.37 | 0.01 |
| C20:4-carnitine§ | -0.06 | 0.16 | 1.00 | 0.86 | 0.78 | 0.05 |
| C18:2-carnitine§ | 0.28 | 0.15 | 1.00 | -0.93 | 0.38 | 0.01 |
| C20:3-carnitine§ | 0.20 | 0.15 | 1.00 | -0.34 | 0.34 | 0.33 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 2-Hydroxybutyrate[ll] | 0.11 | 0.15 | 1.00 | -0.19 | 0.33 | 0.56 |
| Oxoisocaproate[ll] | 0.00 | 0.15 | 1.00 | -0.42 | 0.38 | 0.27 |
| 3-Hydoxybutyrate[ll] | 0.11 | 0.15 | 1.00 | -0.29 | 0.34 | 0.40 |
| Alanine[ll] | 0.35 | 0.16 | 1.00 | -0.15 | 0.37 | 0.69 |
| Carnitine[ll] | 0.20 | 0.15 | 1.00 | -0.61 | 0.38 | 0.10 |
| Choline[ll] | 0.12 | 0.15 | 1.00 | -0.68 | 0.35 | 0.05 |
| Citrate[ll] | 0.28 | 0.16 | 1.00 | -0.47 | 0.37 | 0.21 |
| Creatine[ll] | 0.10 | 0.15 | 1.00 | -0.24 | 0.36 | 0.50 |
| Glucose[ll] | 0.04 | 0.15 | 1.00 | 0.04 | 0.35 | 0.91 |
| Histidine[ll] | 0.24 | 0.15 | 1.00 | -0.14 | 0.36 | 0.70 |
| Isoleucine[ll] | 0.09 | 0.15 | 1.00 | -0.45 | 0.33 | 0.17 |
| Leucine[ll] | 0.23 | 0.15 | 1.00 | -0.36 | 0.36 | 0.32 |
| Ornithine[ll] | 0.29 | 0.15 | 1.00 | 0.24 | 0.34 | 0.49 |
| Phenylalanine[ll] | 0.34 | 0.16 | 1.00 | -0.10 | 0.38 | 0.80 |
| Pyruvate[ll] | 0.13 | 0.15 | 1.00 | 0.41 | 0.35 | 0.24 |
| Valine[ll] | 0.21 | 0.15 | 1.00 | -0.52 | 0.38 | 0.17 |

*The base model is described as $\text{logit}(p) = B_0 + B_D * \text{Dose} + B_M * \text{Metabolite}_i + B_{SOFA} * \text{SOFA} + B_{Age} * \text{Age}$ where p is the probability of mortality in 90 days

†The interaction model is described as $\text{logit}(p) = B_0 + B_D * \text{Dose} + B_M * \text{Metabolite}_i + B_{MD} * \text{Metabolite}_i * \text{Dose} + B_{SOFA} * \text{SOFA} + B_{Age} * \text{Age}$

‡ p-values were calculated upon comparison to the appropriate nested model using the likelihood ratio test; raw p values are not adjusted for multiple comparisons.

§as measured by LC-MS

llas measured by $^1$H-NMR

**Table S-9. All Metabolites Ranked by Descending Maximum Z-statistic**

| Metabolite Predictor | Maximum Z–Statistic | 95% CI |
|---|---|---|
| **C10:1-carnitine** | 3.67 | 2.05–5.29 |
| **C8:1-carnitine** | 3.44 | 2.01–4.87 |
| **C10-carnitine** | 3.06 | 1.44–4.67 |
| **Acetylcarnitine (C2)**[+] | 3.01 | 1.93–4.09 |
| **C8-carnitine** | 2.98 | 1.24–4.72 |
| **C5-carnitine** | 2.74 | 1.76–3.73 |
| Isoleucine | 2.71 | 0.91–4.50 |
| **Valine** | 2.61 | 0.79–4.43 |
| C18-carnitine | 2.53 | 0.88–4.18 |
| **C12-carnitine** | 2.52 | 0.74–4.30 |
| C20:4-carnitine | 2.48 | 0.57–4.40 |
| Leucine | 2.46 | 0.59–4.32 |
| **C18:2-carnitine** | 2.41 | 1.18–3.64 |
| **C14:1-carnitine** | 2.40 | 1.2–3.60 |
| **C16-carnitine** | 2.39 | 0.55–4.23 |
| **C16:1-carnitine** | 2.38 | 0.52–4.23 |
| L-Carnitine[^] | 2.32 | 1.20–3.45 |
| L-Carnitine[+] | 2.29 | 0.71–3.88 |
| Glycine | 2.29 | 0.39–4.18 |
| Acetylcarnitine (C2)[^] | 2.28 | 0.76–3.81 |
| Propylene glycol | 2.24 | 0.70–3.79 |
| C12:1-carnitine | 2.20 | 0.28–4.12 |
| Alanine | 2.18 | 0.28–4.08 |
| Methionine | 2.14 | 0.24–4.03 |
| C14-carnitine | 2.13 | 0.65–3.62 |
| Phenylalanine | 2.11 | 0.24–3.99 |
| C20:3-carnitine | 2.05 | 0.15–3.95 |
| Creatine | 2.04 | 0.21–3.87 |
| C6-carnitine | 1.97 | 0.83–3.11 |
| Proline | 1.92 | -0.02–3.86 |
| Citrate | 1.87 | -0.06–3.81 |
| C20-carnitine | 1.86 | -0.06–3.78 |
| 3-Hydroxybutyrate | 1.83 | -0.11–3.76 |
| 2-Hydroxybutyrate | 1.82 | 0.04–3.61 |
| C20:2-carnitine | 1.79 | -0.12–3.70 |
| C3-carnitine | 1.73 | 0.05–3.41 |
| **Choline** | 1.71 | -0.24–3.67 |
| Glutamine | 1.69 | -0.25–3.64 |
| **2-Oxoisocaproate** | 1.68 | -0.28–3.63 |
| C18:1-carnitine | 1.60 | -0.25–3.46 |
| C20:1-carnitine | 1.59 | -0.33–3.51 |
| C4-carnitine | 1.53 | -0.33–3.40 |
| Histidine | 1.43 | -0.53–3.38 |
| Lysine | 1.40 | -0.56–3.35 |
| Tyrosine | 1.36 | -0.59–3.31 |
| Creatinine | 1.29 | -0.67–3.24 |
| Ornithine | 1.24 | -0.70–3.18 |
| Lactate | 1.15 | -0.81–3.10 |
| Betaine | 1.11 | -0.85–3.07 |
| Glucose | 1.08 | -0.84–3.00 |
| Pyruvate | 0.82 | -1.15–2.79 |

Bolded metabolite predictors are significant in the logistic regression interaction model; see Table 3 and 4 in the main manuscript
[+]as measured by LC-MS
[^]as measured by $^1$H-NMR

## C.5 References


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## Appendix D – Supporting Information for Chapter 5

### A.1 Figures

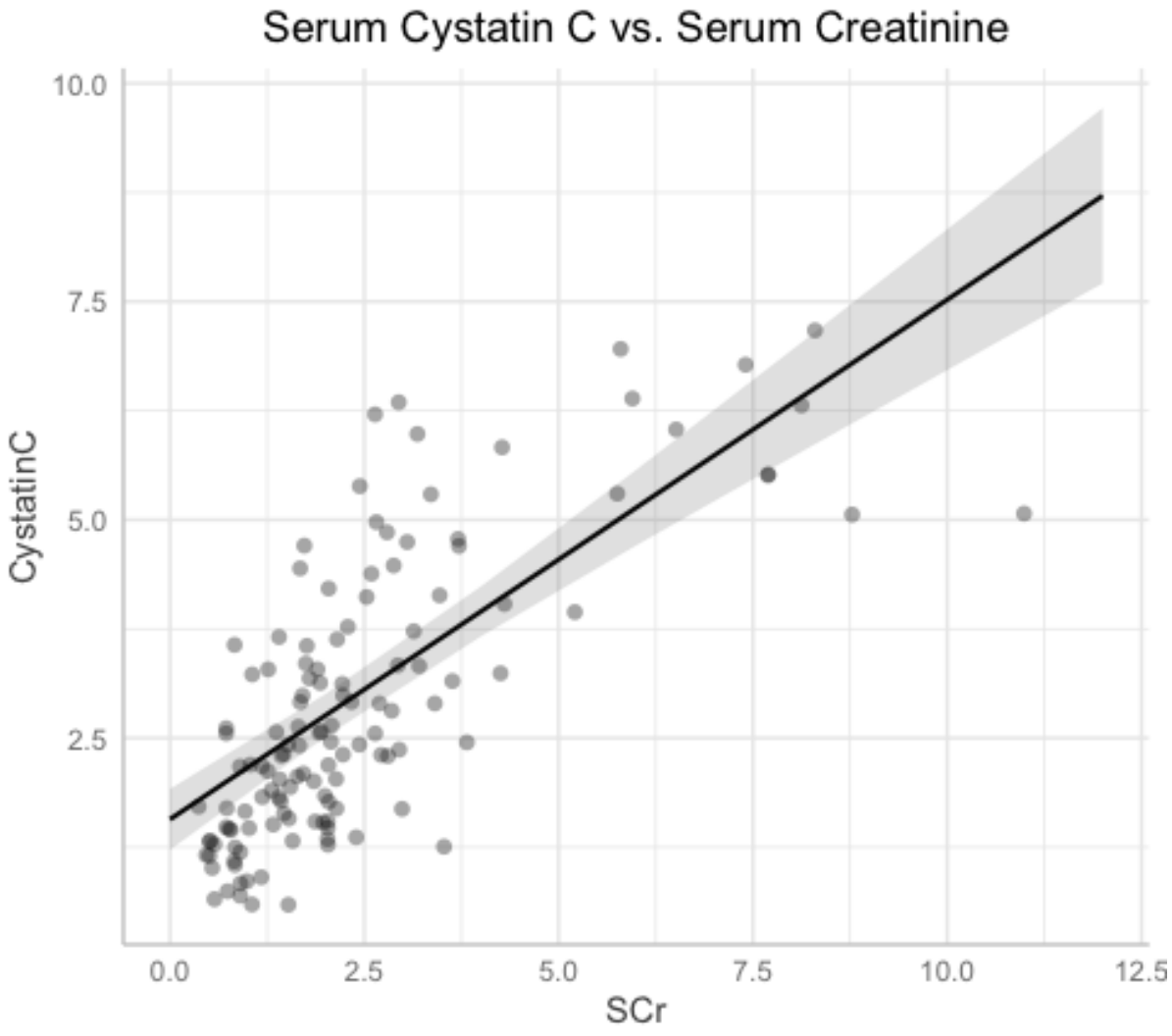


**Figure S-10. Concentrations of cystatin C versus creatinine in baseline serum.**

Residual serum was available to measure cystatin C in 126 (97%) of the patients considered for population pharmacokinetic analysis. This plot shows the concentrations of cystatin C versus clinical measurements of serum creatinine. For patients with missing observations, cystatin C was imputed from a simple linear regression model (shown as the solid, black line) using patient sex, age, weight, and serum creatinine.

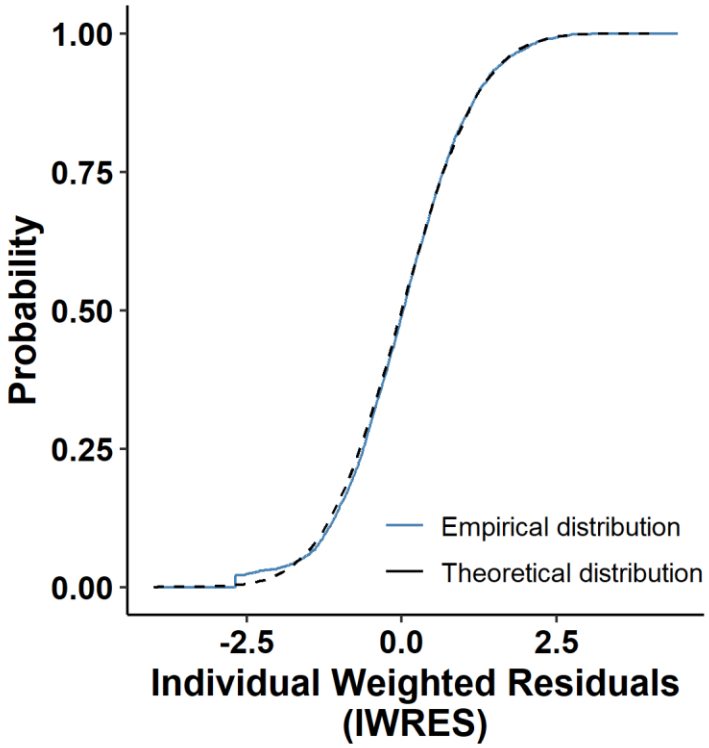


**Figure S-11. Probability of individual weighted residual (IWRES) for the top performing population pharmacokinetic model of high-dose L-carnitine.**

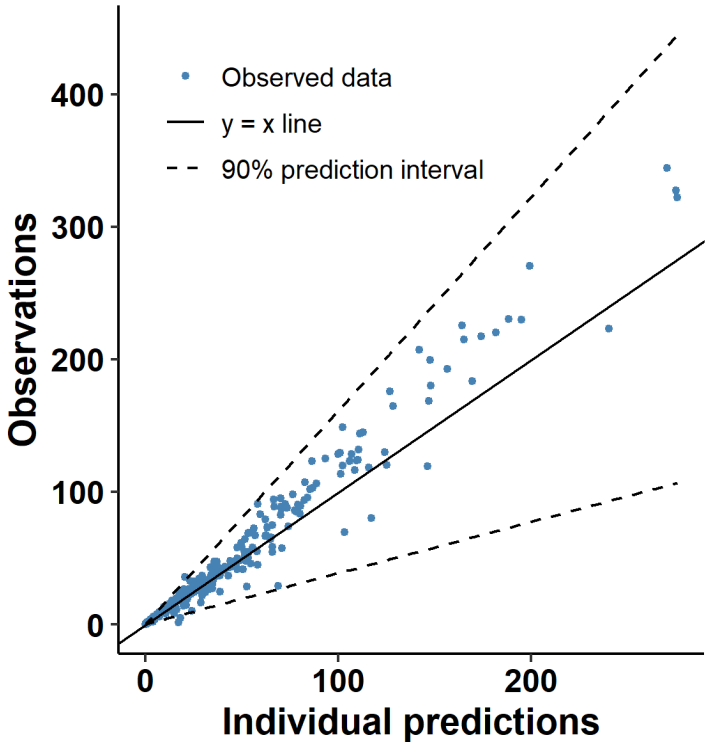


**Figure S-12. Scatter plot of observed vs. predicted concentrations for L-carnitine from the top performing population pharmacokinetic model.**